KILLER FACTORS OF THE GENUS *HANSENULA*, PARTICULARLY *H. SATURNUS*

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PREFACE

It is well known that yeasts possess the ability to antagonise as well as stimulate the growth of other yeasts and micro-organisms. The antagonistic effects are generally non-specific but, with the discovery of a specific killer interaction in yeast, there has been a renewed interest in the field. The killer phenomenon in certain yeast stains is particularly interesting because of its non-chromosomal inheritance and the elaboration of a toxic protein of high specificity - a system analogous to the killers of Paramecium and the colicins. Furthermore, the investigations of various aspects of the killer phenomenon in yeast, a simple eukaryotic organism, might be expected to advance general knowledge in eukaryotic cell biology.

The discovery of a killer factor elaborated by a strain of *Hansenula saturnus*, which differed from the killer factor of *Saccharomyces cerevisiae* in being stable and widely active, stimulated investigations in two main areas:

1. A survey of related yeasts for similar or different killer factors by interaction between pairs of strains on assay media. The killer activities were characterised by determining their physiological effects and biochemical properties.
2. A detailed study of the stable killer factor from *H. saturnus*. This factor was isolated from a minimal medium, purified and characterised. Effects of the purified toxin on cell viability and cytology were examined.

The findings are discussed in relation to current work on the biochemical and physiological aspects of yeast killer factors.

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SUMMARY

Initial investigations had shown that the yeast, *Hansenula saturnus,* when cultured in a chemically defined medium, produced a low molecular weight, pronase-sensitive killer factor of wide activity. In most respects therefore, it differed from the properties of the highly specific killer factor of *Saccharomyces cerevisiae.* This finding led to a detailed study of *Hansenula* yeasts for killer activity comprising two main aspects:

1. Characterisation of the killer activities of *H. saturnus* and its relationship to that found in other *Hansenula* yeasts.
2. Purification and characterisation of the stable killer factor of *H. saturnus.*

Of the six strains of *H. saturnus* tested, five were killer active over a spectrum which was strain and pH dependent. Studies of the filtrate activities of the killer strains grown in complex medium, revealed three distinct killer species based on the differing responses of sensitive yeasts. Toxin-A, elaborated under a wide range of conditions, was thermostable and active over the range pH3.5-7.0, and was analogous to that produced in the chemically defined medium. Toxin-B and toxin-C activities, produced optimally in aerated culture, were thermolabile and active only below pH5.0, but had different optimal values for killer activity.

Survey of a further 22 stains of *Hansenula* for both *H. saturnus* and *Sacch. cerevisiae* killer factor-like activity (tested at pH6.0 and pH4.7, 4.3 respectively), revealed 11 killer strains, three of which displayed considerable killer activity at pH6.0. On the basis of

cross-reactions between 16 killer strains, together with the resistance patterns of non-killer strains, eight classes of killer yeasts were recognised. Filtrates from all but four killer strains greatly reduced the viability of a sensitive strain. Biochemically distinct killer factors of the strains belonging to different killer groups were demonstrated by: membrane diffusibility; inactivation by pH, heat and proteolytic enzymes; effect of pH on biological activity; and electrophoretic behaviour. These analyses also demonstrated that strains belonging to five killer groups produced several toxins. Athough the toxins displayed widely different properties, they had in common a proteinaceous component necessary for activity as for other yeast killer factors. The toxins active at near-neutral pH were generally more stable than those only active at low pH.

<u>H. saturnus</u> killer activity, produced optimally in aerated chemically defined medium supplemented with peptone, was readily isolated by adsorption to Amberlite IR-120 cation exchange resin. The activity was recovered in a small volume of low ionic strength volatile buffer. An aqueous acetic acid extract of the resin eluate concentrate was then eluted from Bio-Gel P-2 and followed by chromatography on Bio-Gel P-10 in aqueous acetic acid to yield three active fractions, all containing toxin-A activity. Rechromatography of the major peak yielded toxin-A purified 3,500-fold on a gravimetric basis and 5-fold with respect to protein. Toxin-A was homogeneous by cellulose acetate membrane and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, with a molecular weight of 11000 1000. Analysis demonstrated at most two hexose residues per polypeptide and 17 amino-acids, of which glycine and the acidic residues were predominant but the proportion of basic and aromatic residues was low.

Initial experiments on the effects of toxin-A against the sensitive yeast *H. anomala* showed that growing cells only were killed, while toxin-induced cell clumping occurred in both growing and resting cell populations. Killing by a single-hit process was indicated and one lethal unit of purified toxin contained 5×10^5 molecules. Cytological examination revealed early structural alterations to the plasma membrane which however appeared to remain intact while nuclear envelope and endoplasmic reticulum damage occurred, but no cell lysis became apparent. The primary site of action remains to be determined.

This study therefore confirms the reported high incidence of killer activity among *Hansenula* yeasts but also demonstrates that related yeasts elaborate biochemically different proteinaceous toxins having broad spectra of activity. In particular, toxin-A contrasts with other reported killer toxins by displaying wide activity and therefore may provide a useful new fungal antibiotic.

DECLARATION

This thesis is submitted for the degree of Doctor of Philosophy in The University of Adelaide.

I delcare that it contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no material previously published or written by any other person, except when due reference is made in the text.

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CHAPTER 1

GENERAL INTRODUCTION

The ability of yeasts to antagonise the growth of other yeasts and micro-organisms has been recognised for some time. However, the recent discovery of a killer character in certain strains of *Saccharomyces cerevisiae* has stimulated new interest in yeast interactions. Specificity of this killer reaction and its cytoplasmic mediated heritability are analogous to killer systems in other micro-organisms, such as the killers in *Paramecium* and colicins, and also more recently described for *Ustilago maydis.* Attention has been mainly focussed on the molecular and genetic aspects of the killer character in *Sacch. cerevisiae* and until recently little was known about killer reactions in other species. This review outlines important investigations into the killer character of *Sacch. cerevisiae* and current work involving other yeasts. Particular attention is paid to the nature and action of the killer factors.

Antagonistic interactions

At various times it has been claimed that yeasts and their fermentation products possess chemotherapeutic power, without clear evidence for belief in their efficacy (FLOREY *et al*, 1949a). Although reports have detailed isolation of various substances possessing anti-bacterial or anti-fungal activity from yeasts (UROMA and VIRTANEN, 1947; MOTZEL and COOK, 1958; BARBER, EYLAN and HEIBER, 1973), systematic studies of yeasts isolated from natural sources and those held in a

culture collection have not revealed any marked anti-microbial activity (KOCH, 1952; Mac WILLIAM, 1959). Yeasts are well known to influence the growth of bacteria and to a lesser extent other yeasts during fermentations (RANKINE, 1954; STRANDSKOV and BOCKELMANN, 1958; RADLER, 1966; FORNACHON, 1968) but a specific lethal interaction was not recognised until the discovery of the killer reaction in *Sacch. cerevisiae* by BEVAN and MAKOWER (1963).

Killer yeasts

MAKOWER and BEVAN (1963) described three phenotypes with respect to the killer trait in *Sacch. cerevisiae:* killer, sensitive and neutral. Killer strains excrete into their environment an unstable toxic substance, the killer factor, to which they are themselves immune but which kills strains unable to produce the factor (sensitives). Neutral strains neither produce the killer factor nor are sensitive to its action. In addition, strains that produce the toxin but are unable to resist it, and are thus suicidal, have also been described (WICKNER, 1974). The killer character of a yeast culture is readily demonstrated on agar culture medium buffered at appropriate pH for killer activity containing sensitive cells and the dye methylene blue to indicate the presence of killed cells (WOODS and BEVAN, 1968). The killer strain in the culture is surrounded by an inhibition zone fringed by blue staining dead cells against a non-staining sensitive cell background. Staining distinguishes killer action from growth inhibition such as G1 arrest caused by the mating pheromone (DUNTZE, STOTZLER, BUCKING-THROM AND KALBITZER, 1973). Both spontaneous and induced blue-staining mutants have been reported which may or may not be killer factor sensitive (MITCHELL and BEVAN, 1973, 1974; AL-AIDROOS and BUSSEY, 1978).

The killer phenomenon, which eluded discovery for so long probably because of the unstable nature of the toxin at pHs normally employed to cultivate yeasts, is widespread among laboratory strains of Sacch. cerevisiae but the high incidence is in part the result of inbreeding (FINK and STYLES, 1972; PHILLISKIRK and YOUNG, 1975). The killer character has also been demonstrated in industrial strains of this yeast as well as in other species (MAULE and THOMAS, 1973; NAUMOV and NAUMOVA, 1973; IMAMURA, KAWAMOTO and TAKAOKA, 1974; PHILLISKIRK and YOUNG, 1975) and also in yeasts of other genera, including Debaromyces, Hansenula, Kluyveromyces, Pichia, Candida and Torulopsis (BUSSEY and SKIPPER, 1975; PHILLISKIRK and YOUNG, 1975; MITCHELL cited by ROGERS and BEVAN, 1978). STUMM, HERMANS, MIDDELBEEK, CROES and DE VRIES (1977) found that of 157 strains belonging to nine genera isolated from natural habitats, 17% were killer yeasts. Coupled to the findings of PHILLISKIRK and YOUNG (1975), it was suggested that the killer phenomenon plays an important role in the ecology of yeasts.

The killer determinant in Saccharomyces cerevisiae

The molecular and genetic aspects of the killer determinants of Sacch. cerevisiae have been reviewed by WICKNER (1976a, 1979) and PIETRAS and BRUENN (1976), and therefore need only be briefly mentioned.

Initial genetic crossing experiments established that the killer phenotype was the expression of a cytoplasmically inherited genetic determinant under control of chromosomal genes (MAKOWER and BEVAN, 1963; SOMERS and BEVAN, 1969; BEVAN and SOMERS, 1969) and together with curing experiments showed that it was independent of the mitochondrial genome (FINK and STYLES, 1972; AL-AIDROOS, SOMERS and BUSSEY, 1973; WICKNER, 1974b; NAUMOVA and NAUMOV, 1974). BERRY and BEVAN (1972) first

described an association between double-stranded (ds) RNA and the killer phenotype. Subsequently, two species of dsRNA were demonstrated: the larger of approximate molecular weight 2.5 x 10^6 denoted P1 or L, and the smaller, of approximate molecular weight 1.4 x 10^6, denoted P2 or M. The latter designations are adopted in the present text. The molecular weight of L dsRNA has recently been revised to 3.5 x 10^6 (HERRING and BEVAN, 1977; HOLM, OLIVER, NEWMAN, HOLLAND, McLAUGHLIN, WAGNER and WARNER, 1978). Both species were present in killer strains but sensitive strains lacked M-dsRNA, thus correlating this species with the killer determinant (BEVAN, HERRING and MITCHELL, 1973; VODKIN, KATTERMAN and FINK, 1974). Furthermore, strains cured of their killer ability also lacked M-dsRNA (see for example YOUNG and YAGIU, 1978) while suppressive strains (sensitives) carried instead a species of dsRNA denoted S, of molecular weight 0.25 to 1.1 x 10^6 depending on the strain (VODKIN, KATTERMAN and FINK, 1974; TZEN, SOMERS and MITCHELL, 1974). Recent investigations suggest that S-dsRNA is a defective species derived entirely from M-dsRNA (BRUENN and KANE, 1978). Several other species of dsRNA are also present in certain killer strains which may or may not remain in cured strains (WICKNER and LEIBOWITZ, 1976a, b; YOUNG and YAGIU, 1978).

The L- and M-dsRNA molecules are associated with virus-like particles (HERRING and BEVAN, 1974; HERRING and BEVAN, 1975; ADLER, WOOD and BOZARTH, 1976), but as they do not appear to possess an infectious cycle they may represent a component system similar to those of other fungal viruses (WOOD, 1973; LEMKE and NASH, 1974; LEMKE, 1976; PIETRAS and BRUENN, 1976, HERRING and BEVAN, 1975). A single-stranded RNA polymerase is associated with isolated particles and it has been shown that their replicative cycle does not follow the host "doubling cycle" (HERRING and BEVAN, 1977; OLIVER, McCREADY, HOLM, SUTHERLAND, McLAUGHLIN

and COX, 1977). L- and M-dsRNA do not show homology with each other (BEVAN, HERRING and MITCHELL, 1973; VODKIN, KATTERMAN and FINK, 1974; SWEENEY, TATE and FINK, 1976; BRUENN and KANE, 1978) and differ chemically from other yeast RNA species (BRUENN and KEITZ, 1976). However, there is disagreement about homology between dsRNA and chromosomal DNA (VODKIN, 1976; WICKNER and LEIBOWITZ, 1977; HASTIE, BRENNAN and BRUENN, 1978). OLIVER et al. (1977) demonstrated three proteins associated with L-dsRNA containing virus-like particles and in vitro translation of denatured L-dsRNA revealed that this species codes for the major capsid protein (HOPPER, BOSTIAN, TIPPER and ROWE, 1977). It has also been reported that part of a major protein obtained by the in vitro translation of denatured M-dsRNA showed homology with the killer toxin (BOSTIAN and ROGERS cited by PALFREE and BUSSEY, 1979).

Characterisation of various mutant strains, containing either killer determinant or chromosomal defects, has provided insight into the control and function of the killer character. Killer determinant mutants studied include (i) neutrals: non-killers possessing the resistance phenotype, (ii) suppressives: non-killers which interefere with propagation of the killer trait (SOMERS, 1973), and (iii) diploid-dependent mutants which are defective for killing action in the haploid state (WICKNER, 1976b). Maintenance of the killer determinant is controlled by a large number of chromosomal genes, some of which also have other known functions (SOMERS and BEVAN, 1969; BEVAN, SOMERS and THEIVENDIRARAJAH, 1969; FINK and STYLES, 1972; WICKNER, 1974a; WICKNER and LEIBOWITZ, 1976a; WICKNER, 1978; and LEIBOWITZ and WICKNER, 1978). Chromosomal genes also affect killer and resistance expression (WICKNER, 1974a; WICKNER, 1976a) and thus, for example, killer strains showing enhanced killer activity may result from chromosomal mutations (TOH-E, GUERRY and WICKNER, 1978; COHN, TABOR, TABOR and WICKNER, 1978) as well

as from an increase in M-dsRNA content (VODKIN, KATTERMAN and FINK, 1974). Cell division cycle mutants have been studied in an attempt to understand control of killer functions (SHALITIN and WEISER, 1977).

Comparison of yeast killer systems: evidence for different types of toxins

The immunity of a killer yeast to the toxin that it excretes implies possession of a specific immunity system. The absence of cross-immunity between different killer yeasts is therefore indicative of dissimilar toxin and immunity systems. Evidence supporting the possession of distinct toxins by yeasts with different killer specificities is provided by (i) the immunity patterns of resistant mutants, (ii) properties of dsRNA species, and (iii) physiological, biochemical and physico-chemical properties of the toxins.

A. Killing and immunity cross-reactions between killer yeasts

Killer strains of the various laboratory *Sacch. cerevisiae* cultures possess analogous killer specificity. However strains of this type and of a wine-making yeast showed no cross-immunity (NAUMOV and NAUMOVA, 1973). Killer and resistance phenotypes of the two groups of strains have been denoted K_1R_1 and K_2R_2 respectively (WICKNER, 1976; see also YOUNG and YAGIU, 1978; WICKNER, 1979). Further differences between the two groups were evident by suppression of the K_2R_2 phenotype by K_1R_1 strains and by the lower pH-optimum for killing by K_2 strains (pH4.2 compared to pH4.7) (NAUMOVA and NAUMOV, 1973). A killer strain of *T. glabrata* also possessed a different specificity to the common killer as shown by reciprocal killing and supported by detailed studies on the killer activity (BUSSEY and SKIPPER, 1975, 1976; SKIPPER and

BUSSEY, 1977).

Cross-reactions between 12 known killer yeasts of 3 different genera revealed 4 groups which ROGERS and BEVAN (1978) call TOX1 to TOX4. TOX1 and TOX2 contained both laboratory and industrial strains of *Sacch. cerevisiae* and strains of *C. albicans* while TOX3 and TOX4 contained a single strain of *T. glabrata* and *Sacch. (Kluyveromyces) drosophilarum* respectively. YOUNG and YAGIU (1978) determined the interaction between 20 killer yeasts (19 strains from the NCYC, PHILLISKIRK and YOUNG, 1975) and found 13 classes of killer yeasts composed of 10 killer types, denoted K_1 to K_{10}, and 10 resistance types denoted R_a to R_j. *Saccharomyces* killer strains and species fell into 3 groups but showed 5 resistance groups (strains of K_1 killers displaying 3 distinct resistance patterns). The remaining 7 killer groups contained non - *Saccharomyces* killers which, with the exception of *T. glabrata* were resistant to all *Saccharomyces* killers. STUMM *et al.* (1977) identified 26 killer strains belonging to two mutually exclusive killer groups, among 157 yeasts isolated from natural sources. WICKNER (1979) has correlated the data from the various studies and described 11 killer and 11 resistance specificities. The position of some killer strains is not yet clear.

B. Resistant mutants

By analogy with the classification of colicins using resistant mutants (DAVIES and REEVES, 1975a, b), the immunity reactions of toxin resistant mutants to toxins from different killer yeasts enables recognition of interrelationships between the yeasts. Although resistant mutants are immune to killing by a different mechanism to that of killer cells (BUSSEY and SHERMAN and SOMERS, 1973; AL-AIDROOS and BUSSEY, 1978) their

immunity patterns to toxins are probably likely to indicate analogous relationships between toxigenic strains as determined by killing and immunity cross-reactions. Thus AL-AIDROOS and BUSSEY (1978), who tested toxin isolates from 5 killer yeasts not showing cross-resistance with a Sacch. cerevisiae killer strain, observed that these toxins could also bypass the resistance mechanism of one group of killer factor resistant mutants. This clearly indicated that these toxins possess a killer specificity different to the killer factor. Furthermore, ROGERS and BEVAN (1978) found that, based on the pattern of immunity exhibited by various killer factor resistant mutants to the toxins isolated from 12 killer yeasts, the 12 yeasts fell into 4 analogous groups as determined by their killer and immunity cross-reactions.

C. Killer determinant

The work of YOUNG and YAGIU (1978) showed that the properties of dsRNA species, found only in Saccharomyces killer yeasts (types K_1, K_2 and K_3), correlated with killer type determined by cross-reaction. Estimated M-dsRNA molecular weight of K_1 strains was approximately 1.3 x 10^6, for K_2, 1.0 x 10^6 and K_3, 0.9 x 10^6. Furthermore M-dsRNA was readily removed from K_2 strains by growth at elevated temperature and from that of K_3 by cycloheximide treatment. Molecular weight of the L species also correlated with killer type. NESTEROVA, MILOVATSKII, SIZOVA and SEMYKINA (1978a, b) have also reported on the properties of the dsRNA species present in killer strains of Saccharomyces.

D. Optimum pH for killing activity

The various killer yeasts have generally been characterised by their optimal pH for killing; different values being indicative of

structurally dissimilar toxins but analogous pH values not necessarily implying that the toxins are of the same type. The toxins produced by the strains studied by ROGERS and BEVAN (1978) all showed analogous optimum pH values for killing within each TOX group, the values being in the range pH4.0 to 4.7. The 56 killer strains studied by PHILLISKIRK and YOUNG (1975) were placed in 4 broad groups on the basis of the effect of pH on their culture filtrate killer activity. For the Saccharomyces strains, at least, those belonging to a particular killer group (see YOUNG and YAGIU, 1978) in general can also be seen to possess a similar pH optimum for killer activity. It is evident from these and other authors that the killer activity of the toxins is confined to a very narrow band of pH (approximately 1 pH unit) with only several strains showing optimal activity at or below pH4.0 or partial activity beyond pH4.9 (WOODS, ROSS and HENDRY, 1974; BUSSEY and SKIPPER, 1975). The upper pH limit for activity of many toxins is due in part to their unstable nature (YOUNG and YAGIU, 1978). The results of ROGERS and BEVAN (1978) suggest that unlike killer activity, immunity does not appear to be pH-dependent though it was recognised that the toxins may have been rapidly inactivated outside their narrow pH range of activity.

Nature of the killer factor and characteristics of other yeast toxins

To date, only the yeast killer factor (Sacch. cerevisiae - produced toxin, K_1) has been extensively characterised. WOODS and BEVAN (1968) partially purified killer factor activity from the culture fluid of Sacch. cerevisiae by ammonium sulphate precipitation, gel chromatography and ultrafiltration. They characterised it to be a papain sensitive thermolabile macromolecule for which activity was rapidly and irreversibly lost both at pHs outside 4.6 to 4.8 and by vigorous shaking, unless stabilised by gelatin. BUSSEY (1972) prepared a

fraction containing killer factor activity by ultrafiltration in which protein and polysaccharide but not nucleic acids were present. Preparations from non-killers also had a similar chemical composition. This extracellular concentrate of killer activity showed similar characteristics as reported by WOODS and BEVAN (1968) and displayed a multicomponent nature by gel chromatography and on a sucrose gradient. Five toxic glycoproteins with protein to polysaccharide ratios of 0.04 - 0.08 and molecular weights from 1.7 to 3.5 x 10^5 were obtained and shown to be homogenous by SDS-polyacrylamide gel electrophoresis (BUSSEY and SKIPPER, 1975; BUSSEY and SKIPPER cited by PIETRAS and BRUENN, 1976). The principal sugar of these glycoproteins was D-mannose. The behaviour of killer factor and other glycoproteins has been studied by specific ion mediated chromatography on substituted agarose gels (BUSSEY, RIMERMAN and HATFIELD, 1975).

Recently PALFREE and BUSSEY (1979) purified the killer toxin obtained in an extracellular medium concentrate by precipitation in poly(ethylene glycol) and chromatography through glyceryl-controlled -pore glass employing urea to split the glycoprotein aggregate while stabilising killer activity with glycerol. A single species of protein of molecular weight 11,470 was shown to be the active component, having greatest stability between pH4.2 and 4.6. Toxin derived from a super killer strain was more stable at 30°C than that from a standard killer. In addition to glycerol, other polyhydric alcohols also stabilise killer activity (OUCHI, KAWASE, NAKANO and AKIYAMA, 1978). The killer toxin for sake yeast displays many analogous physico-chemical properties to the <u>Sacch. cerevisiae</u> killer factor, and also has a similar mode of action (KOTANI, SHINMYO and ENATSU, 1977). It has been reported that this toxin also has the K_1 killer specificity (WICKNER, 1979). WOODS, ROSS and HENDRY (1974) partially characterised a second killer factor

from Sacch. cerevisiae, produced by a killer/sensitive strain, which differed from the K_1 killer factor by showing greater stability to heat and pH.

BUSSEY and SKIPPER (1975) reported on the partial purification of at least three glycoprotein toxins called PEST (pool efflux stimulating toxins) produced by T. glabrata. A crude extracellular concentrate containing PEST activity had polysaccharide to protein ratios of 3:1 to 9:1, absorbancy at 280 nm to absorbancy at 260 nm ratios of 1.2 to 0.9, was rapidly inactivated by boiling, was pronase sensitive and although relatively stable in the range pH3.4 to 7.0, showed optimum killing near pH4 and none at pH6 to 7.

YOUNG and YAGIU (1978) systematically studied the properties of killer activity present in the culture filtrates of some 20 yeasts belonging to 10 killer groups. The tests made on the culture filtrates were; effect of proteolytic enzymes, temperature and pH, and gel chromatographic behaviour. Killer activity of five of the eight killer yeast types tested was inactivated by at least one of three proteases used, the inactivation patterns differing between each killer type. All killer activities were inactivated at 35°C, the non - Saccharomyces - produced toxins being relatively more stable. Toxins from strains within a killer type tended to show similar half-life values. Between pH2 to 4.5 all killer activities remained stable but were completely inactivated at pHs beyond 6. Several broad groups were however recognised by their degree of stability at pHs between 4.5 and 6.0. Gel chromatography of four killer-active concentrates revealed excluded fractions which eluted with protein and polysaccharide, and two of the concentrates also exhibited chromatographically retarded killer species. It was concluded from this study that strains belonging to different killer groups

produced biochemically distinct and hence probably structurally distinct toxins, but all contained a proteinaceous component necessary for activity.

Interaction of killer toxins with cells

Following the preliminary findings of WOODS and BEVAN (1968), BUSSEY and colleagues made a detailed study of the binding of killer toxin to cells and their toxic effects aimed at elucidating the mode of action of the Sacch. cerevisiae killer toxin (herein referred to as "killer factor" to avoid ambiguity with the general usage of "killer toxin"). Investigations have also been made on the killer toxin for sake yeast (IMAMURA, KAWAMOTO and TAKAOKA, 1975; KOTANI, SHINMYO and ENATSU, 1977) and the pool efflux stimulating toxins (PEST) produced by T. glabrata (BUSSEY and SKIPPER, 1975).

A. Binding of toxin to cells

The killing of sensitive cells by killer factor was reported to follow a single-hit process (BUSSEY, 1972), a lethal unit for freshly purified toxin being of the order, 10^4 molecules (PALFREE and BUSSEY, 1979). Killer factor bound irreversibly to sensitive cells and therefore the survival was an effective measure of cell-bound toxin (BUSSEY, 1972). Binding of toxin to cells was complete within 30 min, but these cells were not killed immediately. During this time, a proportion of cells could be rescued by conditions known to inactivate the toxin or by digestion of the cell wall (WOODS and BEVAN, 1968; BUSSEY, 1972). Sphaeroplasts, which bound only a relatively small proportion of killer factor, also exhibited a similar time lag before damage ensued (BUSSEY, SHERMAN and SOMERS, 1973). It was concluded that (i) there were at

least two different toxin binding sites, (ii) the cell wall sites were not essential to the killing process and (iii) the time lag did not represent transport of the toxin across the cell wall.

Resistant mutants have been partially characterised in an attempt to dissect the killing process. The resistant mutant studied by BUSSEY AND SHERMAN (1973) was unable to bind killer factor yet its sphaeroplasts remained fully sensitive to killer factor. This mechanism of resistance was different to that of the killer strain, since killer factor was shown to bind to its sphaeroplasts and cell wall. A large number of killer factor resistant mutants investigated by AL-AIDROOS and BUSSEY (1978), fell into three distinct functional groups, strains of two of the groups being deficient in binding of killer factor to the cell wall. One of these two groups of mutants was cross-resistant to toxins derived from five other killer yeasts, which suggested a common element, possibly carbohydrate in nature, in the cell wall binding site. The other group of killer factor resistant mutants, not cross-resistant to the other yeast toxins, suggested a second element of the cell wall binding site specific for killer factor. Structure of the toxin binding site was compared to that for group E colicins in *Escherichia coli* described by SABET and SCHNAITMAN (1973). The third type of resistant mutant, also showing cross-resistance to the other yeasts toxins bound toxin in wild-type amount. Sphaeroplasting some of the mutants restored killer sensitivity, but the method of sphaeroplast formation appeared to be important in relation to killer toxin sensitivity. In view of the evidence that both killer factor and PEST act at the plasma membrane, (SKIPPER and BUSSEY, 1977), membrane related mutants were expected but not found (AL-AIDROOS and BUSSEY, 1978). Taken together with the findings of ROGERS and BEVAN (1978) it would appear that while the various yeast toxins studied bind to a common element in the cell wall,

toxins from several different yeasts are subject to the killer factor immunity system while others are able to bypass the mechanisms.

B. Effects of toxins on sensitive cells

Sensitive cells were found to be most susceptible to the lethal action of killer factor when growing logarithmically on glucose media, implying the requirement of metabolic activity for the killing process (WOODS and BEVAN, 1968; BUSSEY, 1972). In a culture of sensitive cells treated with killer factor, a lag of some 40 to 50 min occurred before general inhibition of macromolecular synthesis was initiated, by which time 70-80% of cells had bound a lethal dose of toxin (BUSSEY, 1972). The cells continued to take up and maintain pools of leucine, adenine and glucose for at least as long as protein synthesis continued. However, coincident with the co-ordinate cessation of macromolecular sythesis was an increase in efflux of certain compounds from cellular pools (BUSSEY and SHERMAN, 1973; BUSSEY, 1974). During the inhibition period, the cellular ATP pool level fell to exhaustion, although its synthesis continued. Also an efflux of glucose and to a much lesser extent leucine occurred but there was little loss of macromolecules from cells. Sensitive cells loaded with ^{42}K, after a similar lag, were found to rapidly lose the potassium pool, complete depletion being reached by about 180 min (SKIPPER and BUSSEY, 1977). A transient increase in culture turbidity was also observed and appears to be caused by increased light scattering resuting from a reduction in cell volume (BUSSEY, 1974). Killer treated sphaeroplasts also showed the increased turbidity effect. Another sensitive yeast, _T. glabrata,_ showed qualitatively identical changes in metabolic events as did _Sacch. cerevisiae_ following treatment with killer factor (BUSSEY and SKIPPER, 1976). It was concluded that killer factor acts as a specific fungal

antibiotic killing sensitive strains of yeast by a mechanism involving plasma membrane damage.

KOTANI, SHINMYO and ENATSU (1977), studying the effects of the sake yeast killer toxin on a sensitive yeast, found that the survival of toxin-treated resting cells was dependent on the composition of the subsequent growth medium. In rich medium, cell viability sharply decreased with time whereas in minimal medium cells were rescued, for a time, from death. The addition of calcium ions to the rich growth medium prevented killing, and it was shown that it acted by rapidly blocking leakage of ATP from the cell, presumably by acting at the plasma membrane. Protein synthesis was implicated in the recovery process. Addition of ADP to the growth medium however enhanced killing. Thus KOTANI and colleagues proposed that cell death was preceded by a transient state in which calcium ions induced rescue in the presence of protein synthesis, but ADP promoted attainment of the irreversible damaged state. It was suggested that an energised membrane was required to trigger the killing action of this toxin.

SKIPPER and BUSSEY (1977) obtained evidence that the killer factor-induced killing process was linked to energy metabolism by studying the effects of drugs that interfere with the generation or utilisation of energy on cell viability and membrane damage in cultures treated with toxin. Drugs which stopped the growth of glucose-grown cells blocked killer induced potassium efflux, whereas the inhibitors of electron transfer in mitochondria did not affect cell growth or alter potassium efflux. All of the drugs which prevented growth of ethanol-grown cells also blocked the release of potassium from cells. Time-course relationship studies showed that DNP, for example, arrested the killing process (^{42}K efflux) at a stage subsequent to the binding of toxin to

the cell, rapidly blocking potassium efflux in cultures at various stages of potassium depletion. From these results they suggested that the killing process showed two distinct stages: (i) energy independent killer toxin binding to cells and (ii) a delayed energy dependent onset of membrane alteration, the energy being derived from either glycolytic or mitochondrial reactions, an analogy being drawn with the colicin K *Escherichia coli* interaction.

The killer action of PEST, which also involved a single-hit mechanism, like that of killer factor involved alteration of the plasma membrane permeability. PEST induced leakage of cellular potassium, but only a partial dissipation of the ATP pool coincident with a coordinate shutdown of macromolecular synthesis (BUSSEY and SKIPPER, 1975). Extracellular accumulation of AMP and not ATP suggested that PEST depleted the ATP pool by a mechanism different from that induced by killer factor. Furthermore none of the energy poisons tested delayed PEST-induced membrane damage in either glucose- or ethanol-grown cultures, thus contrasting and differentiating its mode of action from that of killer factor (SKIPPER and BUSSEY, 1977).

CHAPTER 2

SURVEY, AND CLASSIFICATION OF HANSENULA KILLER YEASTS, AND PRELIMINARY CHARACTERISATION OF THEIR KILLER FACTORS

INTRODUCTION

During a survey designed to detect antagonistic interaction between yeasts (obtained from Dr. N. Atkinson's laboratory stocks) on standard culture media by a standard agar diffusion method, Hansenula saturnus NA 9 was found to strongly inhibit the growth of various yeasts belonging to several genera. H. anomala NA 10, in particular was highly sensitive. A filtrate of H. saturnus grown in a chemically defined medium (LEDERBERG, 1956) reduced the viability of H. anomala at pH6.0 by more than one hundred-fold. The killer activity was heat stable, pronase sensitive and diffusable through dialysis membrane, suggestive of a novel yeast killer factor (contained in a report submitted in partial fulfilment of the Honours degree at The University of Adelaide, 1971, and reported at The Annual General Meeting of The Australian Society for Microbiology, Adelaide University, 1974).

The aims of the studies reported in this chapter were the partial characterisation of the killer activity of H. saturnus and examination of other strains and species of the genus Hansenula for similar and other types of killer activity. The interaction between representative members of the genus was determined both at a pH near to neutrality (pH6.0) for H. saturnus killer toxin (SKT) like activity and at low pH (initially pH4.7, then pH4.3) for Saccharomyces cerevisiae killer factor-like activities. The killer yeasts were grouped on the basis of

killing and resistance reactions and by the resistance patterns of non-killer strains. Conditions were examined for the production of killer activity in liquid culture media. Killer activity was tested directly by monitoring the viability of a sensitive strain. The killer activities obtained in culture filtrates were characterised by examining inactivation by temperature, pH and proteolytic enzymes, effect of pH on biological activity and electrophoretic behaviour. Diffusibility through membranes was tested on agar culture plates.

MATERIALS AND METHODS

Media

The ingredients are expressed as % (w/v). ME consisted of 3% malt extract (Difco) - 0.5% peptone (Oxoid L39) and MEA in addition contained 1.5% agar (Difco). PDA was Oxoid CM139. MYPGA was composed of 0.3% malt extract - 0.3% yeast extract (Oxoid L21) - 0.5% peptone (Difco) - 1% D-glucose - 2% agar (HAYNES, WICKERHAM and HESSELTINE, 1955). YEPD contained 1% yeast extract - 2% peptone - 2% D-glucose, and YEPDA in addition contained 2% agar (HAWTHORNE and MORTIMER, 1969) and soft-YEPDA 1% agar. Media were made up in distilled water and sterilised by autoclaving at 15 lbf/in^2 (121°C) for 15 min. When a buffered medium was required, 10 ml of 10-fold stock buffer (see below) was added to 90 ml of medium immediately prior to use. Buffered soft-YEPD was conveniently made by mixing five parts molten YEPDA, four parts warmed YEPD and one part stock buffer. Agar medium containing methylene blue was prepared by adding 1% (v/v) of a 0.3% (w/v) methylene blue filter sterilised stock solution at the time of use. Standard 90-mm diamater plastic Petri dishes were used throughout for plate culture and experiments unless otherwise indicated.

Stock buffers

All buffers were prepared as 10-fold concentrated stock solutions so as to produce the required pH when appropriately diluted 10-fold with YEPD, which had a pH of 5.9. Stock 0.081M-sodium potassium phosphate, pH6.0, was the 10-fold concentrated formulation of MUNRO (1970) and stock 0.1M-citrate -phosphate, pH4.7, (final citrate concentration of 1M) was prepared with K_2HPO_4 as described by FINK and STYLES (1972). Stock 0.1M-tartrate -phosphate (final tartrate concentration of 1M) was made similarly but using Na_2HPO_4. Ten-fold concentrated stock buffer solutions of 0.1M-sodium citrate and 0.1M-sodium acetate both of pH4.7 were also prepared by adjusting the pH of the respective acid (final concentration of 1M) but with 40% NaOH. Stock 10-fold concentrated 0.1M-tartrate buffers were made using 40% NaOH so that a 10^{-1} solution in YEPD produced pH3.5, 3.9, 4.3, 4.7 and 5.1. Stock 0.1M-phosphate buffers to produce pH5.5, 6.0, 6.5 and 7.0 were made similarly using KH_2PO_4 (1M final concentration) adjusted with NaOH. The approximate amounts of NaOH required were calculated from the composition tables of BOWER and BATES (1955) and PERRIN and DEMPSEY (1974). It was necessary to prepare some of the buffers in a water bath of about 60°C to prevent crystallisation.

All stocks were sterilised by boiling for 15 min except for the acetate buffer which was prepared from sterile distilled water. Buffer sterility was confirmed by incubating an aliquot in YEPD for three to four days.

Yeast culture maintenance and growth conditions

The yeasts studied are listed in Table 2.2 of the Results. The

non-designated strains referred to in the text were from the collections of Dr. Nancy Atkinson, University of Adelaide, and The Australian Wine Research Institute, South Australia. Yeasts were maintained by subculture at 4 monthly intervals on both YEPDA and MYPGA slopes stored at 4-6°C. Work cultures were generally grown on agar plates incubated at 25°C for 36-48 h. In liquid culture, 10 ml of liquid medium contained in a 100 ml conical flask was lightly inoculated with growth from a plate culture and then incubated at 25°C on a thermostatically controlled reciprocal-action shaker (Paton Industries, South Australia) set at 150 oscillations/min.

Agar-diffusion inhibition test

Initially 10-15 ml test plates of either MEA or PDA were used, but were subsequently replaced by 10 ml of MYPGA (half strength agar concentration) which produced greater test sensitivity. The plates were seeded by spreading 0.1 ml of a 24h ME shake culture and dried for an hour at room temperature. Each plate was then surface inoculated with up to 10 test cultures by a loopful of either 36h ME shake cultures or 48h MEA plate cultures. After incubation at 22-23°C for 48-72h, the plates were examined for zones of inhibition or absence of test culture growth. The width of inhibition zone, edge of the test colony to the edge of the zone, was measured using a vernier calipers. In the case of a poorly defined inhibition zone edge (hazy edge), measurement was made to the point of confluent growth.

Membrane-diffusion agar-plate test

Agar plates (10 ml) of either PDA or MYPGA, or YEPDA buffered at pH4.3 with 0.1M-tartrate or at pH6.0 with 0.081M-phosphate buffer were used.

Cellophane (sold locally as preserve covers) or dialysis membrane (Visking 3.0 in. flat width, cut to use as a single sheet) treated by autoclaving at 15 lbf/in^2 for 7.5 min in distilled water, was rinsed to remove preservative, drained and placed on agar plates. The membrane-plates were dried at room temperature to remove excess moisture. Pre-sterilised 0.22 μm Millipore or Gelman membranes (47 mm diameter) were used on control plates. The membrane-plates were inoculated with up to four test cultures by pipetting about 50 1 of 24h ME or YEPD shake culture. The size of the culture on each plate was standardised as far as practical to facilitate comparison between different membrane-plates. Following incubation at 22-23°C for 48-60h, the colony outlines were marked and the membranes removed to allow pouring of an overlay consisting of half strength-agar medium (5 ml) seeded with 10^6 cells/ml of a 24h shake culture of a sensitive strain. The plates were then examined for zones of inhibition and killing (see below) after incubation at 22-23°C for 48h.

Detection of the killer yeast trait

The method was essentially that of WOODS and BEVAN (1968) using buffered methylene blue agar plates. Vigorously growing cultures (derived from single colonies) were obtained on YEPDA, 36h, (test culture) and by shake culture in YEPD (seeded culture) at 25°C for 5-6h. Total cell counts of each strain to be seeded was made on appropriately diluted YEPD culture using a haemocytometer chamber. Buffered methylene blue containing-soft-YEPDA cooled to 48°C was then inoculated with 0.5-1.0 x 10^6 cells/ml, 12.5 ml of medium being poured per petri dish. The seeded plates were then surface inoculated with test cultures using a bacteriological loop, up to 10 cultures being accommodated per plate. Following incubation at 22-23°C for about 48h,

the plates were examined for zones of killing or inhibition in the seeded agar which were measured using venier calipers. A killer yeast was surrounded by either a clear zone of inhibition fringed with blue stained cells or a blue stained hazy zone.

Preparation of killer yeast culture filtrates

Yeasts were grown in buffered YEPD by either static or shake mode of incubation at 25°C. In static culture, 10 ml of medium contained in a 100 ml flat-sided bottle plugged with cotton wool was inoculated with 10^6 cells/ml and incubated in a horizontal position. For shake culture, 10-15 ml buffered YEPD contained in a 100 ml conical flask was incubated on a reciprocal-action shaker set at 150 strokes/min. A similar ratio of medium to flask capacity was used for larger volumes of culture. After 24-42h shake or 72h static incubation, the cultures were cooled to 4-6°C, clarified by centrifugation at 4000xg for 10 min and sterilised by pressure filtration using 0.22 µm Gelman membranes. The sterile filtrates were stored at 4-6°C. For convenience in many experiments, stock culture-supernatants were adjusted to their initial value of pH (usually pH4.3 or 6.0) with 3-5M-HCl or -NaOH prior to sterile filtration.

An index of culture growth (shake culture only) was determined by measuring the absorbance at 600 nm (10 mm light path) of a 30^{-1} solution in distilled water read against YEPD similarly diluted, using a Hitachi Perkin-Elmer UV-VIS spectrophotometer.

pH measurement and titration

All pH measurements were made at 22-23°C. pH was measured with a

combination type glass electrode (N-68, Schott, Jena) and digital pH-meter (Townson, Australia) two point calibrated with standard phthalate, pH4.00, and standard phosphate,pH6.86, buffers (Beckman). The electrode, after each measurement, was thoroughly rinsed with distilled water followed by buffer to avoid carry-over of toxin activity. Because of the sensitivity to pH of many of the killer yeast culture filtrate activities, extreme care was required in the manipulation of pH. The low activity of several yeast toxin preparations did not allow routine pH adjustment by dilution with buffer to the required pH, so pH was normally adjusted by titration with 1-2M-HC1 or 1-2M-NaOH. Parallel titrations made using 0.5M-tartaric acid or 0.5M-disodium tartrate did not indicate toxin inactivation in the above titration system resulting from possible momentary extremes of pH. In the event of the pH being exceeded in the direction of titration, the sample was discarded. When sample volume was inadequate for titration, 0.5-1.0 ml contained in an autoanalyser cup (Beckman, AAC-2) was adjusted with 3-5M-HCl or NaOH in microlitre amounts using a fine tipped micropipette. This combination of cup and pH electrode allowed reliable pH measurement to be made on a small volume of sample. The strength of titrant was chosen so that the estimated change in volume did not exceed 2%, care being taken to avoid localised high concentrations of acid or base. Even with due care, the latter technique remained open to criticism regarding possible localised areas of extreme values of pH and was therefore used only when titration with less-concentrated titrants was not practical.

Well test - assay

The method used was based on the well test described by WOODS and BEVAN (1968). Buffered soft-YEPDA (1% agar) was seeded to 0.5-1 x 10^6

cells/ml with indicator strain (36h YEPD shake culture kept at 4-6°C for use up to five days)and 12.5 ml poured per 9 cm petri-dish. To six 7 mm wells arranged equidistant from the centre of the plate and also from each other was added 70μl of sample in duplicate. An internal standard was included on each plate to correct for plate variation similarly as described by GROVE and RANDALL (1955, pp. 116-118). Following incubation at 4-6°C for 0-6h, depending on the degree of sensitivity required, the plates were incubated at 22-23°C for 24-48h depending on the indicator strain. Zones of inhibition were measured to 0.1 mm using vernier calipers fitted with needle tipped jaws. In assay, the calibration curve was drawn on semi-log paper by plotting either average inhibition zone diameter or inhibition zone width squared against relative killer concentration (log scale). The plot producing greatest linearity was used to determine the assay. In the case of a non-linear trend, the points were joined with straight lines.

Assay was normally conducted at pH4.3 or 6.0, the media and diluents being buffered with 0.1M-tartrate or 0.081M-phosphate respectively. Calibration curves for each culture filtrate activity assayed were determined at each assay. The calibration sample was usually the control culture filtrate, and following titration to the pH of assay when necessary, was, unless specified otherwise, serially two-fold diluted in appropriate buffer of the same pH. When phosphate buffered samples were assayed at pH4.3, the diluent was 0.081M-phosphate buffer adjusted to pH4.3 with 5M-HCl. Tartrate buffered samples for assay at pH6.0 were treated in an analogous manner. When only small volumes (0.5-1.0 ml) were available, aliquots were contained and diluted in autoanalyser cups, otherwise glassware of appropriate size was used.

Large plate assay

Large rectangular plates of dimensions 12 inches x 12 inches (internal dimensions 28 x 28 x 1 cm) constructed from glass and fitted with aluminium covers were used (LEES and TOOTILL, 1955a). 200 ml of buffered soft-YEPDA (1% agar), cooled to 48°C for seeding (0.5 - 1.0 x 10^6 cells/ml), was poured per plate previously levelled on a tripod platform. Depending on the number of samples for assay, an array of 8 x 8 to 10 x 10 of 7 mm wells were cut in each plate and depending on accuracy required, 70 µl of sample in duplicate to quadruplicate were plated out using the completely random arrangement design described by (LEES and TOOTILL, 1955b). When assay of several different culture filtrate activities were performed on the same plate, samples relating to each activity type were grouped and treated as self-contained assays. The plates were incubated as described for the well test and the inhibition zones measured with the aid of a specially constructed indirectly illuminated viewing cabinet. Assay was determined as described for the well test.

Modification to assay medium

The growth of indicator strain 10, in particular, on soft-YEPDA was very vigorous and rapidly obliterated the inhibition zones by a lush spreading surface growth. This problem was reduced considerably by employing a half-strength YEPDA medium (standard YEPDA diluted with an equal volume of distilled water and appropriately buffered).

Experimental culture filtrate activites

In the following tests, all culture filtrates were, unless otherwise

specified filtrates of stock supernatants obtained from 36h shake cultures of the respective yeast in YEPD buffered at either pH4.3 with 0.1M-tartrate or at pH6.0 with 0.081M-phosphate buffer.

Temperature inactivation

Inactivation by temperature of killer activity in culture filtrate at pH4.3 was assessed by subsequent assay at pH4.3. Duplicate 0.5 ml aliquots of culture filtrate were placed in 5 ml screw cap bottles and heated by immersion in a water bath set at the appropriate test temperature (37-80°C). At various time intervals a bottle was withdrawn, chilled in iced water, the contents mixed, and held at 4-6°C for assay. Control aliquots were kept at 4-6°C for the duration of the experiment. The control was assayed over a series of concentrations, x1, x0.75, x0.5 and x0.25, made by dilution with 0.1-M sodium tartrate, pH4.3, and in the case of phosphate buffered culture filtrates with 0.081M-sodium phosphate buffer adjusted to pH4.3 with 3M-HCl. The heated samples were assayed undiluted. Each sample was assayed in at least duplicate on a large plate of soft-YEPDA buffered at pH4.3.

pH Stability

Culture filtrates were titrated to different values of pH in the range pH3.0-6.0 and after a period of incubation were brought to pH4.3 for assay to determine residual activity. Five ml quantities of culture filtrate were titrated to pH3.0, 4.3, 5.0 and 6.0 with 2M-HCl or -NaOH, pH being monitored with a combination type electrode. In some experiments, smaller volumes contained in autoanalyser cups were pH-adjusted as described above. After a period of 1h at 22-23°C the samples were titrated to pH4.3 for assay at that pH on a large plate.

Activity of the treated samples was compared to the parent culture filtrate (of either pH4.3 or 6.0) held at 4-6°C for the duration of the experiment. Just prior to assay, these controls were, where necessary, titrated to pH4.3 and serially diluted in appropriate buffer.

pH effect on activity

Culture filtrate adjusted to different values of pH were tested for activity at those pHs by the well test. One ml aliquots of culture filtrate were warmed to room temperature for pH adjustment as described above using 2M-NaOH or -HCl and then kept at 4-6°C for assay. Quantities of soft-YEPDA, buffered at pHs3.5, 3.9, 4.3, 4.7 and 5.1 with 0.1M-tartrate and at pH5.5, 6.0, 6.5 and 7.0 with 0.1M-phosphate, were seeded to 0.5-1.0 x 10^6 cells/ml with either indicator strain 10, 560 or 498. Each sample was tested either in duplicate on 12.5 ml petri-plates containing 6 wells or in quadruplicate on 200 ml large plates containing 100 wells. The plates were kept at 4-6°C for 3h before incubation at 22-23°C for 48h. The zones of inhibition were measured to 0.1 mm.

Killing activity

Killer yeast culture filtrate activity was examined by monitoring viability of growing cells in liquid culture medium based on the method of WOODS, ROSS and HENDRY (1974). The incubation medium was 3-fold concentrated YEPD buffered at either pH4.3 with 0.1M-tartrate or at pH6.0 with 0.081M-phosphate (sterilized by membrane filtration) containing log-phase cells of strain 10 or 560 at 3 x 10^6 cells/ml or of strain 498 at 9 x 10^5 cells/ml added immediately before the test. The cells were pregrown by shake culture in YEPD for at least 3h at

22-23°C, collected by centrifugation and taken up in the test medium concentrate. The approximate required cell number, determined by haemocytometer count, was adjusted by appropriate dilution with additional concentrate. The experiment was commenced by adding 6.0 ml of sterile killer or control yeast culture filtrate, previously adjusted to appropriate pH, to 3.0 ml of test medium concentrate which was then incubated at 22-23°C. Culture viability was followed by making suitable dilutions of an aliquot in buffered YEPD and plating out 0.1 ml in triplicate on buffered YEPDA. Buffered YEPD controls (culture filtrate replaced by buffered YEPD) were also included to follow culture growth. The control yeast (indicator strains) culture filtrates were, as for those from the killer yeasts (except where indicated), from 36h shake cultures made at 25°C.

Inactivation by proteolytic enzymes

Protease inactivation of killer yeast culture filtrate activity was studied by incubation with several proteolytic enzymes and assaying for residual activity. Solutions of papain (British Drug Houses), pepsin (Merck, crystallised), pronase (Calbiochem, B grade) and trypsin (Calbiochem, A grade) were prepared by dissolving 1 mg/ml enzyme in deionised distilled water. Papain was activated with 25mM-cysteine and 5mM-versene. Inactivated enzyme was prepared by autoclaving the aqueous enzyme solution at 121°C for 7.5 min before the addition of activating agents where necessary. The enzyme solutions were effectively sterilised by centrifugation at 20000xg for 30 min to remove any particulate material.

Reaction mixtures were prepared by adding an equal volume of culture filtrate of either pH4.7 (pH4.3 in preliminary experiments) or 6.0 to

native or inactivated enzyme solution. Control killer solutions (no added enzyme) were made up with an equal volume of distilled water or solution containing the activating agents where appropriate. Mixtures were incubated at 22-23°C for 24h. The culture filtrate-enzyme mixtures, without further treatment, were then assayed against the respective control culture filtrate mixture, pH4.7 or 6.0 (diluted x1.0, x0.75, x0.5 and x0.25 in appropriate buffer) on large plates of soft-YEPDA buffered at pH4.3 with 0.1M-tartrate for maximum assay sensitivity.

Cellulose acetate membrane electrophoresis

The migration of killer yeast culture filtrate activity by electrophoresis on cellulose acetate membrane (CAM) strips was visualised by bio-autography (BETINA, 1973). The CAM electrophoretic procedure was based on the method of KOHN (1968) for the separation of serum proteins. The conditions described were determined by experimentation to provide optimum resolution within the constraint of adequate sensitivity using neat culture filtrate activity without pretreatment. Initially Gelman Sepraphore III CAM strips of Lot No 80716 were used, but in later experiments the strips were from Lot No 81376. CAM strips (2.5 cm x 15.2 cm) were prepared by soaking in 25mM-sodium tartrate, pH4.3 (measured at room temperature) and pre-electrophoresed in a Shandon Universal Electrophoresis Apparatus at 4-6°C until the current steadied at 1mA/cm-width at about 250 volts. 2.5-15 µl of culture filtrate activity, adjusted to pH4.3, was then applied per 2.5 cm-width of strip using a micropipette and electrophoresed for 5-6h. In some experiments the volume of sample applied was progressively decreased across the CAM strip to obtain optimal resolution. In bio-autographic detection, the strips were

divided in half longitudinally and placed on thin films (approximately 75 ml agar medium per large assay plate) of soft-YEPDA buffered at pH4.3 seeded to 10^5 cells/ml with indicator yeast. After incubation at 22-23°C for at least 5h, the strips were removed and incubation continued for a further 24-36h. The CAM strip sample origin was marked on the agar layer with a pointed instrument. The inhibition zones were either recorded photographically or the assay plate inverted to obtain a graphical record by tracing.

RESULTS

SURVEY

Initial Survey

Following the detection of a growth inhibitory factor produced by *H. saturnus* NA 9, obtained from Dr. Nancy Atkinson's collection, 36 yeasts from the Australian Wine Research Institute Collection were also tested for inhibitory activity by the agar-diffusion inhibition test and the cellophane-diffusion agar-plate test on MYPGA using two highly sensitive yeasts as indicators, *H. anomala* NA 10 and a *Saccharomycodes* yeast NA 14. Of the 51 yeasts tested in total, only the strains of *H. saturnus*, additionally AWRI 354, produced growth inhibitory activity under the conditions of the test. Strain 354 produced comparatively larger inhibition zones than strain 9, but their activity spectra were qualitatively similar. The spectrum of activity of strain 354, summarised in Table 2.1, shows that it was highly active against 19 of 42 yeasts from five genera of Ascomycetous yeasts.

Table 2.1. Inhibitory activity spectrum of H. saturnus AWRI 354[1]

Genus	No. of species	No. of strains	No. of sensitive strains	No. of intermediate strains
Debaryomyces	1	1	0	0
Hanseniaspora	1	1	0	0
Hansenula	2	3	1	0
Kluyveromyces	2	2	1	1
Pichia	2	2	1	0
Saccharomyces	11	27	12	10
Saccharomycodes	2	4	4	0
Schizosaccharomyces	2	2	0	0
Totals	23	42	19	11

[1] Cellophane diffusion agar plate test using MYPG agar, pH approximately 6.0: inhibition zone width >2.0 mm for sensitive strains, and hazy zone or clear zone width of ≤2.0 mm for strains of intermediate sensitivity.

All of the strains of Sacch. cerevisiae tested were to various degrees sensitive as were 10 of 16 strains of other Saccharomyces yeasts. The single species of Sporobolomyces and all asporogenous yeasts tested, strains of Brettanomyces, Candida, Cryptococcus, Kloeckera, Rhodotorula and Torulopsis (total of nine species and strains) were resistant. Insufficient numbers of asporogenous yeasts have been tested to comment on their apparent resistance as a group. It is thought likely that the two strains of H. saturnus have a common origin.

Incorporation of methylene blue (0.003%, WOODS and BEVAN, 1968) into the test agar, deeply stained cells contained in a broad fringe surrounding the inhibition zone indicating killer action, although some viable cells could be cultured from this region (48h plates). A culture filtrate of H. saturnus incubated in a chemically defined medium (described in Chapter 3) proved the killer action, resulting in less than 1% survival of H. anomala after 2h contact (see Table 2.13).

Survey of Hansenula yeasts for killer activity

It was of interest to discover whether other strains of *H. saturnus* possessed killer activity and indeed whether other species of *Hansenula* produced a similar type of killer activity. The strains tested, including those of *Hansenula* used previously are listed in Table 2.2. Killer activities were initially examined on 5 ml methylene blue agar plates (thin-layer plates for high sensitivity) buffered at pH4.7 with 0.1M-citrate -phosphate for killer factor-like activity and at pH6.0 with 0.081M-phosphate buffer for *H. saturnus* -like activity. However, the conditions of this test were revised for two reasons. Firstly, cultivation of many of the *Hansenula* yeasts in citrate-phosphate but not phosphate buffered liquid medium brought about a large change in culture pH (see Table 2.5) and secondly the unexpectedly high incidence of killer activity observed instilled concern over possible false positive results deriving from nutrient deprivation by the more advanced test strain. Of several buffers tested (see Table 2.6), tartrate proved suitable showing adequate buffer capacity and non-toxicity to yeast growth. To eliminate the possibility of false positive results, a basal layer of 10 ml of buffered methylene blue agar was poured prior to the 5 ml seeded layer. As anticipated, a reduction in zone width resulted, but only in several cases were false zones detected and all of those were small hazy non-methylene blue staining zones. Generally there was a wide variation in the intensity of staining and it was occassionally difficult to decide whether the response was killing or inhibition. Subculture from this region was of little assistance since some viable cells were invariably isolated despite cloning of each culture prior to testing (see section F of Discussion).

Table 2.2. Strains of Hansenula used in this study.

Species	Strain designation[1]
H. anomala	NA 10
anomala var. anomala	NCYC 18
	CBS 605
(Candida pelliculosa)	
anomala var. schneggii	CBS 113
	CBS 606
(Mycoderma cerevisiae var. pulverulentum)	
beckii	NCYC 494
beijerinckii	CBS 2564
belgica	NCYC 54
(Pichia membranaefaciens)	
californica	NCYC 496
canadensis	NCYC 497
capsulata	NCYC 498
ciferrii	CBS 111
dimennae	CBS 5762
fabianii	CBS 5640
holstii	NCYC 560
minuta	NCYC 499
mrakii	NCYC 500
polymorpha	NCYC 495
saturnus	NA 9
	NCYC 22
	NCYC 23
	NCYC 57
	AWRI 354
	CBS 5761
silvicola	NCYC 413
subpelliculosa	NCYC 16
wickerhamii	CBS 4307
wingei	CBS 2431

[1] AWRI Australian Wine Research Institute, Adelaide, Australia
CBS Centraalbureau voor Schimmelcultures, Baarn, Holland
NA Dr. N. Atkinson's Collection, University of Adelaide, Australia
NCYC National Collection of Yeast Cultures, Redhill, Surrey, England

The result of interaction (killer and inhibitory) between all 28 Hansenula yeasts (phenotype test) listed in Table 2.2 above is summarised in Table 2.3. The tests recorded were made on 12.5 ml methylene blue agar plates buffered at pH6.0 and at pH4.3 with tartrate buffer, the latter pH being optimal for the majority of killer yeasts detected (see Figure 2.3 and 2.5). Yeasts showing a similar pattern of either killer or sensitivity have been grouped unless the difference(s)

Table 2.3 Killing and inhibitory interaction between strains of Hansenula at both pH 4.3 and 6.0.

Tested strains	pH	Indicator strains																			
		57	9, 354 22, 23 5761	500	496 5762	16	111	10 18	113 606	605	2431 497	2564	560	413	494	495	498	499	54	4307	5640 [a]
9	4.3	-	-	-	±Z	+Z	-	+E	+E	±Z	+E	-	+E	+Z	+E	+E	+E	+	+E	Tr	+
	6.0	-	-	-	Tr	+Z	-	+E	+E	+Z	+E	-	+E	+Z	+E	-	+Z	±Z	+E	-	Tr
354, 22, 23, 5761	4.3	-	-	-	+E	+E	±Z	+E	+E	+Z	+E	-	+E	+E	+E	+E	+E	+	+E	+Z	+
	6.0	-	-	-	±Z	+E	-	+E	+E	+Z	+E	-	+E	+E	+E	-	+Z	±Z	+E	±Z	+Z
500	4.3	-	-	-	-	+	±Z	+	+	±Z	+	+Z	+	+	+	±	+	+	-	+	+
	6.0	-	-	-	±Z	±E	-	±	±	-	+	-	+	±Z	+	-	±Z	±	-	-	-
496, 5762	4.3	-	-	-	-	±E	-	+	+	Tr	+	-	+	Tr	+	-	+	+	-	-	-
	6.0	Tr	-	+*	-	±E	-	+	+	-	+	-	+	Tr	+	-	±Z	+	+	-	-
16	4.3	+Z*	-	-	+E	-	-	±*	-	-	+	-	+	+	+	+E	+	+	-	+Z	+
	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	±E	-	-	-	-	-	-
111	4.3	-	-	-	-	Tr	-	+E	+Z	-	+E	-	+Z	Tr	+E*	-	+Z	+E	-	-	-
	6.0	-	-	-	-	-	-	+Z*	Tr	-	-	-	-	-	-	-	-	-	-	-	-
10, 18, 113	4.3	-	-	-	-	-	-	-	-	-	±E*	-	+	+*	+*	Tr	Tr	Tr	-	-	-
	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2431, 497	4.3	-	-	-	-	-	-	+Z*	-	-	-	-	+Z	-	±Z	-	+Z	+Z*	-	-	-
	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Tr	-	-	-
560	4.3	-	-	-	-	-	-	Tr	-	-	-	-	-	-	-	-	±*	±*	-	-	-
	6.0	-	-	-	-	-	-	Tr	-	-	-	-	-	-	±	-	±*	±*	-	-	-
2564	4.3	Tr	Tr	+Z	±Z	Tr	-	+Z*	+Z*	-	Tr	-	±Z	-	-	-	±Z	+Z	-	-	-
	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57 [b]	4.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ Inhibition zone width > 1.0 mm; ± ≤ 1.0 mm; Tr Trace inhibitory activity; E Hazy zone-edge; Z Hazy zone; *inhibiting activity; [a] Not tested on methylene blue plates; [b] Also yeasts, 606, 605, 413, 494, 495, 498, 499, 54, 4307 and 5640.

was qualitative. Sixteen of the 28 yeasts exhibited killer activity against one or more of the 28 yeasts but no yeast showed either killer or inhibitory activity against itself. Strains 560 and 5640 showed only traces of killer or inhibitory activity and were not considered further. All of the 16 killer yeasts were active at pH4.3, eight of these also being active at pH6.0. Several more showed traces of activity at pH6.0. The six strains of H. saturnus fell into three groups, stain 57 a resistant non-killer, strain 9 a weak killer and the remainder strong killers. Yeasts 496 and 5762, and 10, 18 and 113 produced similar activity patterns. All of the yeasts were sensitive to several or more of the killer yeasts except for strains of H. saturnus and strains 500, 111, and 2564.

Basically three types of zones were apparent (see Figure 2.1A).

A. Strains 500, 496, 5762, 16, 10, 18 and 113 produced clear zones with sharply defined edges. Methylene blue staining was usually confined to a narrow band of cells surrounding the killer zone, but the intensity of staining varied widely between sensitive strains.

B. The killer zones produced by the strains of H. saturnus and strain 111 were also clear, but showed a broad hazy edge which usually stained blue over the entire hazy region.

C. Strains 2431 and 497 produced large hazy zones of increasing haziness towards their poorly defined edges. Strain 2564 only produced solid hazy zones which usually were stained intensely.

Interaction between killer yeasts

On the basis of killer activity interaction among the 16 strains of Table 2.3 (strain 560 not considered), seven groups of killer yeasts were apparent. Considering also the resistance patterns of the non-killer strains, killer strains 10, 18 and 113, and 2431 and 497

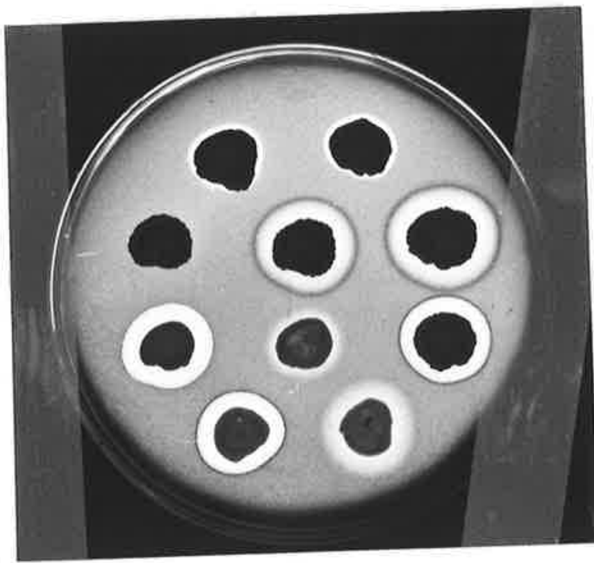
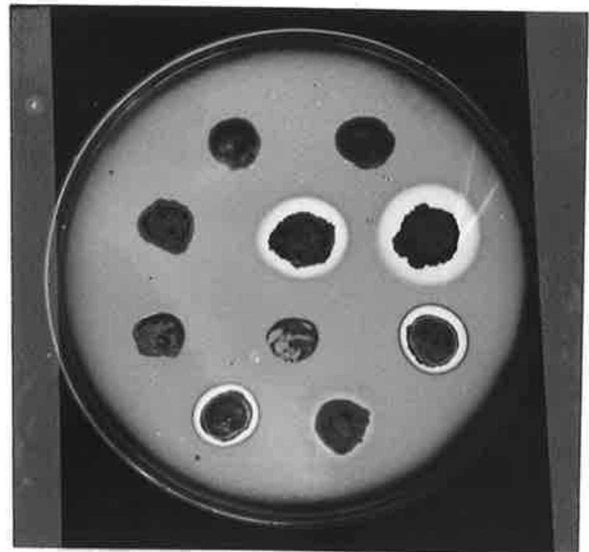
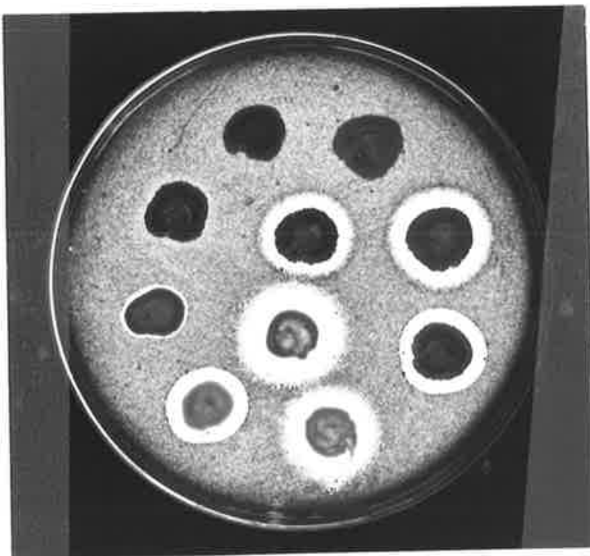
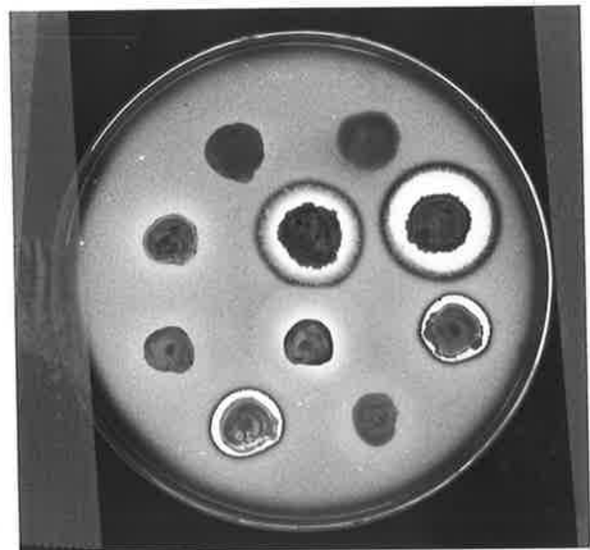





Figure 2.1A. Test plates showing the activity of <u>Hansenula</u> killer yeasts against a background of sensitive cells.
Agar plates buffered at pH4.3 (left column) and pH6.0 (right column), and seeded with indicator strains 560 (upper row) and 10 (lower row) were inoculated with killer strains and incubated at 22-23°C for 2 days. The killer strains are in analogous position on each plate and are, beginning with the upper row, from left to right, 10, 113, 57 (non-killer) 9, 354, 16, 111, 500, 5762 and 2431.

showed different killer specificities, and hence 8 killer types were recognised. They are denoted HK_1 to HK_8 as shown in Table 2.4. (Note that inhibitory cross-reactions, weak or no staining, are included in the table, but were not considered in the grouping of the killer strains). Type HK_1 consisted of the five killer strains of *H. saturnus,* NA 9, AWRI 354, NCYC 22 and 23, and CBS 5761, but was subdivided because strain 9 showed a different interaction pattern to the remaining four strains. Type HK_2, exhibited by *H. mrakii* NCYC 500, was the most extensive killer type, to which only HK_1 strains were resistant. The two species, *H. californica* NCYC 496 and *H. dimennae* CBS 5762, showed HK_3 killer activity, and was the only group of yeasts to be active against strain 500. The single strains *H. subpelliculosa* NCYC 16 and *H. ciferrii* CBS 111 exhibited types HK_4 and HK_5 respectively. Type HK_6 was displayed by three strains of *H. anomala* (NA 10, NCYC 18 and NCYC 113) and HK_7 by the two species *H. wingei* CBS 2431 and *H. canadensis* NCYC 497. Neither HK_6 or HK_7 strains showed definite killing activity (by this test) against the other killer yeasts but had dissimilar inhibitory activity patterns. The solid hazy zone killer type HK_8 was exhibited only by *H. beijerinckii* CBS 2564.

Different strains or species belonging to a particular killer group also exhibited analogous resistance patterns. Since the resistance patterns of strains between killer groups were different, there were 8 resistance types, denoted HR_1 to HR_8 (corresponding with each killer type). Thus, in all there are eight classes of killer yeasts among the strains examined. Strains of HR_1 were resistant to all killer types, whereas the strains belonging to HR_7 were only definitely resistant to two killer types. Strain 113 of HR_6 was much less sensitive than the remaining strains of this resistance type, but the resistance pattern was not considered incompatible with type HR_6 (see Table 2.3).

Table 2.4. Interaction between killer yeasts of the genus *Hansenula*

Toxin producing strains			Indicator strains							
			9, 354 22, 23 5761 HR_1	500 HR_2	496 5762 HR_3	16 HR_4	111 HR_5	10 18 113 HR_6	2431 497 HR_7	2564 HR_8
H. saturnus	9	HK_{1a}	–	–	Z	Z	–	+	+	–
H. saturnus	354,22, 23, 5761	HK_{1b}	–	–	+	+	Z	+	+	–
H. mrakii	500	HK_2	–	–	Z	+	Z	+	+	Z
H. californica H. dimennae	496 5762	HK_3	–	+*	–	+	–	+	+	–
H. subpelliculosa	16	HK_4	–	–	+	–	–	+*	+	–
H. ciferrii	111	HK_5	–	–	–	–	–	+	+	–
H. anomala	10,18, 113	HK_6	–	–	–	–	–	–	+*	–
H. wingei H. canadensis	2431 497	HK_7	–	–	–	–	–	Z*	–	–
H. beijerinckii	2564	HK_8	–	Z	Z	–	–	Z*	–	–

+, Clear zone killing; Z, Hazy zone killing activity; *, Growth inhibition: non-killing;
–, No killing or inhibitory activity; HK, Killer group; HR, Resistance group.

KILLER ACTIVITY PRODUCTION CONDITIONS

Production of killer activity in liquid culture

Initially the production of activity in liquid culture was examined by growing killer yeasts in buffered YEPD to stationary phase by shake (aerobic) culture. The medium was buffered at pH6.0 with 0.081M-phosphate and at pH4.7 with 0.1M-citrate- phosphate buffer. Measurement of the culture supernatant pH from several of the killer yeast cultures buffered at pH4.7 revealed values of pH exceeding 7.0, whereas the supernatant pH of cultures buffered at pH6.0 did not exceed pH6.5. It was found that all of the yeasts producing the excessive pH drift in the citrate-phosphate buffered medium can aerobically assimilate citrate (Table 2.5). This observation was supported by the even greater pH drift resulting when phosphate, which buffers strongly between pH6-7, was omitted from the citrate buffered medium (see Table 2.6). Furthermore culture growth (turbidity) was considerably greater in the citrate medium compared to the phosphate medium, indicating the metabolism of citrate as a carbon source.

The range of non-metabolisable and non-toxic substances possessing good buffering capability in the range pH4-5 is limited, and includes acetate, phthalate and tartrate salts. These were tested and some of the results are given in Table 2.6. Acetate and to a much lesser extent phthalate were toxic to citrate metabolising yeasts, but tartrate, particularly in conjunction with phosphate adequately stabilised culture pH. However, because the presence of phosphate appeared to reduce production of killer activity, possibly by the reduction of growth, tartrate alone was chosen as the buffer for low pH culture.

Table 2.5. Correlation between culture pH drift and citrate assimilation.[a]

Strain	0.1M-citrate/phos. pH4.7 Culture turbidity[b]	pH	0.081M-phos. pH6.0 Culture turbidity	pH	Aerobic assimilation of citrate[c]
Initial value	0.0	4.68	0.0	5.85	
22	0.22	4.62	0.195	6.10	?
354	0.30	4.62	0.35	6.05	?
500	0.33	4.60	0.35	5.82	-
496	0.36	4.90	0.38	5.90	?
16	0.55	8.10	0.40	6.35	+
10	0.44	8.15	0.39	6.40	+
497	0.40	7.45	0.35	6.20	+
560	0.46	6.95	0.31	6.30	+

[a]Yeasts were grown in YEPD, buffered as indicated, by shake culture at 25°C for 36h; [b]Culture turbidity is absorbance measured at 600 nm on the culture diluted 30^{-1} in water; [c]From BARNETT and PANKHURST (1974): +, positive; -, nil; ?, unknown.

Effect of culture pH on killer yeast activity production

The effect of pH on killer yeast growth and activity production was examined in YEPD buffered with 0.1M-tartrate buffer for culture at low pH and with 0.081M-phosphate buffer at pHs nearing neutrality. The filtrates derived from shake culture incubations at 25°C for 36h were tested for activity against indicator strains 10 and 560 at pH4.7 and 6.0 using the well test. The results are shown in Table 2.7.

Table 2.6. The effect of buffering species on yeast culture.

Strain	Culture parameter[a]	Buffer species					
		Cit-K_2HPO_4	Na Cit	Na Ac		Na Tart	Tart-PO_4
		0.1M[b]	0.1M	0.05M	0.1M	0.1M	0.1M[c]
	Initial pH	4.63	4.70	4.71	4.71	4.67	4.69
354	Culture turbidity	0.39	0.42	0.41	0.44	0.38	0.37
	Final pH	4.66	4.75	8.55	8.80	4.72	4.72
	Relative activity(%)	100	147	65	68	94	68
16	Culture turbidity	0.53	0.55	0.48	0.032	0.42	0.41
	Final pH	7.90	8.68	8.75	5.03	5.11	5.03
	Relative activity(%)	100	400	9	15	750	250
10	Culture turbidity	0.44	0.44	0.40	0.06	0.36	0.32
	Final pH	7.95	8.82	8.65	4.97	4.92	4.89
	Relative activity(%)	100	200	< 1	< 1	120	27
560	Culture turbidity	0.46	0.45	0.30	0.015	0.41	0.37
	Final pH	6.92	7.60	8.15	4.94	5.14	5.02

[a] See Table 2.5 for explanation of parameters: inhibitory activity assayed by well test at pH4.7 with indicator strain 560; [b] Final concentration of citrate; [c] Final concentration of tartrate.

HK_1 strains represented by strain 354, shows that pH had little effect on yeast growth or on killer activity production, whether assayed at pH4.7 or 6.0 by either indicator strain 10 or 560. A non-killer strain of H. saturnus , strain 57, produced no detectable activity in culture over the range pH4.35-7.0. Activity produced by the HK_2 strain was detectable only at low assay pH and increased in activity with decreasing culture pH while growth was not affected. The activity of strain 496, HK_3, with respect to culture pH depended on the assay strain and pH. Large hazy zones were obtained when phosphate buffered culture filtrates were assayed at pH6.0 whereas clear zones were obtained by assay at pH4.7. Culture filtrate activity of strains of the remaining groups, HK_4 to HK_8 was virtually undetectable in phosphate buffered culture filtrates, but activity increased with decreasing pH in tartrate buffered media. Activity was measured only at pH4.7 since these strains showed little or no activity by the plate phenotype test made at pH6.0 (Table 2.3). The activity of strain 16 was much greater against indicator 560 than 10 whereas the reverse was true for strain 111. Group HK_6 strain 113 produced barely detectable activity in contrast to strains 10 and 18 (data for the latter strain is not shown). Killer strains 2431, 497, and 2564 produced only weak activity (small hazy zones) in liquid culture.

These results indicated that killer yeasts produced greatest low pH-active substance at pHs near pH4.3 and near-neutral pH active substance at pHs near pH6.0. These conditions were employed in experiments to be described below. No further attempts were made to optimise killer yeast activity production with respect to culture pH.

Table 2.7. The effect of pH on killer yeast growth and activity production.

Killer group	Strain	Culture[a] analysis	Culture buffer and pH			
			0.1M-NaTart		$0.081M\text{-}NaKPO_4$	
			4.26	4.69	5.92	6.72
-	57	Growth	0.40	0.375	0.375	0.385
		Final pH	4.30	4.76	6.13	7.07
		Well test, 10:4,7,6.0	-	-	-	-
		560:4.7,6.0	-	-	-	-
HK_1	354	Growth	0.38	0.38	0.37	0.40
		Final pH	4.30	4.73	6.13	6.97
		Well test, 10:4.7	4.05	4.15	4.30	4.15
		10:6.0	5.35	5.85	5.85	4.75
		560:4.7	4.65	5.00	5.35	4.90
		560:6.0	4.10	4.10	4.60	3.60
HK_2	500	Growth	0.42	0.44	0.44	0.43
		Final pH	4.22	4.61	5.87	6.80
		Well test, 10:4.7	4.75	4.65	2.75	2.0
		10:6.0	-	-	-	-
		560:4.7	5.05	4.85	2.80	1.70
		560:6.0	-	-	-	-
HK_3	496	Growth	0.36	0.38	0.38	0.41
		Final pH	4.28	4.68	5.90	6.80
		Well test, 10:4.7	1.80	1.70	0.80	0.65
		10:6.0	-	-	3.6z	3.9z
		560:4.7	1.05	1.25	3.10	3.10
		560:6.0	NT	NT	NT	NT
HK_4	16	Growth	0.45	0.42	0.43	0.44
		Final pH	4.38	5.13	6.25	7.10
		Well test, 10:4.7	2.1	0.85	1.15	-
		560:4.7	5.25	4.80	3.55	-
HK_5	111	Growth	0.32	0.27	0.22	0.29
		Final pH	4.44	5.22	6.37	7.25
		10:4:7	6.60	6.05	2.65	
		560:4.7	3.5z	-	-	-
HK_6	10	Growth	0.41	0.39	0.38	0.37
		Final pH	4.37	4.91	6.33	7.30
		Well test,560:4.7	3.80	3.55	1.15	-
	113	Growth	0.37	0.38	0.35	0.30
		Final pH	4.32	4.81	6.40	7.18
		Well test,560:4.7	1.0z	0.5z	-	-

[a] Growth, absorbance at 600 nm of culture diluted 30^{-1} in distilled water, Well Test strain number and pH of test: inhibition zone in mm; z, Hazy zone; NT, Not tested.

Table 2.7. continued.

Killer group	Strain	Culture analysis	Culture buffer and pH 0.1M-NaTart 4.26	4.69	0.081M-$NaKPO_4$ 5.92	6.72
HK_7	2431	Growth	0.36	0.31	0.28	0.30
		Final pH	4.43	5.03	6.32	7.15
		Well test, 10:4.7	1.5z	1.5z	-	-
		560:4.7	1.5z	1.5z	-	-
	497	Growth	0.40	0.38	0.39	0.39
		Final pH	4.32	4.85	6.22	7.07
		Well test, 10:4.7	1.5z	1.5z	0.5z	-
		560:4.7	1.5z	1.5z	0.5z	-
HK_8	2564	Growth	0.43	0.43	0.45	0.47
		Final pH	4.27	4.69	5.99	6.89
		Well test, 10:4.7	1.5z	-	-	-
		560:4.7	-	-	-	-

Effect of shake and static modes of culture

The physical mode of culture, that is shake (aeration) or static, effects both growth rate and cell yield, and hence the titer of toxin. However, the yield of toxin, may be low in shake grown culture despite the stimulating effect of aeration on cell titer if it is sensitive to inactivation by surface action (WOODS and BEVAN, 1968). Comparison between shake and static mode of culture on killer activity was therefore examined.

Killer yeasts were cultured in YEPD buffered at pH4.3 or 6.0 with 0.1M-tartrate or 0.081M-phosphate respectively by shake or static mode of incubation at 25°C. Culture filtrate activity was tested at either pH4.3 or 6.0 by the well test using indicator strains 10 and 560 (see Table 2.8) and in addition the activity of culture filtrates of killer strains 9 and 354 was assayed by indicator yeasts 413, 495, 498, and 499 (see Figure 2.1B).

Table 2.8. The effect of mode of culture, shake or static on the production of activity by killer yeasts in buffered YEPD media.[a]

Killer group	Strain	Culture pH	Condition of assay : inhibition zone width (mm)							
			Assay at pH4.3				Assay at pH6.0			
			Strain 10		Strain 560		Strain 10		Strain 560	
			Shake	static	Shake	static	Shake	static	Shake	static
HK_{1a}	9	4.3	see Appendix One and Figure 2.1B.							
		6.0								
HK_{1b}	354	4.3								
		6.0								
	22	4.3	5.85	5.75	6.30	6.05	NT	NT	NT	NT
		6.0	5.25	5.05	6.10	5.75	5.70	6.00	5.50	5.85
	23	4.3	5.50	5.35	6.05	5.80	NT	NT	NT	NT
		6.0	5.85	4.95	6.65	5.45	6.40	5.80	6.00	5.65
	5761	4.3	5.90	5.65	6.45	5.90	NT	NT	NT	NT
		6.0	5.45	5.00	6.40	5.70	5.70	5.80	5.65	5.75
HK_2	500	4.3	5.90	5.70	6.60	6.40	1.25	–	2.25z	–
HK_3	496	4.3	3.70	3.15	4.25	3.30	–	–	–	–
		6.0	1.90	1.55	3.5z/1.5	3.5z/1.5	2.5z	2.5z	–	–
	5762	4.3	2.80	2.65	3.00	3.00	–	–	–	–
		6.0	1.60	1.75	3.5z/1.0	2.5	2.5z	1.5z	–	–
HK_4	16	4.3	1.50	0.25	5.80	6.05	–	–	3.0z	3.25z
HK_5	111	4.3	8.0z/5.5	7.0z/3.0	4.0z	–	–	–	–	–
HK_6	10	4.3	–	–	5.15	1.15	–	–	0.75	–
	18	4.3	–	–	3.90	0.25	–	–	–	–
	113	4.3	–	–	1.25	–	–	–	–	–
HK_7	2431	4.3	2.5z	2.5z	1.5z	1.5z	–	–	–	–
	497	4.3	1.5z	1.0z	–	–	–	–	–	–
HK_8	2564	4.3	–	–	–	–	–	–	–	–
–	57[b]	4.3	–	–	–	–	–	–	–	–
		6.0	–	–	–	–	–	–	–	–

[a] Yeasts were grown in YEPD buffered at pH4.3 or 6.0 under shake or static culture incubated at 25°C for 36h. Culture filtrate activity was measured by the well test in YEPDA buffered at pH4.3 or 6.0 and using the indicator strains 10 and 560; NT, Not tested; –, activity not detectable; z, Hazy zone activity; [b] Also strains 54 and 560 tested only at pH.4.3

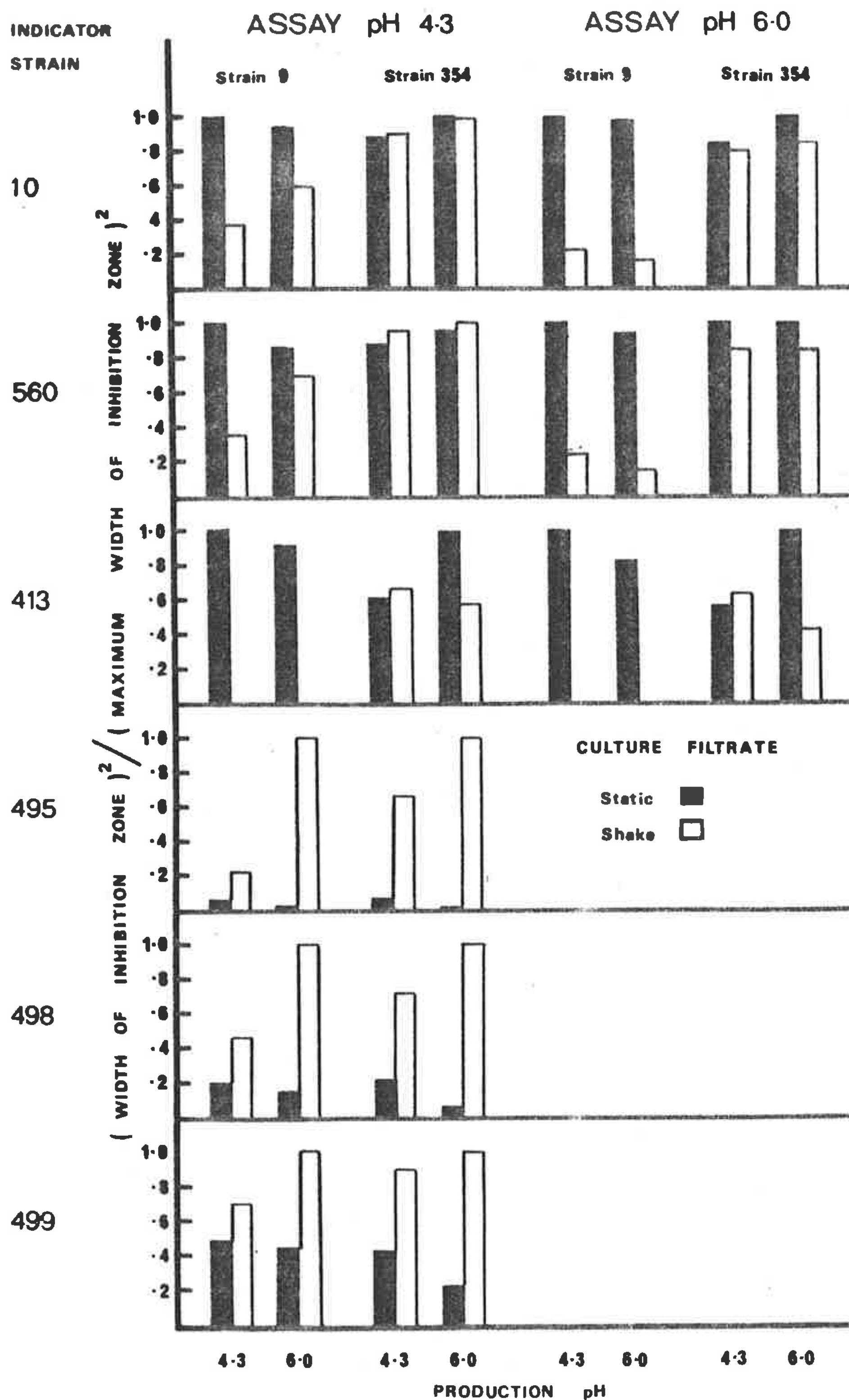


Figure 2.1B. Assay of strain 9 and 354 culture filtrate killer activity produced by various conditions of culture.
Static or shake culture filtrates produced at pH4.3 or 6.0 were assayed with each of six indicator strains in YEPDA buffered at pH4.3 or 6.0.

Broadly, two groups of indicator responses were apparent in the assay of activity present in the culture filtrates of H. saturnus strains. The indicator strains 10, 560, and 413 exhibited similar patterns and are referred to as group 1 indicators and strains 495, 498 and 499 as group 2 indicators. The activity produced by the H. saturnus strains in culture filtrates was dependent on the mode of culture and to a much lesser extent the pH of culture. The apparent activity of HK_{1a} (strain 9) filtrates, but not those of HK_{1b} (strain 354), when assayed by group 1 indicators, was dependent on the pH of assay. Indicator strains 10 and 560 were highly sensitive but 413 was only weakly sensitive. Group 2 indicators were only sensitive to killer activity at low pH (below pH5), strain 495 being less sensitive and strain 498 indicating in addition weak hazy zone activity at pH6.0 (inhibitory).

The assay of H. saturnus culture filtrate activity provided evidence for at least two different killer activities: type A detected by group 1 indicators at neutral pH and type B detected by group 2 indicators only at low pH. Group 1 indicators, 10 and 560 were sensitive to both types of killer activities at low pH and hence only the most diffusable or most potent activity could be assayed at this pH when both types were present together. Accepting this interpretation, killer strain 354 produced roughly similar amounts of type A activity under all cultural conditions studied, whereas type B activity was produced only in shake culture and more so at neutral rather than at low pH. Assay with group 1 strains at pH4.3 indicated the non-additive summation (predominance assay) of type A and type B activities produced in culture filtrates. The remaining killer strains of killer type HK_{1b} (strains 22, 23 and 5761) displayed a similar pattern to that of strain 354. However, the culture filtrate activity of strain 9 differed markedly from those of

HK_{1b} strains on a quantitative basis. While producing similar amounts of type A activity (assayed at pH6.0) to HK_{1b} strains in static mode of culture only very low amounts were produced in shake culture. Also the disparity of type B production with respect to culture pH was very marked in killer strain 9, being much reduced at low pH. Evidence from experiments presented below indicates that the type B activity produced by strain 9 at pH4.3 differed (different optimum pH for activity) from that produced at pH 6.0 and is therefore referred to as type C. The activity produced at pH6.0 contained both type B and type C, type B being predominant.

Mode of culture had no effect on HK_2 activity production as indicated by assay at pH4.3 but in assay at pH6.0 some killer activity was detected in shake culture filtrates. HK_3 activity produced at pH4.3 was only detected at that pH whereas culture filtrates made at pH6.0 showed reduced clear zone activity by assay at pH4.3 but additional hazy zone killer activity against indicator strain 560 at pH4.3 and against strain 10 at pH6.0. Mode of culture had no marked effect. As for type HK_2 activity, mode of culture of the HK_4 yeast had no definite effect on activity production. Activity was comparatively greater in the shake produced filtrate of the HK_5 strain, there being no activity detected by indicator strain 560 in the static culture filtrate. Except for strain 10, none of the filtrates of HK_5 to HK_8 yeasts showed activity by assay at pH6.0. HK_6 activity was vastly greater in shake culture filtrates while HK_7 activity production at pH4.3 was little affected by mode of culture. The inhibition zone edges produced by HK_6 and HK_7 strains were only weakly or not stained by methylene blue. In addition to strain 2564, strains 57, 54 and 560 were tested, but no activity could be detected in their culture filtrates under the conditions employed.

PROPERTIES OF KILLER FACTORS

Membrane diffusibility

The diffusion of killer yeasts' activities through membranes having different pore size provides an indication of the molecular size of the active substances. Standard cellophane and dialysis membranes were used, and in an attempt to control sensitivity of the test, a bacterioproof membrane was included since the results are highly dependent on the sensitivity of the indicator organisms used (see for example ROGERS, 1976).

The results are shown in Table 2.9 and are compared to the responses obtained by the agar diffusion killer test (phenotype test, Table 2.3). The activity of both HK_1 and HK_2 strains was diffusible through all three membranes tested at both pH4.3 and 6.0. The pattern of membrane diffusibility of HK_3 strains was complex depending both on pH of test and the indicator strain, strain 10 being more sensitive than strain 560. The anti-strain 10 activity produced at pH4.3 was weakly diffusible through dialysis membrane and at pH6.0 was only detectable on the control membrane plate. HK_4 activity (pH4.3) against indicator strain 560 was diffusible through all three membranes but not against strain 10. This may be due to the very weak sensitivity of strain 10 to HK_4 activity. The pattern of HK_5 activity against the two indicator strains was the reverse of that of HK_4. HK_6 strain 10 activity was diffusible through all three membranes whereas strain 113 activity was only detectable on the control membrane plate. The hazy zone activity of HK_7 strains tested on membrane plates was only weak or undetectable and did not stain with methylene blue. Strain 2431 produced the only detectable dialysis membrane diffusible activity, visualised by strain

Table 2.9. Diffusibility of killer yeast activity through membranes in agar plate culture.

Killer group	Strain	pH	Indicator strain 10: Agar diffusion test[a]	Indicator strain 10: 0.22 μm membrane	Indicator strain 10: Cellophane	Indicator strain 10: Dialysis membrane	Indicator strain 560: Agar diffusion test	Indicator strain 560: 0.22 μm membrane	Indicator strain 560: Cellophane	Indicator strain 560: Dialysis membrane
HK_1	9,	4.3	+	+	+	+	+	+	+	+
	354	6.0	+	+	+	+	+	+	+	+
HK_2	500	4.3	+	+	+	+	+	+	+	+
		6.0	±	+	NT	+Z	+	NT	NT	NT
HK_3	496,	4.3	+	+	+	+Z	+	+	Tr	−
	5762	6.0	+	+	−	−	+	Tr	−	−
HK_4	16	4.3	±*	+*	Tr	−	+	+	+	+
		6.0	−	NT	NT	NT	−	NT	NT	NT
HK_5	111	4.3	+	+	+	+	+Z	−	−	−
		6.0	−	NT	NT	NT	−	NT	NT	NT
HK_6	10	4.3	−	NT	NT	NT	+	+	+	+
		6.0	−	NT	NT	NT	−	NT	NT	NT
	113	4.3	−	NT	NT	NT	+	+	−	−
		6.0	−	NT	NT	NT	−	NT	NT	NT
HK_7	2431	4.3	+Z*	+Z*	+Z*	+Z*	+Z	Tr	−	−
		6.0	−	NT	NT	NT	−	NT	NT	NT
	497	4.3	+Z*	−	−	−	+Z	−	−	−
		6.0	−	NT	NT	NT	−	NT	NT	NT

[a] Response and symbols of Table 2.3; +, Inhibition zone; *, Weak dye staining; Tr, Growth thinning; −, No inhibition zone; Z, Hazy zone; NT, Not Tested.

10. Indicator strain 560 was only weakly sensitive to HK_7 activity by the phenotype test. None of the killer activities excluded by cellophane were sufficiently strong on control plates to provide evidence for the involvement of particles.

Differences in the activity spectrum between HK_{1a} and HK_{1b} strains of H. saturnus at pH4.3 and 6.0, determined by the agar diffusion killer phenotype test, suggested the involvement of multiple killer activities. Six indicator strains were therefore selected and their responses examined by the membrane-diffusion plate test. Only strain 354 of HK_{1b} strains was tested. The results, shown in Table 2.10 compares their responses to those obtained by the agar diffusion test. The responses of the four indicator strains, 10, 560, 413 and 498 to the membrane diffusible activities of both strain 9 and 354 were similar except that strain 498 was only weakly sensitive at pH6.0 (small hazy non-staining zones) and strain 413, which was only weakly sensitive on the control membrane plates (small zones) was not sensitive to strain 9 activity on the dialysis membrane plate. This latter result may be due to the comparitively weaker sensitivity of strain 413. The two indicator yeasts 495 and 499 which were only sensitive at pH4.3 by agar diffusion test were also only sensitive on the membrane plate tests at this pH. Strain 495, which was only weakly sensitive on the control membrane plate, was not sensitive to cellophane or dialysis membrane diffusible activity as was strain 499. Again the killer activity of strain 9 was relatively less active than that of strain 354.

These results suggest that both killer strains produce two activities: (i) that active near neutral pH against strains 10, 560 and 413 was dialysis membrane diffusible, while (ii) that active against indicator strains 495 and 499 at low pH was only weakly diffusible through

Table 2.10. Diffusibility of *H. saturnus* activities through membranes during agar plate culture[a]

Indicator strain	pH	Group HK_{1a} : Strain 9				Group HK_{1b} : Strain 354			
		Agar diffusion test	0.22 µm membrane	Cellophane	Dialysis membrane	Agar diffusion test	0.22 µm membrane	Cellophane	Dialysis membrane
10	4.3	+	+	+	+	+	+	+	+
	6.0	+	+	+	+	+	+	+	+
560	4.3	+	+	+	+	+	+	+	+
	6.0	+	+	+	+	+	+	+	+
413	4.3	+Z	+	+Z	–	+	+	+	+
	6.0	+Z	+	+Z	Tr	+	+	+	+
495	4.3	+	+	–	–	+	+	Tr	–
	6.0	–	–	–	–	–	–	–	–
498	4.3	+	+	+Z	–	+	+	+Z	+Z
	6.0	+Z	+Z	+Z	–	+Z	+	+Z	+Z
499	4.3	+	+	+	–	+	+	+	+
	6.0	±Z	–	–	–	±Z	–	–	–

[a] See Table 2.9 for explanation of symbols.

dialysis membrane. The activity of strain 9 was weaker than that of strain 354.

Effect of temperature

Culture filtrates of killer yeasts, adjusted to pH4.3, were incubated at different temperatures, and at time intervals the residual activity was compared to that of the controls (filtrates kept at 4-6°C) by assay at pH4.3. The results are summarised in Table 2.11. None of the culture filtrates, except those from HK_3 strains made at pH6.0 lost activity at 22-23°C over a period of 24h.

The culture filtrate activity from HK_1 strains showed differing stability patterns with respect to temperature depending on culture filtrate production conditions and the assay indicator strain (group 1, strain 10, or group 2, strain 498) used. Irrespective of production pH, the activity of shake culture filtrates assayed against strain 10 was thermolabile (stable at 37°C but rapidly inactivated at 80°C) while that of static filtrates was thermostable. Assay employing indicator strain 498 revealed that the activity in the culture filtrates produced under all conditions tested was thermolabile. The patterns of stability for the activities of both killer strains 9 and 354 were analogous but differed quantitatively with respect to residual thermostable activity in shake culture filtrates detected by indicator strain 10. The residual activity approximated to 5-10% of the total for strain 9 and 25-50% for strain 354. On the basis of assay indicator strain specificity (see Figure 2.1B), the thermolabile activity corresponded to type B and the thermostable to type A. Thus the distribution and proportion of types A and B in the various culture filtrates can be seen to be similar to that shown by differential assay (Figure 2.1B).

Table 2.11. Temperature inactivation, at pH4.3, of killer yeast culture filtrate activity.

Killer group	Killer culture filtrate[a]	Time (min.) for 50% inactivation assayed with:					
		Strain 10		Strain 560		Strain 498	
		37°C	80°C	37°C	80°C	37°C	80°C
HK_{1a}	9	>30	<5[b]			>30	<5
	9 (6.0)	>30	<5[b]			>30	<5
	9 (static)		>90			>30	<5
	9 (static, 6.0)		>90			>30	<5
HK_{1b}	354	>30	<5[b]			>30	<5
	354 (6.0)	>30	<5[b]			>30	<5
	354 (static)		>90			>30	<5
	354 (static, 6.0)		>90			>30	<5
HK_2	500	45	<10[c]	45[b]	<10[c]		
HK_3	496	15-30		15-30			
	496 (6.0)	5		5			
	5762	15-30		15-30			
	5762 (6.0)	5		5			
HK_4	16	30-40	<5	50	<5[b]		
HK_5	111	>90	<10	20			
HK_6	10				>90		
	113			>30	<10		

[a] All culture filtrates were from shake cultures buffered at pH4.3 unless indicated otherwise; [b] Underlying heat stable (80°C) activity detected (see text); [c] T-half at 50°C.

The time for 50% inactivation of the HK_2 strain killer activity at 37°C was 45 min, 95% of this activity being inactivated at 50°C in <10 min while the remainder was heat stable. Stability of the killer activities produced by HK_3 strains was dependent on the culture filtrate production pH; a half-life of 15-30 min for the activity produced at pH4.3 and 5 min for that produced at pH6.0. No reason was known for the wide difference in half-life values between different batches of pH4.3 culture filtrates, although hazy zone activity, as produced by HK_3 strains at pH6.0, proved difficult to assay reliably. HK_4 activity, which was very weak against indicator strain 10 (inhibitory), appeared to be inactivated more rapidly than the anti-strain 560 killer activity, the residual of which (about 5%) was stable to heating at 80°C. HK_5 activity against strain 10 was stable at 37°C though quickly inactivated at 80°C whereas the anti-strain 560 activity was much more heat labile, having a half-life of 20 min at 37°C. HK_6 strain 10-produced activity was stable at 80°C whereas strain 113-produced activity was quickly inactivated at that temperature. The culture filtrate activities produced by killer yeasts belonging to HK_7 and HK_8 were not sufficiently active to determine their stability but appeared to be moderately stable at 37°C.

The results of these experiments show that :-

(i) The thermal stability of activities produced by yeasts of different killer groups are different and may be broadly grouped as heat stable and heat labile activities.

(ii) Killer strains of types HK_1 to HK_5 produced at least two activities having different thermal stabilities.

Effect of pH on stability

The effect of pH on the stability of killer yeast culture filtrate activity following incubation at 22-23°C for 60 min was assessed by comparison to that of control samples kept at 4-6°C. The controls were maintained at the pH of culture filtrate production for the course of the experiments but all test and control samples were subsequently assayed at pH4.3. The results are illustrated in Figure 2.2.

Group HK_1 yeast culture filtrate activities showed three patterns of pH-related stability which depended on both the culture filtrate production pH and the assay indicator strain employed. The three types of responses observed in the range pH3.0-6.0 tested were: 1. complete stability, 2. complete stability except below pH4, and 3. stability only near pH4. pH related stability type 1 was only observed on indicator strain 10 (of group 1 indicator yeasts), being displayed by the activities of all filtrates irrespective of culture production mode, except for the shake culture filtrates of killer strain 9. On the other hand indicator strain 498 (of group 2 indicator yeasts) revealed that the activities of both killer strains 9 and 354 culture filtrates, irrespective of static or shake mode of culture, when produced at pH6.0 showed stability type 2 response while those produced at pH4.3 appeared to be less stable at pH6.0 (type 3 response, see below).

As noted above, the activity of killer strain 9 that was not of stability type 1 when assayed by indicator strain 10, did however display analogous stability types on indicator strains 498 and 10. Except for the pH4.3 shake culture filtrate activity of killer strain 9, all the activities showing type 3 stability, while being rapidly inactivated at pH6 to 25-75% of their initial activity, were stable to

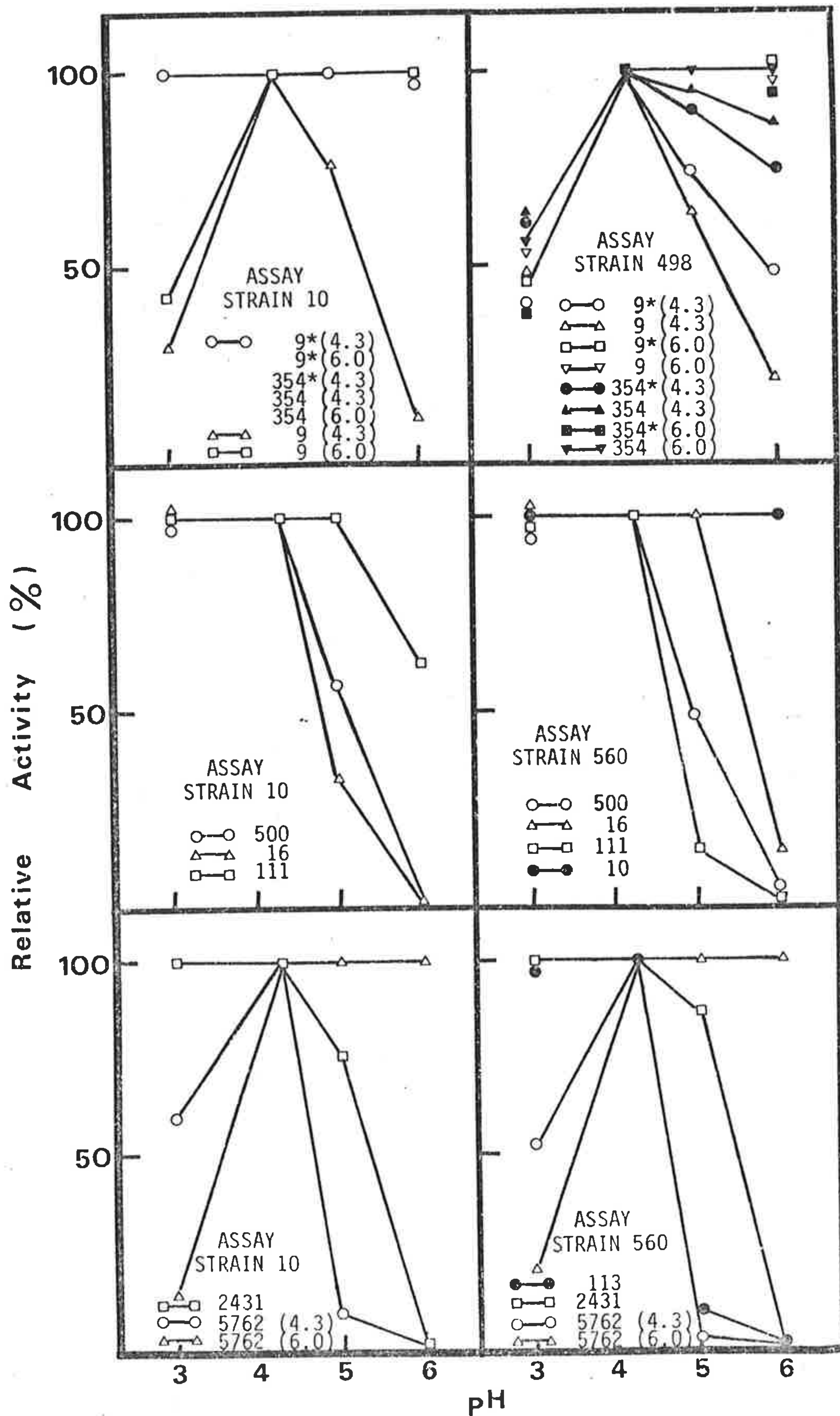


Figure 2.2. The effect of pH on the stability of killer active culture filtrates at 22-23°C for 60 minutes.
An asterisk indicates that the culture filtrate was produced by static incubation and the value in parenthesis indicates culture production pH.

further inactivation with respect to time. Presumably this was due to the presence of "underlying" type 2 activity which was stable at pH6.

Thus it can be inferred that all culture filtrates showed mixed activity types except for the shake culture filtrates of strain 9, which depending on production pH, showed predominantly either type 2 or 3 activity. All of the remaining filtrates showed a combination of type 1 and either type 2 or 3, depending on filtrate production pH, and those containing type 3 also apparently possessed underlying smaller amounts of type 2. H. saturnus culture filtrates heated at $50^{o}C$ for 15 min to remove type B activity resulted in abolition of all assayable profiles from indicator strain 498 and the non-type 1 profiles of strain 9 against indicator strain 10. Thus the heat stable type A activity corresponded to type 1 (complete stability at all pHs), whereas the thermolabile activity was composed of two different pH stability types.

Groups HK_2 strain 500, HK_4 strain 16 and HK_5 strain 111 activity was stable at and below pH4.3 but was inactivated at higher pH. HK_5 activity against indicator strain 10 and HK_4 activity against indicator 560 was relatively more stable at higher pH, showing residual activity stable at pH6.0, as was also shown by strain 500. The residual activities were stable at pH6.0 for at least 3h. HK_6 activity shown by strain 10 was fully stable over the range pH3.0-6.0, while strain 113 activity was rapidly inactivated beyond pH4.3. The stability of HK_3 activity (strain 5762), dependent on culture filtrate production pH, was shown by both indicator strains 10 and 560. The pH4.3-produced activity was unstable at all pHs excepting near pH4.3 while the pH6.0-produced activity, also unstable below pH4.3, was relatively more stable at pH6.0. HK_7 activity (strain 2431) was stable below pH5 but totally inactivated at pH6.0.

Effect of pH on killer activity

The effect of pH on the activity of killer yeast culture filtrates active against several indicator yeasts was studied in YEPDA buffered at values of pH between 3.5 to 5.1 with 0.1M-tartrate and 5.5 to 7.0 with 0.1M-phosphate. Experiments employing pH adjusted unbuffered YEPDA were also made, but the data is treated separately. For each killer yeast, two to five independently prepared batches of culture filtrates were tested. The growth of indicator yeasts 10 and 560 was little affected in buffered YEPD between pH 3.5 to 7.0 but the growth rate and yield of strain 498 was reduced at pH3.9 and further restricted at pH3.5.

The effect of pH on the culture filtrate (shake and static culture at both pH4.3 and 6.0) killer activities of HK_{1a} strain 9 and HK_{1b} strain 354 assayed with both group 1 (strains 10 and 560) and group 2 (strain 498) indicator yeasts is illustrated in Figure 2.3 and detailed in Appendix One. Interpretation of the results indicated three killer activities showing different pH optimum values and pH ranges for activity: Type A, no definite optimum pH, range of at least pH3.5-7.0; type B, optimum of pH4.3-4.7, range of pH<3.5-5.5; and type C, optimum near pH4.3 or less, range of pH<3.5-5.0.

Type B and C activities were most readily seen by the responses of indicator strain 498 which was insensitive to type A. However type C is obscured by the presence of type B. Shake culture filtrates of killer strain 354 show type B (possibly obscuring type C) while static-produced culture filtrates displayed type C activity. However in strain 9, only the pH6.0-produced shake culture filtrate exhibited type B activity, also apparent by assaying against indicator strain 10, which in addition revealed a lesser amount (underlying) of type A activity apparent by the

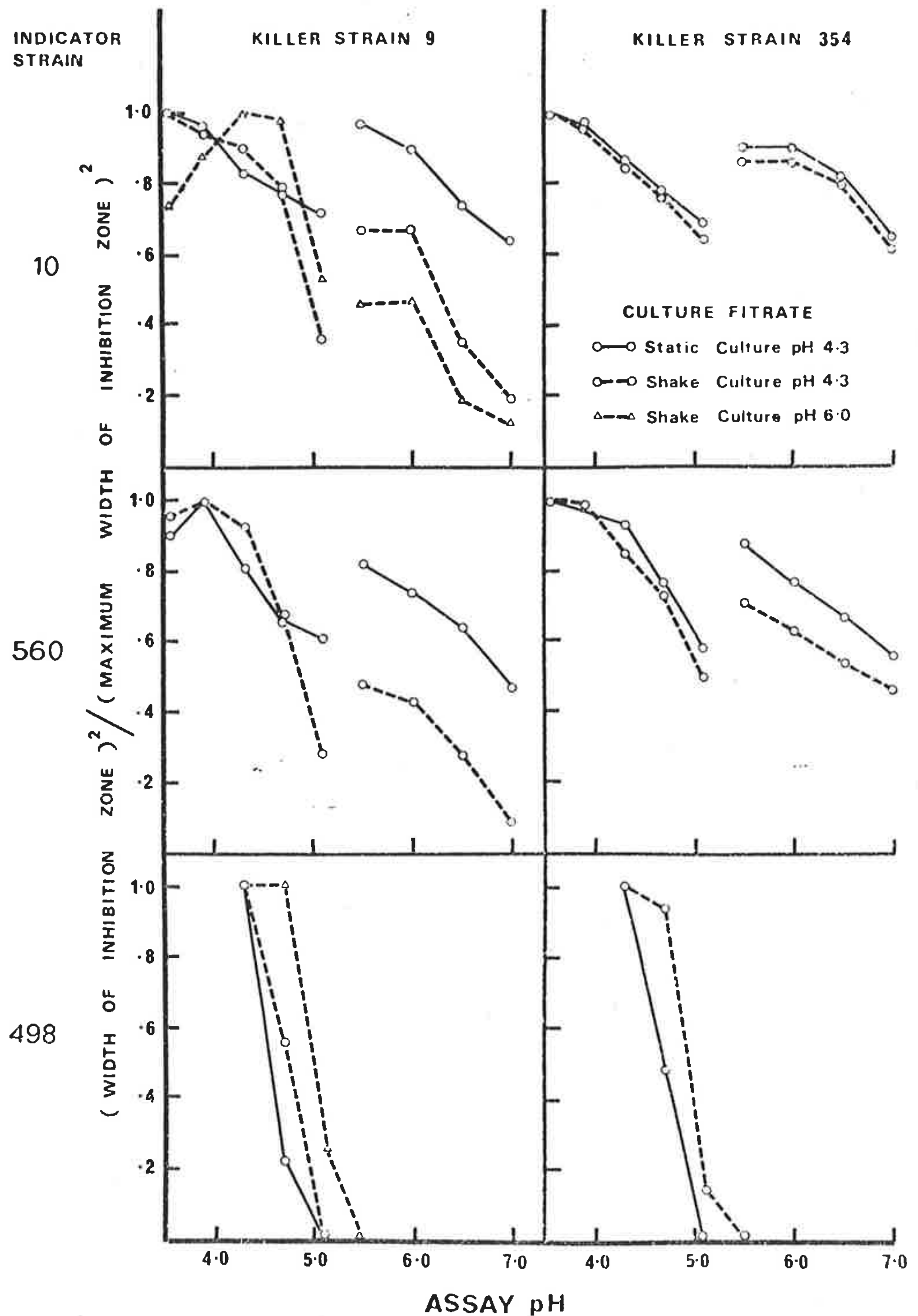


Figure 2.3. The effect of pH on the activity of culture filtrates of killer yeast strains 9 and 354.
Static or shake culture filtrates produced at pH4.3 or 6.0 were assayed with each of three indicator strains in YEPDA buffered at pH3.5 to 5.1 with tartrate and at pH5.5 to 7.0 with phosphate buffer. Excepting where shown (△--△) the profiles for culture filtrates produced at pH6.0 were analogous to the respective filtrates produced at pH4.3. The complete data is given in Appendix One.

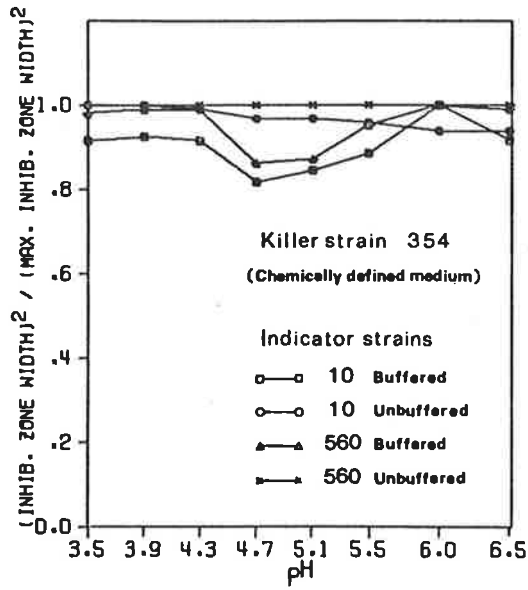
shoulder of activity extending to pH7.0. The remaining culture filtrates from killer strain 9 exhibited type C activity and in particular the pH4.3 produced shake culture filtrate of strain 9 also showed type C against indicator strain 10, in combination with a slightly lesser amount of type A activity (compare strain 9 shake pH4.3 pH-activity profile to that of strain 354). The pH-activity profiles of killer strain 354 culture filtrates determined with indicator strain 10 and 560 represent the resultant overlapping profiles of type A with type B or type C. Removal of type B and C by heating ($50^{o}C$ for 15 min) abolished all response by indicator strain 498 and decreased that against strain 10 and 560 at low assay pH resulting in a more uniform response with respect to pH (data not shown). A similar pH-activity profile was shown by a culture filtrate derived from strain 354 (or strain 9) grown by shake culture in a chemically defined medium (see Methods, Chapter 3)shown only to contain type A activity by an absence of activity against group 2 indicator strains (Figure 2.4A).

Killer activities from the remaining killer strains of group HK_{1b}, though not examined in detail (see Figure 2.4B), were, by inference from assay of their culture filtrate activities using group 1 and 2 indicator yeasts (Table 2.8) of a similar type and distribution to those from strain 354. Thus, on accepting the above interpretation, the results show that under analogous cultural conditions the killer activities produced by strains of HK_{1a} and HK_{1b} primarily differ in a quantitative manner. All five _H. saturnus_ killer strains, under all cultural conditions tested, produced type A activity whereas the distribution of type B and C activity was more complex, being produced optimally in shake culture filtrates and in only trace amounts in static culture.

The type of buffer used in assay affected activity, as seen by a discontinuity at the buffer change between pH5.1 (tartrate) and 5.5 (phosphate) (see Figure 2.4A). This effect was not observed in pH adjusted unbuffered media. No other conditions were examined in an attempt to reduce this problem.

The effect of pH on the culture filtrate activity of killer yeasts of groups HK_2 to HK_7 is shown in Figure 2.5. These pH-activity profiles were grouped into four broad categories, including the type B displayed by H. saturnus killer strains. Type B, which exhibited a broad pH-optimum from pH3.5 to at least pH4.3 and sharply declined to little or no activity at pH4.7-5.5 was shown by killer strains 500, 16 (against strain 560), 111 and 113. Filtrates of the killer strains, 500, 16, (both against indicator strain 560), and strain 111 (against strain 10) also exhibited a plateau of activity near pH6.0. Killer yeasts producing type D response included HK_3 yeasts 496 and 5762 (pH4.3 shake culture filtrates) and strain 16 (against indicator strain 10). Activity of this type was optimum at pH4.3, considerable at pH3.5 but abolished at pH5.1. Type E, in which little or no activity was apparent at pH3.5, was optimum near pH4.7 and declined to zero at pHs of 5.5 or greater, included the yeasts of HK_3 grown at pH6.0 and the HK_7 yeasts 2431 and 497. Type F, strain 10, exhibited an optimum at or below pH3.5 with a gradual decline in activity with increasing pH. The weak culture filtrate activity of HK_8 strain 2564 was not tested. The inhibition zone edges produced by the culture filtrate activities of all the killer yeasts tested stained with methylene blue except for those of strains 16, 2431 and 497 (against indicator strain 10) and strains 111, 10 and 113 (against strain 560) which were only weakly stained.

The pH-activity profiles determined on pH adjusted unbuffered media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(Chemically defined medium)
Indicator strains
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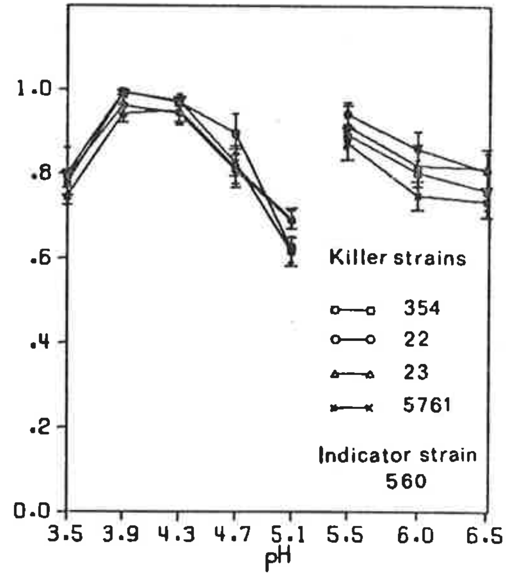
560 Buffered
560 Unbuffered
pH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23
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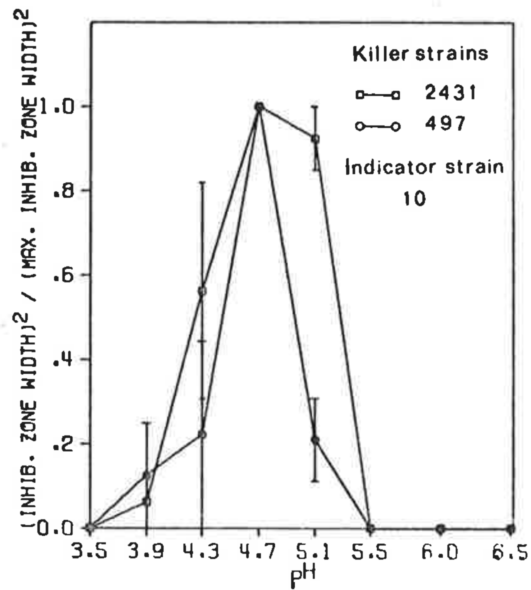
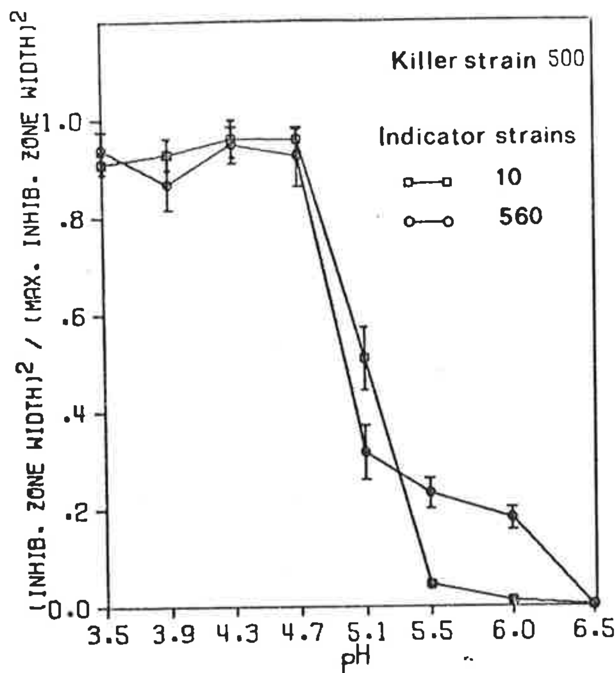
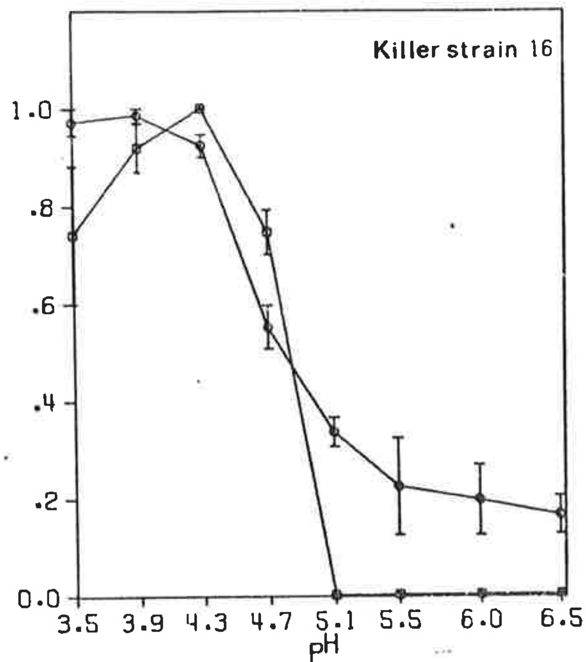
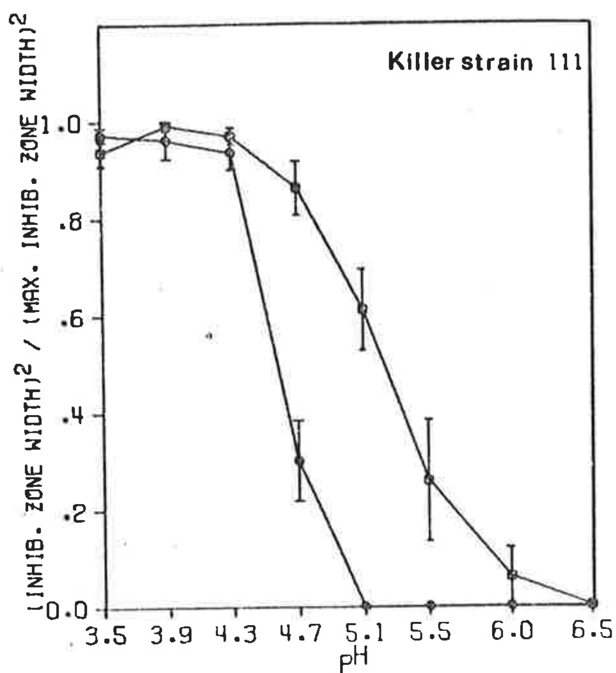
Indicator strain
560
pH

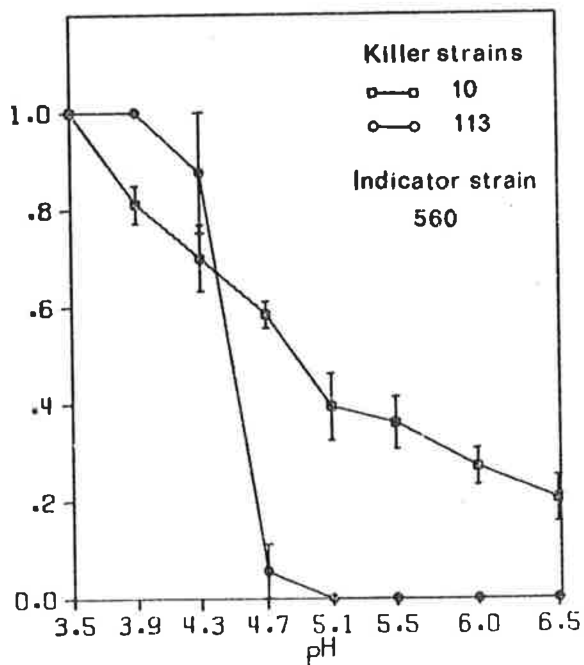

(INHIB. ZONE WIDTH)2 / (MAX. INHIB. ZONE WIDTH)2
Killer strains
2431
497
Indicator strain
10
pH

BUFFERED ASSAY MEDIUM









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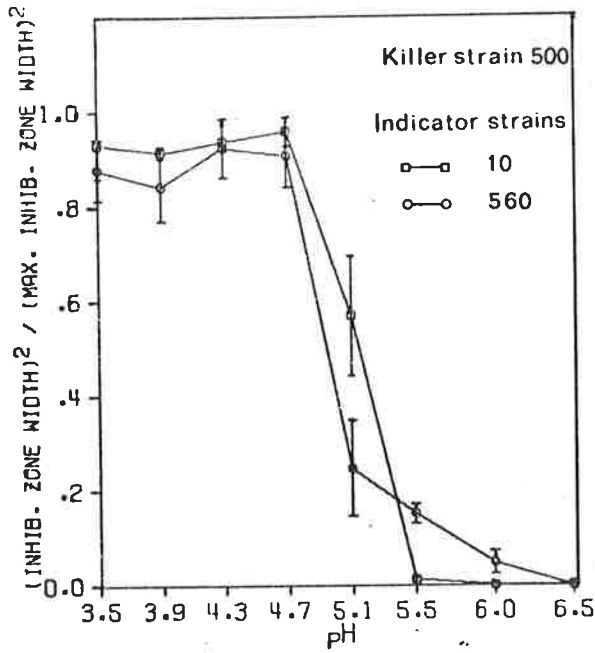

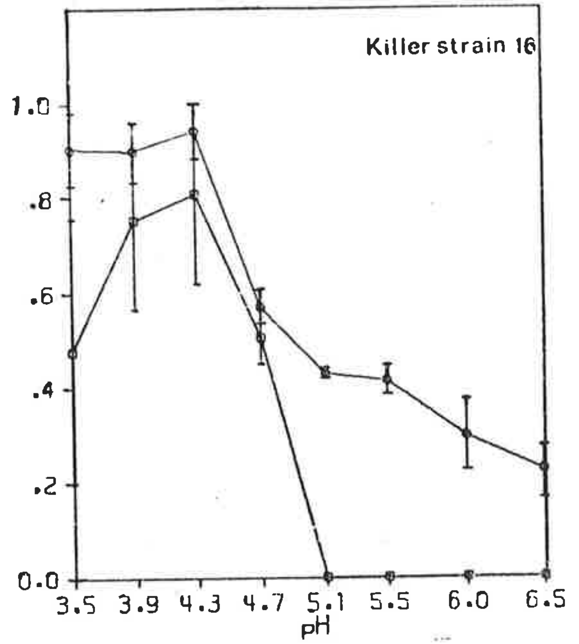

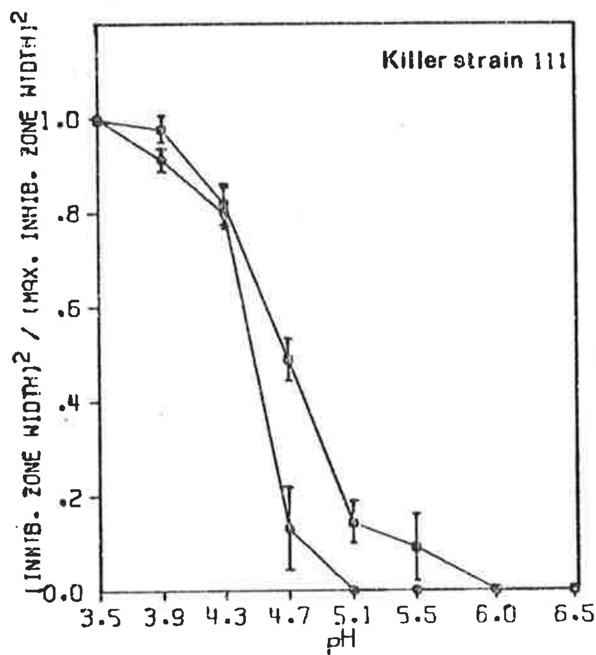

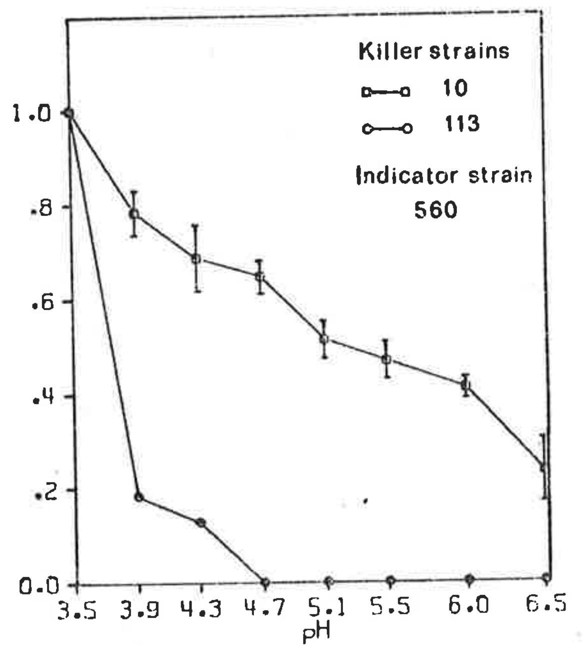

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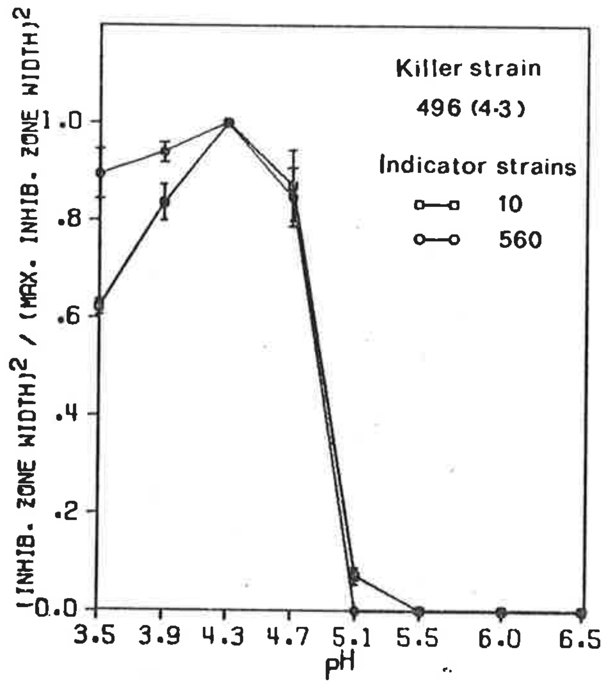
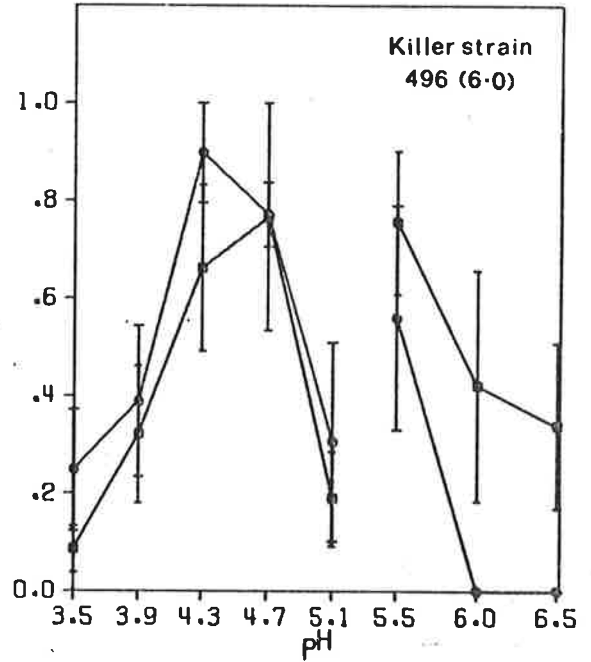
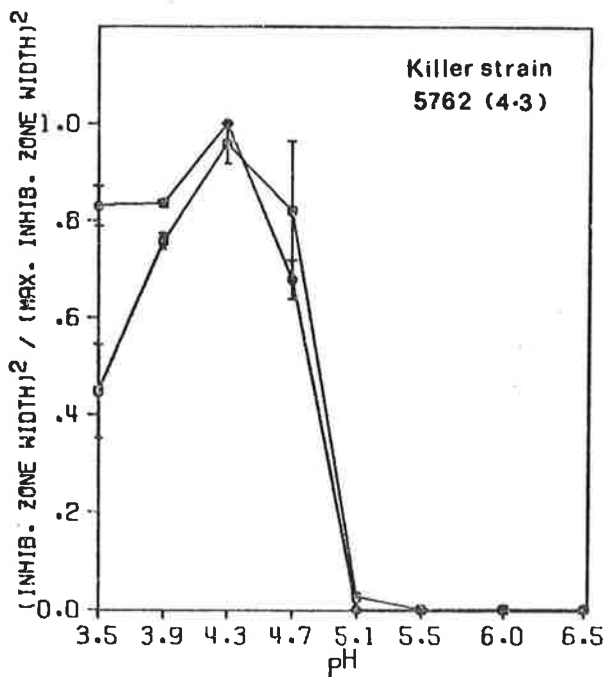
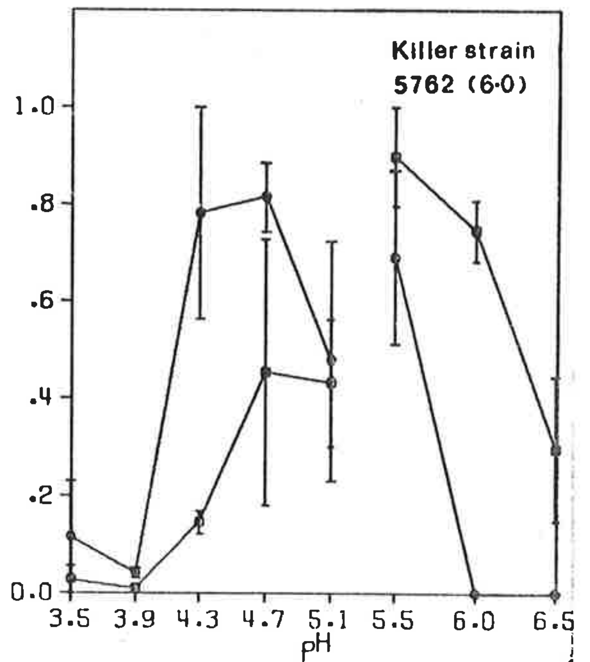
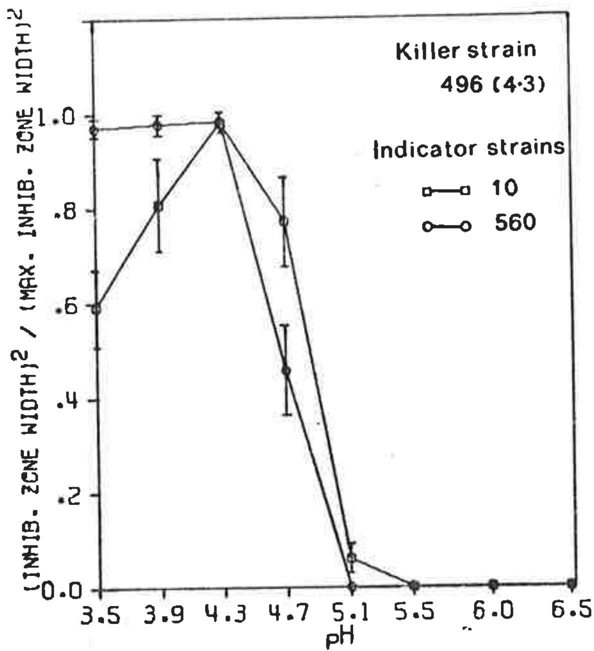


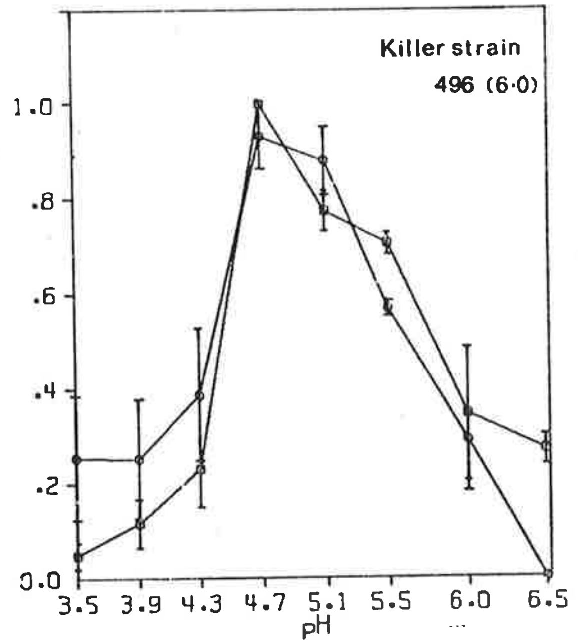


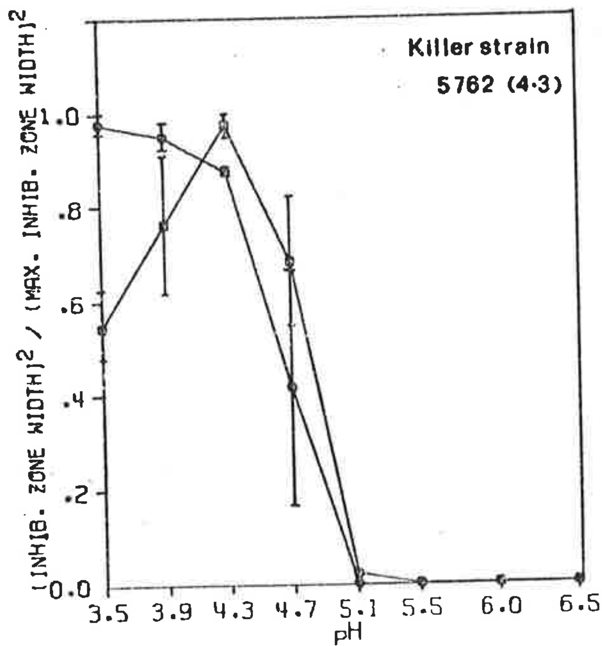


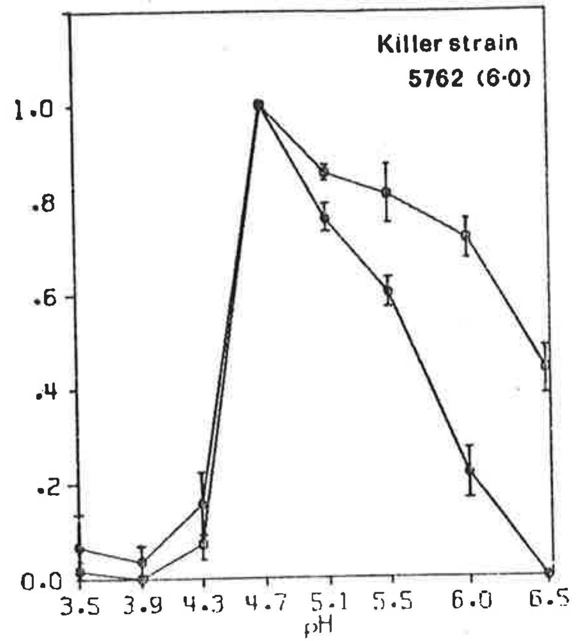


UNBUFFERED ASSAY MEDIUM









exhibited the same pH optimum and profile type as determined on buffered media. However, the activities of HK_3 yeasts revealed several small differences which may reflect either a greater sensitivity to an inevitable pH drift in unbuffered media or interference by the type of buffering species.

These results, summarised in Table 2.12, provide evidence that like the killer strains of H. saturnus, yeasts of the killer groups HK_2 to HK_6 each produce at least two different types of toxin activities, exhibiting different pH optimal values or profiles. The majority of killer yeasts' activities show an optimum for killing between pH3.5-4.3, but culture filtrates from five yeasts (strain 500, 16 and 10 against indicator strain 560, HK_3 yeasts and strain 111 against strain 10) also show a plateau or shoulder of activity in the range pH5.5-7.0.

Table 2.12 pH optima of killer yeast culture filtrate activities[1].

Killer group	Killer strain	Indicator strain	pH-activity profile type[a]	pH optimum for killing
HK_1	9, 354	10, 560	A	4-6
		498	B	<4.3-4.7
		498	C	<4.3
HK_2	500	10, 560	B	3.5-4.7
		560		(5.5-6.0)[b]
HK_3	496 (4.3) 5762 (4.3)	10, 560	D	4.3
	496 (6.0) 5762 (6.0)	10, 560	E	near 4.7
HK_4	16	10	D	4.3[c]
		560	B	3.5-3.9
		560		(5.5-6.5)[b]
HK_5	111	10	B	3.9-4.3
		560	B	3.5-4.3[c]
HK_6	10	560	F	<3.5[c]
	113	560	B	3.5-3.9[c]
HK_7	2431, 497	10	E	4.7[c]

1 Determined on buffered YEPDA; a See text for explanation; b Apparent pH range; c Weak staining with methylene blue.

Effect on cell viability

The killing activity of killer yeast culture filtrates was examined by determining the viability of log-phase cells in culture medium, to which killer solutions had been added. Controls included culture filtrates of non-killer yeasts and the test medium. Preliminary time course-killing experiments using shake-produced culture filtrates of killer strains 354, 500 and 111 showed that maximal killing of strain 10 occurred between 4-6h. The effect of HK_1 to HK_7 yeast culture filtrates on selected indicator yeasts was examined and the results of cell viability at 5h is given in Table 2.13. The growth of the indicator strains in the test medium is shown, and indicates that the non-killer culture filtrates had only a small or no inhibitory effect on growth.

Culture filtrates of HK_1 strains 9 and 354 reduced the survival of sensitive strains 10 to 0.1% and 498 to 0.01% depending on the origin of the killer solution. Killing by type A, present in culture filtrates having mixed activities, was shown by a reduced survival of strain 10 at pH6.0. A culture filtrate obtained from strain 354 grown in the chemically defined SKT production medium (see Chapter 3), known to contain activity of type A only, showed a similar degree of killing at both pHs 4.3 and 6.0. The quantitative effect of the tartrate and phosphate buffers on killing at each pH was not examined. Indicator strain 498 shows killing by type B and/or type C activities, present in shake culture filtrates. Static culture filtrates, which contain only comparatively small amounts of type B and C relative to shake filtrates, showed much reduced killing of strain 498. The shake culture filtrates of killer strain 9 produced at pH4.3 and 6.0, in which type C and type B activities were found to predominate (see Figure 2.3) respectively,

Table 2.13. The effect of killer and non-killer yeast culture filtrates on the survival of sensitive yeasts[1].

Group	Culture filtrate[b]	% survival[a] Strain 10 pH4.3	Strain 10 pH6.0	Strain 560 pH4.3	Strain 498 pH4.3
Control	YEPD - buffer control	800	500	500	200
	10	700	400		
	560			500	
	57	500			100
	57 (6.0)	450	350		
HK_{1a}	9	0.3			<0.01
	9 (6.0)	0.8	1.0		<0.01
	9 (static)	0.1			0.6
HK_{1b}	354	0.1			<0.01
	354 (6.0)	0.1	0.2	0.4	<0.01
	354 (static)	0.2			0.2
	354[c]	0.2	0.2		
HK_2	500	<0.01		<0.01	
HK_3	496	<0.01			
	496 (6.0)	5	100		
	5762	<0.01			
	5762 (6.0)	2	80		
HK_4	16	200		<0.01	
HK_5	111	<0.01		60	
HK_6	10			13	
	113			150	
HK_7	2431	300			

[1] Colony-forming units/ml (CFU/ml) surviving 5h incubation at 22-24°C enumerated on buffered YEPDA; [a] Cell survival is expressed as the percentage of initial CFU/ml, where 100% is the number immediately before culture filtrate addition which was 0.9 - 1.5 x 10^5 CFU/ml except for strain 498 which was 3.0 - 3.6 x 10^5 CFU/ml; [b] Culture filtrate produced by shake culture at pH4.3 unless otherwise indicated; [c] Culture filtrate from strain 354 grown aerobically in the chemically defined medium of final pH,5.5 (see Methods of Chapter 3).

produced a high degree of killing of strain 498. The presence of type A in these solutions is shown by the reduced survival of strain 10 at pH6.0.

Group HK_2 activity reduced survival of indicator strains 10 and 560 to less than 0.01% as also did HK_3 yeast culture filtrates, produced at pH4.3, against indicator strain 10. The pH6.0 culture filtrates, from HK_3 yeasts, while reducing the viability of strain 10 to <10% at pH4.3, were only inhibitory to growth at pH6.0. HK_4 and HK_5 yeast activities resulted in <0.01% survival of indicator strains 560 and 10 respectively, but were only inhibitory or produced a small degree of killing of strains 10 and 560 respectively. Killer strain 10 of HK_6 reduced survival by about 10-fold whereas strain 113 was only inhibitory. The activity of strain 2431, group HK_7, was only slightly inhibitory to the growth of indicator strain 10.

Cellulose acetate membrane electrophoresis

Electrophoresis of culture filtrate samples applied to cellulose acetate membrane (CAM) strips, equilibrated at pH4.3 with tartrate buffer, at 250V for 5-6h resulted in migration of the active principles by up to 2 cm and in two cases to 4-6 cm, as detected by bio-autography. The volume of sample, applied, 2.5-10μl per 2.5 cm-width of strip, was adequate for detection of the major active principles and had minimal effect on the rate of migration. Sample composition however slightly affected migration, which was marginally increased in the presence of phosphate when compared to tartrate buffered samples. But because many of the activities were dialysable and since the present technique was investigated principally for an aid to identification of the active principle(s), no attempt was made to solve this problem. Longer

electrophoretic times increased migration distances but this was partly off-set by the greater diffusion of the activities, resulting in little effective increase of separation. However in the case of incomplete separation, resolution was enhanced by applying a graded volume of sample across the CAM strip which resulted in a wedge-shaped inhibition zone (see Figure 2.6). Use of a citrate-phosphate, and an acetate buffer did not improve separations.

Killer activity present in culture filtrates of strains 9 and 354, produced by static and shake culture at pH4.3 and 6.0, were tested in an attempt to determine the number and distribution of active principles. To facilitate this aim, bio-authographic detection was carried out on thin-layer agar plates buffered at either pH4.3 or 6.0 and seeded with one of six indicator yeasts, 10, 560, 413, 495, 498 and 499, previously shown to respond variously to different activities present in the culture filtrates from H. saturnus strains. A summary of the results is illustrated in Figure 2.7. No killer zones of activity were detected on plates seeded with strain 413 at the sample loadings used and the pattern of killer zones produced on strain 499 was similar to that for strain 498.

The results indicated that three different activities were present in cultures of strains of H. saturnus. The most rapidly migrating activity was present in all culture filtrates produced by all four conditions of culture tested. It was detected as a narrow-width band on plates seeded with either strain 10 or 560 buffered at either pH4.3 or 6.0 but was not detected by the three strains, 495, 498 and 499 at either pH4.3 or 6.0, and therefore corresponded with type A. At high sample loadings (>15 µl), slight growth inhibition was observed on the latter two strains. At least two lesser mobile activities were observed depending on the

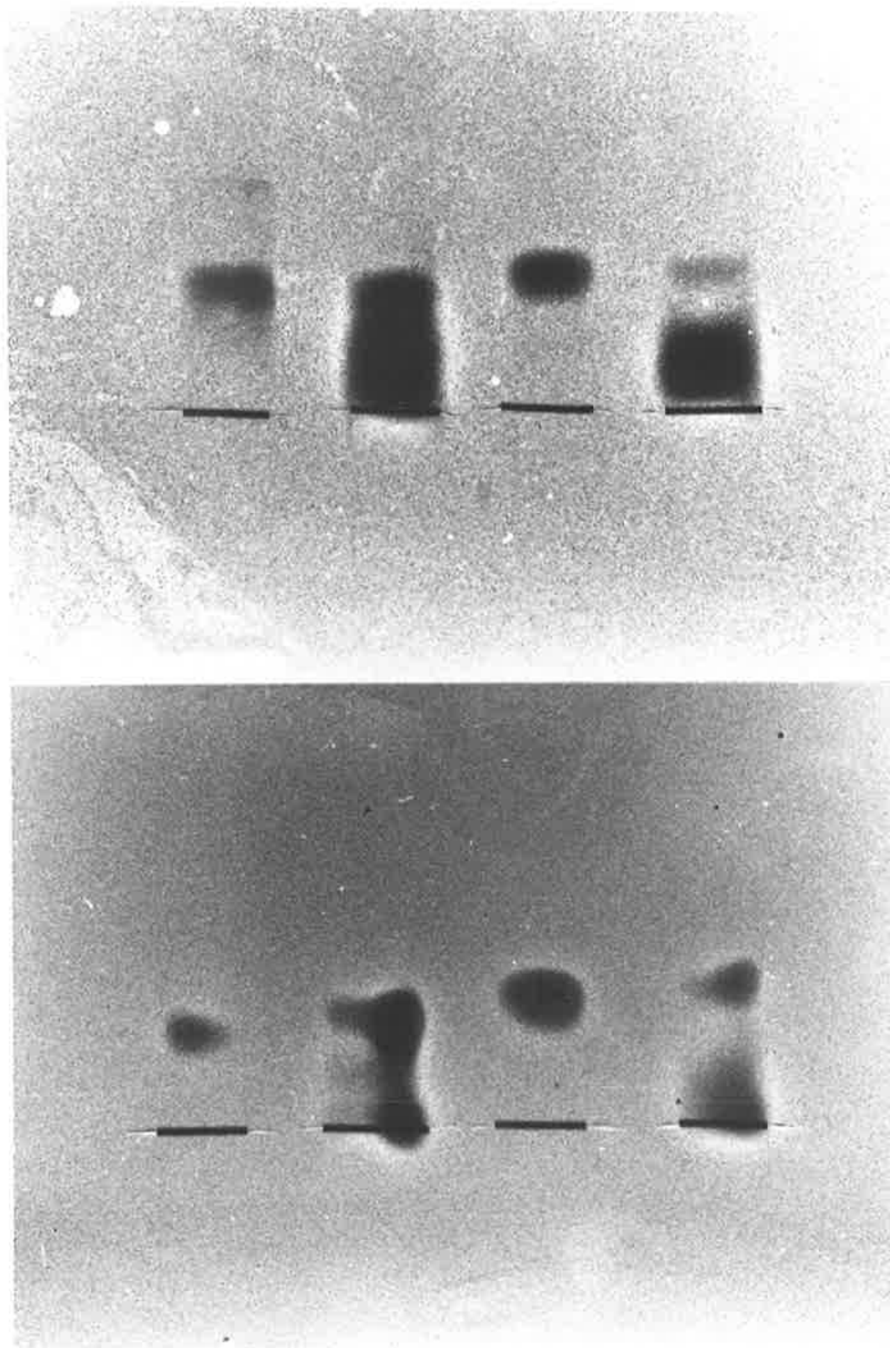


Figure 2.6. Bio-autographic plates showing the electrophoretic migration of killer activities in culture filtrates of H. saturnus AWRI 354. Approximately 5µl of sample, pH4.3, was applied to CAM strips buffered at pH4.3 and electrophoresed at 1mA/cm width of strip for 6h at 4-6°C. Longitudinally divided CAM strips were then placed on thin-layer pH4.3 buffered agar plates seeded with indicator yeast and developed at 22-23°C for 40h. Bio-autographic plate A, seeded with H. anomala NA10, shows the result of sample applied evenly across the CAM strip whereas in Plate B, seeded with H. holstii NCYC 560, graded application of sample produced "wedge-shaped" zones. From left to right, the samples were culture filtrates produced under the following conditions: static culture at pH4.3; shake, pH4.3; static, pH6.0; and shake, pH6.0. Migration was towards the cathode.

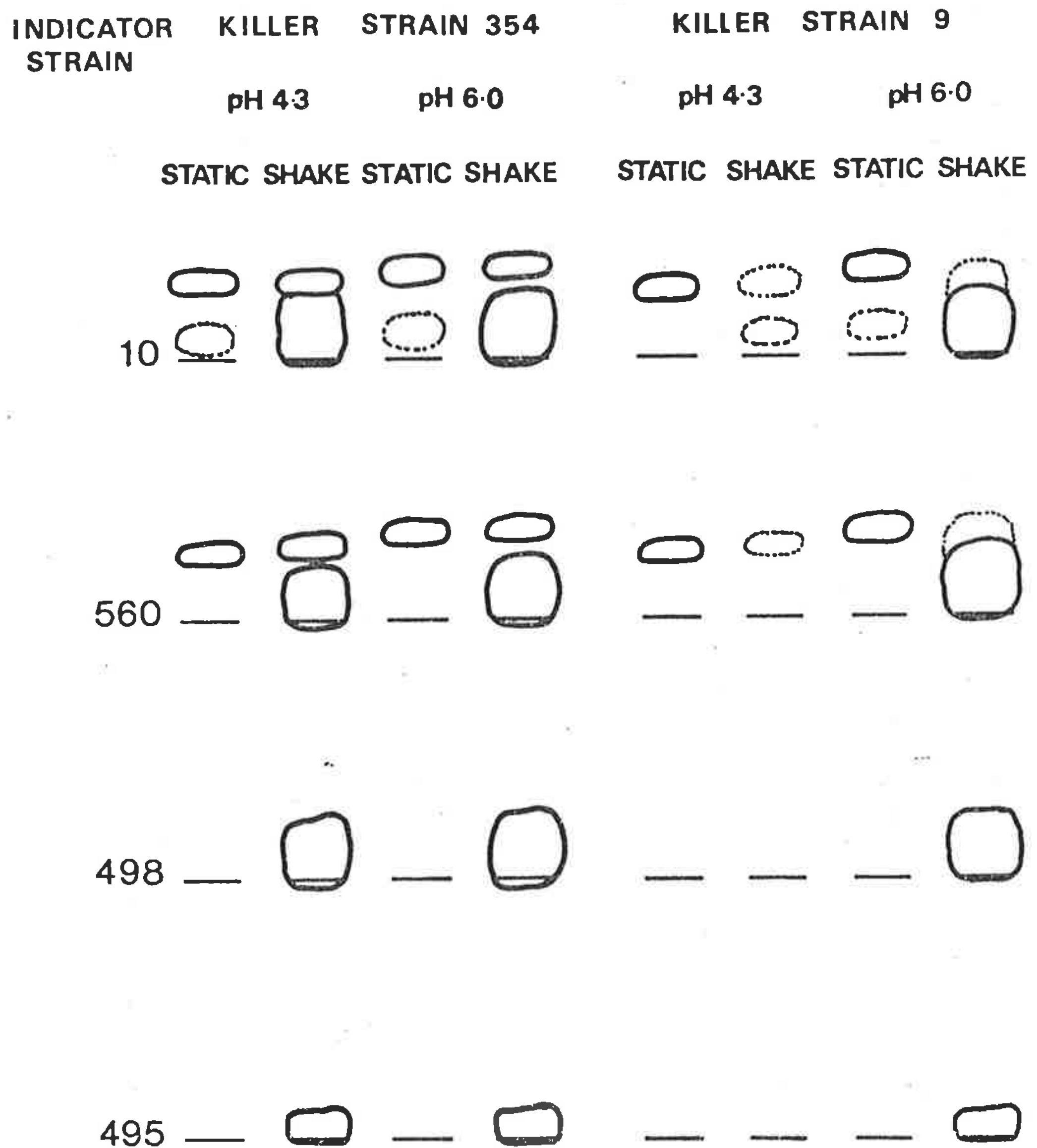


Figure 2.7. Diagrammatic representation of bio-autographic plates, containing indicator strains 10, 560, 498 and 495, showing the electrophoretic migration of killer activities present in culture filtrates from H. saturnus strains 354 and 9 made under various conditions.
The experimental conditions were as described in Figure 2.6. The horizontal bar indicates the origin of the applied sample, and migration was towards the cathode. A solid line indicates the outline of the killer activity zones and the broken line denotes a hazy zone (weak activity) and/or a hazy zone edge.

type of culture filtrate, static or shake, and indicator strain used. The first, present in only shake culture filtrates, showed only slight migration from the origon and was the sole activity detected by strain 495. The other, the slow migrating activity present in all static culture filtrates irrespective of pH, with the exception of the pH4.3 filtrate of killer strain 9, showed slightly increased mobility over the activity detected on indicator 495. This activity, detected as a hazy edged zone, which, in several preparations was just detectable, probably indicated that it was present only in very low concentrations. The shake culture filtrates, which produced a broad killer zone extending from the sample origin nearly to the most rapidly migrating activity observed, was shown to be composed of two overlapping activities (see Figure 2.6).

Killer strain 9 culture filtrates showed qualitatively similar electrophoretic patterns, except that the activity of the pH4.3 shake culture filtrate was very weak (trace growth inhibition) and the rapidly migrating activity of the pH6.0 shake filtrate was either present as a hazy zone or absent. Heat treatment of _H. saturnus_ culture filtrates (50°C for 15 min) resulted in abolition of all activities excepting the rapidly migrating principle detected by indicator strains 10 and 560 at either pH4.3 or 6.0.

The electrophoretic patterns of HK_2 to HK_6 yeast killer activities, present in pH4.3 shake culture filtrates, and detected by bio-autography using indicator strains 10 and 560 are illustrated in Figure 2.8. Migration is relative to the pH4.3 shake culture filtrate activities of killer strain 354. Killer strain 500 activity showed a large zone on both indicator strains 10 and 560, but in addition, a more rapidly migrating zone was also present on the latter indicator, and in several

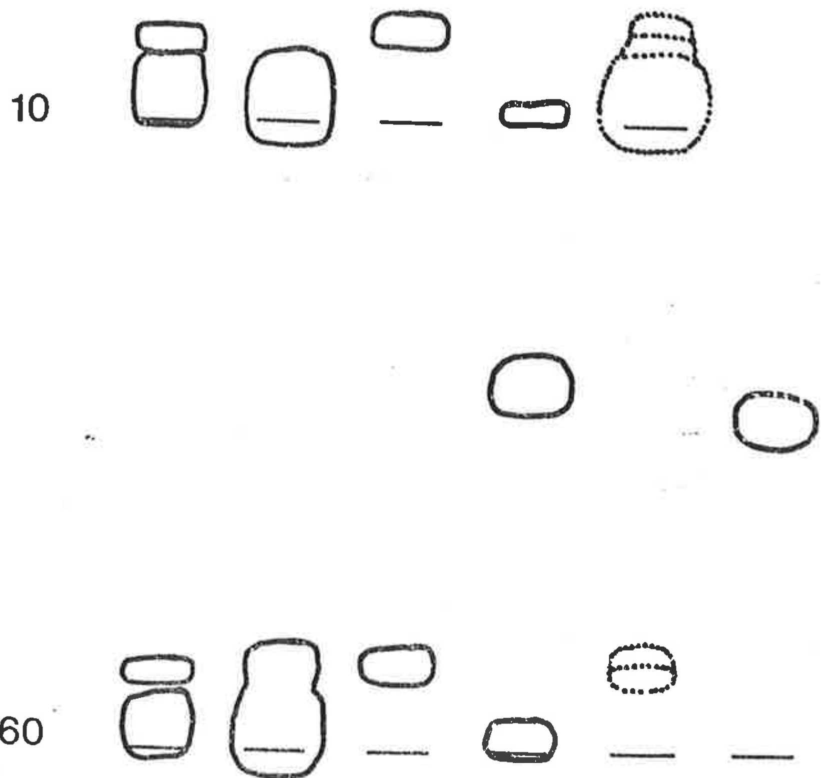


Figure 2.8. Bio-autography on indicator strains 10 and 560 showing the electrophoretic migration of activities present in pH4.3 shake culture filtrates of killer yeasts.
Experimental conditions were as described in Figure 2.6.

preparations a trace of activity (inhibitory), in this position, was also observed on strain 10. Group HK_3 (strains 496 and 5762) preparations showed a single zone of similar migration rate to the rapidly migrating activity of strains 354 and 500. The pH6.0 culture filtrate activity of HK_3 strains was not sufficiently active at the sample loadings used to describe its mobility pattern. Killer strain 16 showed a killer zone near the origin on both indicator strains used as well as a very rapidly migrating zone on indicator strain 560. The single active species of killer strain 10 was also highly mobile. Killer strain 111 displayed a large doubled-edged zone only on indicator strain 10 and 1 to 2 more rapidly migrating hazy zones on both indicator strains. The zone edges only weakly stained with dye. The activity of strain 113 (group HK_6) and of HK_7 were too weak to be detected following electrophoresis.

The results reported here were obtained on Gelman Sepraphore 111 strips of Lot No. 81376. However during preliminary tests, in which strips of Lot No. 80716 were used, migration distances were greater under the same conditions, and the double-edged activity of killer strain 111 migrated toward the anode. The latter Lot No. is no longer available and so these differences could not be investigated.

Inactivation by proteases

Solutions of proteolytic enzymes were added to killer culture filtrates and after incubation for 24h, the activity of mixtures containing native or denatured (head) enzyme were compared with control mixtures containing enzyme activators or distilled water added to the killer yeast filtrates. Initially culture filtrates were treated with native enzyme at pH4.3 but because of little or no apparent enzyme

activity, culture filtrates were adjusted to pH4.7 before enzyme addition. All reaction mixtures irrespective of pH were assayed directly without prior pH adjustment on YEPDA buffered at pH4.3 with 0.1M-sodium tartrate for maximal assay sensitivity. Control and experimental reaction mixtures were expected to be affected similarly. Because the activity of several culture filtrates against certain indicators on 2 to 4-fold dilution were extinguished, these could not be adequately tested. Also, papain reaction mixtures could not be assayed on indicator strain 560 because of a large inhibition zone produced by the control cysteine-versene enzyme mixture. The results of protease inactivation of HK_1 activities is shown in Table 2.14 and of HK_2 to HK_6 activities in Table 2.15.

None of the culture filtrate activities of killer strains 9 or 354 were inactivated by enzymes at pH4.7. However, the activities in shake culture filtrates produced and tested at pH6.0 were susceptible to the action of all four proteases tested, with the exception of strain 354 shake culture activity against indicator 10, which was not affected by papain. The activities of HK_2, HK_3 and HK_5 yeasts were inactivated by papain, but additionally HK_2 and HK_5 activity was susceptible to the action of pronase. The activities of HK_4 and HK_6 yeasts were inactivated only by pronase. In many of these, the susceptible activities were only partially inactivated, that is, by between 10 to 50% of total activity.

Table 2.14. Inactivation of H. saturnus strain 9 and 354[1] culture filtrate activity by native (N) and heat denatured (D) proteolytic enzymes.

Killer solution	Treatment pH	Assay with strain 10 Papain N	Papain D	Pepsin N	Pepsin D	Pronase N	Pronase D	Trypsin N	Trypsin D	Assay with strain 498 Papain N	Papain D	Pepsin N	Pepsin D	Pronase N	Pronase D	Trypsin N	Trypsin D
STRAIN 9																	
Static(4.3)	4.7	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-
Shake (4.3)	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Static(6.0)	6.0	0	0	0	0	+	0	0	0	-	-	-	-	-	-	-	-
Shake (6.0)	6.0	++	0	++	0	++	0	++	0	++	0	++	0	++	0	++	0
STRAIN 354																	
Static(4.3)	4.7	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-
Shake (4.3)	4.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Static(6.0)	4.7	0	0	0	0	+	0	0	0	-	-	-	-	-	-	-	-
Shake (6.0)	6.0	0	0	++	0	++	0	++	0	++	0	++	0	++	0	++	0

[1] All mixtures were assayed directly on plates buffered at pH4.3 with 0.1M-sodium tartrate and seeded with either indicator strain 10 or 498; ++, > 50% inactivation; +, To 50% inactivation; 0, No inactivation; -, Not tested; N, Native enzyme; D, Denatured enzyme.

Table 2.15. Inactivation of killer yeast activities by proteolytic enzymes at pH4.7.[1]

Killer group	Killer solution	Assay with strain 10								Assay with strain 560					
		Papain		Pepsin		Pronase		Trypsin		Pepsin		Pronase		Trypsin	
		N	D	N	D	N	D	N	D	N	D	N	D	N	D
HK_2	500	+	0	0	0	+	0	0	0	0	0	+	0	0	0
HK_3	496	+	0	0	0	0	0	0	0	0	0	0	0	0	0
	5762	+	0	0	0	0	0	0	0	0	0	0	0	0	0
HK_4	16	0	0	0	0	+	0	0	0	0	0	+	0	0	0
HK_5	111	+	0	0	0	+	0	0	0	-	-	-	-	-	-
HK_6	10	-	-	-	-	-	-	-	-	0	0	+	0	0	0

[1] Killer solutions at pH4.7 were treated with native or denatured enzyme and after 24h assayed directly at pH4.3; +, Inactivation; 0, No inactivation; -, Not tested; N, Native enzyme; D, Denatured enzyme.

DISCUSSION

A. Survey and categorisation of killer yeasts

Following the initial discovery of the antifungal activity of *H. saturnus* NA 9 from Dr. Nancy Atkinson's collection, a survey of 35 strains obtained from The Australian Wine Research Institute's collection revealed that only *H. saturnus* AWRI 354, antagonised the growth of the two indicator strains *H. anomala* NA 10 and *Saccharomycodes* NA 14 on standard culture media. The sensitive strains belonged to several genera of Ascomycetous yeasts: 22 of the 27 *Saccharomyces* strains tested, including all nine strains of *Sacch. cerevisiae*, were sensitive to various extents, as were the four strains of *Saccharomycodes.* In addition, some 17 of the 25 *Hansenula* yeasts obtained from The National Collection of Yeast Cultures (NCYC), England, and The Centraalbureau voor Schimmelcultures (CBS), The Netherlands, were variously sensitive. Strains belonging to Asporogenous yeast genera (eight strains from six genera) were however resistant, although insufficient numbers were tested to make a definite conclusion regarding their resistance as a group.

The finding that the two strains of *H. saturnus* produced a strong killer reaction at near neutral pH (pH6.0) on methylene blue containing plates, prompted a survey of other strains and species of *Hansenula* for a similar killer reaction and also for *Saccharomyces* yeast killer factor-like activity in low pH media. Of the 28 strains of *Hansenula* tested in total by interaction in all paired combinations, 16 strains produced a killer reaction. All 16 strains were active at low pH.

Eight, of which five were strains of *H. saturnus*, were killers at neutral pH.

Thus, beside the killer strains of *H. saturnus*, only three other strains (all species of *Hansenula*) of the 76 strains tested were antagonistic to the growth of yeasts on standard culture media. This result was not unexpected since a survey of 153 yeasts from the NCYC did not reveal significant anti-fungal activity on yeast media (MacWILLIAM, 1959). The extensive survey of NCYC yeasts (964 strains from 28 genera) employing a single indicator strain test system at low pHs, conducted by PHILLISKIRSK and YOUNG (1975), revealed that the two genera, *Saccharomyces* and *Hansenula*, provided a high incidence of killer strains, but many strains of the former genus were inbred. Among the 29 strains (11 species) of *Hansenula* tested, 12 killer strains were present. It is possible that some killer strains, not active against the single indicator strain used, were overlooked. STUMM *et al.* (1977) surveying yeasts isolated from natural habitats (175 strains belonging to nine genera) also detected killer strains in *Hansenula* yeasts (two strains) and in the related *Pichia* yeasts (15 strains). These findings therefore support the conclusion of PHILLISKIRK and YOUNG (1975) that yeasts belonging to genera other than *Saccharomyces* produce a killer factor mediated reaction, which as suggested by STUMM and colleagues (1977) may be an important factor in the ecology of yeasts. Although wider surveys are needed to draw a definite conclusion, the findings to date are suggestive that yeasts belonging to the genera *Hansenula*, *Pichia* and *Saccharomyces* harbour a relatively large proportion of killer strains.

Both RODGERS and BEVAN (1978), and YOUNG and YAGIU (1978) demonstrated the utility of classifying killer yeasts on the basis of killer and

resistance phenotypes determined by interaction between the various killer strains. Because a killer yeast is immune to the toxin(s) it produces, a toxin to which it is susceptible must be structurally dissimilar to enable by-pass of the particular immunity system(s). The toxins of a multi-toxigenic strain may not be structurally related and hence the producer strain is likely to possess independent immunity systems for each type of toxin. Resistant mutants have also been used to indicate relationships between different killer yeasts (AL-AIDROOS and BUSSEY, 1978; ROGERS and BEVAN, 1978).

The killer strains identified in this study have been categorised in a similar manner to that described by YOUNG and YAGIU (1978) but as it is impossible at this stage to incorporate their scheme, a nomenclature was devised which distinguishes the present classification from others and indicates its application to Hansenula killer yeasts. The Hansenula killer types are denoted HK_x and the resistance phenotype HR_y, where x and y are randomly assigned numerals.

Categorisation of the 16 Hansenula killer strains according to their killer and resistance phenotypes determined by interaction of all killer and non-killer strains at both pH4.3 and 6.0 revealed eight classes of killer yeasts, composed of eight killer types denoted HK_1 to HK_8 and their corresponding resistance types, denoted HR_1 to HR_8. Type HK_1, which contained all H. saturnus killer strains, was subdivided into two groups because the activity spectrum of strain 9 differed from that of the remaining strains. Strain 9 however displayed the same resistance pattern (HR_1) and was shown capable of elaborating similar toxins (in liquid culture media) but with quantitative differences. The non-killer H. saturnus 57 also showed the HR_1 phenotype, but genetic manipulation is required to establish whether it carries the killer strain immunity

system(s). HK_2 (H. mrakii) was the most extensive killer type, killing all other killer yeasts except HK_1 strains. All of the Hansenula yeasts tested by YOUNG and YAGIU (1978) were also killed by this yeast. They also tested NCYC 16, finding all but one non-Saccharomycete were resistant. In the present survey the majority of killer strains were also resistant to killing by this yeast.

On the basis of the dye staining reaction, strains of both types HK_6 and HK_7 were apparently unable to kill any of the killer stains examined. However several non-killer strains were susceptible, showing more intense staining, enabling differentiation of the strains of the two killer groups. Partial characterisation of their culture filtrate activities supported differentiation of the HK_6 and HK_7 strains. These two groups of strains, however, exhibited reciprocal inhibitory activity and indirect evidence suggests that they were killer reactions in which staining by methylene blue was weak or not apparent (see Section F. below). YOUNG also classified two strains of H. anomala, one of which had an analogous killer phenotype to that of strain 16 (NCYC 434), thus distinguishing it from the strains of HK_6. On the basis of available phenotype data it is not possible to relate the second strain of H. anomala (NCYC 435) to the HK_6 strains but the properties of their respective killer factors suggest that they are different (see Section D. below).

Resistance patterns of non-killer strains supported the grouping of killer strains determined by their interaction. That is, strains within a killer group had analogous patterns of activity against non-killers while patterns between killer groups were different.

The killer factors present in culture filtrates of strains belonging

to different groups were shown to differ with respect to biochemical properties. The tests also demonstrated that strains from five killer groups produced multiply-active culture filtrates. It was therefore inferred that the killer and resistance phenotypes of these strains were composite and hence it is possible that relationships between killer strains producing toxins in common were not recognised. The use of resistant mutants could be employed to demonstrated such relationships, which would ultimately be confirmed by a structural analysis of the individual killer factors.

B. The killer toxins of H. saturnus

Differences in the killer activity spectra of H. saturnus strains at neutral and low pH suggested involvement of more than one killer factor showing different pH optima. The killer spectrum of H. saturnus NA 9 (denoted type HK_{1a}) was less extensive than those of the remaining H. saturnus strains AWRI 354, NCYC 22, NCYC 23 and CBS 5761 (denoted type HK_{1b}), but because the two groups were cross-resistant, the difference was likely to be only quantitative.

Assays of the killer activity of strains from both killer types in different culture filtrates revealed, in addition to quantitative differences between the two groups, the presence of at least two distinct toxic principles. At low assay pH (pH4.3) but not at near neutral pH (pH6.0), group 2 indicator strains (strains 495, 498 and 499) were sensitive to a toxin of high activity in shake culture filtrates. Filtrates from cultures at pH6.0 were more active than those from culture at pH4.3. At neutral pH, group 1 indicator strains (strains 10, 560 and 413) were sensitive to a different activity which was predominant in static culture filtrates irrespective of culture pH.

Killer strains of type HK_{1b} also produced a similar amount of this activity in shake culture filtrates irrespective of production pH, but this activity was much reduced in the shake filtrates of HK_{1a} strain 9. That the two groups of indicator strains under these conditions responded to different toxic principles was shown by the abolition of activity against group 2 indicator strains following heat treatment (50^oC for 15 min) of the culture filtrates, while the responses of group 1 indicator strains remained unaltered. However, at low pH, group 1 indicator strains 10 and 560 were sensitive to both the thermolabile and thermostable toxins. Thus the response to a culture filtrate containing relatively more thermolabile to thermostable toxin activity following heat treatment was reduced to a second but stable value representing the level of thermostable activity. Therefore assay under the appropriate conditions revealed that both HK_{1a} and HK_{1b} stains, under analogous cultural conditions, produced broadly similar amounts of the thermolabile and thermostable toxin activity. However killer strain 9 (HK_{1a}) shake culture filtrates, irrespective of pH, exhibited comparatively much less thermostable toxin activity, and with respect to pH, contained less thermolabile toxin activity at low pH.

The membrane diffusion plate test, employing assays under appropriate conditions to distinguish the killer activities, provided an indication of the molecular size of the two toxins. Toxin diffusible through standard dialysis membrane was active on group 1 indicator strains at near neutral pH and therefore corresponded to the thermostable active principle. Thus its molecular weight is not likely to exceed 10000-15000. The results were not clear-cut for the toxin (thermolabile) active against group 2 indicator strains. The activity produced by HK_{1b} strains was considerably less diffusible through dialysis membrane than through the control membrane whereas the toxic

activity produced by HK_{1a} strain 9 was not diffusible through the dialysis membrane. The apparent non-diffusibility of the strain 9 toxin may, in part, be due to reduced toxin biosynthesis which, coupled with probable low membrane-diffusability could not be detected by the test plate. Evidence for reduced production of this toxin compared to HK_{1b} strains was seen by the relatively smaller amount of this activity in culture filtrate produced at low pH (see Figure 2.1). It was concluded therefore that the toxin corresponding to the thermolabile active principle was at the threshold of diffusibility through dialysis membrane, indicating a molecular weight well in excess of 10000-15000.

Evidence for the presence of three distinct killer activities in various culture filtrates of H. saturnus was apparent from the effect of pH on activity against key indicator strains. The killer factors in both the chemically defined and static produced culture filtrates, which were active against only group 1 indicator strains, exhibited strong activity across the entire pH range 3.5 to 7.0. The active principle, which was thermostable, is designated toxin-A. The activity of toxin-A was fully stable to changing pH in this pH range (by subsequent assay at either low or near neutral pH). Tartrate and phosphate buffers (or counter ions) appeared to interfere with toxin-A activity as was evident by the near-uniform activity exhibited on unbuffered media over the same range of pH.

The thermolabile activity, which was almost exclusively produced in shake culture filtrates and could be assayed in the presence of toxin-A by group 2 strains, showed two different pH-activity profiles depending upon the origin of the culture filtrate. This was most readily observed in the low and near neutral pH shake culture filtrates of killer strain 9 when assayed against strain 498. Activity present in the filtrate

produced at low pH was maximal at or below pH4.3 but declined sharply to little or no activity at pH5.0. The active principle is designated toxin-C. On the other hand, activity of the filtrate made at neutral pH exhibited a plateau of activity at and below pH4.7, which declined sharply to zero at pH5.5. When assayed with indicator strain 10, it also displayed optimal activity at pH4.3-4.7, but because of the presence of a small proportion of toxin-A, a plateau of activity was shown at assay values exceeding pH5.5 (see Figure 2.3). The active substance is designated toxin-B. Supportive evidence for this interpretation was obtained by the nature of pH-activity profiles following culture filtrate heat treatment which eliminated all activity exhibited against strain 498 and resulted in near uniform response with respect to pH against strain 10 due to residual thermostable toxin-A activity. The effect of changing pH on the stability of toxin activities in the low and near neutral pH culture filtrates of strain 9, containing predominantly toxins-C and -B respectively, indicated that they were different. Activity of the former was most stable near pH4.3 whereas that of the latter was stable between pH4-6. Both the low and near neutral pH-produced shake culture filtrates of strain 354 assayed on strain 498 showed similar pH-activity profiles (type B) with near or complete stability of the killer activity at pH6.0. This indicated that either toxin-B and -C were present in similar amounts or that toxin-B predominated. The partial loss of activity from the pH4.3-produced filtrate when exposed to pH6.0 suggested that toxin-C may predominate and thus revealed underlying stable toxin-B activity.

Electrophoretic analysis of killer activity present in the various culture filtrates of H. saturnus, carried out on cellulose acetate membrane strips and identified by subsequent bioautography on appropriate indicator strains, largely supported interpretation

regarding the distribution of the three active components. By a combination of both their activity against key indicator strains and response to heat treatment these components were, in part, correlated with the three toxins identified from their pH-activity profiles and by pH and temperature inactivation experiments. The most rapidly migrating electrophoretic component was detected on plates of group 1 indicator yeasts (strains 10 or 560) at either pH4.3 or 6.0 but not on plates of group 2 strains (strains 495, 498 and 499). It was unaffected by heat treatment (50°C for 15 min) and therefore corresponded with toxin-A. As was also demonstrated by direct assay of the various culture filtrates, toxin-A was highly active in the culture filtrates of H. saturnus strains produced by all cultural conditions investigated except for the shake filtrates of strain 9 in which this activity was comparatively low. On the other hand, the lesser migrating broad band of activity was composed of two overlapping components which have not yet been rigorously indentified. Their general identity on bioautographic plates, corresponding to toxin-B and -C activities, was disclosed by activity against the highly sensitive group 2 indicator strains 498 and 499, in addition to activity against strains 10 and 560 at low pH. Furthermore their activity against all four indicator strains was abolished following heat treatment. Confirming the distribution of toxin-B and -C activity in culture filtrates of H. saturnus strains as determined by direct assay, their activities were high in shake but not static culture filtrates, except for the low pH shake culture filtate of strain 9 in which activity was much reduced.

Although no assay system was developed to differentiate toxin-B from toxin-C, the indicator strain 495, when compared with strains 498 and 499, had little or no sensitivity to toxin-C. This was evidenced by the reduced assay of activity in the shake culture filtrate of stain 9

(designated toxin-C) produced at pH4.3 when compared to that of the pH6.0 shake-produced filtrate (which contained predominantly toxin-B) (see Figure 2.1B). Accepting this, indicator strain 495, which was sensitive only to the electrophoretic component appearing nearest the origin, was therefore presumably sensitive only to toxin-B. This assumption appeared reasonable since only solutions which contained toxin-B produced a band of activity on strain 495. The less active partially resolved electrophoretic component of intermediate mobility to those of toxins-B and -A (on bioauthographic plates of strains 10 and 560), therefore presumably corresponded with toxin-C. This toxin, which was essentially free of toxin-B activity in only the low pH culture filtrate of killer strain 9, was not of sufficient activity following electrophoresis to be detected on plates containing strain 498 to confirm its electrophoretic mobility.

Obviously further experimentation is required to more fully characterise toxin-B and toxin-C activities and to confirm their electrophoretic identity. A more potent solution of toxin-C would facilitate this work. A differential assay for toxin-B and -C appears possible by exploiting (i) the reduced sensitivity of strain 498 to toxin-C at pH4.7 compared with pH4.3 and (ii) the absence of sensitivity of strain 495 to toxin-C. Greater electrophoretic resolution of toxins-B and -C might be obtained at another value of pH, bearing in mind the unstable nature of toxin-C.

Killer activity of the toxins present in culture filtrates of *H. saturnus* was confirmed by the loss of viability in log-phase sensitive cultures. Culture filtrates of *H. saturnus* strain 57, a strain which did not elicit killing on methylene blue plates, manifested only slight ihibitory activity against sensitive indicator strains at either pH4.3

or 6.0. Culture filtrates of other non-killer stains were similarly non-toxic. Killing by toxin-A was demonstrated by the lethal effect of static culture filtrates, which contained only small amounts of other toxin activities, and by the loss of viability at pH6.0, at which only toxin-A was active. The chemically defined medium culture filtrate of killer strain 354, which contained only toxin-A by assay, resulted in comparable killing of sensitive strain 10 at both pH4.3 and 6.0. It must be pointed out that either or both tartrate and phosphate or their cations interfered with the activity of toxin-A, as was evident from the pH-activity profile determined on media containing these buffers, whereas activity was near uniform with respect to pH in unbuffered media. Inorganic ions and buffers affect the activity of various antibiotics and toxins, for example, polyenes (LIRAS and LAMPEN, 1974) amino-glycosides (DONOVICK, BAYAN, CANALES and PANSY, 1948) and colicins (REYNOLDS, 1966 cited by REEVES, 1972; KOPECKY, COPELAND and LUSK, 1975) by affecting either their binding to the target cell or receptor, or some subsequent event. Since solutions containing toxin-B also contained lesser amounts of toxin-C and vice versa, it was not possible to conclusively demonstrate killing by the individual toxin. However, the shake culture filtrates of strain 9, which, when produced at low and near neutral pH contained predominantly toxin-C and -B respectively, were both highly lethal to the sensitive strain 498 at pH4.3. These two components, when partially separated on CAM strips, exhibited the killer reaction on bioautographic plates.

Certain native, but not denatured proteases, inactivated toxin-A and toxin-B. Toxin-A activity, which was predominant in static culture filtrates, was inactivated only by pronase and was unaffected by papain, pepsin and trypsin. Toxin-A, obtained from culture in a chemically defined medium was only sensitive to the action of pronase. On the

other hand, toxin-B, predominant in pH6.0 shake culture filtrates, was inactivated by all four proteases tested. The nature of toxin-C could not be deduced because of the apparent resistance of the toxins present in low pH culture filtrates buffered with tartrate. Papain and pronase, at least, were known to be active in tartrate buffered culture filtrates at low pH as was evident by the inactivation of toxins from several other Hansenula species. However, it was possible that (i) an enzyme inhibitor was either produced in only low pH culture filtrates of H. saturnus or that it was only active at low pH or (ii) toxin comformation changes, aggregation, or binding of ions at low pH prevented access of the cleavage sites to the protease molecules. YOUNG and YAGIU (1978) also reported differences in the susceptibility of toxins in culture filtrates to inactivation by proteases at different pHs.

C. Killer toxins of Hansenula yeasts active at pH6

Of the additional 18 species of Hansenula (22 strains) tested for H. saturnus -like killer activity at pH6.0, three species (strains 500, 496 and 5762) exhibited considerable activity and a further five yeasts displayed weak inhibitory activity. The three killer strains, like those of H. saturnus, killed at least 8 of 19 Hansenula species at pH6.0, but the spectrum of activity of each killer was different, except for the strains 496 and 5762 which showed similar spectra. These two strains were cross-resistant suggesting that their toxins are similar. Because strains 496 and 5762 were weakly sensitive to the pH6.0 active toxins of the H. saturnus groups and of strain 500, their toxins were likely to differ from these. H. saturnus strains and strain 500 were cross-resistant, suggesting a possible similarity between their pH6.0 active toxins.

Because the activities of the culture filtrates of killer strains 500, 496 and 5762 were weak at pH6.0, few characteristics could be determined. The activity of strains 496 and 5762, unlike those of strain 500 and toxin-A, were not diffusible through dialysis membrane and hence were probably macro-molecular toxins. The pH6.0 active toxin produced by strain 500, while stable at pH6.0 was inactive at pHs exceeding 6.5. A thermostable component was present which amounted to less than 10% of the total activity when assayed against the indicator strain 560 following heat treatment to inactivate the heat labile, low pH active killer principle. Because of inadequate assay sensitivity following CAM eletrophoresis this component could not be correlated with the pH6.0 stable and active component. In contrast, the toxins produced by strains 496 and 5762 in culture filtrate at pH6.0 were slowly inactivated at room temperature (pH6.0), possibly by the presence of a protease, and were rapidly inactivated at 37°C (only tested at pH4.3). Their pH optima for killing was near pH4.7 but they elicited considerable activity against the highly sensitive strain 10 to a pH of 6.5. The toxins of both strains 496 and 4762 were lethal to log-phase sensitive cells at pH4.3, but were only inhibitory at pH6.0. Total cell count was not monitored and hence killing is not eliminated by this test. This contrasted with the strong killer activity exhibited at pH6.0 on agar-plates suggesting that, like the killer factor of <u>Sacch. cerevisiae,</u> the toxin was relatively unstable in solution unless appropriately stabilised.

Although not apparent from plate culture tests, shake culture filtrates of killer strains 16 (HK_4) and 10, but not 113 (both HK_6), were active against strain 560 at pH6.0. The toxin activity of strain 10 was thermostable, and while being maximally active at low pH, displayed considerable activity beyond pH6.0. Electrophoretic analysis

revealed a single component with a mobility about five-fold that of toxin-A. Strain 16 produced two toxin components as was evident by pH and temperature inactivation experiments and electrophoretic analysis (see Section D.). Whether either of these two toxins or a third minor component (revealed by thermal inactivation experiments) produced the plateau of activity near pH6.0 has not yet been determined. This could be tested by bioautography at pH6.0 following CAM electrophoresis.

These results therefore demonstrate that, in addition to *H. saturnus*, at least three other species of *Hansenula* produced toxins exhibiting appreciable killer activity at pH6.0. These toxins (including that of strain 10) resemble toxin-A in being stable and active over a wide range of pH. With the exception of those from type HK_3 strains (strain 496 and 5762), they are diffusible through dialysis membrane. The toxins of the latter two strains were similar, if not identical, and differed from toxin-A in having a definite pH optimum for killer activity (near pH 4.7). Those of killer strains 10 and 16 were probably structurally unrelated to toxin-A since the producer strains were not cross-resistant with *H. saturnus* or in fact with strains of killer types HK_2 and HK_3. However, the killer activity of strain 10 resembled that of toxin-A in being stable to heat and active over a wide range of pH. The absence of killer activity by strains 10 and 16 in plate culture at pH6.0 is difficult to explain but may be related to increased culture growth through greater aeration and availability of nutrients in shake liquid culture.

D. Low-pH active killer toxins

Strains of *H. saturnus* produced three toxins, all of which were active at low pH: toxin-A, highly active at pH3.5-7.0; toxin-B, optimum

pH4.3-4.7; and toxin-C, optimum pH at or below 4.3. Their distribution and approximate proportion in various culture filtrate preparations was determined both by direct assay and by bioautography following CAM electrophoretic separation using indicator strains 10 and 498 under appropriate conditions. Thus the quantitative differences in toxin production in liquid culture by strain 9 compared with HK_{1b} strains suggests a basis for the observed differences between the activity spectra of the two groups on culture plates. It is probable that each toxin would show its respective pH optimum on solid media, so that the seeded yeasts in plate tests sensitive at pH6.0 would reveal only toxin-A activity, whereas strains exclusively sensitive at low pH demonstrate only toxin-B or -C, or both killer activities. Since the activity spectra of HK_{1a} and HK_{1b} differed simultaneously at low and neutral pH (compare response of strains 496, 16, 413, 4307 and 5640 to HK_{1a} and HK_{1b} killer activities, Table 2.3), this suggested that the reduced production of toxin-A activity by strain 9 observed in both low and neutral shake culture filtrates would account for these differences. There may be other explanations.

HK_2 activity was more extensive at pH4.3 (18/27 strains) than at pH6.0 (14/27 strains). All but two strains (strains 496 and 5762) sensitive at pH6.0 were also sensitive at pH4.3. Both electrophoretic analysis and assay following heat treatment suggested that at least two different toxins were produced. The major component (>90% of total activity) was optimally active between pH3.5-4.7, and was inactivated at higher values of pH. The lesser component(s) (see Section C.) was heat stable and active at both pH4.3 and 6.0. YOUNG and YAGIU (1978) have also characterised the killer activity of strain 500, finding two active components in similar proportion by gel chromatography; a high molecular weight component which was associated with both protein and carbohydrate

while the chromatographically retarded component was only accompanied by traces of these. The latter component may correspond with the dialysis membrane diffusible component of these studies. It would therefore appear that the two components reported by these workers were not resolved by CAM electrophoresis, which did however allow detection of a third minor component. Diffusion on the bioautographic plates, as was the case for toxins -B and -C, may have obscured recognition of the two components separable by gel chromatography. It seems likely that the indicator strains used in this study were sensitive to the same killer activity studied by YOUNG and YAGIU (1978) as it was also found to be thermolabile and inactivated at pH6.0. One difference reported here is partial inactivation by pronase. The presence of at least two different toxins probably accounts for this difference, different production and assay systems resulting in altered proportions of and sensitivity to each component.

The extent of HK_3 activity on plates at both pH4.3 and 6.0 was similar except against strains 500 (inhibitory activity) and 54, which were only sensitive at pH6.0. However, culture filtrates of HK_3 killer yeasts produced at pH4.3 and 6.0 showed vastly different properties; in the first, killer activity was optimal at pH3.5-4.3, depending on the indicator strain, and was inactivated at pHs beyond this range. On the other hand, activity produced at pH6.0 was optimally active at slightly greater pH showing little activity at pH4.3 or below, was stable between pH4.3-6.0 and was more thermolabile than the lower pH active component. The low pH-active toxin revealed only one component electrophoretically and differed from the low pH active toxins of H. saturnus and strain 500 which it most resembled by being pronase resistant.

The effect of pH on the culture filtrate activity of strain 16, HK_5,

when assayed against the two indicator organisms, strains 10 and 560, revealed two distinct toxins. The major toxin, which was optimally active against strain 560 between pH3.5 to 4.3, was inactivated at pH6.0 and was only slowly inactivated at 37°C (pH4.3), appears to correspond to a toxin from this yeast studied by YOUNG and YAGIU (1978). Although the toxin studied by these workers was pronase resistant, the partial inactivation of activity by pronase observed in these experiments may be due to inactivation of the second component which was active against both indicator strains 10 and 560, and possibly not detected by their assay system. This component was non-mobile during electrophoresis and had an optimum pH of 4.3 for activity. It was also inactivated at pH6.0 but was more thermolabile than the major component. A minor component, active against strain 560, was apparent by residual activity following heating or inactivation at pH6.0 of the major toxins (see Section C above).

The H. anomala strains 10 and 113 (HK_6), although exhibiting indistinguishable activity spectra by the plate phenotype test, produced toxins of contrasting properties in culture filtrates. The former was by contrast stable to heat and changing pH and showed activity at pH6.0, but both were optimally active at low pH. The toxins from two different strains of H. anomala NCYC 434 and 435, studied by YOUNG and YAGIU (1978), appear to most resemble that of strain 113 in being thermolabile and inactivated at pH6.0. The culture filtrate activity of strain 113 was however only extremely weak and therefore was only partially characterised.

CAM electrophoretic analysis of the culture filtrate activities of killer strain 111, HK_5, demonstrated at least two active components, one highly active against indicator strain 10 and another more rapidly

mobile toxin (possibly two unresolved components) active against both strains 10 and 560. The component active only against strain 10 exhibited a characteristic clear zone surrounded by a wide but well defined hazy zone which only weakly stained with methylene blue. The survival assay however demonstrated strong killer activity. This toxin, showing a broad optimum for activity at pH3.5-4.7, with some activity at pH6.0, was fully stable between pH3.0-5.0 and lost little activity at 37°C; it was rapidly inactivated at 80°C. The toxin(s), that were only weakly active against strain 560, resulted in only about a 40% reduction in viability. In contrast with the major component, it displayed a lesser range of pH for optimal activity (pH3.5-4.3) being rapidly inactivated at pH5.0, and was relatively more thermolabile.

The culture filtrate activities of the hazy zone killers of HK_7 and HK_8 were either only weak or undetectable and could not therefore be readily characterised. As discussed below in section F, the filtrate of strain 2431 may contain the killer component: total cell count was not monitored in the survival assay and hence killing is not ruled out; inhibition zones against non-killers stained with dye. Should the killer component be demonstrated in the culture fluid, it may correspond with the component that was stable below pH5.0 and optimally active near pH4.7. Clearly these two groups of yeasts require further study to characterise the killer factors involved.

E. The effect of culture conditions on the production of killer activity

The finding that citrate assimilating yeasts brought about a dramatic increase in pH of the culture medium buffered with citrate-containing buffers emphasised the importance of monitoring and control of pH in

uncharacterised killer systems, particularly in view of the irreversibly unstable nature of many killer factors with respect of pH. Sodium tartrate proved to be a suitable replacement buffer (pH drift < 0.3 pH units at 0.1M-tartrate concentration) while permitting comparable yeast growth (comparison of citrate negative strains buffered with either 0.1M-citrate or 0.1M-tartrate) and toxin production. The potassium salt is preferred, but it is doubtful whether the sodium salt at the concentrations employed in this study would significantly affect results.

In general, assay of a killer yeast culture filtrate by a particular indicator strain revealed a killer zone with analogous definition to that exhibited by plate culture suggesting that the same killer factor(s) was responsible. However, among the killer strains active in agar plate culture at pH6.0, only strains of H. saturnus produced a high titre of pH6.0 active toxin (toxin-A) relative to that displayed by culture on agar medium. These strains also produced similar amounts of toxin-B at pH6.0 as at low pH even though this toxin is only active at pHs below 5.0. All of the strains which produced toxins active only at low pH on agar medium, with the exception of strains 113 (HK_6), 2431 and 497 (HK_7) and 2564 (HK_8), yielded culture filtrates showing strong killer activity. The latter three strains exhibited hazy zone activity on agar plates and only weak hazy zone activity in their culture filtrates, which was unaffected by different modes of culture. Assay of their culture fluid supernatants following centrifugation discounted inactivation by filtration and furthermore these activities were stable at room temperature in liquid medium. It may be possible to obtain more active solutions of these killer factors by expression from agar plate cultures or by the addition of a stabilising component in liquid culture. The apparent weak activity may be due to the unavailability of

indicator strains of greater sensitivity. PHILLISKIRK and YOUNG (1975) reported that of the 59 killer yeasts examined, three failed to show killer activity in their culture filtrates, two of these being strains of Hansenula. Some bacteriocins produced by, for example E. coli or Streptococci, while being produced (detected) on agar plates were not produced (detected) in liquid medium even when in some cases the medium was supplemented with agar, dextran or glycerol (REEVES, 1972; ROGERS, 1976). The killer factor of Sacch. cerevisiae, while being stable in liquid culture under static mode conditions, was rapidly inactivated by surface action, but this could be reduced by the addition of gelatin (WOODS and BEVAN, 1968). Other substances, including glycerol poly(vinyl alcohol), poly(ethylene glycol), also stabilise Saccharomyces killer activities (KOTANI and colleagues, 1977; PALFREE and BUSSEY, 1979).

The effect of culture mode on toxin production in liquid medium indicated that, at least comparable and in many instances, greatest yield of toxin was obtained under shake (aerobic) conditions, possibly through stimulation of cell growth. However, toxin production could not be related to cell number since many of the yeasts produced strong pellicle growth by static culture. This problem could be overcome by determining the total dry weight of the culture. The results also suggested that the toxins were probably not susceptible to inactivation by surface action.

A study of factors affecting toxin production by strains 9 and 354 may provide useful information concerning toxin-A biosynthesis and excretion. Under analogous conditions strains 9 produced similar amounts of toxin-B and -A, except in shake culture in which toxin-A

activity was greatly reduced (pH independent effect). No experiments were made to examine this observation, but a possible explanation might involve an inhibitor (production of which is stimulated by aeration, or alternatively is normally produced but only activated in shake culture conditions) or a biochemically altered species of toxin-A showing susceptibility to surface inactivation. Inactivation by a protease would appear to be eliminated as the toxin was stable for at least 24h at 25°C. Alternatively, toxin-A excretion may be controlled by the oxygen tension in culture.

F. Relationship between methylene blue staining and killing

The blue staining of dead cells or cell colonies with the dye methylene blue is reasonably specific since viable cells are able to reduce the dye and therefore do not appear to be stained. The application of this reaction to the detection of killer factors and the general acceptance of the technique suggests that there is a high specificity, however exceptions are known. MITCHELL and BEVAN (1973, 1974) have studied mutant yeasts which visibly stain in their viable state, that is, the cells take up larger amounts of dye and possess a reduced ability to decolourise the dye and on the other hand ULACZEWSKI, WOODWARD and CIRILLO (1978) observed that the methylene blue-staining technique under-estimated non-viability, at least, by the technique that they employed.

In the present survey, the intensity of staining of inhibition zone edges produced by strains 16 111, 10, 18, 113, 2431 and 497 was generally weak and widely variable between susceptible indicator strains (strains of H. anomala in particular stained only weakly) causing some difficulty in their interpretation. In view of the possible lack of

specificity with methylene blue staining, caution was exercised in interpretation of the staining reaction. Weak or indefinite staining was recorded as an inhibitory reaction until killing was demonstrated by an independent technique. Thus it is possible that some apparent inhibitory reactions were in fact killing, and the reverse might also be possible. Strain 10 (HK_6) for example produced only weak staining at best and yet its culture filtrate reduced survival of the susceptible strain 560. The definition of the inhibition zone on assay medium was analogous for each, suggesting that the filtrate contained the same active component as produced in culture on plates. An explanation for the absence of staining might involve delayed killing, in which the indicator and producer colony growth was well advanced relative to cell death and thus depleted the dye to a concentration level insufficient to show visible staining of the toxin killed cells.

The killer ability of active filtrates (scored as killer zones on assay plates containing dye) obtained from all but one group of killer yeasts (HK_7) was confirmed by monitoring viability of a sensitive strain. Killer strains 16 and 111 produced only a weak staining reaction on plates of the indicator strains 560 and 10 respectively, yet these culture filtrates, which produced only weak staining, were highly lethal to the same strains, as was evident by determination of survival. HK_7 activity, and that of additional components produced by killer strains 496 and 16 were to some degree growth inhibitory, but since viability was not related to total cell counts, it is not possible to distinguish inhibitory activity from killing in these instances. The weak culture filtrate activity of strain 2564 (HK_8) was not tested.

Strain 2431, HK_7, which produced only weakly staining hazy zone activity against HK_6 strains produced more intense staining against

non-killer susceptible strains. Its culture filtrate activity was only weakly inhibitory to strain 10 (survival assay made at pH4.3) but since the total cell count was not followed and the experiment was not made at its pH optimum of pH4.7-5.1, killing of strain 10 (HK_6 strain) cannot be ruled out. Definition of the hazy zone against strain 10 was similar to those which stained more intensely on other strains suggesting that the same killer factor was involved, but this needs to be demonstrated by, for example, comparative inactivation experiments.

G. Techniques used to characterise toxins

The various biochemical techniques adopted differed somewhat in their ability to differentiate between the various toxins examined. The use of at least two different indicator micro-organisms greatly facilitated characterisation of individual toxins in mixtures.

The physiological properties of the toxins of each killer group were unique, supporting the basis for categorisation of the producer strains. The physiological characteristics examined were: (i) killer zone type and (ii) effect of pH on activity.

(i) The killer zone definition on assay media, that is clear or hazy zone with either sharp or hazy zone edges, was generally analogous whether the killer yeast was cultured on agar or in liquid medium of the same composition. Thus it was assumed that the killer activity produced in liquid culture was analogous to that observed in plate culture. A notable exception was HK_3 strains which at pH6.0 on agar medium produced a clear zone whereas the liquid culture filtrates produced hazy lightly stained zones, sometimes with a small clear inner zone. The killer zone produced by strain 111 (against indicator strain 10) was

distinctly characteristic, being a clear central zone surrounded by a wide hazy zone.

(ii) The pH-activity profiles of killer yeast culture filtrates fell into six groups. Strains, belonging to four killer-groups, which exhibited more than one profile type when assayed against different indicator yeasts were interpreted as being the result of at least two distinct killer activities. In some instances a composite profile resulting from the action of more than one killer activity against a particular indicator strain, was demonstrated. The effect of pH on the stability of culture filtrate activity indicates that the pH-activity profile is a characteristic of the toxin and not the result of an interplay of interference effects, such as proteolytic inactivation. The absence of activity by some toxins at pH6.0 for example, is due to rapid irreversible inactivation at that pH. The use of different indicator yeasts in this study appears to be, at least in part, responsible for the fact that other groups of pH-activity profiles in Hansenula yeasts can not be detected by the assay system of PHILLISKIRK and YOUNG (1975).

Physicochemical properties, while assisting in characterisation of the toxins, also provided important data concerning toxin stability. The properties determined were, (i) thermal stability, (ii) pH-related stability and (iii) diffusion through membranes.

(i) The toxins fell into two major groups with respect to thermal stability: those stable at 25°C but inactivated at 37°C and those stable at 37°C but slowly inactivated at 80°C. In general the toxins showing activity at pH6.0 were thermostable whereas those only active at low pH (<pH5.0) were thermolabile. Toxins of the four Hansenula strains

studied by YOUNG and YAGIU (1978) were inactivated at 35°C and although not tested for activity at pH6.0, all were inactivated by exposure to pH6.0 for 4h at 20°C. The toxins of killer yeasts of other genera are also generally characterised as being thermolabile (YOUNG and YAGIU, 1978).

(ii) Four patterns of pH-related stability in the range pH3.0-6.0 were observed: A. complete stability, B. stable below pH5.0, C. stable above pH4.3, and D. only stable near pH4.3 (approximately pH4.0-4.5). The second category was predominant. The toxins inactivated at high pH (pH6.0-7.0) did so rapidly (<5 min) ruling out proteolytic action. All of the toxins produced by Hansenula which were studied by YOUNG and YAGIU (1978) were of the second category described as were the toxins isolated from yeasts of other genera.

(iii) The membrane-diffusion plate tests indicated that at least one toxin from strains of six killer groups was of sufficiently low molecular weight to exhibit partial diffusion through dialysis membrane. Comparison of these with control plates (0.22 μm membrane) suggested that many of these toxins diffused only at a low rate through dialysis membrane. This may also be interpreted as indicating that low molecular weight toxins, in native form however, exist as aggregates and only the monomer is able to penetrate the membrane. Gel chromatography of extracts made from the culture medium of various other killer yeasts suggested that some killer strains produced only single high molecular weight toxins (or aggregated species) while others produced multiple forms (aggregates or separate species) (BEVAN and WOODS, 1968; BUSSEY, 1972; YOUNG and YAGIU, 1978). BUSSEY and SKIPPER (1975, 1976) reported that the toxins present in cell-free isolates of both Sacch. cerevisiae and T. glabrata were glycoproteins. In the former a low molecular

weight protein was shown to be the active component (PALFREE and BUSSEY, 1979).

Two biochemical techniques, namely cellulose acetate membrane electrophoresis and protease digestion, provided further characterisation of the various toxins; the former technique in addition provided further evidence for the elaboration of more than distinct toxin species by strains of five killer groups. Under the conditions employed, toxin mobility was generally low (0-0.5 cm/h) for many of the toxins and hence resolution in some instances was incomplete, but the technique was sensitive and was fairly rapid. It is also possible to quantitate the method (BETINA, 1973). Resolution may perhaps be improved by altering the pH at which electrophoresis is conducted and by dialysis of the sample against buffer using low molecular weight cut-off tubing. In several cases, bioautographic detection sensitivity was inadequate and a method of toxin concentration will need to be found to further characterise these toxins.

Toxins produced by the majority of killer strains (eight strains from six killer groups) were inactivated by either or both papain and pronase and were resistant to pepsin and trypsin. Killer activities from five of the eight killer groups studied by YOUNG and YAGIU (1978) were sensitive to various proteases, pronase, pepsin and papain. The results of the present study strengthens their conclusion that most, if not all, killer factors contain a proteinaceous component necessary for killer activity. In contrast to the results reported in this study, the killer activity of strains 16 and 500 were not inactivated by pronase, but these differences may be due to different conditions of the test and assay system. In view of the observation that both strains 16 and 500 produce at least two different toxins, the indicator strains used in

this work may be sensitive to a toxin (pronase sensitive) not detected by their indicator strain (NCYC 1006).

Conclusion

While further work is required to more fully interpret the results obtained, which in some cases are still incomplete, several conclusions may be drawn which provide a foundation for more detailed investigations.

The results confirm the finding of PHILLISKIRK and YOUNG (1975) that *Hansenula* yeasts show a high incidence of killer ability and support the conclusions reached by YOUNG and YAGIU (1975) that (i) yeasts of the same genus produce distinct proteinaceous killer factors, many of which share properties in common and (ii) biochemical tests support the classification of killer yeasts on the basis of cross-reactivity. Additionally, the reaction of non-killer strains resulted in the recognition of analogous groups of killer yeasts determined by their interaction. This finding largely supports the suggestion of AL-AIDROOS and BUSSEY (1978) and ROGERS and BEVAN (1978), using resistant mutants, that the toxins have both unique and common sites of attachment or attack on sensitive cells.

In summary:

1. Yeasts of the genus *Hansenula* show a high incidence of killer ability exhibiting a relatively wide spectra of activity against strains of *Hansenula.*
2. Eight classes of killer yeasts were recognised on the basis of cross-reactions between killer strains supported by the resistance patterns of non-killer strains. Each class was composed of a

single killer type and its respective resistance phenotype, but five of these contain mixed phenotypes.

3. Physiological, biochemical and physico-chemical tests provided evidence for structurally distinct toxins produced by strains belonging to different killer types.
4. Filtrates from cultures of all but four killer strains detected in agar plate culture showed readily demonstrable killer activity (reduced sensitive strain viability).
5. Strains from five killer groups produced more than one toxin, being readily demonstrated by cellulose acetate membrane electrophoresis in which killer activity was detected by bioautography against appropriate indicator strains.
6. Strains from at least three killer groups produced toxins exhibiting considerable activity at near-neutral pH while all other toxins were active within the range pH3.5-5.0. Six patterns of the effect of pH on killer activity were observed.
7. Generally, killer factors which were active at near-neutral pH were more stable and smaller molecules than those active only at low pH which were thermolabile and irreversibly inactivated at pH6.0. This indicates that there are biochemically two major classes of killer factors.
8. A killer component from eight strains was to some degree diffusible through dialysis membrane, but none were conclusively shown to be excluded by a cellophane membrane, indicating that they are either low molecular weight or macromolecular toxins.
9. Killer factors from eight strains were inactivated by a proteolytic enzyme, indicating that a proteinaceous component is needed for activity.

CHAPTER 3

H. saturnus KILLER ACTIVITY: ASSAY, PRODUCTION AND PROPERTIES

INTRODUCTION

This chapter is concerned with the quantitation, production in liquid medium and partial characterisation of *H. saturnus* killer toxin (SKT) activity, with a view to the development of a procedure for purification of the active principle(s). The work reported in this chapter, which concerns the stable and widely active killer activity produced by *H. saturnus*, was carried out prior to the identification of the low pH active toxins that are also produced by H. saturnus killer strains (see Chapter 2).

Various assay procedures are available for quantitative estimation or comparison of antibiotics in culture fluids; these include the end-point assay performed in either solid or liquid media, turbidimetric assay and the agar diffusion assay, all of which have been described with numerous modifications. The agar diffusion technique, in which the zone of inhibition is related to the concentration of the active substance, is widely employed. However it is subject to numerous factors which, apart from drug concentration, also affect the size of the inhibition zone. However under controlled conditions the technique is capable of reliable quantitative assay of good precision (LEES and TOOTILL, 1955a; SIMPSON, 1963; HEWITT, 1977). WOODS and BEVAN (1968) described an agar diffusion assay (well test) for quantitation of the killer factor, which under the conditions adopted showed a linear relationship between the square of

the width of the inhibition zone and the log of killer factor concentration, at high concentrations.

Conditions were defined for a reliable sensitive assay of SKT activity using the highly sensitive yeast *H. anomala* NA 10 as the indicator organism. The conditions examined, which mainly affect assay sensitivity, were composition of the agar medium, density of seeding, pH and incubation regime. Two methods of assay using the large plate technique were adopted for routine assay.

Factors affecting the production of SKT in liquid medium by the *H. saturnus* killer strains 9 and 354 were studied using chemically defined media. The effect of incubation temperature, pH and mode of culture were examined, and a minimal medium for SKT production (by strain 354) was developed by modification of the chemically defined medium of Wickerham. A stock of SKT active filtrate derived from the culture of strain 354 in a 20 litre capacity laboratory fermentor was prepared for the experimental work of Chapter 4.

The culture filtrate SKT activity was partially characterised with a view to evolving a suitable method for its purification. The properties examined were, stability with respect to pH and temperature, isoelectric point, inactivation by proteases, approximate molecular size and solubility in different chemical milieu.

MATERIALS AND METHODS

Media

The composition of MEA, PDA, MYPGA and YEPDA is given in Materials and Methods of Chapter 2. The formulation of medium No.12 was as given by GROVE and RANDALL (1955; p. 221). SKT assay agar was composed of BBL PDA (19.5g), Difco ME (12.5g) and Oxoid Nutrient Broth No.2 (2.5 g) prepared in one litre of distilled water. The medium was dissolved by boiling, dispensed in 200 and 320 ml quantities and sterilised by autoclaving at 15 lbf/in^2 for 15 min.

The Lederberg yeast chemically defined medium (LEDERBERG, 1956: based on the medium described by OLSON and JOHNSON, 1949) contained per litre of medium: D-glucose, 15g; $NH_4H_2PO_4$, 6g; KH_2PO_4, 0.2g; $MgSO_4.7H_2O$, 0.25g; Na Citrate, 10g; 10 ml of vitamin solution; and 10 ml of trace metals solution. The vitamin solution contained per 100 ml: biotin, 0.2 mg; Ca pantothenate, 10mg; Thiamine-HCl, 10 mg; para-aminobenzoic acid, 10 mg; inositol, 200 mg; and Pyridoxine, 10 mg. The solution of trace metals contained per 100 ml: $ZnSO_4$, 160 mg; ferrous ammonium sulphate, 100 mg; and $CuSO_4$, 5 mg. The latter two solutions and glucose (20% (w/v) solution) were sterilised separately by autoclaving.

Composition of Wickerham's chemically defined medium was as described by VAN DER WALT (1970). Stock solutions of each component had the following composition per 100 ml of solution: Ammonium sulphate, 10.0 g; D-glucose, 20.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 1.0 g; $MgSO_4$, 0.5 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g. A solution containing the compounds supplying trace elements was 100-fold concentrated, the vitamins 20-fold

concentrated and the amino acids 20-fold concentrated. Additional chemicals used per 100 ml of solution were: Ammonium acetate, 10.2 g; Ammonium citrate, 12.27 g; and Potassium nitrate, 15.3 g. Except for the solutions of glucose and vitamins which were filter sterilised (0.45 μm, Gelman), the solutions were sterilised by autoclaving (15 lbf/in^2 for 15 min). All chemicals were of the highest purity obtainable.

Procedure for the assay of SKT activity.

Factors affecting the SKT calibration curve were studied using a well test technique performed in standard petri-dishes (GROVE and RANDALL, 1955; WOODS and BEVAN, 1968). The source of SKT activity was derived from *H. saturnus* NA 9 grown aerobically in a chemically defined medium.

A. Inoculum

H. anomala NA 10, grown in 10 ml YEPD (contained in a 100 ml conical flask) with vigorous shaking at 25°C for 24h, was stored at 4-6°C for use up to seven days.

B. SKT sample

The well-test reference standard sample of SKT was a culture filtrate of strain 9 grown aerobically in 500 ml of Lederberg medium at 28°C for 50h (see Methods below). The filtrate was adjusted to pH6.0 with 1M-NaOH, dispensed in 10 ml quantities and stored at -18°C. For each test the standard was serially 2-fold diluted (5.0 ml volume) in 0.081M-sodium potassium phosphate buffer, pH6.0 (MUNRO, 1970).

C. Preparation of assay plates

Molten agar medium, dispensed in appropriate volumes (usually 12.5 ml) was cooled to 48°C, inoculated with the required volume of indicator yeast culture (routinely to 5 x 10^5 cells/ml) and immediately poured into a 90 mm diameter standard plastic-petri dish. The plates were hardened at 4-6°C for several hours. Each plate contained six 7 mm wells arranged in a hexagonal array. Following the addition of sample (70 µl for 12.5 ml agar plates) to each well, the plates were pre-incubated at 4-6°C for 0-6h (depending on required sensitivity) and incubated at either 22-23°C or 28°C until the zones of inhibition were stable. Their diameters were measured to 0.1 mm with vernier calipers.

D. Assay technique

In the study of factors affecting assay, each dilution of the reference standard was tested in duplicate on two separate plates and the inhibition zone diameters averaged. In quantitative assay, each plate contained an internal standard (usually the crude preparation of SKT derived from killer strain 354, pH6.0) and two test or unknown samples, all in duplicate. The inhibition zone diameters for each sample were averaged and corrected using the internal standard (GROVE and RANDALL, 1955). A standard curve was constructed by plotting corrected average inhibition zone diameter of the standard against its relative concentration (log scale).

E. Large plate assay methods

The two large-plate-assay designs used, the completely random

arrangement and the Youden square design, were as described by LEES and TOOTILL (1955a,b). In the former, 12 in x 12 in glass plates (internal dimensions 28 cm x 28 cm x 1 cm) containing 200 ml of seeded SKT-assay agar were used and in the latter, 15 in x 15 in plates (internal dimensions 35.5 cm x 35.5 cm x 1 cm) contained 320 ml of the assay medium. The agar medium contained 0.1 - 1 x 10^6 cells/ml *H. anomala* depending on required assay sensitivity. The preparation of materials was as described for the petri dish assay.

In the completely random arrangement design, a pattern of eight rows by eight columns to 10 rows by 10 columns of 7 mm wells was made. These designs enabled from 16 to 25 samples (standards and unknowns) to be tested in quadruplicate. The samples were randomised, and plated out according to LEES and TOOTILL (1955a). On the 15 in square plates, two sets of 11 x 5 designs were employed allowing two Youden square assays each of 11 samples in total to be performed (LEES and TOOTILL, 1955b).

The plates were incubated and inhibition zones measured as described for the petri dish assay. In routine assay, by either large plate method, the plates were seeded to 5 x 10^5 cells/ml with indicator strain 10 and either incubated at 22-23°C for 30-36h or at 4-6°C for 4h followed by 28°C for about 20-24h. In the random arrangement designs, the inhibition zones diameters were averaged, and in assay, potency was determined from standards included in the test. The routinely employed reference standard was a culture filtrate derived from strain 354 grown is SKT production medium, and was assigned a potency of 100 arbitary units (a.u.) per millilitre. Potency determination in the Youden square design was performed as described by LEES and TOOTILL (1955b). A Fortran computer programme was written to facilitate potency calculation, and for assay in the range 20-100

a.u./ml of SKT, the assay curve was calculated by the least squares method (CHILTON, 1967). A test was included in the programme to detect replicate measurements showing a wide discrepancy, such as resulting from a distorted well or error in plating out the solution. Where possible an average value was inserted or that sample re-assayed.

Effect of growth conditions on SKT production

The effect of temperature, pH and mode of culture on SKT production by killer strain 9 was determined in the chemically defined medium of LEDERBERG (1956). A chemically defined minimal medium for production of SKT by strain 354 was determined by a systematic modification of the medium described by Wickerham (VAN DER WALT, 1970).

A. Preparation of media and culture conditions

Media of required composition (10.0 ml final volume) were made by pipetting, under sterile conditions, appropriate volumes of the required stock solutions. Each was inoculated (in 0.2 ml) to 0.1 - 1.0 x 10^6 cells/ml (total cell count) with *H. saturnus* 9 or 354, pregrown in YEPD, washed twice in sterile distilled water and resuspended in water to produce approximately 0.5 - 5.0 x 10^7 cells/ml. Duplicate cultures contained in 100 ml conical flasks plugged with nonabsorbant cottonwool were incubated at 25-35°C on a reciprocal action shaker (Paton Industries, Australia) set at 150 strokes/min.

B. Culture analysis

Cell growth was monitored by either total cell count (average of five counts on an appropriately diluted sample using a haemocytometer) or

turbidity measurement. Initially the absorbance at 650 nm (10 mm cell) of an undiluted culture sample read against a filtrate of the culture was made. Otherwise a sample of culture was diluted 30^{-1} to 40^{-1} with distilled water and its absorbance measured at 550 nm. pH was measured on the culture supernatant (10 min at 4000xg), which for SKT assay, was adjusted to pH6.0 and sterilised by filtration through an 0.45 μm pore-size Gelman or Millipore membrane.

C. Mode of culture conditions

250 ml quantities of Lederberg medium, contained in 1 litre flasks, were inoculated with washed cells of *H. saturnus* 9 to 1 x 10^6/ml of medium. The cultures were incubated either by shaking (120 strokes/min) (i) exposed to air or (ii) to a continuous flow of 5% CO_2 in N_2, or (iii) by static culture in an incubator. The temperature was 30°C and aliquots of culture were withdrawn at various time intervals to determine culture growth and SKT activity. In the anaerobic culture, sampling was effected by clamping the gaseous exit tube which expelled culture through a second tube which was normally clamped. The static culture was thoroughly shaken before sampling for SKT accumulation, but no reliance can be placed on the turbidimetric measurement of cell growth because of pellicle formation.

Production of culture filtrate SKT

A stock of culture filtrate SKT, derived from the culture of killer yeast 354 in SKT production medium (composition given in Table 3.1), was prepared in a 20 litre capacity laboratory fermentor on loan from Professor D.O. Jordan (University of Adelaide). The fermentation chamber (18 litre) was spherical in shape and attached to a vertical

cylinder (2 litre) which supported the external heating elements, and contained a thermistor (to monitor temperature), a sparging adaptor and several sampling ports. The main chamber contained a stirring paddle and foam "breaker", a gas exit port equipped with a condenser and several other ports, used to admit solutions. Compressed air was admitted to the fermentor via a filter and humidifying flask and distributed through the sintered glass membrane sparging adaptor. Temperature was controlled by a digital controller operating the heating elements.

In use, a suitable volume of distilled water was sterilised by boiling in the fermentation chamber while the components of the SKT production medium (see Table 3.1) were sterilised separately; glucose and tri-ammonium citrate, each dissolved in one litre of distilled water, were sterilised by filtration (0.45 m membrane) and the remaining salts, each in 10 ml of solution, and trace metal salts in 500 ml of solution were autoclaved at 15 lbf/in^2 for 15 min. In a typical production run, 10 litres of medium containing 1 x 10^6 cells/ml of <u>H. saturnus</u> 354 was incubated at 29°C with aeration (5 litres/min) and stirring (500 rev./min). The culture was harvested at about 5h following the attainment of stationary phase (total cell count) by continuous flow centrifugation in an MSE high speed 18 continuous action rotor operated at 12000 rev./min. The supernatant liquid was passed through a 0.45 m pore-size membrane (Gelman or Millipore) and stored at 4-6°C. This preparation is referred to as culture filtrate SKT.

<u>Properties of culture filtrate SKT</u>

The tests performed to characterise culture filtrate SKT are briefly described in the results or have been described in 'Chapter 2. The

activity of a 15 litre batch of culture filtrate SKT (derived from strain 354) was used as an assay standard and was assigned a potency of 100 arbitrary units (a.u.) per millilitre, at pH6.0.

RESULTS

Factors affecting assay

Of the many factors known to affect microbiological assay, those studied principally relate to assay sensitivity: composition of the assay medium; density of the indicator organism; regime of incubation (temperature); and pH of the sample and medium. The yeast highly sensitive to SKT activity, *H. anomala* NA10, was the assay indicator organism. Although this yeast produces a dense pellicle in broth culture, when grown in shake culture it produces an even turbid growth with few clumps of cells. The dose-response curve was prepared employing culture filtrate SKT (derived from *H. saturnus* NA 9 grown in Lederberg's medium), adjusted to pH6.0 and serially two-fold diluted in phosphate buffer. The following parameters were chosen from previous unreported work using PDA medium, and formed the basis for further development: 12.5 ml per petri dish of PDA, seeded to 1 x 10^6 cells/ml with *H. anomala*, contained six 7 mm wells filled with 70 µl of sample. Following incubation at 22-23°C for 30-36h, neat SKT activity produced an inhibition zone diameter of 19 to 20 mm.

A. Effect of assay medium composition

The media tested were PDA (1.5% agar), MEA (1.5% agar), MYPGA (2% agar), YEPDA (1% and 2% agar) and Medium No.12 (2.5% agar). Each, where necessary, was brought to pH6.0 with 1M-HCl or 1M-NaOH. Using the experimental parameters given above, PDA provided greatest assay sensitivity, the end point being 2^{-7} dilution of neat SKT solution, while sensitivity of the remaining media fell between 2^{-5} to 2^{-6}, except for Medium No.12 which was near 2^{-4} dilution. Definition of

the inhibition zone was sharp on all media except for the latter two. Although the PDA medium provided greatest assay sensivity, seed growth was poor. Addition of ME stimulated growth while resulting only in a small reduction in sensitivity of the assay. The effect of agar concentration between 0.75% to 2.5% (w/v) (prepared by adding agar to a mixture of equal volumes of PDA and ME) showed that zone size, but to a lesser extent sensitivity, increased with decreasing agar concentration. The lowest concentration of agar, 0.75% (w/v), was of sufficient strength to withstand distortion, and was chosen for routine assay. This medium is referred to as SKT assay medium.

B. Effect of seeding density

Seeding density of *H. anomala* (determined by total cell count) was tested in the range 1 x 10^5 to 1 x 10^7 cells/ml. Doubling cell density, over this range, resulted in approximately 1 mm decrease in the inhibition zone diameter with no significant change to the assay slope. Thus the sensitivity of assay was approximately proportional to seeding density. A cell density of 0.5-1.0 x 10^6/ml was chosen for routine assay, allowing adequate sensitivity of between 2^{-6} to 2^{-7} dilution units of neat culture filtrate SKT activity. Cell density proved to provide the best control over assay sensitivity, the lower limit being about 5 x 10^4 cells/ml for inhibition zones of sharp definition. The lower seeding densities required a longer incubation period.

C. Temperature

Preincubation of the plates at 4-6°C for 0-24h before incubation at 22-23°C resulted in both an increase to assay slope and sensitivity, the latter being much less affected. Preincubation for 5h at 4-6°C

followed by incubation at 30°C reduced the seed development time by about 4-5h for inhibition zones of comparable size to those obtained by incubation at 22-23°C. This regime was conducted with an electronic timer and conventional incubator placed in a cold room.

D. Effect of pH

SKT assay agar medium, adjusted to pHs in the range 4 to 9, containing samples applied at pH6.0, resulted in an increase of inhibition zone diameter with increasing pH. The relative increase was sharp between pH4.0 to pH5.5-6.0 with only a marginal increase to pH8, at which pH the normally sharp inhibition zones had hazy edges. Several buffers (0.05 - 0.1M) at pH6.0 were tested and all resulted in a decrease of inhibition zone size. The addition of potassium chloride to the medium also reduced zone size. pH of the sample (culture filtrate SKT) showed a much greater influence on the inhibition zone size, being for example 13.2 mm at pH3.0, 17.0 mm at pH4.0, 18.7 mm at pH5.0 19.2 mm at pH6.0 and 19.3 mm at pH7.0. Assay sensitivity was little affected at sample pHs between pH5.5 to 7.0. Samples were routinely assayed at pH6.0 on SKT assay medium without pH adjustment.

Large plate assay

Employing the conditions for assay established by the petri dish well test, the nature of the dose-response curve for crude SKT (culture filtrate SKT derived from strain 354 and arbitrarilly assigned a potency of 100 arbitrary units (a.u.) per ml) was examined by the completely random arrangement assay design. This was carried out using an 8 x 8 quasi-Latin square design for 16 samples at four replications. The assay curve, shown in Figure 3.1, was linear at SKT concentrations

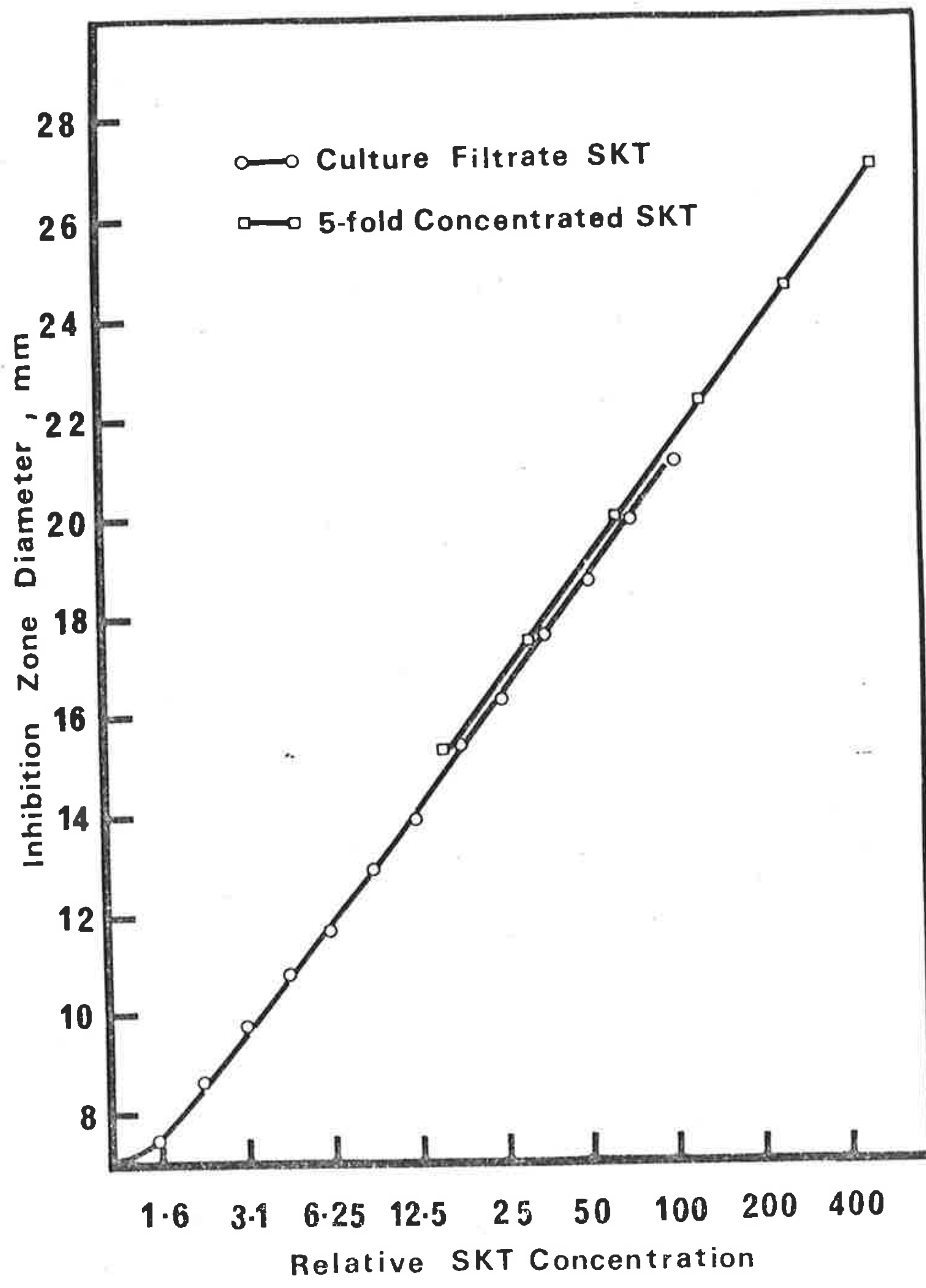


Figure 3.1. Dose-response curve of H. saturnus killer toxin (SKT) activity.
Neat or 5-fold concentrated culture filtrate SKT, serially diluted in phosphate buffer of pH6.0, was assayed in SKT assay medium, seeded with H. anomala 10 (1×10^6 cells/ml) and incubated at 22-23°C for 32h.

exceeding 10 a.u./ml when the averaged response, inhibition zone diameter, was plotted against relative SKT concentrations on a logarithmic scale. A similar curve was obtained by the Youden square assay design for the same concentration range of SKT (data not shown). The dose-response curve remained near linear for crude SKT to, at least, 500 a.u./ml (concentrated by incomplete lyophilisation). Following the practice of KAVANAGH (1972; 1976), dose-response curves exceeding a range of five-fold concentration of the standard were constructed by joining the individual points rather than calculating the line of best fit. This practice was particularly important at levels of SKT activity below 10 a.u./ml, where the dose-response relationship was non-linear.

Factors affecting production in chemically defined media

Initially, factors affecting SKT production were studied in the chemically defined minimal medium described by LEDERBERG (1956) using *H. saturnus* NA 9. In preliminary experiments, the effect of temperature and pH were examined in shake (aerobic) culture grown to mid-stationary phase. Incubation at 25°C and 30°C did not affect SKT production, which was reduced by 60% at 35°C. The effect of pH in the range pH3.0 to 8.0 was tested by adjusting the pH of the medium with 10% (w/v) lactic acid or 1M-NaOH. pH had little effect (100% ± 15%) on SKT production between pH4.0 to 6.5, but at more extreme pH values (pH3.0 and 8.0) only trace amounts of SKT were detectable although yeast growth was affected to a much smaller extent.

The kinetics of SKT production were examined under aerobic (vigorous shaking), static (no shaking) and anaerobic (shaking in an atmosphere of 5% CO_2 in N_2) growth conditions. The results are illustrated in Figure 3.2. The apparent extended lag-phase of culture growth was due

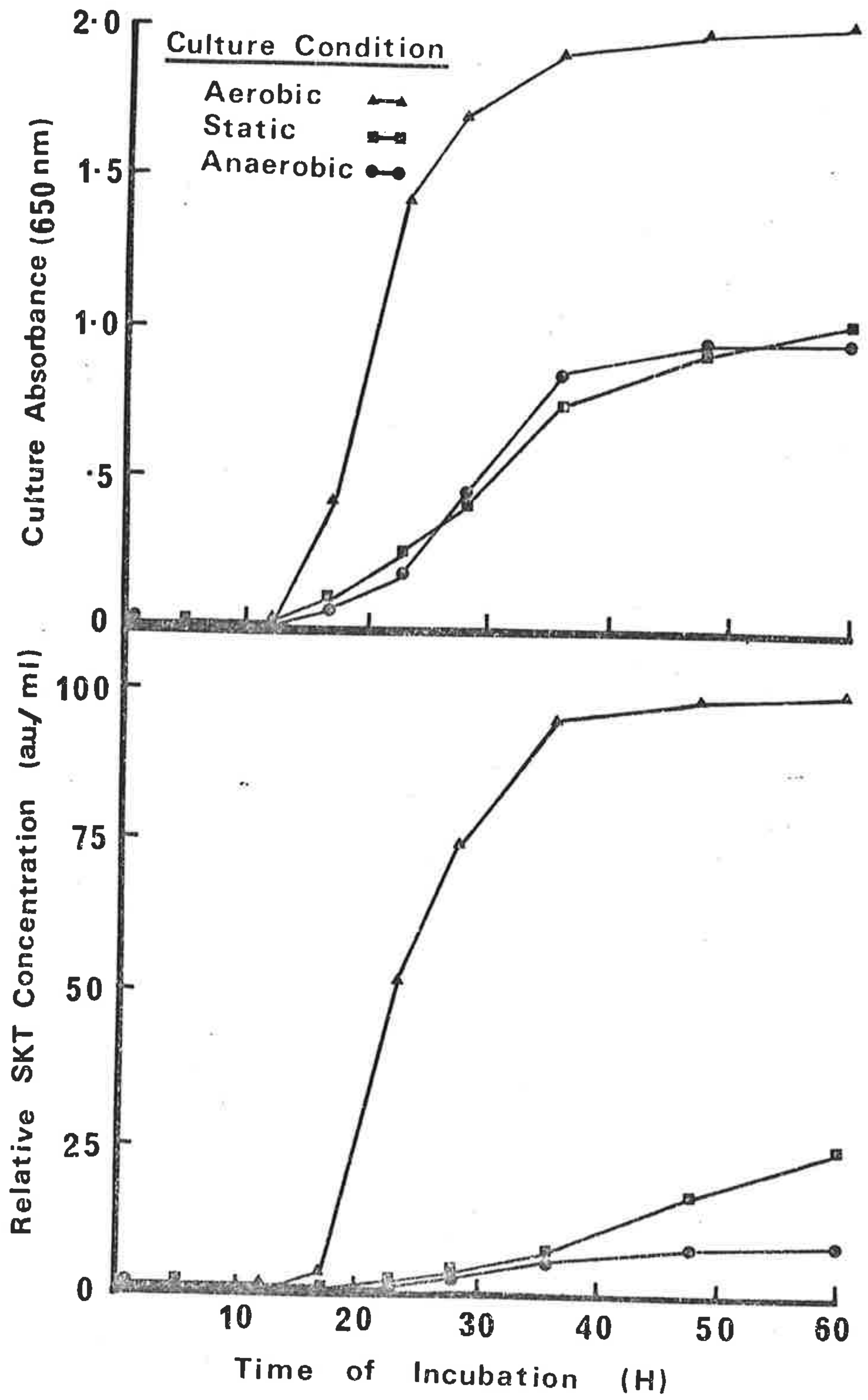


Figure 3.2. Effect of culture conditions on the growth of *H. saturnus* 9 and on SKT activity production in Lederberg's medium.

to the low sensitivity of cell growth measurement. Under anaerobic growth conditions, growth rate was minimal and entered stationary phase after 40h of incubation by which time 80% of the total SKT activity produced had accumulated in the culture fluid. Culture under static conditions stimulated cell growth, and SKT production exceeded that of the anaerobically grown culture by more than two-fold at 60h. Aerobic conditions greatly stimulated cell growth (total cell count of 4 x 10^8), being four-fold that produced in the anaerobic culture. SKT production was also greatly stimulated by aerobic conditions, approximately 80% of which was produced during the log and early stationary phases of culture. At 48h, SKT had accumulated to 10-fold the amount produced in anaerobic culture.

Formulation of the production medium

A. Chemically defined minimal medium for SKT production

The chemically defined medium described by Wickerham (VAN DER WALT 1970) was systematically modified to produce a minimal medium for optimal SKT production by *H. saturnus* AWRI 354. The Wickerham medium was intially modified to contain an excess of glucose (5.0 g/l), and the amino acids were omitted. Each test medium (10 ml), prepared from stock solutions of each component, was inoculated to 1 x 10^6 cells/ml with strain 354 and incubated with vigorous shaking for 40h (mid-stationary phase) at 28°C. Culture turbidity and pH was measured and the culture supernatant was assayed for SKT activity at pH6.0.

Pilot experiments using Wickerham's medium, although supporting similar levels of growth to that obtained with the Lederberg medium, resulted in low (30%) SKT activity production. The final pH was very

low (pH2.2), being well outside the range optimum for SKT production. Phosphate buffer was added to maintain pH in the range pH4.0 to 6.5, but the amount required interfered with culture growth. To overcome this problem, the nitrogen source counter ion , sulphate, was replaced by citrate. An initial pH of 6.0 resulted in a final pH of 5.0 and SKT activity production was approximately 1.3-fold that produced in the Lederberg medium. The effect of tri-ammonium citrate concentration on the culture of strain 354 is illustrated in Figure 3.3, which shows that, for pH's above 4.0, SKT activity production was proportional to cell growth.

To study the influence of culture medium components on SKT production, nitrogen limitation was employed to restrict yeast growth. The concentration of tri-ammonium citrate used throughout was 60 mM (14.6 g/l) and the initial pH of the medium was adjusted to 6.5. Omission of the vitamin solution did not reduce yeast growth or SKT production by more than 5% and was therefore omitted from the following experiments.

Potassium nitrate was tested both as the sole source of nitrogen and in conjunction with tri-ammonium citrate. As the sole source of nitrogen, growth and SKT production was maximal at 250 mM, (final pH of 4.9) at which growth was 50% and SKT 40% that obtained with 100 mM tri-ammonium citrate alone. Increasing concentrations of potassium nitrate were toxic to yeast growth. When combined with 45 mM tri-ammonium citrate, potassium nitrate in the range 10 to 250 mM stimulated growth by up to 20% but simultaneously decreased SKT production by up to 60% (maximum effects at 250 mM, final pH of 4.7).

The minimum limit concentrations, for maximal SKT production, of the other components of the medium were determined. The final composition

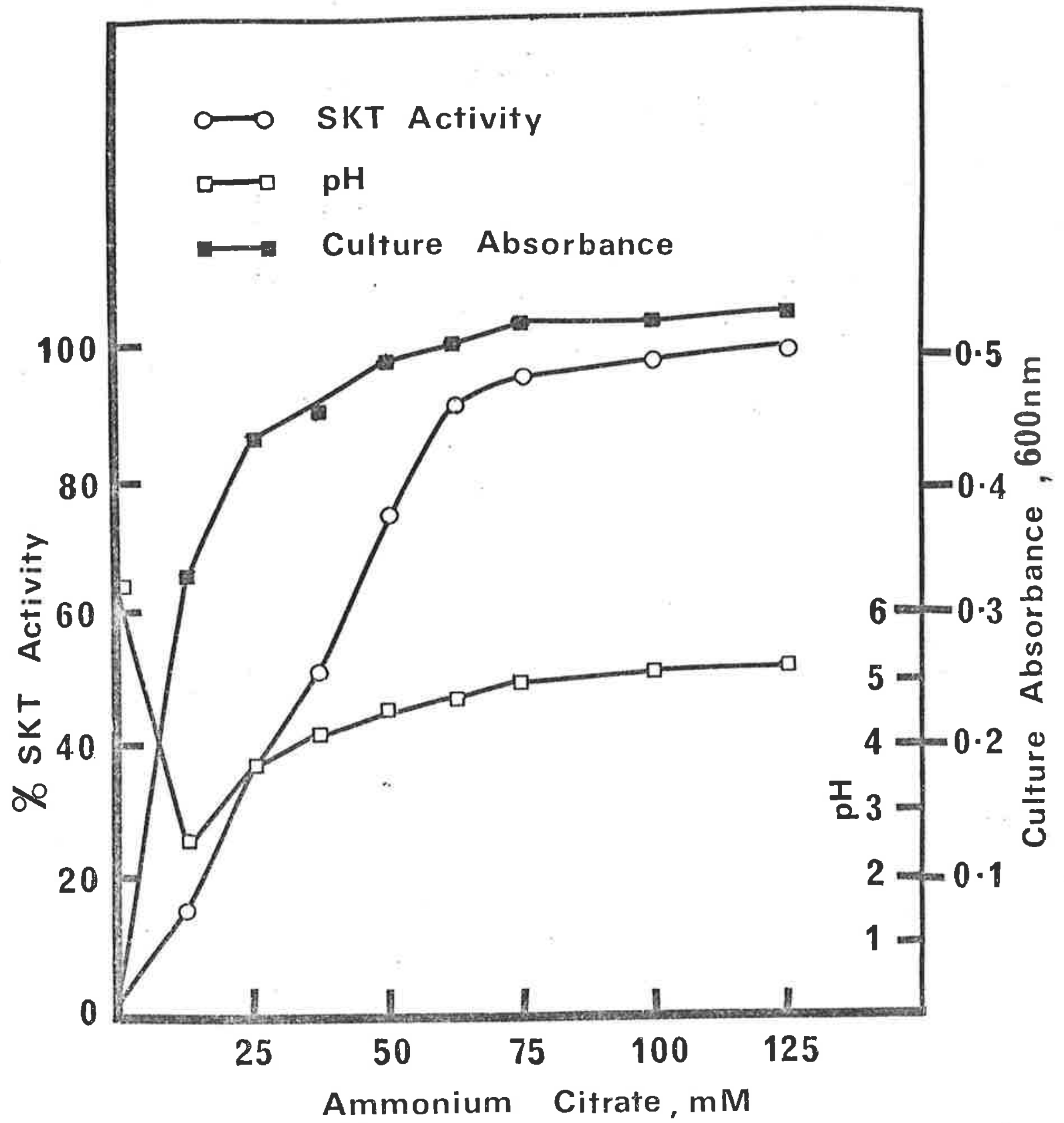


Figure 3.3. Effect of ammonium citrate on the growth of _H. saturnus_ 354 and SKT activity production in Wickerham's medium.

of the minimal medium for optimum SKT production is given in Table 3.1. This medium is referred to as SKT production medium.

Table 3.1. Composition of the chemically defined minimal medium for SKT productions by *H. saturnus* AWRI 354.

Component	Concentration (mM)
Tri-ammonium citrate	60
D-glucose	140
KH_2PO_4	2.5
$MgSO_4$	1.0
$CaCl_2$	0.05
Trace elements	as per Wickerham (VAN DER WALT, 1970)

B. Effect of pH

The initial pH of the SKT production medium between pH4.0 to 6.0 did not affect the production of SKT whereas at greater values of pH, yeast growth and SKT production were markedly reduced (see Table 3.2.) confirming the results obtained with the Lederberg medium. The initial pH of the medium was routinely adjusted to pH6.0. This was conveniently done by adjusting the pH of the tri-ammonium citrate stock with concentrated HCl prior to sterilisation.

Table 3.2. The effect of initial pH on SKT production.

Initial[1] pH	Final pH	Culture[2] turbidity	%SKT of maximum
3.96	3.22	0.325	100
5.08	4.35	0.330	100
6.03	5.21	0.355	100
7.04	5.55	0.350	77
8.02	6.95	0.010	3.3

[1] Adjusted with 5M-HCl or 5M-NaOH; [2] Absorbance at 550 nm (10 mm cell) of culture diluted 40^{-1} with distilled water.

C. Complex components

The effect of yeast extract, peptone and selected amino acids on SKT accummulation in SKT production medium was examined. See Table 3.3 for the results. As similarly observed in a previous experiment using a mixture of vitamins, yeast extract had no definite effect on SKT production although yeast extract markedly stimulated growth. Peptone, on the other hand, stimulated SKT production but not culture growth. However, yeast extract antagonised the stimulatory effect of peptone. The amino acids tested, alone or in mixture with peptone or yeast extract neither affected culture growth or SKT production. No further experiments were made to determine whether amino acids present in the peptone was responsible for its stimulatory effect on SKT production. YEPD medium (2% D-glucose) supported growth to a similar extent as did the control medium but SKT accumulation never exceeded 50% that of the control. Both the growth yield of killer strain 9 and production of SKT in the control medium was approximately 80% that of strain 354.

Table 3.3. The effect of yeast extract and peptone on SKT activity production by H. saturnus 354 in SKT production medium.

Components added to SKT production medium, pH6.0.	Final pH	Culture turbidity[1]	%SKT of control
Control (no additions)	5.16	0.35	100
0.1% YE	5.15	0.385	100
1.0% YE	5.51	0.41	105
1.0% YE (Oxoid)	5.62	0.43	95
0.03% Peptone	5.18	0.36	115
0.1% Peptone	5.16	0.36	135
1.0% Peptone	5.16	0.36	135
0.1% YE + 0.1% P	5.11	0.37	130
1.0% YE + 0.1% P	5.15	0.385	115
1.0% YE + 1.0% P	5.54	0.38	80
Amino acids	5.18	0.355	100
AA + 1.0% YE	5.47	0.40	90
AA + 1.0% Peptone	5.17	0.35	135
YEPD, pH5.9	7.62	0.34	44
YEPD, 0.08M-phosphate, pH6.0	6.28	0.33	40
Control, Strain NA 9.	5.11	0.30	83

Control medium, see Table 3.2; YE, Yeast extract (Difco); P, Peptone (Difco); AA, Amino acids: 10 μg/ml l-Histidine monochloride, 20 μg/ml dl-methionine and dl-Tryptophan; [1] Absorbance at 550 mm (10 mm cell) of culture diluted 40^{-1} in distilled water.

Larger scale production

Production of SKT activity in larger volumes of SKT production medium was examined. Two litre volumes of medium contained in 6 litre conical flasks were inoculated to 10^6 cells/ml with H. saturnus 354 and incubated at 28°C with vigorous shaking. Figure 3.4 shows the relationship between total cell number and accumulation of SKT in the culture fluid. SKT activity production paralled culture growth, reaching 60 to 70% of the maximum titre attained, at the transition from log to stationary phase of growth. During the log-phase of culture growth, SKT activity was approximately proportional to cell titre. SKT activity was stable during both mid and late stationary phase.

larger batch culture carried out in a 20 litre fermenter containing 10-16 litres of medium stirred and aerated with 0.5 culture volume of air per minute, showed a similar pattern of SKT production. A main difference was a more extended transition to the stationary phase, possibly through limiting aeration, which had the effect of increasing the time of attainment of maximum SKT activity production.

The maximum titre of SKT activity obtained in batches of 10-16 litres was similar to that obtained in 10 ml shake cultures (5%) in which the total cell population also reached 3-4 x 10^8 /ml. However, a 10 litre batch supplemented with 0.02% peptone yielded 20% greater SKT than achieved in 10 ml cultures. This finding was not followed up.

Properties of the killer activity

A. Effect of pH on killer activity

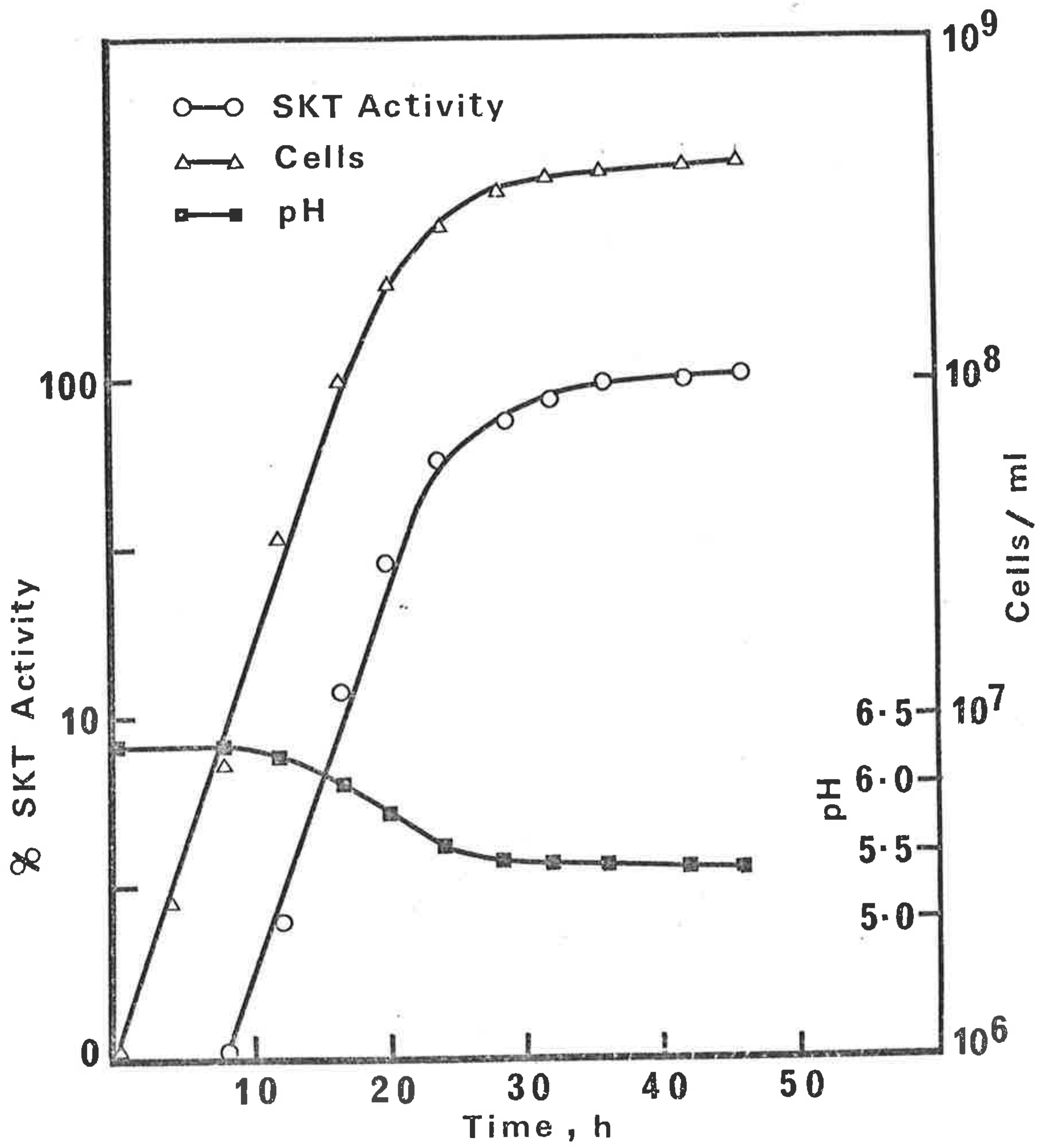


Figure 3.4. Production of SKT activity by *H. saturnus* 354 in SKT production medium.

Incorporation of 0.003% methylene blue in assay plates of pH4.0-8.0 indicated that SKT was highly active over the entire pH range (see Figure 2.4A of Chapter 2).

B. stability with respect to pH and temperature

250 ml of culture filtrate SKT brought to 23°C, was titrated with 2.5M-HCl or-NaOH to values of pH in the range 0.5 to 12.0. An aliquot was removed and immediately back-titrated to pH6.0 for assay. Two additional 5 ml portions were also removed, one kept at 23°C for 24h and the other for heating at 100°C for 15 min. All test samples (pH6.0) were assayed against a sample of SKT (at pH6.0) kept at 4-6°C for the duration of the experiments to determine residual SKT activity.

At 23°C, SKT was stable between pH0.5 and 11.0 for at least 15 min and between pH2.0 and 9.0 for 24h. Boiling for 15 min reduced activity by about 50% between pH3.0 and 7.0 where it was most stable but completely inactivated SKT activity beyond pH1.0 and 9.5 (see Figure 3.5).

C. Isoelectric point

The approximate isoelectric point was determined by electrophoretic behaviour of SKT on cellulose acetate membranes (Sepraphore III) at different pHs in 0.05M-Citrate phosphate buffers. SKT was detected by bio-autography against _H. anomala_ 10 in SKT assay agar. Experimental conditions were otherwise similar to those described in Materials and Methods of Chapter 2. A single band of SKT activity was detected which was cationic at pH4.0, non-mobile at pH5.0 to 6.0 and anionic at pH7.0.

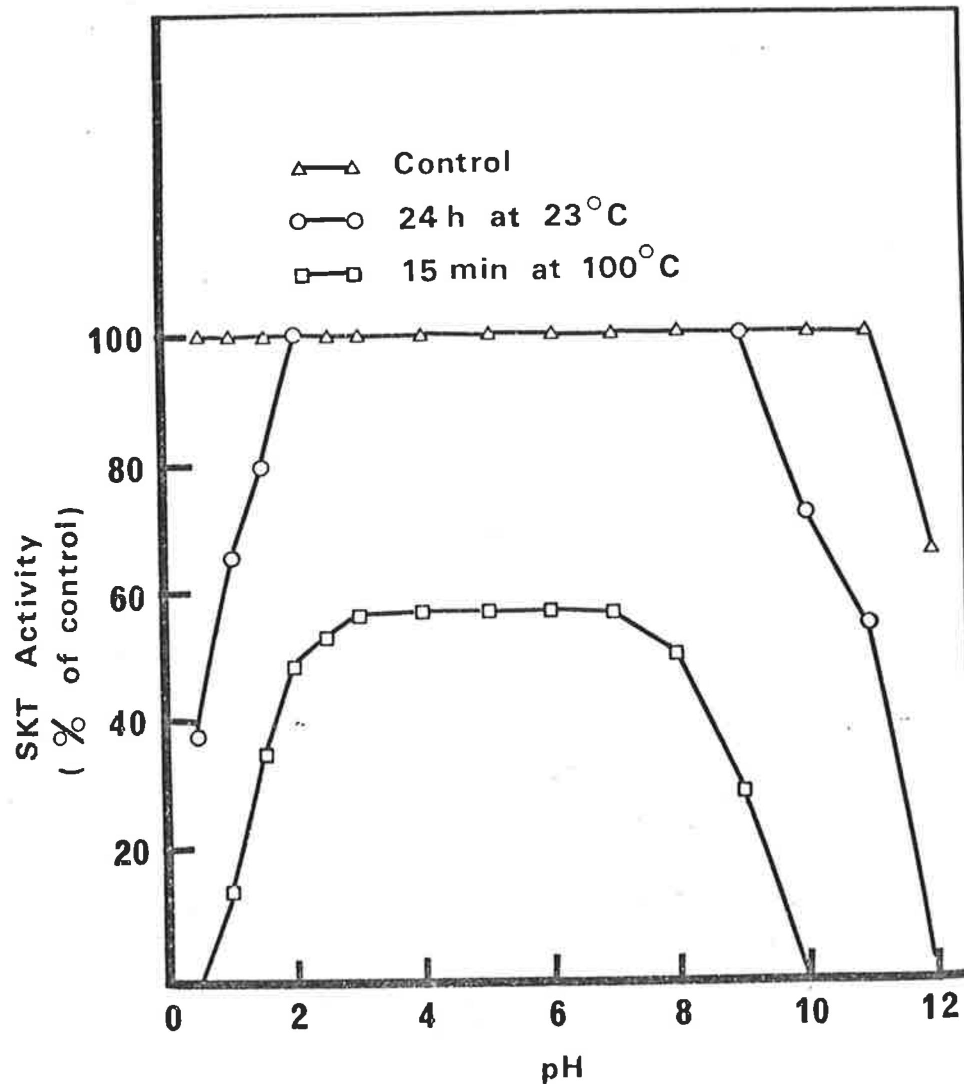


Figure 3.5. Effect of pH and temperature on the stability of SKT activity.
Culture filtrate SKT was titrated to different pH's and a portion immediately back-titrated to pH6.0 for SKT activity assay (Control). Other portions were incubated at 23°C or 100°C before being back-titrated for assay.

D. Inactivation by proteolytic enzymes

Four proteolytic enzymes were tested: papain and pepsin at pH4.0 and pronase and trypsin at pH6.5. These were prepared as described in Materials and Methods of Chapter 2. An equal volume of culture filtrate SKT at appropriate pH was mixed with an equal volume of enzyme solution and incubated at 37°C. Aliquots were withdrawn periodically and assayed for SKT activity. Only native pronase inactivated SKT activity, greater than an 80% reduction occurring within 60 min.

E. Precipitants

The effect of trichloroacetic acid (TCA), perchloric acid (PCA), phosphotungstic acid (prepared and used by the method of CHASE and WILLIAMS; 1968; p.251) and absolute ethanol on the solubility of SKT in culture filtrate was tested. Neither TCA (final concentration to 20%) or PCA (one molar final concentration) precipitated detectable SKT activity at either 23°C or 4°C. The possibility that these agents irreversibly inactivated SKT activity could not be discounted. Phosphotungstic acid at a final concentration of 2% produced a copious precipitate (SKT activity was not determined), however a similar precipitate formed with the uninoculated SKT production medium. Ethanol, at a concentration of 50% (v/v) produced a light precipitate overnight at 4°C. Assay of the precipitate, collected by centrifugation at 4°C and dissolved in 0.1M-sodium acetate, pH6.0, indicated that it contained 55-60% of the original culture filtrate activity. It may be possible to increase the recovery (or decrease denaturation) by precipitation with ethanol at lower temperature (see for example KAUFMAN, 1971).

F. Solubility in organic solvents

Solubility of SKT activity in organic solvents was tested by shaking culture filtrate directly with (i) organic solvents or (ii) inferred from its chromatographic behaviour on paper.

(i) Extraction

An equal volume of culture filtrate, at either pH2.0, 6.0 or 10.0, was shaken with one of nine solvents for a period of 15 min at 23°C and centrifuged to facilitate phase separation for sampling. The mixture was then shaken for an additional period of 2h before sampling again. The aqueous phase was tested for loss of SKT activity by assay at pH6.0 and the solvent phase was tested for the acquisition of activity by placing 100 μl on a 12.5 mm paper disc (Whatman No.3), evaporating the solvent in a stream of air and testing on an assay plate.

None of the following solvents, diethyl ether, petroleum spirit of b.p. 40-60° and b.p. 100-120°, benzene, toluene, chloroform, carbon tetrachloride, butan-1-ol, and ethyl acetate, extracted detectable SKT activity at either of the three values of pH tested. The aqueous phase from several mixtures, particularly at pH2.0 and 10.0 showed a reduction of activity, presumably due to partial inactivation of SKT.

To test water-miscible organic solvents, 1.0 g of lyophilised culture filtrate material was dispersed in 5 ml of solvent for 1h and the supernatant liquid tested for SKT activity by the paper-disc test. The solvents were, methanol, acidified methanol (to 1M with 11.5M-HCl), ethanol, acidified ethanol, butan-1-ol, acidified butan-1-ol, ethyl acetate, amyl acetate and acetone, none of which were effective.

Furthermore, none of these solvents reduced SKT activity of the residue (dissolved in 5 ml distilled water and adjusted to pH6.0) by more than 50%.

(ii) Paper chromatography

Culture filtrate SKT was applied to Whatman paper number 1, dried and then chromatographed in various solvents. The chormatograms, dried in a stream of air (room temperature) were examined under u.v. light, and either dipped in 0.25% ninhydrin (TOENNIES and KOLB, 1951) or bioautographed on SKT assay agar seeded with strain 10 (5 x 10^5 cells/ml) and incubated for 40h at 22-23°C.

Of the wide range of solvents and mixtures tested, only mixtures of an alcohol (for example, methanol, ethanol and butan-1-ol), glacial acetic acid and water (2:1:1 by volume respectively) partially eluted SKT activity (Rf of 0.4-0.5) while a portion of the activity remained at the origin. There was no definite or characteristic u.v. or ninhydrin positive material associated with the mobile SKT activity.

G. Ultrafiltration

Concentration of culture filtrate components by reduced pressure dialysis (method of HOFSTEN and FALKBRING, 1960) using Visking tubing (0.390 in flat width) did not fully retain SKT activity. On a five-fold reduction of the retained volume, less than 90% of total activity was retained and following a 50-fold reduction of membrane permeable components (reduced to 1 ml and made up to 10 ml with 0.08M-phosphate buffer, pH6.0) less than 50% of the total activity was retained.

Retention of SKT activity during ultrafiltration by various Amicon membranes was examined using an Amicon model 52 cell operated at a pressure of 40 lbf/in^2 (276 kP). Approximately 90% of the total activity was retained during a 10-fold reduction in volume on either a PM-10 or UM-10 membrane, but on dialysis (150-fold dilution of membrane permeable components) against 0.1M-acetate buffer, further activity became membrane permeable. SKT activity was fully retained on both UM-05 and UM-2 membranes under similar conditions.

DISCUSSION

The killer activity of the culture filtrate, derived from H. saturnus grown in a chemically defined liquid medium, was both stable and active over a wide range of pH. The H. saturnus killer toxin (SKT) activity was strongly hydrophilic as was evident by its insolubility in the common organic solvents and their mixtures, unless made polar by the addition of, for example, acetic acid. Although two chromatographic components were detected, only a single electrophoretic entity, a weakly acidic amphoteric species, was observed. SKT was generally soluble in the commonly employed protein precipitants, except ethanol. The diffusibility of SKT through membranes of different molecular size rejection properties suggested that it was a small molecule, of the order 10^4 molecular weight.

The chemical identity of SKT was not elucidated by the methods used. The absence of a correlation with a chemical component on chromatograms may have been a result of inadequate test sensitivity in relation to the biological activity of SKT. Sensitivity to pronase, which possesses a wide side-chain specificity, including the hydrolysis of various peptides, acylamino acids, acylpeptides, amides and esters (LASKOWSKY

and SEALOCK, 1971), coupled with resistance to other proteases, provided little additional information on its chemical nature. The possibility that these proteases were not active in the culture filtrate milieu could not be discounted. However these data are not inconsistent with SKT being a polypeptide molecule. The effect of pH on the activity of SKT in relation to its apparent isoelectric point of 5-6 suggests that the nett charge of the molecule is relatively unimportant to its killer action determined on agar media.

The dose-response curve, for crude culture filtrate SKT at high concentration was linear when the assay response was plotted against log SKT concentration. The change in slope at low concentrations may result from the removal, by dilution, of an interfering substance. The dose-response relationship is different to that exhibited by the killer factor described by WOODS and BEVAN (1968) but this may be due to the different conditions of assay. The large plate method is particularly suited to the simultaneous assay of many samples against a common standard and this proved to expedite assay compared with the petri dish method. The Youden square design was preferred since it eliminates both positional and temporal effects which may produce bias of assay by the completely random arrangement. In the present experiments, employing a crude reference standard, statistical treatment of the results was inappropriate; however reproducibility of assay was good, having a standard error of better than ±5%.

The killer activity of the chemically defined culture medium, containing growing cells of *H. saturnus*, increased approximately in proportion to the cell number. About 70% of the total SKT activity produced accumulated during log-phase. Toxin production by a sake killer strain, in a non-shaken culture was reported to parallel cell

growth (KOTANI and colleagues, 1977). However, killer factor production by *Sacch. cerevisiae* in a moderately shaken culture was not linearly related to the viable-cell titre, indicating that either the rate of toxin excretion varied with culture age or the toxin was inactivated disproportionately with toxin concentration (PALFREE and BUSSEY, 1979). OUCHI and colleagues (1978) have studied conditions which affect the stability of killer factor in liquid media. SKT was however fully stable under the conditions of culture.

Evidence that SKT was excreted into the culture medium comes from the effect of different conditions on cell growth and SKT activity production. Aerobic culture stimulated SKT production by more than two-fold (related to total cell number) than in anaerobic culture, although the possibility that anaerobically grown cells adsorbed SKT could not be discounted. Unlike killer factor production (WOODS and BEVAN, 1968; PALFREE and BUSSEY, 1979), yeast extract did not produce any significant effect on SKT production, possibly because *H. saturnus* is able to grow in vitamin free media (WICKERHAM, 1970). However, peptone stimulated SKT to a small extent, but further experimentation is required to establish whether the presence of amino acids or a complex nitrogen source was responsible for this effect. *Hansenula* yeasts are able to utilise nitrate as the sole nitrogen source but this reduced cell yield and toxin elaboration. Nutrients added to stationary phase cells did not stimulate toxin production in the absence of cell growth. It therefore seemed unlikely that SKT was released from lysed cells as is the case for bacterial endotoxins (RAYNAUD and ALOUF, 1970) or many bacteriocins (REEVES, 1972, pp. 33-45) but rather was synthesised and excreted by metabolically active cells.

CHAPTER 4

ISOLATION, PURIFICATION AND CHARACTERISATION OF TOXIN-A

INTRODUCTION

Characterisation and studies on the action of the H. saturnus killer toxin, SKT, could be expected to be greatly facilitated by the preparation of a highly purified fraction. A method was therefore sought for the preparative primary isolation of SKT from the chemically defined culture medium, and a procedure for its subsequent purification developed.

Procedures for the isolation of toxins from two killer yeasts, Sacch. cerevisiae and T. glabrata have been described. WOODS and BEVAN (1968) achieved a 40-fold purification of the killer factor derived from a supplemented chemically defined culture medium by a combination of ammonium sulphate precipitation, gel chromotography and dialysis. The unstable, papain sensitive killer activity was stabilised in the presence of gelatin. BUSSEY (1972) obtained a cell-free concentrate of killer factor activity by ultra-filtration and dialysis, and confirmed the finding of Woods and Bevan that the unstable macromolecule showed a hetero-disperse nature by both gel filtration and sucrose-gradient centrifugation. The concentrate contained 1-4 x 10^{11} killing units for protein concentrations of 2-2.5 mg/ml (BUSSEY and SHERMAN, 1973).

Recently PALFREE and BUSSEY (1979) reported the purification of killer factor activity present in a carbohydrate rich extra-cellular

concentrate made by ultrafiltration. The killer activity, precipitated in 10% poly(ethylene glycol), was dissolved in buffer containing 4M-urea to dissociate the toxin from aggregates and then loaded on a column of glyceryl-controlled- pore glass equilibrated with urea and also glycerol to stabilise the killer activity. The protein toxin, eluted with salt, was purified 79,000-fold over that present in the culture fluid. This preparation, obtainable only from killer strain cultures, was homogeneous by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and was correlated with the killer activity by their coelution from SP-Sephadex C-25.

BUSSEY and SKIPPER (1975) reported the partial purification of at least three components having killer activity from an extracellular concentrate derived from T. glabrata. Killer activity present in a poly(ethylene glycol) precipitate was chromatographed firstly on a double-column of Sephadex A-25 and Sephadex C-25, then on a column of hydroxyapatite and finally on a column of Sephadex A-50. A number of fractions containing killer activity of different specific activities were obtained, and were found to be glycoproteins when examined by SDS-gel electrophoresis.

The present indication for the chemical nature of SKT was a stable low molecular weight hydrophilic molecule, the activity of which was destroyed by pronase. The techniques employed for the macromolecular yeast toxins were therefore not appropriate. The small molecular size of SKT made its isolation by ultrafiltration (using for example an Amicon UM-2 membrane) except on an analytical scale, largely impractical. Ethanol precipitation was also considered unsuitable for the primary isolation of SKT from large volumes of culture filtrate. The strongly hydrophilic nature of SKT ruled out extraction by solvents. Therefore

gel filtration and the use of various adsorbants were investigated, with the aim of arriving at an isolation procedure suitable for a preparative scale.

As the chemical nature of SKT was not known with certainty, an index of SKT purity based on total substance was initially adopted. The dry weight-method was employed but is subject to interference by, for example, added non-volatile solutes. This problem was overcome by using buffers composed of volatile components. Gradient elution chromatography experiments indicated an association between SKT activity and Folin-Ciocalteu positive material by the Lowry method for proteins providing a more specific means for the estimation of SKT purity. Although this method is subject to well-known limitations, particularly interference by non-protein substances (CHASE and WILLIAMS, 1968; BONITATI, ELLIOTT and MILES, 1969) it is highly sensitive and applicable to routine assay.

The experimental section of this chapter is divided into two parts: the first is concerned with development of a preparative procedure for the isolation of SKT activity and the second with its purification and subsequent characterisation.

MATERIALS and METHODS

Materials

Sephadex G-10 (medium), G-50 (medium) and Blue-Dextran 2000 were from Pharmacia. Bio-Gel P-2, P-4 and P-10 (100-200 mesh) were the products of Bio-Rad Laboratories. Amberlite IRC-50(H) AG, Amberlite IR-120(H) AG, Dowex 21K(Cl) and Dowex 2-X8(Cl), all 14-52 or 20-50 mesh, were obtained from British Drug Houses (B.D.H.). Acrylamide was from Eastman, N,N'- methylenebisacryl amide from B.D.H., TEMED from Canalco and 2-mercapto-ethanol from Sigma Chemical Co.

Cytochrome C, type 11A, lysozyme, grade 2, ribonuclease A, type 11-A, and trypsin inhibitor, type 11-S were from Sigma Chemical Co. Insulin (purified from pork, Actrapid MC, 200 i.v./ml) and glucagon Novo, 1 i.v./vial containing 108 mg lactose were obtained from Novo Industri, A/S, Copenhagen. Bacitracin, 55.3 units/mg micronised powder came from Upjohn Pty Ltd. Trypsin was Calbiochem A grade. Pepsin was 10,000 E/g Merck. Bovine serum albumin fraction V was obtained from The Commonwealth Serum Laboratories, Australia.

Ethylenediamine, Unilab, came from Ajax chemicals. Standard 0.100M-HCl and 0.100M-NaOH were CVS, B.D.H. All other salts and solvents were analytical reagent grade from B.D.H. or Merck.

Preparation of standardised stock solutions for preparing ethylenediamine - acetic acid buffer mixtures

Composition of the constant ionic-strength buffer mixtures is given in Appendix Three.

0.20M-acetic acid

Residue free acetic acid was obtained by fractional distillation of the glacial acid (Analar, B.D.H.) at 118°C (atmospheric pressure). Approximate 0.20M-acetic acid was prepared by dissolving 12.044 g redistilled acid in freshly deionised distilled water to a final volume of 1000 ml. The exact strength was determined by titration with standard 0.100M-NaOH to the phenolphtholein end point and the stock diluted to 0.200 ± 0.002M.

0.20M -ethylenediamine

The starting material was ethylenediamine, 98.5% pure and containing 0.1% non-volatile matter. 500 ml of impure ethylenediamine was refluxed with 25 g solid NaOH (Pro Analysis, Merck) for 2h (PERRIN, ARMAREGO and PERRIN, 1966). The hot ethylenediamine (65°C) was decanted and distilled under CO_2 free air at atmospheric pressure, the fraction boiling at 117.0°C being collected. This was redistilled and stored for up to three months in airtight dark glass at room temperature. Approximate 0.20M-ethylenediamine was prepared by making up 12.042 g to 1000 ml with freshly deionised distilled water. The exact strength was determined by titration with standardised HCl. The titration-pH curves were plotted and the equivalence points determined from first derivative curves. The stock solution was then diluted to 0.200 ± 0.002M.

Assay of SKT activity

Exploratory test. The quantitative assay of numerous samples, such as column effluent fractions, consumed considerable materials and time, and

was therefore preceded by a semi-quantitative test performed to locate and estimate the extent of dilution required for quantitative assay. An aliquot (0.5 ml) of each fraction, adjusted to approximately pH6.0 as described in Methods of Chapter 2, was tested for SKT activity on a large plate containing several dilutions of the reference standard culture filtrate.

Quantitation. The Youden square method (one to two self-contained assays per plate) was generally used except when the simultaneous assay of more than 12-14 samples was required, in which case the completely random arrangement was employed (see Methods of Chapter 3). The samples were titrated to pH6.0±0.1 unless the volume was insufficient in which case the pH was adjusted with 3M-HCl or 3M-NaOH as described in Methods of Chapter 2. Samples contained in aqueous acetic acid were made pH6.0 by dilution with an appropriate volume of approximately 0.05M -ethylenediamine. Samples were diluted in 0.081M-phosphate buffer, pH6.0, unless noted otherwise, to fall within the range 25-100 a.u./ml. Each assay was calibrated against the reference standard culture filtrate SKT (pH6.0, refrigerated at -18°C in small volumes) serially two-fold diluted in phosphate buffer.

Protein estimation

Lowry method. Protein was estimated by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) using bovine serum albumin as calibration standard. Absorbance was measured at 750 nm in 10 mm cells.

U.v. absorbance. Protein was estimated from u.v. absorbancy measurements in 10 mm quartz cells using a Hitachi UV-VIS spectrophotometer. The protein concentration was calculated by the

method of WARBURG and CHRISTIAN (LONG, 1961);

$$\text{Protein (mg/ml)} = 1.55\ A_{280} - 0.76\ A_{260}.$$

Dry weight

Lyophilisation. This method was used when a small amount of material was present in a large volume or when a non-destructive method was required. The sample volume was reduced to about 10 ml under reduced pressure by rotary film evaporation with the water both set at 35°C. The sample was freezedried in a 50-150 ml flask and kept over P_2O_5 *in vacuo* until constant weight was achieved. The dried material was then removed by dissolution in appropriate solvent and made up volumetrically for further analysis.

Destructive method. Aliquots were dried in 0.5 ml or 5.0 ml capacity aluminium planchettes to constant weight at 105°C and cooled in a dessicator prior to weighing. The small volume planchettes were weighed on a Microforce balance (Mark 2B, C.I. Electronics Ltd, England) and the larger planchettes on a Mettler analytical balance (Model H20T). The weight of a hydroscopic sample was corrected back to zero time.

pH measurement

pH was measured with a glass combination electrode (N-65, Jenaer Glaswerk, Schott & Gen., Mainz) and Townson digital pH-meter (Townson, Australia) two point calibrated with primary standard phthalate buffer, pH4.00 (Beckman), and phosphate buffer, pH6.86 (Beckman) at 22-23°C.

The pH of solutions, of low ionic strength (<0.025I), were read by inserting the electrode and taking the reading after one minute had

elapsed, by which time the pH value had stabilised.

Conductivity

Conductivity measurements were made at room temperature with a Townson Conductivity Meter (Model 2103A, T.P.S. Pty. Ltd., Australia) and modified probe (K = 1.015). The probe, normally operated by immersion in about 20 ml of solution was modified to measure smaller volumes (3.0 ml) by sealing two of the three external communicating ports and applying the solution to be measured via the remaining port. The meter was calibrated with standardised 0.020M-KCl taken to have a conductivity of 2.50 mS/cm at 20°C with a correction factor of +0.05 units per degree in the range 15-25°C. Sample temperature was measured and all values are reported for a temperature of 22°C.

Gel chromatography

The technique of chromatography on columns of xerogels has been thoroughly documented: MALE, (1967); DETERMANN (1968); FISCHER (1969); REILAND (1971).

A. Column Chromatographic equipment and technique

Exploratory work was carried out in small glass columns, 1 cm x 30 cm (Quickfit CR 12/30) packed with either Bio-Gel P gels (100-200 mesh) or Sephadex G type gels (medium) to a bed volume of 25 ml, according to the manufacturers' recommendations. The column dead-space was reduced by packing with 1 mm glass beads. For analytical evaluation and preparative experiments larger columns of bed volume 130 ml (2 cm x 40 cm, Fischer & Porter) and 200 ml (2.6 cm x 40 cm, Pharmacia K26/40) were

used. The columns were packed and operated at room temperature (constant 22-23°C) and eluted at 10 cm/h controlled either by a constant head reservoir or eluant pump (Peripump, Townson & Mercer, Australia; Holter pump, Model 904, Extracorporeal Medical Specialities Inc., U.S.A.).

The column effluent was continuously monitored at 280 nm using a quartz flow- through cell (5 mm optical path length, 0.6 ml capacity) attached to a Hitachi Perkin Elmer 139 UV-VIS spectrophotometer equipped with a 10 in strip chart recorder (Rikadenki B-140) operating in the transmittance mode. The chromatographic profiles reported show "% optical transmission" for a 5 mm optical path length. The effluent was then collected by either a Paton (Paton, Australia) or Gradipore (Townson, Australia) fraction collector. The sample was applied to the drained gel bed as described by MAITLAND (1967).

The change in eluant composition was carried out in the column. Several centimetres of gel was removed to allow for a change in swelling and subsequently replaced as required. Three to four total column volumes of buffer were then passed through the column before commencement of the experiment.

B. Calibration

A variety of parameters have been advanced to describe the elution behaviour of a solute in gel chromatography (DETERMANN, 1968): Kav was employed in the present work, and is defined;

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

where Vt is the bed volume, Vo the excluded or void volume and Ve the

elution volume of the experimental solute. Vt was measured from column geometry but was checked at the conclusion of an experimental series by measuring the weight of water held in the column. Vo was measured with Blue Dextran 2000 (Pharmacia) according to the manufacturer's recommendations. Ve was determined from the volume of collected fractions and corrected for dead space volumes. When sample size exceeded 2% of Vt, Ve, measured from the point of entry of the sample into the gel bed, was corrected by subtraction of half the sample volume.

Pre-treatment of ion-exchange resins

In the first instance, each resin was pretreated with acetone in a manner similar to that described by HIRS, MOORE and STEIN (1953). Prior to each use, fines were removed by decantation after the bulk of resin had settled in water. The cation exchange resins were cycled once through the sodium form and the anion exchangers through the hydroxy form as described below. All aqueous solutions used throughout were prepared in deionised distilled water. Amberlite IRC-50 was cycled by the method of HIRS, MOORE and STEIN (1953) and IR-120 in a manner similar to that described for Dowex 50 by HIRS, MOORE and STEIN (1952). Dowex 21K and Dowex 2-X8 resins were cycled and prepared in the chloride form as described by SCHROEDER (1967b) for Dowex 1. The resin was finally collected on a fritted glass funnel, drained of excess liquid by drawing air through the resin bed for 15 min, and stored in a sealed glass container at room temperature.

The swollen resins used, of 14-52 mesh, in addition to containing a relatively constant amount of water of hydration for a particular ionic state, also contain a variable amount of external or adherent liquid.

In quantitative experiments this was determined by the centrifugation method of HELFFERICH (1962) using modified conical centrifuge tubes. Centrifugation was made at 500 xg for 15 min. In the present text, "wet wt." refers to ion exchange resin containing adherent liquid and "swollen wt.", the weight of resin following removal of the liquid by centrifugation.

SDS-polyacrylamide gel electrophoresis

The method was the modified procedure of WILLIAMS and REISFELD (1964) described by NICHOLAS and DEERING (1976) except that the gel was 10% polyacrylamide and the large-pore gel was omitted.

Solutions used in polyacrylamide gel electrophoresis

The stock solutions were kept at 4°C. All other solutions were prepared immediately prior to use, unless indicated otherwise.

Solution A (stock)

Tris	36.3
IN-HCl	48 ml
Water to	100 ml
pH7.9	

Composition of 10% polyacrylamide gel

Solution A	1.25 ml
SDS, 10% (w/v)	1.00
Catalyst	0.10
TEMED	0.015
Solution C	3.57
Water	4.065

Catalyst

freshly prepared

$(NH_4)_2S_2O_8$	1.0 g
water	9 ml

Solution C (stock)

Acrylamide	28.0 g
N, N'-methylene-bisacrylamide	0.735 g
Water to	100 ml

Electrode buffer, pH7.0

Barbitone	5.52 g
Tris	1.0 g
SDS	1.0 g
Water	1.0 litre

Sample mixture (reduced and denatured)

per 100 µg sample:

0.025M-Tris-HC1,pH7.5	100 µl
10% (w/v) SDS	5 µl
2-mercapto-ethanol	1 µl

Protein staining solution (stock)

0.05% (w/v) Coomassie brilliant blue R250

10% (v/v) Acetic acid

20% (v/v) Iso-propan-2-ol

De-staining solutions (stock)

1. 0.005% (w/v) Coomassie brilliant blue R250

   10% (v/v) Acetic acid

2. As in 1. but omit the Coomassie stain.

Sample preparation

Fractions containing SKT were dried by lyophilisation and dissolved in sample buffer (0.025M-Tris-HCl at pH7.5) to a concentration of 1.0 µg/µl. the concentration of standard proteins (see Table 4.16) used to calibrate the gel for molecular weight determination was 1 µg/µl buffer with the exception of trypsin inhibitor (1.67 µg/µl) and insulin. The latter, which was obtained as a 200 i.u./ml solution in acetate buffer, was adjusted to pH7.5 and 0.33 µl used per 0.67 µl sample buffer. To each mixture was added 0.5% (w/v) SDS and when required, 1% 2-mercapto-ethanol. To ensure complete denaturation, the mixtures were heated in boiling water for 3-5 min (SCHNAITMAN, 1973). When the comparative mobility of proteins was required for molecular weight determination, mixtures were made by adding equal volumes of the prepared denatured samples. The density of the sample mixture was increased by adding 4% (w/v) sucrose, and 0.1% bromophenol blue was added as the tracker dye.

Electrophoresis

The gels were cast in detergent-rinsed glass tubes (7 mm x 65 mm), and a Conalco electrophoretic unit employed. Typically 50-100 µl sample was layered on to the gel surface under the electrode buffer. A current of 5 mA/gel was applied immediately and when the sample (bromophenol blue) had just entered the gel the current was reduced to 3 mA/gel. Electrophoresis was continued for 2.5 - 3h, by which time the tracker dye had migrated about 45 mm.

Staining

After removal of the gel from the tube, the position of the tracker dye was marked with indian ink and the gel stained for protein overnight with Coomassie brilliant blue (protein staining stock solution) followed by de-staining in de-staining solution 1 for 6-8h and in several changes of de-staining solution 2.

Measurement of relative mobilities

Gels were scanned directly with a Conalco Model J micro-densitometer from which the migration distance of proteins and bromophenol blue marker was obtained by measurement. Mobilities were expressed relative to the bromophenol blue marker.

RESULTS

PART A. ISOLATION OF KILLER ACTIVITY

Initially several techniques were assessed for applicability to

preparative isolation of SKT activity from the chemically defined culture medium filtrate. The methods examined were charcoal extraction, gel filtration and ion exchange.

Charcoal extraction

This method was examined principally as a means of concentrating SKT activity (FLOREY et al., 1949a, pp. 94-109). Of three different grades of charcoal tested, Oxoid L9 bacteriological charcoal most effectively adsorbed all detectable activity at a concentration of 0.05% (w/v). pH in the range 2.5-10 had no effect on the adsorption efficiency of SKT.

Elution of SKT was tested by treating 0.2 g (wet weight) of distilled water-washed charcoal with 1 ml of eluant for 4-24h at 22-23°C. The eluate was tested for activity by the paper disc method (described in Chapter 3) using SKT assay agar buffered at pH6.0 with 0.08M-phosphate. As anticipated, no activity was detected in organic solvents and their mixtures (16 tested) but several buffers and culture media (5 tested) eluted traces of SKT activity. No activity was detected in mixtures of various alcohols-acetic acid-water as was expected from paper chromatography experiments. A test on the recovered charcoal, washed in distilled water and placed on an assay plate, revealed that the SKT activity remained adsorbed to the charcoal. While both grades of charcoal having lesser affinity for SKT and other eluants could be tested, the results indicated that this approach did not merit further consideration.

Gel filtration

Pilot experiments, in which 0.5-2.5 ml samples of culture filtrate

(2-10% Vt) were applied to small columns (1 cm x 30 cm) of medium to highly cross-linked Sephadex or Bio-Gel gels using phosphate buffered eluants, revealed that only trace amounts of SKT activity could be detected in the column effluents. Concentration of the sample five-fold, under reduced pressure, however allowed the Ve of SKT to be determined: on Sephadex G-10 and Bio-Gels P-2 and P-4 the bulk of SKT was eluted near Vo ahead of the culture filtrate derived salts, whereas on Sephadex G-50 and Bio-Gel P-10, SKT eluted together with the salts near the Vt of the column. Bio-Gel P-2 was chosen for a more detailed assessment as a possible means of isolating SKT from the bulk of culture medium components.

Effect of eluant on the desalting properties of Bio-Gel P-2

The aim of these experiments was to define conditions which provided the greatest separation volume between SKT activity and salts enabling a large sample volume to be processed, enabling a preparative isolation of SKT activity.

A column of bed dimensions 2.6 cm x 38 cm (in 0.1M-phosphate buffer, pH6.0) containing Bio-Gel P-2 was used in these experiments. The samples were 10.0 ml volumes (5% Vt) of culture filtrate concentrate (five-fold) adjusted to the pH of the eluant with 5M-HCl or 5M-NaOH. The flow rate was 30 ml/h and the fraction size was 3-5 ml.

The effect of pH and ionic strength of the eluant was initially examined using phosphate buffers. A typical elution profile in which 0.1M-potassium phosphate was the eluant is shown in Figure 4.1. SKT was eluted at the Vo together with a peak of u.v. absorbing and Lowry positive material just ahead of the large peak of culture filtrate

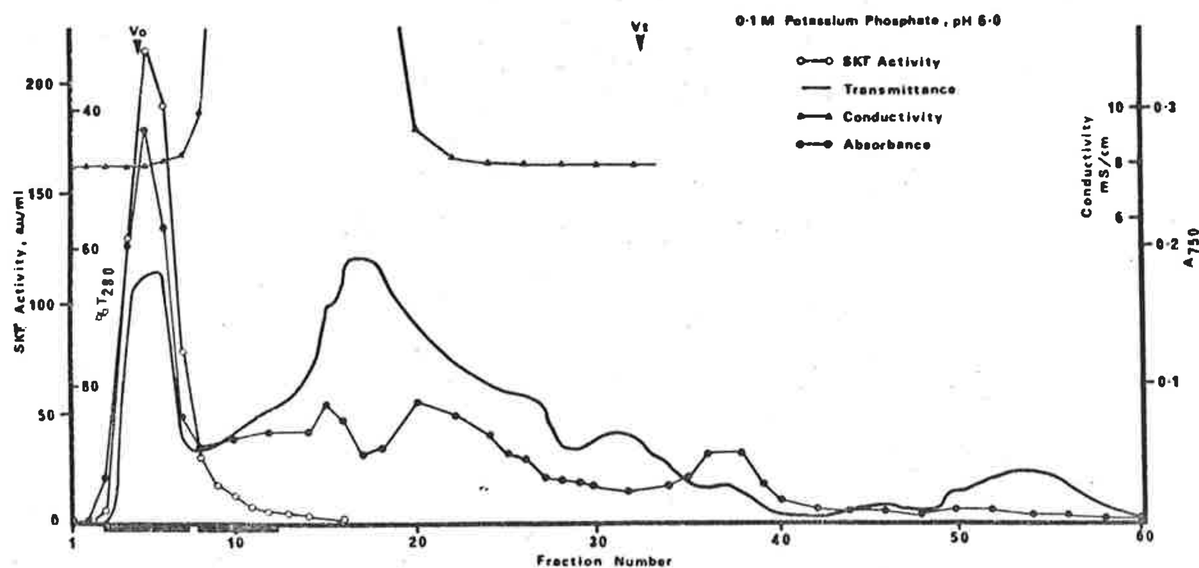


Figure 4.1. Gel filtration of culture filtrate on Bio-Gel P-2 in potassium phosphate eluant.
10.0 ml of a culture filtrate concentrate (five-fold) was eluted (30 ml/h) from a column (2.6 cm x 38.0 cm) of Bio-Gel P-2 (100-200 mesh) with 0.1M-potassium phosphate buffer at pH6.0. Fractions of 5.1 ml were collected and the gel column Vo and Vt was 60.9 and 203 ml respectively. Transmittance was measured in a 5 mm cell.

salts. Equilibration of the column in and elution with buffers composed of 0.02M and 0.4M-potassium phosphate of pH6.0, 0.1M-phosphate of pH6.0 containing 0.5M-KCl, 0.1M-phosphate of pH8.0 and 0.1M-citrate -phosphate of pH4.0 had no definite effect on the elution profile between Vo and a K_{av} of about 0.5, but did vary the pattern of inactive trailing peaks beyond this point. The yield of SKT in these experiments was 85-90%.

The effect of acidic pH on the elution of SKT activity was examined using aqueous acetic acid as the eluant. Concentrations of 0.05M to 0.4M-acetic acid had no definite effect on the elution profile between a K_{av} of 0.0 to about 0.7 but influenced the number and position of trailing SKT-inactive peaks. SKT activity eluted near the Vo as a relatively broad peak but was not directly associated with the relatively more sharply defined excluded peak of u.v. absorbing and Lowry positive material as appeared in phosphate eluates (see Figure 4.2). The culture filtrate salts were eluted with a greater K_{av} (0.5) than in phosphate eluates (K_{av} 0.3). The elution profiles shown in Figure 4.2 demonstrate the effect of sample pH on the resolving power of the column in 0.2M-acetic acid eluant, but sample pH did not affect the elution of SKT and other near-excluded peaks of inactive substances. The total recovery of SKT activity was 70-80%.

Elution at alkaline pH (0.1M-glycine-NaOH pH9.5) while providing a sharply defined peak of SKT activity (92% recovered), achieved only partial separation from the culture filtrate salts (Figure 4.3).

When distilled water was the eluant, although u.v. absorbing and Lowry positive material was eluted at the Vo, SKT did not appear in the eluate until Kav 0.5, in conjunction with the culture filtrate salts (Figure 4.4). Only about 10% of SKT applied to the column was

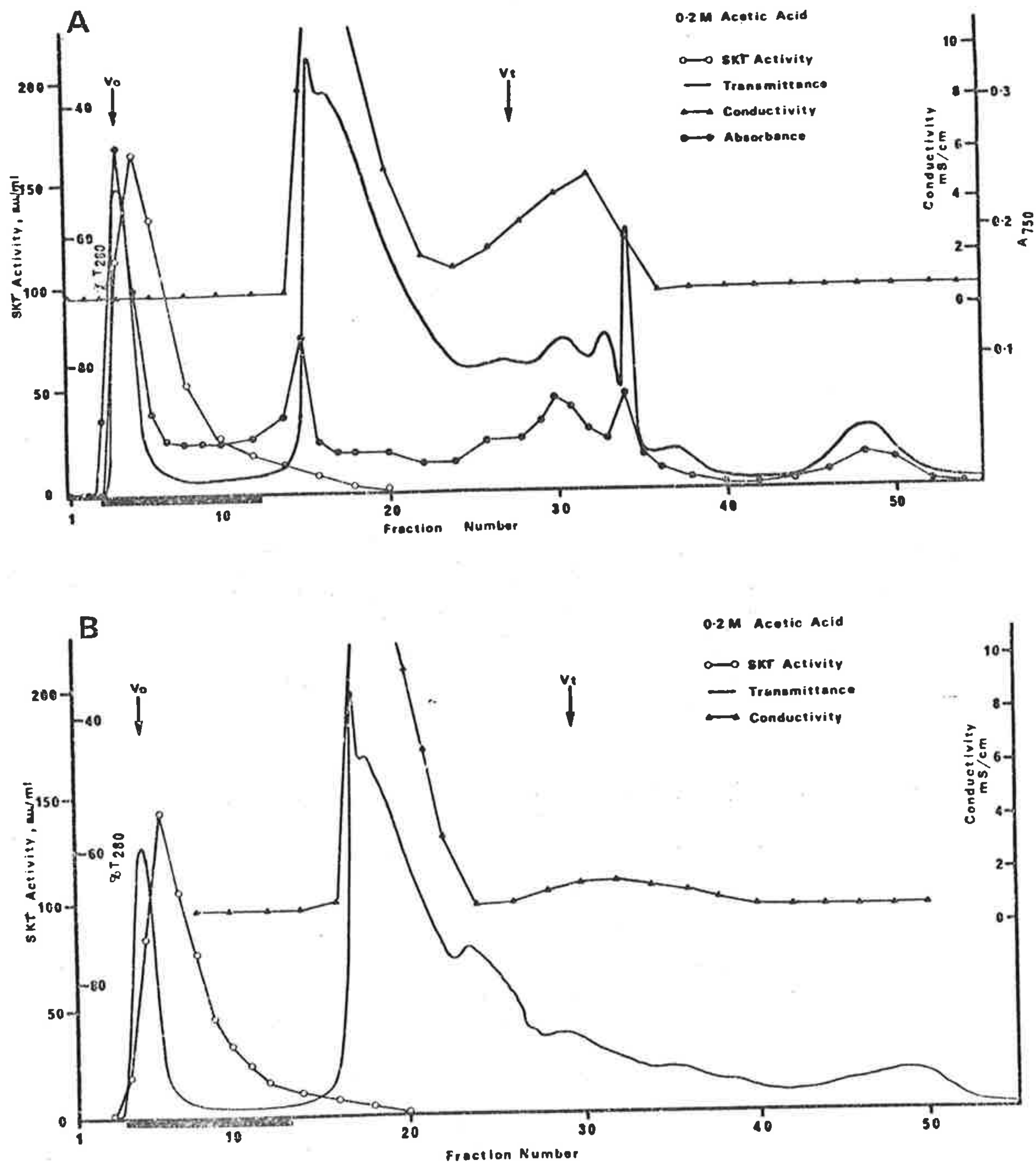


Figure 4.2. Gel filtration of a culture filtrate concentrate of (A) pH5.5 and (B) pH3.5, in 0.2M-acetic acid eluant.
The conditions were as described in figure 4.1 except that fractions of 5.8 ml were collected in (A), 5.4 ml in (B), and the column Vo and Vt was 61.0 ml and 197 ml respectively.

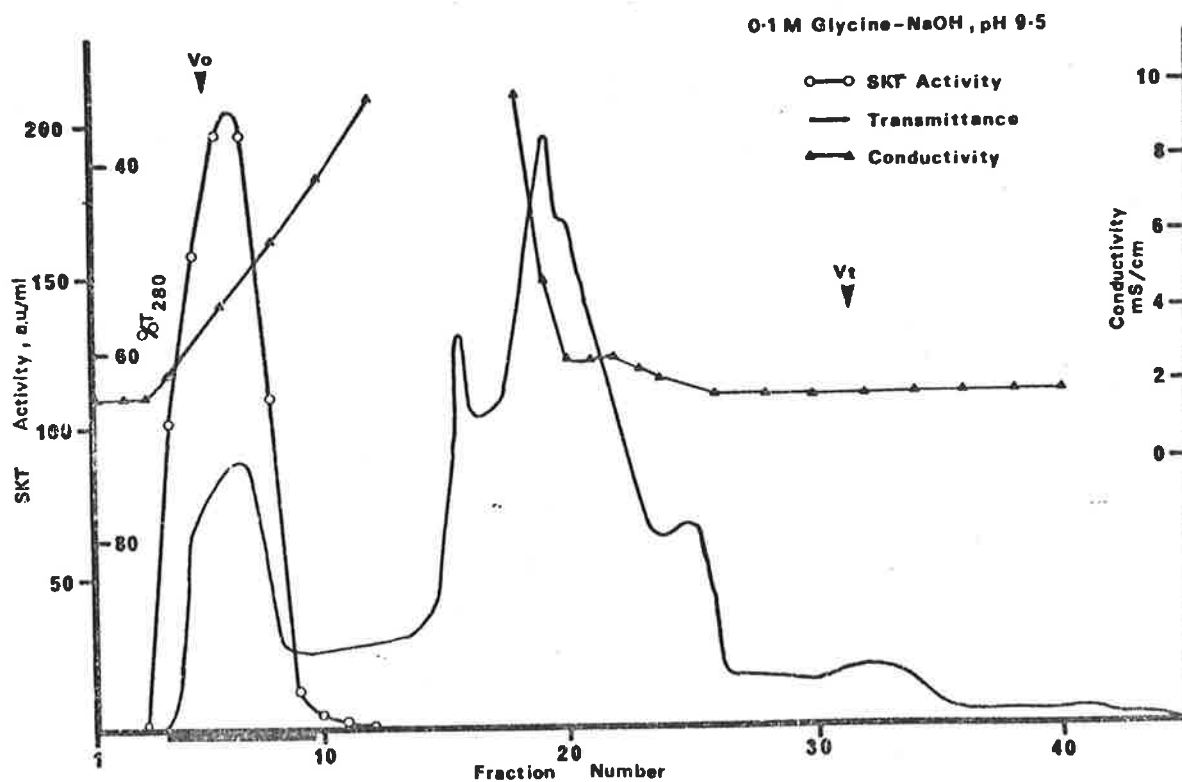


Figure 4.3. Elution of culture filtrate components from Bio-Gel P-2 with 0.1M-glycine -NaOH, pH9.5.
The conditions were as described in Figure 4.1 except that fraction size was 5.0 ml and the column Vo was 64.2 ml and the Vt, 194 ml.

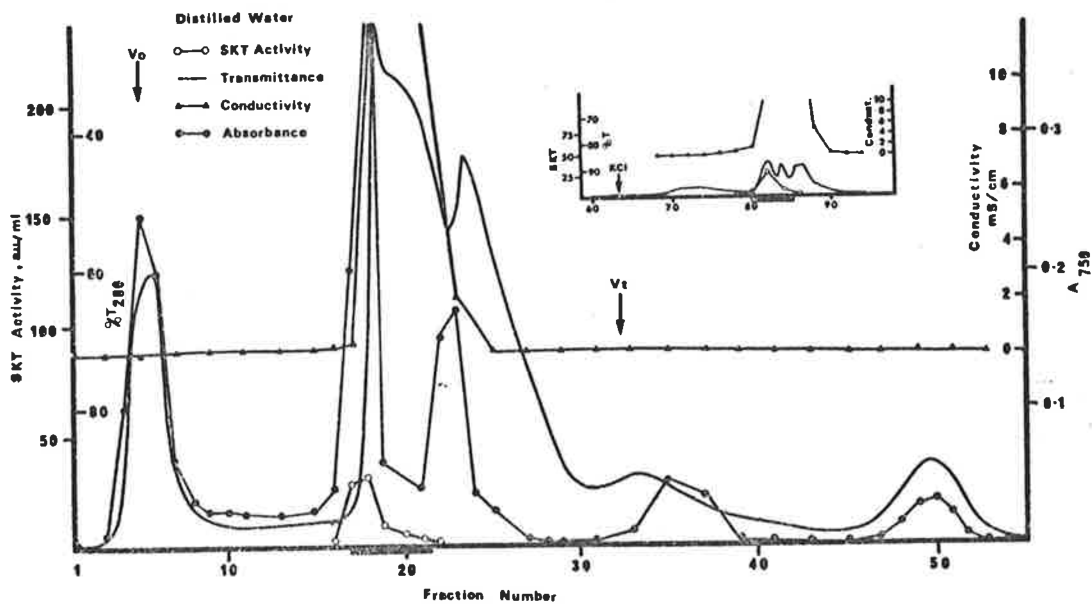


Figure 4.4. Gel filtration of a culture filtrate concentrate (pH5.5) on Bio-Gel P-2 in distilled water eluant.
The conditions were as described in Figure 4.1 except that the fraction size collected was 5.0 ml and the gel column Vo and Vt was 61.2 ml and 201 ml respectively. Insert shows subsequent column elution with 4M-KCl (5 ml).

recovered, but some of the adsorbed SKT could be eluted with a zone of 4M-KCl. Increasing the sample size to 30 ml resulted in about 30% of the applied SKT being eluted just ahead of the salts and the overall yield was greatly improved (70%).

Yield and purity of desalted SKT activity.

These experiments were performed using two different batches of culture filtrate, their activities and specific activities, based on both dry wt. (lyophisation) and protein, are given below. Protein was measured by the colourimetric procedure on an aliquot of culture filtrate following exhaustive dialysis against 0.1M-sodium acetate, pH6.0, using Visking tubing (0.39 in flat width).

Components in 10.0 ml of five-fold concentrate	Culture filtrate concentrate	
	CFC_a	CFC_b
SKT activity (a.u.)	4800	5000
Dry wt. (mg)	685.5	666
Specific activity (a.u./mg)	7.00	7.51
Protein (mg)	1.24	1.02
Specific activity (a.u./mg)	3870	4900

Column effluent fractions were pooled as indicated on the respective elution profile to determine yield and purity of desalted SKT activity. Dry wt. of the pooled fractions was determined by lyophilisation and total protein was estimated colourimetrically. Yield and specific activity of SKT activity, eluted in three different eluants, is summarised in Table 4.1. The excluded fraction in phosphate eluate contained 83-84% of total activity while the tailing fractions accounted

Table 4.1. Effect of eluant on the yield and specific activity of SKT in pooled selected fractions eluted from Bio-Gel P-2.

Eluant	0.1M-potassium phosphate, pH6.0				0.2M-acetic acid, pH2.8		Distilled water		
CF concn.	CFC_a		CFC_b		CFC_a	CFC_b	CFC_a	CFC_b	
Peak pooled[a]	Excluded	Tailing	Excluded	Tailing	Excluded + part tailing	Excluded + part tailing	Total	Total	KCl eluate
Volume pooled (ml)	28.0	28.0	25.5	25.5	57.6	58.0	20.4	25.0	40.5
SKT activity (a.u.)	3976	406	4208	383	3197	3480	265	350	446
SKT yield (%)	82.8	8.1	84.2	7.7	66.6	69.6	5.5	7.0	8.9
Dry wt. (mg)	N.D.	N.D.	17.60[b]	285.1	11.9	15.15	583.4	858.1	N.D.
SKT specific act. (a.u./mg)	-	-	238.5	1.34	268.7	229.7	0.45	0.41	-
Purification (fold)	-	-	31.8	0.18	38.4	30.6	0.06	0.05	-
Protein (mg)	0.622	0.678	0.694	0.255	0.858	0.806	1.173	0.678	0.081
SKT specific act. (a.u./mg)	6390	599	6060	1500	3730	4320	266	516	5510
Purification (fold)	1.65	0.16	1.24	0.31	0.96	0.88	0.06	0.11	1.12

[a] Refer to Figures 4.1, 4.2 and 4.4; [b] Corrected for buffer salts; N.D., Not determined.

for another 8%. With the acetic acid eluant, pooled excluded and tailing SKT up to the commencement of the eluted salts accounted for 67-70% of total applied SKT activity. Less than 10% of total SKT appeared in the distilled water eluate and a further 9% was eluted with a zone of KCl.

The specific activity of SKT with respect to dry wt. in either phosphate or acetic acid eluates were similar, 230-269 a.u./mg, representing an increase over the parent culture filtrate of 31-38 fold. Specific activity of SKT in the distilled water eluate was much lower (18-fold) than the starting material, presumably due to the comparatively low recovery of SKT activity. SKT specific activity based on total protein was greatest in the excluded fraction with phosphate eluants, being 6060-6390 a.u./mg, compared with 3730-4320 a.u./mg of the combined excluded and part tailing fractions of the acetic acid eluant. The former showed a 1.24-1.65-fold increase over the specific activity of culture filtrate SKT, whereas the latter showed a small decrease of 0.88-0.96-fold. Protein-based specific activity of SKT was again very low in distilled water eluates, but was similar to the starting material in the KCl eluate.

SKT yield was greatest in neutral to alkaline eluates but resulted in a minimal separation volume between SKT and retarded components, thus limiting the application of larger sample volumes. These eluants however afforded SKT of increased specific activity. Aqueous acetic acid provided a larger separation volume and although this was partly off-set by a lower recovery of activity, the sample volume could be increased 2.5-fold while maintaining a 70% yield of desalted SKT. Application of a more concentrated culture filtrate sample was restricted by viscosity effects which degraded chromatographic

resolution. Therefore, on a preparative scale, for example, a 2 litre column would be required to desalt the equivalent of 1.25 litre of neat culture filtrate, or several runs made on a column of smaller diameter.

Ion exchange resins

Ion exchange resins were investigated for application to the isolation of SKT activity from culture filtrate. They have a high capacity for small molecules and consequently have been extensively employed in the isolation and purification of amino acids, peptides, drugs, hormones and particularly antibiotics (WATCHEL and STILLER, 1957; BORDERS, 1975; UMEZAWA and KONDO, 1975). In use, the ion exchange resin is either stirred directly into the culture liquor and the pH adjusted for maximum adsorption of the substance of interest, or the liquor, at the appropriate pH, is passed through a bed or resin contained in a column. Following various washing procedures to remove sorbed solutes and inert substances, the resin is then eluted with a solvent, usually one which allows greatest recovery rather than purity, and can be readily removed by evaporation or lyophilisation.

Adsorption and elution conditions

Adsorption of SKT activity

A pilot experiment was made to determine whether SKT activity could be adsorbed to ion exchange resin directly from the culture filtrate. It was anticipated from the pI of SKT that it would adsorb to cation exchangers below pH5 and to anion exchangers beyond pH6. Four types of resin were tested: Amberlite IRC-50(H), a weakly acidic cation exchanger; Amberlite IR-120(H), a strongly acidic cation exchanger;

Dowex 21K(Cl), a strongly basic anion exchanger; and Dowex 2-X8(Cl), a less strongly basic anion exchange resin.

A stirred mixture of resin and culture filtrate (10.0 g of swollen resin to 100 ml of filtrate) was titrated to different values of pH with either 5M-HCl or 5M-NaOH as described below. When the pH of the mixture remained stable for at least 5 min, an aliquot of liquid was removed, centrifuged to sediment any resin particles and brought to pH6.0 for SKT assay. Control culture filtrates were titrated to the same values of pH at which sampling was made to determine by difference the proportion of SKT adsorbed to the resin.

Neither anion exchange resin Dowex 21K or Dowex 2-X8 reduced SKT activity of the liquid phase in the range pH5-10. Both cation exchangers adsorbed SKT activity below pH6.0: IRC-50, maximally between pH3.0-4.0 and IR-120 adsorbed all detectable activity between pH2.0-4.0. The loss of activity from the liquid phase was assumed to indicate adsorption to the resin and not inactivation, since SKT was fully stable between pH2.0-10.0 for the 5h duration of each experiment. Although IRC-50 has a two-fold greater ion exchange capacity than IR-120, under these conditions it adsorbed only 85% of total SKT. However the carboxylic acid functional group is not fully ionised below pH6.0, whereas the strong acid sulphonate group of IR-120 has full capacity down to pH2.0, thus accounting for the results. The effect of pH on the adsorption of SKT to cation exchangers is therefore consistent with the electrophoretic mobility of SKT, but the reason for non-adsorption to anion exchangers is not known. A possibility is the high ionic strength of the culture filtrate medium which contains a high proportion of citrate.

Elution of SKT activity

Quantities of resin containing adsorbed SKT were shaken with buffer at different pHs and the supernatant fluid assayed for SKT activity. A batch of IRC-50 and of IR-120 resin containing SKT were prepared by adsorption at pH3.5 using 1.0 g of resin to 10 ml culture filtrate. The resin was washed in water and 1.0 g (wet wt.) quantities were eluted with 2.0 ml of 0.1M-sodium citrate-phosphate buffer, pH4.0-8.0 (McKENZIE, 1972). After ensuring that the mixture had reached equilibrium with respect to pH, which was hastened by gentle agitation, the supernatant SKT activity was determined.

Increasing pH promoted elution of SKT from both resin types. For IRC-50 resin, detectable SKT eluted at pH4.5 and was maximal at pH7.0 whereas for IR-120, an eluate at pH4.0 contained detectable SKT activity which was maximal at pHs exceeding 6.0.

Optimisation of adsorption conditions

Amberlite IR-120 resin was selected for further study because of its greater capacity for SKT directly from culture filtrate medium. Factors affecting the adsorption of SKT were examined to determine optimal conditions. Because strong acid cation exchangers establish equilibrium conditions rapidly (HELFFERICH, 1962) this could be readily studied by a titration procedure as used above. Thus resin, stirred in culture filtrate of different compositions was titrated to different values of pH to determine the distribution of SKT between the solid and liquid phases.

In an attempt to standardise the ionic composition of various batches

of culture filtrate, the pH was adjusted to 5.30 with 10M-HCl or 40% NaOH, and when necessary the filtrate was diluted so that the conductivity of a two-fold dilution in distilled water was 6.0 mS/cm at 22-23°C, neat culture filtrate being about 10.5 mS/cm.

A. Titration

To 10.0 g swollen wt. (10.5 to 11.0 g wet wt.) of IR-120(H) resin contained in a 250 ml beaker, was added 100 or 200 ml of 100% or 50% (v/v) standardised culture filtrate. The mixture was stirred with a magnetic bar and the pH of the liquid was measured with a combination type pH electrode. Titrant, 40% NaOH (4.0 g NaOH added to 10.0 ml deionised water), was added dropwise while vigorously stirring the mixture. To reduce the formation of resin fines, once the pH of the solution steadied (2-3 min) the speed of stirring was reduced. The establishment of equilibrium was indicated by a final constant value of pH. After a brief pause in stirring to allow the resin to settle, an aliquot of liquid (1 ml) was removed and centrifuged. 0.5 ml of supernatant fluid was adjusted to pH6.0 for SKT assay and the remaining fluid returned to the titration mixture.

B. Results

Three sets of conditions were examined by titration, and were per 10.0 g IR-120(H) resin: A. 100 ml of 100% culture filtrate (neat, standardised); B. 100 ml of 50% culture filtrate and; C. 200 ml of 100% culture filtrate. The titration and associated SKT distribution curve determined for each treatment is illustrated in Figure 4.5.

The titration and resultant SKT distribution curve for neat culture filtrate containing 10% (w/v) IR-120(H) resin is represented by Curve A of Figure 4.5. Initial equilibrium of the mixture resulted in a pH of 2.1 at which a small amount (4% of total) of SKT was detectable in the liquid phase. On titration, the mixture initially showed high buffering capacity to pH2.5 (approximately 100 meq NaOH consumed) after which the titration curve became linear (consuming 47 meq NaOH per pH unit) to the limit pH tested of 6.0. No SKT activity was detected (assay sensitivity 1.8 a.u./ml) between pH of 2.25 to 3.25 but became measurable toward pH4.0 whereupon the liquid phase activity increased sharply to 100% at pH5.25.

When the ratio of culture filtrate to resin was doubled (resin, 5% w/v of filtrate) but the concentration of the filtrate solution halved, titration resulted in Curve B of Figure 4.5. The initial equilibrium pH was 2.3, greater than in A, due to a 50% reduction in culture filtrate ionic strength, which also reduced the amount of titrant consumed compared with that under the conditions of A. SKT was not detected in solution until pH3.5-3.75 and was not eluted sharply until pH4.5, whereupon its elution was complete by pH5.5. Curve B therefore demonstrates the effect of reducing ionic strength on the distribution of SKT between resin and culture filtrate, with respect to pH; that is at lower ionic strength, the resin has a greater capacity for SKT activity and binds to the resin at a higher value of pH.

Doubling the ratio of 100% culture filtrate to resin (resin, 5% w/v of filtrate), on titration, produced curve C of Figure 4.5. The initial equilibrium pH value was 2.4. The titration curve was much reduced because of the greater neutralisation capacity of the culture filtrate for the resin acidity, hence less titrant was required. SKT was not

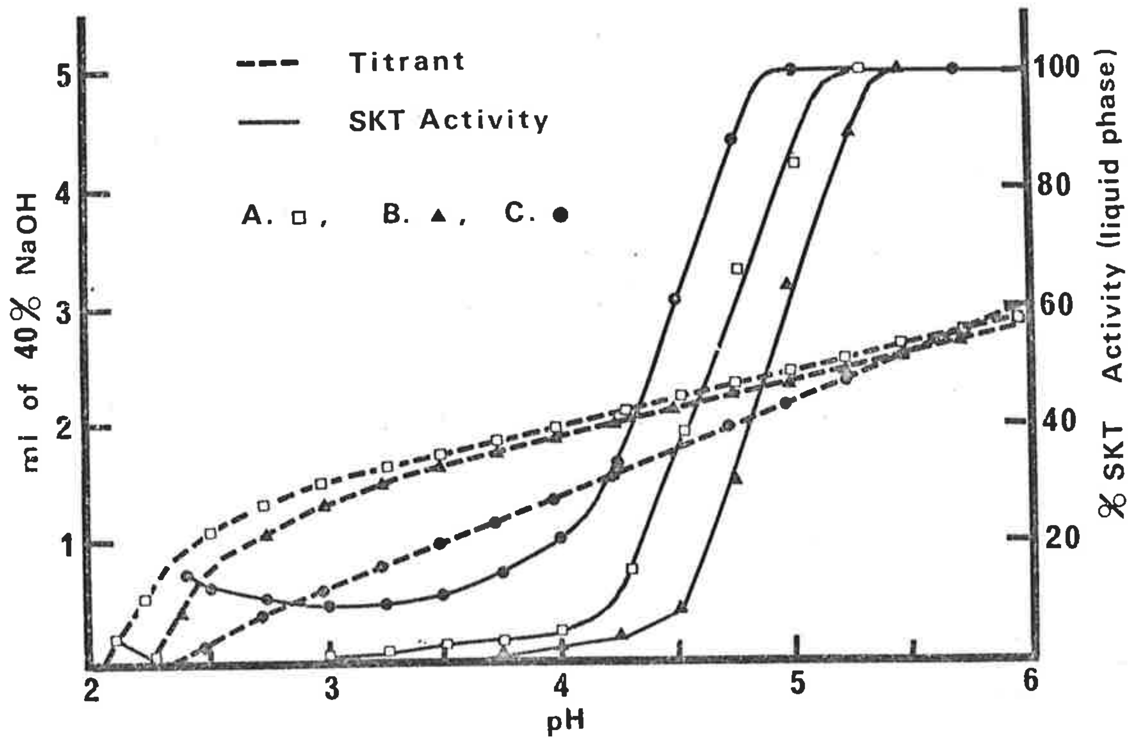


Figure 4.5. Distribution of SKT activity in mixtures of culture filtrate and IR-120(H) resin titrated with 40% NaOH. The mixtures consisted of 10.0 g swollen wt. of resin (14-52 mesh) per : A., 100 ml of 100% culture filtrate; B., 100 ml of 50% filtrate and; C., 200 ml of 100% filtrate. SKT activity in the liquid phase is expressed as a percentage of that originally present in the culture filtrate.

completely adsorbed at any pH, a maximum of 90% occurring between pH3.0 to 3.25. No SKT activity adsorbed to the resin beyond pH5.0.

These curves therefore demonstrate the distribution of SKT activity between the culture filtrate and resin phases, with respect to pH, for different ratios of filtrate to resin. SKT activity was shown to be quantitatively adsorbed to and eluted from IR-120 resin in the range pH2-6. To select optimal conditions for SKT adsorption, the greatest value of pH allowing total activity to be adsorbed from the culture filtrate by the largest ratio of culture filtrate to resin is required. For neat culture filtrate at a ratio of 10:1 (Curve B) the optimal pH for SKT adsorption was 3.0 to 3.5 (>98 SKT being resin bound), whereas for diluted filtrate (50%) at a ratio of 20:1, pH3.75 to 4.0 was optimal. Further dilution would allow an even greater ratio of filtrate to resin ratio at this pH for quantitative adsorption of SKT. For larger scale operation, such as 5-10 litres of filtrate, dilution would result in excessive volumes, so it was decided to carry out experiments using neat culture filtrate. The optimum conditions for these and culture filtrates with different composition could best be determined by titrating mixtures, containing various ratios of liquid to resin, to pH3.5 and selecting the mixture allowing the greatest ratio consistent with maximal SKT adsorption.

Column isolation

The adsorption of a solute by an ion exchanger contained in a column is generally more efficient than an equivalent batch method. Although the conditions for optimal adsorption of SKT to a column of resin are likely to differ to those determined by the batch titration procedure, it was assumed that SKT adsorption from neat filtrate would also be

maximal near pH3.5. Thus an experiment was made using a column of IR-120(H) resin, charging with standardised culture filtrate at pH3.5 and assaying the column effluent for SKT activity. A run using filtrate at the pH of production (pH5.3) was initially made.

5.00 g of swollen IR-120(H) was packed into a column (1.0 cm x 30 cm) producing a bed height of 9.5 cm in distilled water. Culture filtrate at either pH5.30 (6.0 mS/cm of a two-fold dilution) or pH3.50 was pumped through the column at 10 ml/h and the effluent collected in 5.0 ml fractions.

Analysis of the column effluents for the two experiments are shown in Figure 4.6. The break-through volume of SKT in culture filtrate of pH5.30 was 40-50 ml while that of Lowry positive material was less than 10 ml. Further charging of the column with culture filtrate resulted in enrichment of the effluent in SKT, presumably through elution by incoming filtrate solution of pH5.3. The break-through volume was greater for culture filtrate of pH3.50, being 70-80 ml, but that of the Lowry reactive material remained low.

From these experiments it was calculated that 1000 a.u./g of swollen resin was adsorbed at pH5.3 (breakthrough volume of 50 ml, SKT activity 100 a.u./ml) and 1600 a.u./g of resin at pH3.5. This compares with 1000 a.u./g swollen resin by the batch method for an adsorption pH of 3.5. Maximum capacity by the column procedure will be greater than these values but is obtained at the expense of an overall decreased adsorption yield of SKT. Other factors such as ionic strength and resin particle size could also be expected to improve capacity of the resin for SKT activity (see Discussion).

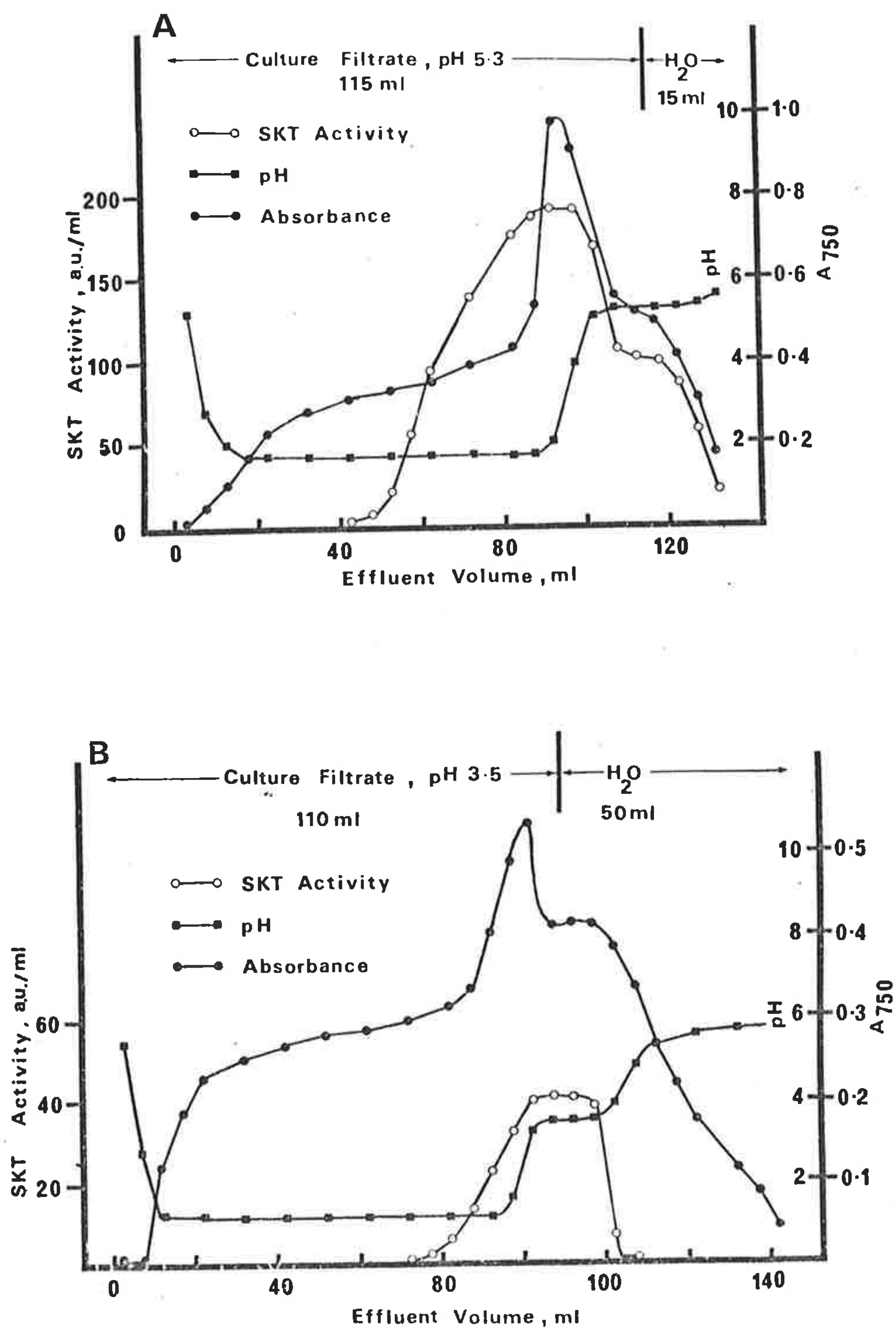


Figure 4.6. Elution profile of a column of IR-120(H) resin charged with culture filtrate of A. pH5.3 and B. pH3.5.
Standardised culture filtrate at either pH5.3 or pH3.5 was applied to a column (1.0 cm x 9.5 cm) of IR-120(H) resin (5.00 g swollen wt.) at 10 ml/h. Fractions of 5.0 ml were collected for analysis.

Volatile eluants.

Since the available methods for desalting SKT activity were limited, it was desirable to recover SKT in a volatile eluate which could readily be removed by lyophilisation. This would also facilitate the estimation of SKT purity on a gravimetric basis. Commonly employed volatile eluants include HCl, acetic acid, ammonia, pyridine, pyridine-acetic acid and ammonium formate, the choice depending primarily on the stability of the substance of interest (HIRS, MOORE and STEIN, 1952, 1954; WATCHEL and STILLER, 1957; SCHROEDER, JONES, CORMICK and McCALLA, 1962; HIRS, 1967; TANIYAMA, SAWADA and KITAGAWA 1971; UMEZAWA and KONDO, 1975).

Initially dilute HCl was tested as an eluant since SKT was markedly more stable in acidic than basic media; it is easily removed and does not interfere in analytical tests. A column of resin containing adsorbed SKT was prepared as described in Figure 4.6B using 100 ml of culture filtrate of pH3.50 followed by 50 ml of deionised water. The column was then eluted stepwise with 50 ml volumes of 0.05-1.0M-HCl at 10 ml/h. No activity was detected in eluants at levels below 0.1M-HCl but 25-30% of the activity applied to the column was eluted with 0.5M-HCl. Low recovery may have been due to partial inactivation of SKT. A weak acid such as acetic acid may provide greater recovery but was not tested.

It was decided to investigate the use of volatile buffers with which the problem of SKT instability is eliminated. Pilot experiments using a citrate-phosphate buffer indicated that a buffer spanning the range pH4-8 was required, the actual pH being dependent on ionic strength. Volatile buffers are composed of a volatile weak acid and weak base, and

mixtures which fall within this range of pH include: a combination of either ammonia, triethylamine or trimethylamine with formic acid for pH3-5 or with acetic acid for pH4-6; acetic acid and pyridine, pH4-6; ethylenediamine and acetic acid, pH4-8; collidine and acetic acid, pH5.5-7 (McKENZIE, 1972; PERRIN and DEMPSEY, 1974). Factors which influence the choice of a buffer include: interference with biological and biochemical assay; polarity of buffer species in relation to exchanger polarity; pH-ionic strength relationship; buffer stability and purity; ligand stability. Buffers composed of acetic acid and either pyridine or ethylenediamine appeared potentially most suitable. Neither bases (0.1M) when neutralised to pH6.0 with 1M-acetic acid were toxic in the assay system, and did not significantly interfere in assay as judged by the co-linearity of dose-response curves of SKT (lyophilised eluate obtained from Bio-Gel P-2 in 0.2M-acetic acid) in these buffers and 0.1M-phosphate, pH6.0. Pyridine precludes u.v. spectrophotometric analysis and since it has low buffer capacity at pHs exceeding 6.0, near the expected elution maximum for SKT, ethylenediamine-acetic acid buffer was chosen. However this buffer mixture has a disadvantage in that it produces a precipitate with the Folin-Ciocalteu reagent thus reducing the colour yield in the Lowry method for proteins, necessitating its removal by lyophilisation in quatitative estimations This problem was not appreciated at the time this work was commenced.

JOUSTRA and LUNDGREN (1969) noted that an impurity present in commercial batches of ethylenediamine resulted in a change to the chromotographic behaviour of their system. Ethylenediamine was therefore double-distilled before use and stored in an air-tight dark glass container for a limited period of time. Since a comprehensive composition table for constant ionic strength ethylenediamine-acetic acid (EN-AA) mixtures was not available, this was determined empirically

(see Appendix Three).

The effect of ionic strength and pH on the elution of SKT activity

The effect of ionic strength and pH of EN-AA buffer on the elution of SKT from IR-120 resin was evaluated in terms of elution efficiency and specific activity based on gravimetric determinations. By using low ionic strength eluants it was anticipated that elution of resin adsorbed culture filtrate salts would be minimal.

50 g of swollen resin containing SKT adsorbed from 500 ml of neat culture filtrate (to pH3.5) was prepared by the batch method as described above. The resin was thoroughly washed with deionised water and drained. To 1.00 g wet wt. (0.6 g swollen wt.) of resin was added 2.50 ml of 0.0075-0.06I EN-AA buffer, pH4.5-7.5. Stock 0.06I buffers were prepared freshly in deionised water and the buffers of lower ionic strength were made by dilution of the stocks. Equilibration of the resin-buffer mixtures was hastened by gentle agitation and where necessary, the pH was returned to its former value by the addition of 0.2-0.5M -ethylenediamine. Additions did not exceed 3% (v/v). The eluates were withdrawn (about 2.7 ml) and the supernatants divided, 2.0 ml for dry wt. determinations and the remainder adjusted to pH6.0 for SKT assay.

The results are summarised in Table 4.2. The onset of maximal elution of SKT activity was dependent on both pH and ionic strength; being near pH6.6 at 0.0075I and about pH6.0 at 0.06I. Calculation of elution efficiency indicated that 50-65% of the adsorbed SKT was recovered. The apparently low yields were not due to incomplete elution of SKT since recovery was not increased by the addition of NaCl (to 0.5M) to the

Table 4.2. Elution efficiency and specific activity of SKT eluted from IR-120 resin with 0.0075 - 0.06I ethylenediamine-acetic acid buffer in the range pH4.6 - 7.5.[a]

eluant pH series	Ionic strength	Equilibrium pH	Final pH	SKT activity (a.u./ml)	Dry wt. (mg/ml)	Specific activity (a.u./mg)	Elution efficiency (%)
4.64	0.0076	4.68	4.68	-	0.25	-	-
	0.015	4.67	4.67	-	0.55	-	-
	0.03	4.65	4.65	-	1.20	-	-
	0.06	4.64	4.64	5.8	2.05	2.8	2.8
4.93	0.0075	4.60	4.90	-	0.25	-	-
	0.015	4.64	4.96	-	0.55	-	-
	0.03	4.67	5.09	12.5	1.25	10.0	5.9
	0.06	4.71	5.05	47.0	2.25	20.9	22.3
5.53	0.0074	4.71	5.51	8.8	0.35	25.1	4.2
	0.015	4.82	5.55	28.0	0.55	50.9	13.3
	0.03	4.90	5.59	82.0	1.20	68.3	39.0
	0.06	5.18	5.53	123.0	2.40	51.3	58.4
5.97	0.0074	4.62	5.94	72.0	0.35	206.0	34.2
	0.015	4.81	5.96	105.0	0.60	175.0	45.2
	0.03	4.94	5.95	120.0	1.25	96.0	57.0
	0.06	5.10	6.04	140.0	2.40	58.3	66.5
6.52	0.0074	4.70	6.59	100.0	0.40	250.0	47.5
	0.015	4.83	6.48	115.0	0.65	176.9	54.6
	0.03	5.24	6.45	130.0	1.20	108.3	61.8
	0.06	5.71	6.46	140.0	2.35	59.6	66.5
7.12	0.0076	4.81	6.95	100.9	0.50	200.0	47.5
	0.015	5.22	6.96	120.0	0.75	160.0	57.0
	0.03	6.00	7.05	130.0	1.30	100.0	61.8
	0.06		7.04	145.0	2.85	50.9	68.9
7.53	0.0077	5.09	7.48	105.0	0.50	210.0	49.9
	0.015	5.76	7.42	115.0	1.00	115.0	54.6
	0.03	N.D.					
	0.06		7.50	140.0	2.70	51.9	66.5

N.D., Not determined; [a] 1.00 g wet wt. (0.62 g swollen wt.) of IR-120 resin containing at least 977 a.u./g (swollen wt.) SKT was eluted with 2.50 ml (total liquid volume of resin-eluate mixture, 2.88 ml) of EN-AA buffer. Elution efficiency (%) was calculated by (see Appendix Two, equation 4),

$$= \frac{2.88 \text{ ml} \times \text{eluate SKT (a.u./ml)} \times 50.0 \text{ g}}{500 \text{ ml} \times (100 - <2.3 \text{ a.u./ml}) \times 0.62\text{g}} \times \frac{100}{1}$$

$$\leqslant \text{eluate SKT (a.u./ml)} \times 0.475.$$

eluant or by titration to pH8.5. Comparison of the dose-response curve of SKT eluted and diluted in EN-AA buffer and the culture filtrate standard showed that they were not parallel. It was estimated that eluate SKT activity was under-estimated by about 20%. This is described below (see Figure 4.19). Therefore, the values obtained may represent near-quantitative yield since SKT was fully stable at the values of pH employed and in titration experiments (see Figure 4.5) it was shown that SKT activity could be quantitatively adsorbed to and eluted from IR-120 in culture filtrate.

The specific activity of SKT in the eluates, illustrated in Figure 4.7, was inversely related to ionic strength of the eluant. Furthermore, maximum specific activity was attained near the lowest value of pH at which SKT elution was maximal. Thus both increasing the pH and ionic beyond the optimal values promoted elution of additional inactive material. However this experiment does not provide any information on the nature of the inert material.

Development of a batch isolation procedure

Batchwise elution experiments indicated that the bulk of SKT activity could be recovered with a substantial increase in purity by elution with low ionic strength buffer. Stepwise elution can be expected to further increase purity but in addition provides an opportunity to concentrate the product by recovery in a much reduced volume of eluant. A series of experiments were therefore carried out to define the experimental conditions. The general outline of the proposed procedure was:

(i) Batch adsorption of the standardised culture filtrate at pH3.5 using 10% (w/v) swollen resin.

(ii) Pre-treatment of the resin with buffer near threshold

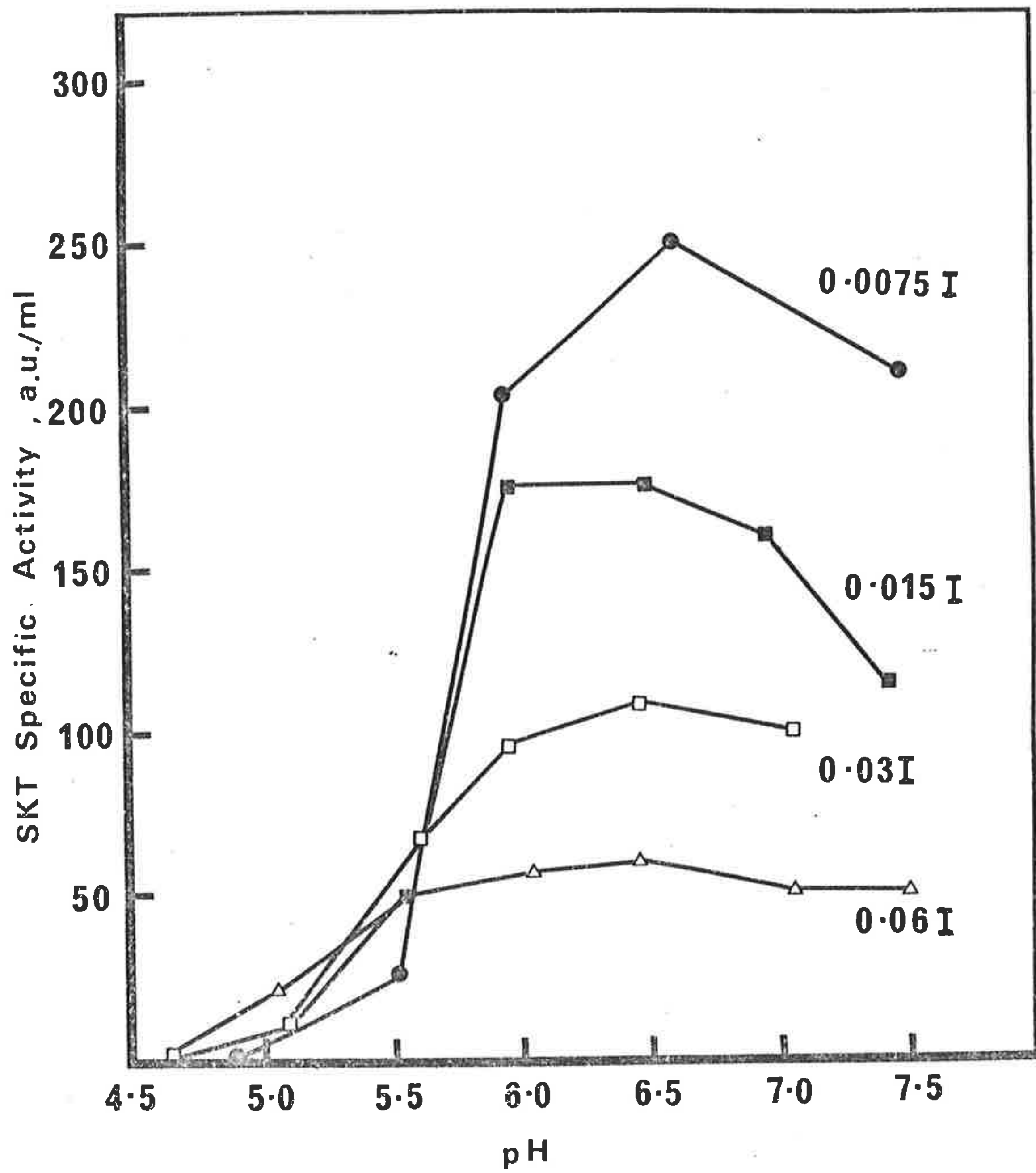


Figure 4.7. Specific activity (gravimetric) of SKT eluted from IR-120 resin with 0.0075-0.06I ethylenediamine-acetic acid buffer.

conditions for SKT elution.

(iii) Elution of SKT in a small volume of low ionic strength EN-AA buffer.

Elution conditions

Two preliminary experiments were carried out to define the threshold pH value for SKT activity elution at 0.06I and the lowest pH value permitting maximal recovery of SKT in 0.0075I buffer. The experiments were carried out as described above. The threshold pH value for SKT elution was found to be 4.5 at 0.06I (Table 4.3) and the maximal recovery of SKT was attainable at pHs exceeding 6.8 in 0.0075I EN-AA buffer (Table 4.4). Therfore in the following experiments, the resin containing adsorbed SKT, following washing with deionised water, was equilibrated in 10-fold (v/w) 0.06I buffer, pH4.52. SKT was then recovered from the washed resin in 0.0075I EN-AA buffer, pH7.0, brought to pH6.8-7.0 with 0.2M-ethylenediamine.

Effect of eluant to resin ratio on yield and purity

The final concentration of SKT in the eluate depends on the ratio of adsorption volume (Av) to the elution volume (Ev) per unit wt. of resin and the efficiency of the process is given by;

$$\text{SKT concentration ratio} = \frac{Av}{Ev} \times \frac{\%\ \text{yield}}{100}$$

Since the ratio of culture filtrate to resin in the adsorption phase was fixed at approximately 10:1 (v/w), the maximum overall concentration ratio was dependent on the relative size of the eluate volume. As the practical lower limit was about 0.5 ml of eluant per gram swollen resin (14-52 mesh), the maximum concentration ratio attainable would be 20 for a quantitative recovery of SKT activity.

Table 4.3. Elution of SKT activity from IR-120 resin in 0.06I EN-AA buffer at pH4.36 - 5.05.[a]

Eluant pH	Equilibrium pH	SKT activity (a.u./ml)	Extraction efficiency (%)
4.37	4.36	< 1.6	< 1.6
4.52	4.51	2.8	2.8
4.64	4.63	7.4	7.4
4.75	4.72	14.9	14.9
4.93	4.85	26.8	26.9
5.17	5.05	59.5	59.7

[a] 1.30 g wet wt. (1.04 g swollen wt.) of washed resin containing adsorbed SKT (at least 984 a.u./g swollen resin, 25 g of resin used) was eluted with 10.0 ml (total liquid volume of mixture, 10.26 ml) of 0.06I EN-AA, pH4.37-5.17. Elution efficiency was calculated with equation 4 (Appendix Two)

$$= \frac{10.26 \text{ ml} \times \text{eluate SKT (a.u./ml)} \times 25 \text{ g}}{250 \text{ ml} \times (100 - <1.6, \text{ a.u./ml}) \times 1.04\text{g}} \times \frac{100}{1}$$

$$\leqslant \text{eluate SKT (a.u./ml)} \times 1.003\%$$

Table 4.4. Elution of SKT activity from IR-120 resin in 0.0075I EN-AA buffer at pH5.97 - 7.41.[a]

Eluant pH	Equilibrium pH	Final pH	SKT activity (a.u./ml)	Elution efficiency (%)
5.96	4.61	5.97	48.0	22.3
6.17	4.73	6.12	62.0	28.8
6.39	4.80	6.30	76.0	35.3
6.56	4.85	6.55	83.0	38.6
6.70	4.86	6.71	97.5	45.3
6.82	4.88	6.86	110.0	51.2
6.93	4.86	6.97	110.0	51.2
7.32	4.96	7.41	115.0	53.5

[a] Duplicate 1.00 g wet wt. (0.63 g swollen wt.) of washed resin containing adsorbed SKT (at least 980 a.u./g swollen resin, 15.0 g resin) was eluted with 2.50 ml (total liquid volume of resin buffer mixture of 2.87 ml) of 0.0075I EN-AA, pH5.96 to 7.32. The pH was returned to its former value with 0.5M-ethylenediamine (not greater than 100 µl added). Elution efficiency was calculated with equation 4 (Appendix Two)

$$= \frac{2.87 \text{ ml} \times \text{eluate SKT (a.u./ml)} \times 15.0 \text{ g}}{150 \text{ ml} \times (100 - <2.0 \text{ a.u./ml}) \times 0.63 \text{ g}} \times \frac{100}{1}$$

$$\leq \text{eluate SKT (a.u./ml)} \times 0.465\%$$

Several experiments were therefore performed using different ratios of resin to eluant to evaluate the effect on both overall recovery (extraction efficiency) and purity of SKT. The experimental conditions and calculations are recorded in Appendix Five, and the results are presented in Table 4.5. SKT elution was carried out in 0.0075I buffer at pH7.0 (Experiment 1), pH8.0 (Experiment 2) and in 0.06I buffer at pH6.0 (Experiment 2) for resin to eluate ratios in the range 0.2:1 to 1.6:1 (w/v), the latter ratio being the practical upper limit.

Elution efficiencies, which ranged 64.7 - 74.9%, did not vary greatly over the range of resin to eluate mixtures examined, and neither eluant ionic strength (0.0075 or 0.06I) or pH(7 or 8) showed any appreciable effect on recovery. This suggested that SKT elution was complete in eluates of different resin to eluate ratios and that elution at pH7.0 (actual value 7.15 - 7.23) resulted in maximal SKT recovery from pretreated resin.

Specific activity of SKT in the eluates is seen to increase for greater resin to eluate ratios for a given eluate condition (pH or ionic strength). Also there was little or no difference in specific activity between 0.0075I eluates at pH7.0 or 8.0 for a given resin to eluate ratio, but at 0.06I specific activity was approximately four times less. Since the elution of SKT was apparently complete for each mixture tested, specific activity was a direct function of eluate dry wt. The reason for a resultant decrease in eluate dry wt. with respect to greater resin to eluate ratios is not known, but is presumably due to insufficient buffer cation (eluant and titrant) to promote the same degree of elution of inert materials at high ratios (1.6:1) as at a lower ratio (0.4:1) of resin wt. to eluate volume.

Table 4.5. The effect of the ratio of resin wt. to eluate volume on the elution of SKT.[a]

Elution condition	0.0075I, pH7.0			0.0075I, pH8.0				0.06I, pH8.0	
Resin/eluate ratio (w/v)	0.4:1	0.7:1	1.6:1	0.2:1	0.4:1	0.7:1	1.6:1	0.4:1	1.6:1
Resin (swollen wt., g)	0.96	1.91	4.78	0.48	0.96	1.92	4.80	0.96	4.80
Titration pH	7.15	7.22	7.23	8.30	8.12	7.93	7.94	7.83	7.92
Titrant volume (ml)	0.04	0.095	0.25	0.02	0.05	0.11	0.28	0.03	0.29
Total eluate vol. (ml)	2.584	2.683	2.970	2.54	2.59	2.69	2.98	2.57	2.99
SKT specific activity (a.u./mg)	595	1120	1980	352	615	1190	1760	132	415
Elution efficiency (%)	64.7	66.9	67.4	67.4	71.1	71.3	69.5	74.9	68.4
Extraction efficiency (%)	59.2	61.2	61.6	61.4	64.8	65.0	63.3	68.3	62.3
Volume concentration ratio	3.72	7.12	16.09	1.97	3.86	7.43	16.78	3.89	16.72
SKT concentration ratio	2.20	4.36	9.91	1.21	2.50	4.83	10.62	2.66	10.42

[a] See Appendix Five for experimental conditions and calculations.

Extraction efficiency ranged 59.2 - 68.3%, being slightly less than elution efficiency since some activity remained non-absorbed from the culture filtrate and a small amount was eluted in the resin pretreatment buffer at pH4.52.

The maximum volume concentration ratio achieved was 16 to 17-fold for a resin to eluate ratio of 1.6:1, while the maximum SKT concentration achieved was about 10.5-fold due to the apparent extraction efficiency being about 65%. Since SKT activity was underestimated by the assay system, this figure represents the minimum concentration of SKT activity obtainable.

Effect of resin pretreatment at 0.015I on purity

In the previous experiments, the quantity of titrant required to bring the resin from pH4.5 to 7.0 (in 0.0075I buffer) was large compared to the eluant buffer capacity, being about 0.02 meq/g swollen resin. Equilibration of the resin in 0.015I buffer at a pH value near to the threshold for SKT elution would be expected to reduce this greatly and also elute additional inert material.

SKT-containing resin, equilibrated in 0.06I EN-AA, pH4.52, was washed with deionised water and portions (1.00 g wet wt.) were equilibrated in 10.0 ml of 0.015I EN-AA buffer, pH 5.56-7.08. From the results, Table 4.6, the threshold pH for SKT elution was about pH5.3. The effect of pHs near to the threshold elution value were then examined on the recovery and purity of SKT eluted at pH7.0 in 0.0075I buffer. The experimental data is given in Appendix Six, and is summarised in Table 4.7. Resin pretreated with 0.015I buffer, when equilibrated in 0.0075I elution buffer, yielded values of pH within 1.0 pH unit of their former

value of 7.0, compared to a difference exceeding 2 units in the control. The amount of 0.20M-EN titrant was therefore markedly reduced, being <10 μl/g resin (<0.002 meq/g resin) compared with four-fold this amount in the control.

Table 4.6. Elution of SKT activity from IR-120 resin with 0.015I EN-AA buffer of pH5.56 - 7.08.[a]

Eluant pH	Equilibrium pH	Eluate conductivity (mS/cm)	SKT activity (a.u./ml)	Elution efficiency (%)
5.56	5.16	0.78	< 1.5	< 1.7
5.96	5.31	0.78	< 1.5	< 1.7
6.19	5.44	0.82	3.2	3.6
6.43	5.68	0.87	17.0	19.1
6.60	5.92	0.91	29.7	33.3
6.75	6.38	0.96	56.0	62.8
6.87	6.62	0.98	56.0	63.4
7.08	8.28	1.05	58.0	65.0

[a] 25.0 g resin containing SKT, equilibrated in 0.06I buffer, pH4.52, and washed and drained was prepared as described in Appendix Six. 1.00 g wet wt. (0.963 g swollen wt.) was equilibrated in 10.0 ml (10.037 ml total volume) of 0.015I EN-AA buffer (1.0 mS/cm) of pH5.56-7.08. The elution efficiency was calculated:

$$= \frac{10.037 \text{ ml} \times \text{eluate SKT (a.u./ml)} \times 25 \text{ g}}{250 \text{ ml} \times (100 - 7.0^{*}, \text{ a.u./ml}) \times 0.963 \text{ g}} \times \frac{100}{1}$$

$$= \text{eluate SKT (a.u./ml)} \times 1.121\%.$$

* Equivalent of 7.0 a.u./ml: SKT lost from the resin during both adsorption at pH3.5 and pretreatment at pH4.52.

Table 4.7. The effect of resin pre-treatment with 0.015I EN-AA buffer of pH5.56 - 6.43 on the specific activity of SKT eluted in 0.0075I EN-AA buffer of pH7.03.[a]

0.015I pre-treatment buffer series (pH) :	5.56	5.96	6.19	6.43	Control, deionised water
0.0075I, pH7.03 eluant:					
Equilibrium pH	6.39	6.66	7.02	7.28	4.85
Titration pH	7.23	7.31	7.28	7.28	7.21
Titrant, 0.20M-EN (ml)	0.007	0.003	0.001	-	0.042
SKT specific activity (a.u./ml)	817	783	685	655	627
Elution efficiency (%)	<59.1	<59.0	57.5	59.7	59.9
Extraction efficiency (%)	53.7	53.6	50.7	41.0	54.4

[a] SKT-containing resin (0.91 g swollen wt.) was equilibrated in 0.015I buffer as described in Table 4.6. Control resin was washed in deionised water. Each resin treatment was washed quantitatively with 3 x 20 ml changes of water and the excess liquid removed by suction through a pasteur pipette. The resin adherent liquid content was determined on three control resin batches. SKT was eluted in 2.50 ml of 0.0075I EN-AA buffer, pH7.03, titrated with 0.20M-EN. See Appendix Six for experimental data.

Pre-treatment with 0.015I buffer at different pHs had no significant effect on SKT elution efficiency, which was similar to that of the control. However, it reduced extraction efficiency where pre-treatment pH exceeded the threshold value (pH5.3) for SKT elution, due to some "loss" of SKT from the resin. Eluted SKT specific activity was greater in the eluates from resin pre-treated at pHs below the threshold of SKT elution than at greater values, which are in turn marginally greater than the specific activities of control resin eluates. This result may be explained by the disproportionate elution of SKT compared with the dry wt. material, with respect to pH of the 0.015I pretreatment

buffers.

These results demonstrate that the pre-treatment of resin in 0.015I buffer both reduces titrant (10-fold) in the final elution of SKT and produces a 1.3-fold increase in SKT specific activity compared with the control.

Detailed description of batch isolation procedure

The aforegoing series of experiments established optimal conditions for the isolation of SKT activity from culture filtrate using IR-120(H) resin. An outline of the procedure used to isolate SKT from 4 litres of culture filtrate is shown in Figure 4.8. The scheme consists of four main stages, each of which are separated by a step in which the resin is washed with deionised water to reduce the volume of lower-ionic strength buffer needed in the proceeding step.

400 g of cycled resin was stirred into 4000 ml of culture filtrate, previously diluted to a conductivity of 10.5 mS/cm at pH5.3, contained in a 6 litre flask. The mixture was shaken, except during titration, when it was stirred using a magnetic bar, as vigorous stirring produced resin fines which reduced SKT yield. When the pH approached 2.0, the stirred mixture was carefully brought to pH3.50 by slowly adding 40% NaOH, about 60 ml being required. When the pH remained stable (after 15 min) the liquid was removed by pouring the mixture into a large scintered glass funnel. An aliquot of fluid was kept for assay to determine the amount of non-adsorbed SKT. This was done for each step.

The resin was washed with deionised water, under restricted flow until the effluent conductivity fell to about 50 μS/cm, and transferred to a 2

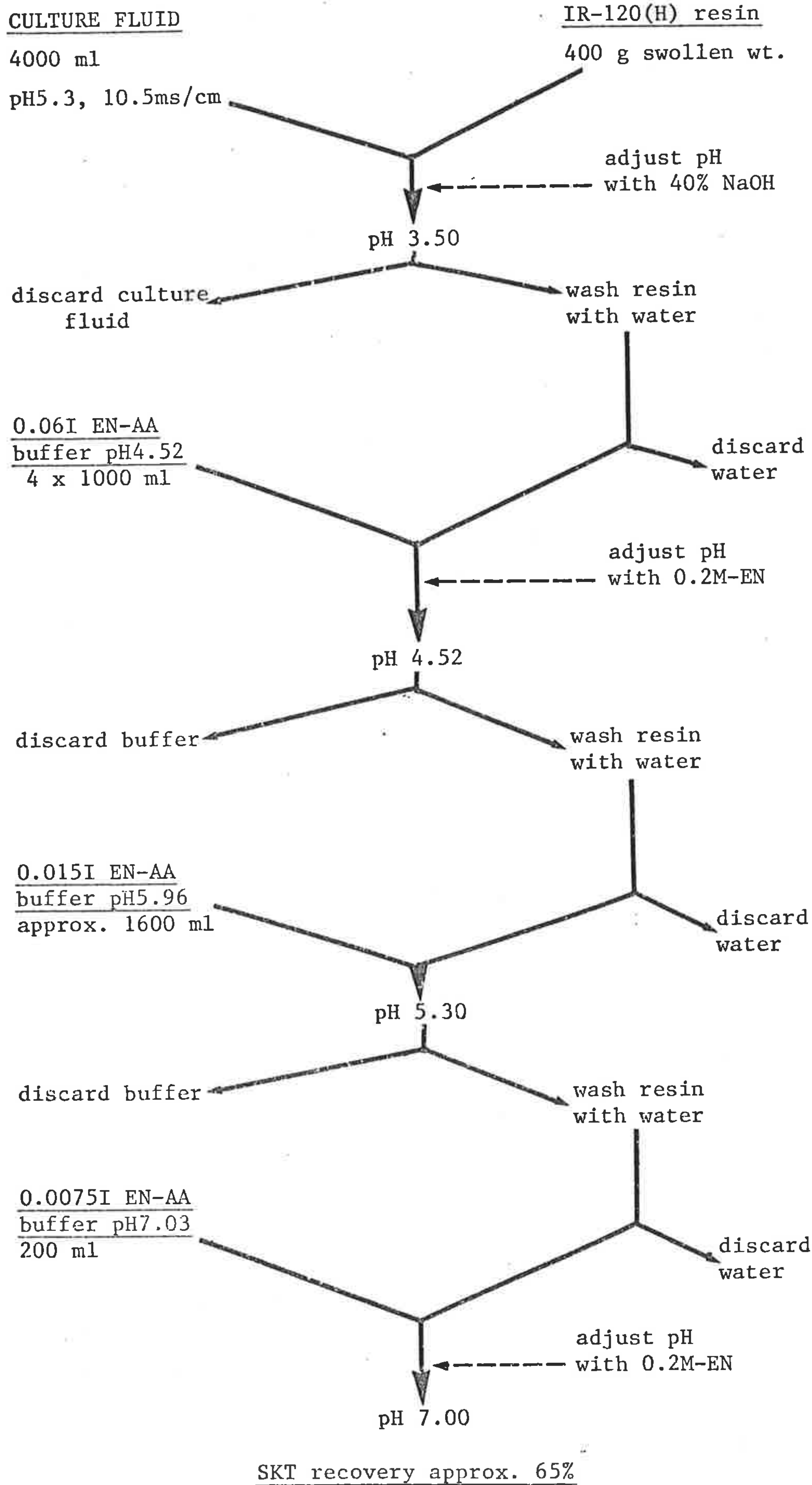


Figure 4.8. Procedure for the batch islation of SKT from culture fluid using Amberlite IR-120(H) resin.

litre flask. The resin was equilibrated in 1 litre of 0.06I EN-AA buffer of pH4.52 (3.5 mS/cm) and the pH brought to 4.55 with 0.2M-ethylenediamine. The buffer was removed by decantation and that trapped in the resin was removed by suction using a 5 ml pipette inserted to the bottom of the bed of resin. This procedure was repeated with 3 x 1 litre portions of buffer. The pH and conductivity of the final equilibration liquid was 4.54 and 3.3 mS/cm respectively.

Following washing of the resin with deionised water by decantation and suction, it was treated with two portions of 0.015I EN-AA buffer, pH5.96 conductivity 1.0 mS/cm. Firstly the resin was equilibrated in 1 litre of buffer, and after removal of the buffer by decantation, 100 ml portions of buffer were added with thorough mixing until the pH reached and remained at 5.30 - 5.35; conductivity was approximately 0.75 mS/cm. About 600 ml of buffer was required. The buffer was removed and the resin thoroughly washed with water until the conductivity was less than 50 μS/cm.

After removal of the excess water by suction, 200 ml of 0.0075I EN-AA buffer, pH7.30, 0.56 mS/cm, was added to the resin. With constant stirring, the mixture was adjusted to pH7.0 by dropwise addition of 0.2M-ethylenediamine; approximately 4 ml. pH of the mixture was monitored by a combination type electrode. When the pH of the liquid remained steady at 7.0 for 15 min, the mixture was poured into a glass column (4.6 cm x 60 cm) and the eluate displaced with 50-100 ml of buffer, pH7.03, followed by deionised water. The total effluent collected (350 ml) was reduced to about 50 ml under reduced pressure by rotary film evaporation at 40°C. The concentrate was clarified by centrifugation (20000 xg) and either lyophilised and kept *in vacuo* over P_2O_5 or stored frozen at -18°C.

Table 4.8 shows the recovery and specific activity of SKT isolated from some four different batches of culture filtrate. 64.7% of the activity present in culture filtrate was isolated, but only 73.9% of the total could be accounted for by assay. This discrepancy is largely accounted for by the under-estimate of SKT potency through the use of the culture filtrate reference standard. Furthermore, SKT present in pre-treatment washes at levels near that of the assay sensitivity (approximatly 2 a.u./ml), when present in large volumes may also contribute a significant discrepancy. In early experiments, up to 10% of the total culture filtrate activity remained resin-bound following elution with 0.0075I buffer and displacement by water. This could be reduced to only several percent by using buffer instead of water to displace the eluate.

The average specific activity of SKT isolated from four different batches of culture filtrate was 5342 a.u./mg dry wt. representing an 862-fold purification of SKT. The actual increase is thought to be greater because of the underestimate of SKT potency by assay.

Table 4.8. Recovery and specific activity of SKT isolated from culture filtrate using IR-120 cation exchange resin.[1]

Stage of treatment	SKT activity in liquid phase		
	Mean %		Range
Culture filtrate	100.0		–
Adsorbed culture filtrate at pH3.5	2.2	(7)	N.D. - 4.0
pre-SKT elution, 0.06I at pH4.52-4.55	2.1	(7)	1.0 - 2.6
pre-SKT elution, 0.015I at pH5.3	N.D.	(5)	N.D. - 1.6
SKT elution 0.0075I at pH7.0-7.5	64.7	(7)	55.6 - 73.0
post-SKT elution 0.06I at pH6.7-8.5	4.2	(5)	2.0 - 10.5
TOTAL SKT activity recovered[a]	73.9	(4)	69.3 - 76.5
SKT Specific activity (a.u./mg dry wt.)	5340	(4)	4720 - 6580
Purification (-fold)	862	(4)	829 - 960

[1] The results are the average of data from 4 to 7 experiments each using a different batch of culture filtrate stock. The purification index was determined for each respective batch of SKT and the individual values averaged; [a] Total activity accounted for by assay; Value in parenthesis is the number of experiments: N.D., Not detectable, corresponding to about 0.005% of total culture filtrate activity for an assay sensitivity of 2 a.u./ml.

Gradient elution chromatography

Gradient elution ion exchange chromatography, by which a high degree of resolution of the components in a mixture can be achieved, was employed to indicate a correlation between SKT activity and a biochemical component. The conditions employed were based on those established in the previous batchwise experiments.

Initially it was decided to isolate SKT on a column of IR-120(H) resin, which provides maximal adsorption efficiency, and following washing of the column with buffer (0.0075I EN-AA, pH4.6) eluate SKT with a gradient of 0.0075I EN-AA buffer of pH4.6-7.0. However, titration of the resin acidity from approximately pH3.5 to 4.6 required an impractically large volume of low ionic strength buffer. This difficulty was overcome by employing batch adsorption conditions after which the resin was washed with deionised water, suspended in EN-AA buffer of pH4.6 and titrated to pH4.6 with 0.2M-ethylenediamine. The resin was then resuspended in a small volume of EN-AA buffer pH4.6 of appropriate ionic strength and packed into a column for the gradient elution procedure.

Three conditions of gradient elution were used: (i) 0.0075I EN-AA, pH4.7-7.0; (ii) 0.06I EN-AA, pH4.6-7.0 and (iii) 0.0075I EN-AA, pH4.7 to 0.06I EN-AA, pH7.0. The gradients were produced by mixing limit buffer with starting buffer, each contained in vessels of appropriate shape to obtain the required gradient (PETERSON, 1970; Sephadex ion exchangers, Pharmacia). The eluant volume was 16 - 20-fold the weight of resin. Gradients (i) and (iii) were produced by mixing 300 ml of limit buffer contained in a 500 ml small diameter beaker with 350 ml of starting buffer contained in a 500 ml conical flask from which the eluant was drawn. The convex-shaped pH gradient produced was necessary to compensate for the relatively high buffering capacity of the column. Gradient (ii) was produced by the linear mixing of 400 ml of limit buffer with 400 ml of starting buffer, both contained in 500 ml beakers. This gradient was slightly concave with respect of pH owing to the relatively greater buffer capacity of the starting buffer. The gradients were pumped at 25 ml/h through a 40 g (swollen wt.) bed of

prepared-resin contained in a column of dimensions 2.0 cm x 30 cm. The column effluent was monitored continuously at 280 nm and fractions of 11 ml were collected. The fractions were assayed for SKT activity and for protein by the method of LOWRY et al. (1951). No attempt was made to correct for the interference caused by the presence of ethylendiamine in the effluent fractions, but since no precipitation occurred, the concentration of ethylenediamine was low and hence interference was assumed to be minimal. The BSA protein assay reference was therefore prepared in 0.1M-acetate buffer, but it must be pointed that protein may be underestimated by these conditions. Fractions corresponding to the major peak of SKT activity were pooled to determine both the recovery of SKT and dry wt. (by lyophilisation). Total protein, estimated by the Lowry method, was determined on the lyophilised material, dissolved in distilled water, to avoid interference by ethylenediamine.

Typical elution profiles obtained are shown in Figures 4.9, 4.10 and 4.11. At low ionic strength (0.0075), SKT activity was eluted maximally at pH6.0 (effluent pH) and was associated with a small peak of Lowry positive material. The main peak of SKT contained a weakly active tailing shoulder, the elution of which, was complete at an effluent pH of 8.0. The reason for the effluent pH exceeding that of the eluant may be due to the very low buffering capacity of the eluant. pH gradient elution made at greater ionic strength (0.06I) resulted in elution of SKT at a lower pH, maximally at pH5.0 (see Figure 4.10). This peak also contained a tailing shoulder of SKT activity. The major peak of SKT was again associated with a small peak of Lowry reactive substance. The dual gradient (increasing pH and ionic strength) eluted SKT activity as a sharp peak at an effluent pH of 5.6 and was associated with both u.v. absorbing and Lowry positive material. No tailing SKT activity was detected. In none of these experiments was additional SKT activity

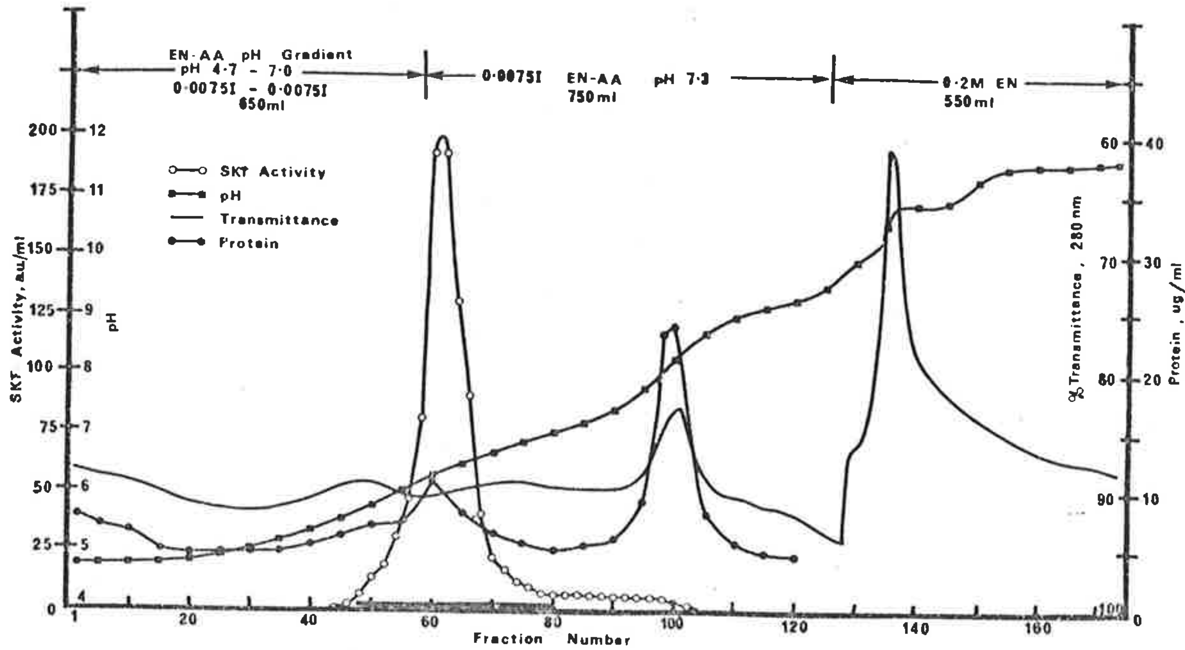


Figure 4.9. A profile showing the elution of culture filtrate components from a column of IR-120 resin by a 0.0075I ethylenediamine-acetic acid gradient of pH4.71 - 7.03. Elution was completed with 0.0075I EN-AA at pH7.33, and 0.2M-EN. 400 ml of culture filtrate (pH5.30, 10.5 mS/cm) was adsorbed with 40 g (swollen wt.) of IR-120(H) resin (14-52 mesh) at pH3.60 after which the resin was firstly equilibrated in 0.06I EN-AA at pH4.64 and then in 0.0075I EN-AA at pH4.71. The resin was packed into a column (bed, 2.0 cm x 25.0 cm) and eluated with a 650 ml gradient of 0.0075I EN-AA pH4.71 - 7.30 followed by 750 ml of 0.0075I EN-AA at pH7.33 and 550 ml of 0.2M-EN. The flow rate was 25 ml/h and fractions of 11.2 ml were collected.

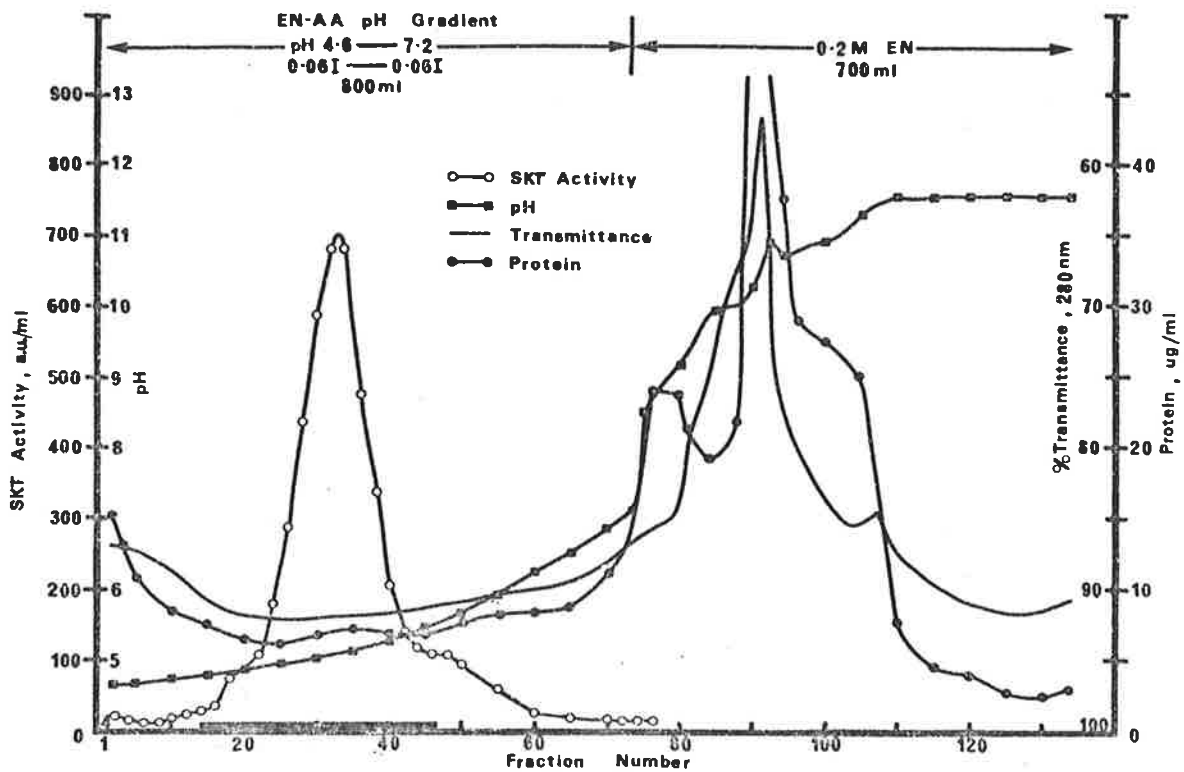


Figure 4.10. Elution of culture filtrate components from a column of IR-120 resin by a 0.06I EN-AA gradient of pH4.64 - 7.23. The conditions were as described in Figure 4.9 except that equilibration of the resin in 0.0075I EN-AA at pH4.71 was omitted and the pH gradient was made with 800 ml of 0.06I EN-AA at pH4.64 - 7.23. Fraction size was 11.0 ml.

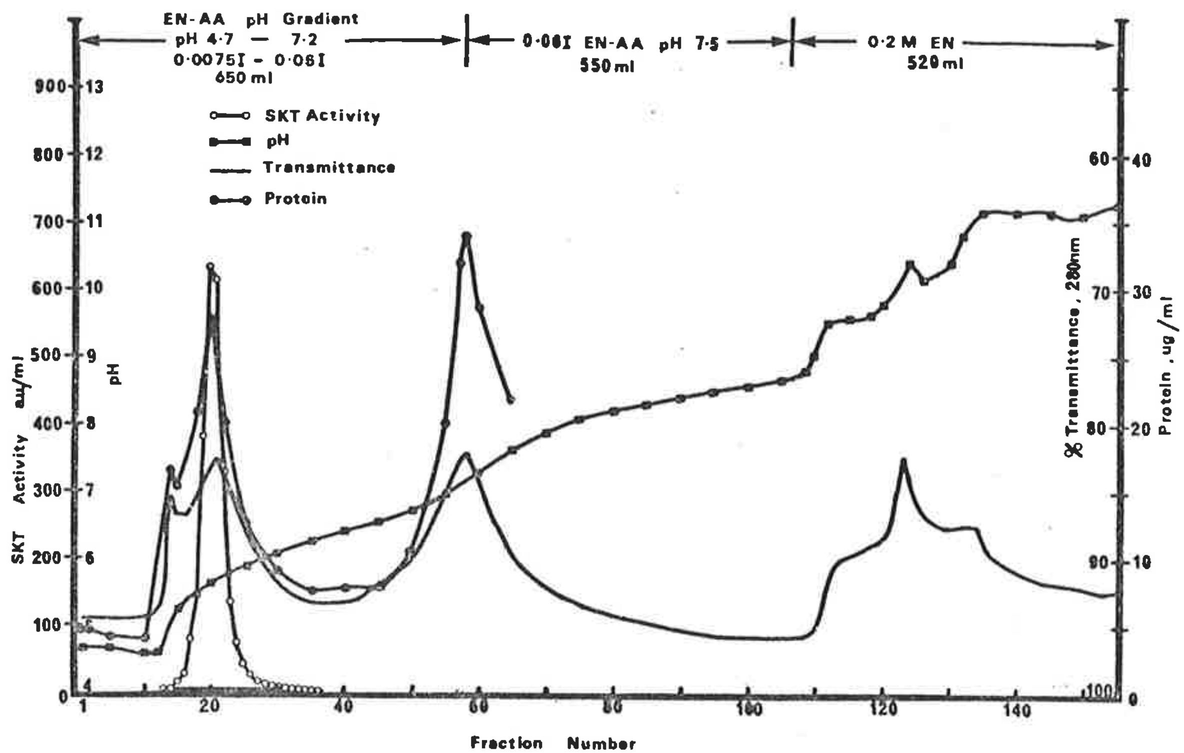


Figure 4.11. Elution of culture filtrate components from a column of IR-120 resin using a dual gradient of EN-AA buffer, 0.0075I at pH4.71 to 0.06I at pH7.23.
The conditions were as described in Figure 4.9 except that the gradient was formed by mixing 350 ml of 0.0075I EN-AA at pH4.71 with 300 ml 0.06I EN-AA at pH7.23 as described in the text. Fractions of 11.2 ml were collected.

detected in the effluent to a pH of 11 to 12, although at such pH it would probably be inactivated (see Figure 3.5).

The properties of SKT activity eluted by the three gradient conditions are shown in Table 4.9 (detailed in Appendix Four). Yield of SKT ranged from 40 to 66.7%, being maximal by dual gradient elution. The method of selection of SKT active fractions for pooling is in part responsible for the difference in SKT recovery between gradient types. The apparent overall low recovery of SKT resulted from an approximate 20% underestimate in assay through the use of culture filtrate SKT as the reference standard (see Figure 4.19). All three gradient conditions yielded SKT of increased purity, the dual-gradient providing SKT of greatest specific activity with respect to both dry wt. (18-fold) and protein (2.1-fold). It is clear from these results that a large amount of non-Lowry reactive material also eluted with SKT.

The experiments suggested that SKT activity was associated with protein since a small peak of Lowry positive material co-eluted with SKT under each of the three chromatographic conditions. CAM electrophoretic analysis of the material obtained by lyophilisation of the major and shoulder peaks showed single active bands which when dissolved in culture filtrate co-migrated with culture filtrate SKT. The tailing activity was therefore considered to be derived from the major SKT peak.

<u>Acetic acid extract</u>

The lyophilised material obtained from the batch resin eluates was not fully soluble in either distilled water, phosphate buffer containing 0-5M-KCl, or 1-10% acetic acid, but while being completely soluble in aqueous ethylenediamine or NaOH, SKT was inactivated and the material

Table 4.9. Yield and specific activity of SKT in pooled fractions obtained under three conditions of pH gradient elution.[1]

Gradient	Expt. No.	Pooled vol. (ml)	Total SKT (a.u.)	Dry wt. (mg)	Specific activity (a.u./mg)	Protein (mg)	Specific activity (a.u./mg)	SKT yield[2] (%)
-	Culture filtrate	-	40000	6442.0	6.21	10.28	3890	100.0
0.0075I pH4.71 - 7.03	A1 a)	369.6	17926	166.1	107.9	2.920	6139	45.8
	b)	246.4	1725	N.D.	-	-	-	4.4
	A2 a)	347.2	15659	149.8	104.5	2.118	7393	40.0
0.06I pH4.64 - 7.23	B1 a)	442.8	19483	N.D.				49.7
	B2 a)	363.0	20328	356.3	57.1	2.251	9031	52.1
	b)	154.0	1694	N.D.	-	-	-	4.3
0.075I, pH4.71 to 0.06I, pH7.23	C1	118.8	23522	N.D				60.5
	C2	179.2	26163	241.2	108.5	3.118	8391	66.7
	C3	179.2	23296	207.6	112.2	2.491	9352	59.6

[1] See Appendix Four; [2] SKT yield (elution efficiency) corrected for losses during adsorption and pre-gradient elution; a) Major peak of SKT activity; b) SKT tailing shoulder; N.D. not determined.

reprecipitated on neutralisation. Concentration of the resin eluate to <20 ml, under reduced pressure, also resulted in the formation of insoluble material which, by difference, contained 5-10 of the total SKT activity and behaved similarly to the lyophilised insoluble material. For both cases, both the insoluble material and SKT showed greatest solubility in 5-10% acetic acid, in which insoluble SKT was estimated at about 2%.

Since gel chromatography was employed in the further purification of SKT activity, it was desirable to reduce the resin eluate volume to less than 10 ml for application to a gel column. Thus the lyophilised material (60-80 mg) was treated with 5 ml of 10% (v/v) acetic acid, made up to 10 ml with distilled water and clarified by centrifugation (20000 xg for 20 min). Resin eluates that were concentrated directly to about 10 ml under reduced pressure at 35°C, were acidified to 10% (v/v) with glacial acetic acid and clarified by centrifugation. In analysis of the supernatant fluid, an aliquot was diluted 100-fold in 0.1M-sodium acetate pH6.0 for both SKT assay and protein estimation and 10-fold in 0.11M-acetic acid for u.v. absorbance measurement by reading against 0.20M-acetic acid in 10 mm quartz cells.

Comparison of SKT isolated from standard and peptone supplemented cultures

Large batches of culture filtrate, derived from the chemically defined SKT production medium, although less active than the peptone supplemented-derived culture filtrate, were used in the development of the SKT isolation procedure because of a possible difficulty in the subsequent removal of the added peptone (0.02% Bacto-peptone). Properties of SKT activity in the standard culture filtrate (CF_s),

peptone supplemented filtrate (CF_p), and in their derived batch resin eluates and subsequent acetic acid extracts is summarised in Table 4.10.

The activity of SKT was about 60% greater in CF_p than CF_s and consequently its specific activity in CF_p was also greater. SKT specific activity, on a protein basis, was presumably relatively lower in CF_p than expected because of the added peptone (protein was measured, following dialysis in Visking tubing 0.390 in flat width, by the Lowry method). SKT derived from either CF_s or CF_p isolated by the batch resin procedure, had a similar specific activity on a gravimetric basis but SKT present in the acetic acid extract and derived from CF_p was greater than that from CF_s. This indicated that the resin isolation procedure did not reflect the greater specific activity of SKT, in CF_p, on a dry wt. basis but did so with respect to protein. Since the SKT eluate (pH7) derived from CF_p was displaced from the resin by buffer (0.0075I, pH7.03) whereas those from CF_s were displaced by distilled water, it is possible that the displacement buffer eluted additional inactive material and thus accounted for the apparent reduced SKT purity on a gravimetric basis. This effect was observed in the experiments on the effect of the ratio of resin to eluate on SKT purity (see Table 4.5).

Protein was not estimated directly on the resin eluate because of possible interference to assay by ethylenediamine, but that estimated on the acetic acid extract may not directly reflect the protein content of the resin eluate through possible partial loss of protein to the acid insoluble fraction.

A detailed analysis of two SKT preparations derived from CF_p is presented in Table 4.11. Of note is the u.v. absorbance ratio (280

Table 4.10. Yield and specific activity of SKT in filtrates, IR-120 resin eluates and subsequent acetic acid extracts derived from standard and peptone-supplemented cultures.

Treatment	Culture filtrate		IR-120 eluate		5% acetic acid extract	
Sample origin	CF_s	CF_p	IR_s	IR_p	IR_s-aa	IR_p-aa
Number of preparations	5	1	4	2	2	2
SKT activity (a.u./ml)	94 (82-105)[a]	156				
Yield (%)[b]	100	100	62.7 (55.6-69.2)	67.9	60.7	66.5
Specific activities:						
Dry weight (a.u./mg)	6.46 (5.73-7.84)	10.13	5340 (4720-6580)	5660	N.D.	N.D.
Purification (-fold)	-	-	862	559	-	-
Protein (a.u./mg)	4209 (3306-5147)	5474	N.D.	N.D.	8800	11360
Purification (-fold)	-	-	-	-	2.09	2.08

Subscripts s and p designate preparations derived from standard and peptone supplemented cultures respectively; N.D. Not determine; [a] Range of values; [b] Resin SKT eluates (pH7) derived from CF_s were displaced with water whereas those from CF_p were displaced with buffer.

Table 4.11. Properties of SKT in two preparations of IR-120 resin eluates and subsequent acetic acid extracts derived from a peptone supplemented culture filtrate.

IR-120 eluate preparation	IR_p-1	IR_p-2
SKT activity (a.u.)	446500	401000
Yield (%)	71.6	64.3
Dry weight (mg)	79.55	70.22
Specific activity (a.u./mg)	5610	5710
Purification (-fold)	544	564
5% acetic acid soluble fraction		
Volume (ml)	10.0	10.0
SKT activity (a.u.)	438000	392000
Protein (Lowry method, mg)	36.95	36.10
Specific activity (a.u./mg)	11850	10860
Purification (-fold)	2.17	1.98
Absorbance, 280 nm	6.42	6.30
Absorbance ratio, 280 nm/260 nm	1.05	1.04
Protein (Warburg method, mg)	53.15	51.67
Specific activity (a.u./mg)	8240	7590

nm/260 nm) of the acetic acid preparation and the protein estimate derived from it. This suggests that the greater proportion of u.v. absorbing material can be accounted for as protein. It is important to realise however that protein estimates by both methods used different standards (bovine serum albumin compared with yeast enolase and nucleic acid) and both are subject to interference by non-protein substances which may be present in these crude preparations.

PART B. PURIFICATION AND CHARACTERISATION OF THE ISOLATED KILLER ACTIVITY

Bio-Gel P-2 filtration

A. Preliminary findings

Chromatography of the batch resin eluate concentrates on a column of Bio-Gel P-2 in aqueous acetic acid eluant produced a broadly similar elution pattern as obtained with culture filtrate concentrates (compare Figures 4.2 and 4.12). SKT eluted near the Vo, overlapping excluded u.v. absorbing and Lowry positive material. These peaks were followed by two large peaks of inactive u.v. absorbing but weakly Lowry positive substances. Aqueous acetic acid was the preferred eluant because it delayed the elution of gel-included substances enabling the application of highly active SKT preparations, and was readily removed by lyophilisation. In preliminary experiments, in which the resin eluate concentrates of pH7.0 were chromatographed in 0.1M-acetic acid, SKT was retarded and eluted near K_{av}0.7, coinciding with the second peak of u.v. absorbing material. Acidification of the sample to 2-5% (v/v) acetic acid resulted in its near-exclusion from the gel as for culture filtrate preparations.

Although the bulk of SKT (60-70%) eluted between Vo and the following u.v. absorbing inactive peak (that is between K_{av}0.0-0.5), when the column loading of SKT activity was large (>50000 a.u.) a considerable proportion of activity eluted as an extended tailing shoulder, up to a K_{av} of 1.7. Such tailing was not observed with the culture filtrate concentrate (compare Figures 4.2 and 4.12), possibly because of the much lower SKT activity applied to the column and was therefore not detected.

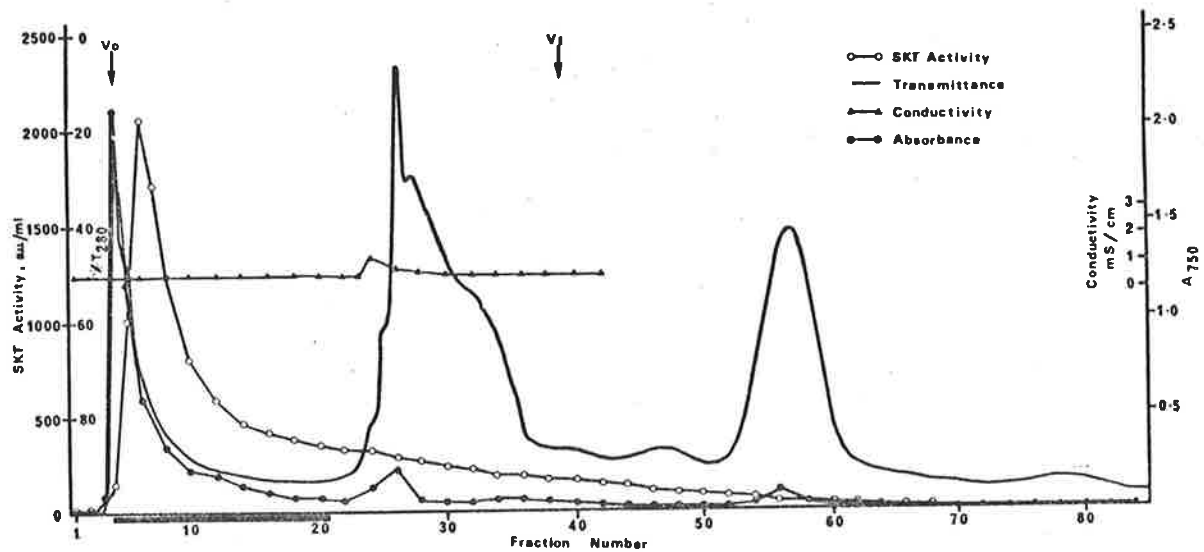


Figure 4.12. Bio-Gel P-2 column chromotography of a highly active SKT acid extract in 0.2M-acetic acid.
The 4.0 ml sample, containing 175000 a.u. SKT (derived from CF_p), 31.82 mg dry wt. and 1.48 mg protein, was eluted from a column of Bio-Gel P-2 (2.6 cm x 38.0 cm) with 0.20M-acetic acid at 30 ml/h, and fractions of 3.9 ml were collected. Column Vo and Vt was 62 ml and 198 ml respectively.

Rechromatography experiments suggested that the tailing SKT was not different from the near-excluded fraction. Pooled post-K_{av}0.5 SKT activity re-eluted as a near-excluded peak and associated tailing shoulder (see Figure 4.13), and the K_{av}0.0-0.5 fraction showed an analogous SKT profile as obtained in the primary run, except that the following u.v. absorbing peaks were absent. SKT, recovered from both the near-excluded and tailing fractions by lyophilisation and taken up in culture filtrate, co-migrated with culture filtrate SKT when electrophoresed on CAM strips at pH4.3.

B. Procedure

A maximum of 4.0 ml of concentrate (acetic acid extract) was applied to a column of Bio-Gel P-2 of dimensions 2.6 cm x 38 cm and eluted with 0.2M-acetic acid. The rate of elution was 30 ml/h and fractions of 3-5 ml were collected. Samples from each fraction were conveniently prepared for SKT assay by diluting 200 µl with 400 µl of diluent consisting of 1 part 0.20M-ethylenediamine to 3 parts distilled water. It was however necessary to check and adjust the pH of samples prepared from fractions of K_{av} near 0.5 which showed increased conductivity and hence buffer capacity. A pH of 6.0±0.2 was tolerable in the assay of column effluent fractions.

SKT activity was recovered from the Bio-Gel P-2 eluate by pooling SKT active fractions lying between Vo and about K_{av}0.5, that is, up to the two preceding fractions showing an increase in conductivity and a coincident increase in u.v. absorbancy (corresponding to fractions 4-20 in Figure 4.12). In early experiments, using preparations derived from CF_s, the first two SKT active fractions that eluted, which contained the bulk of gel-excluded proteinaceous material, were excluded from the

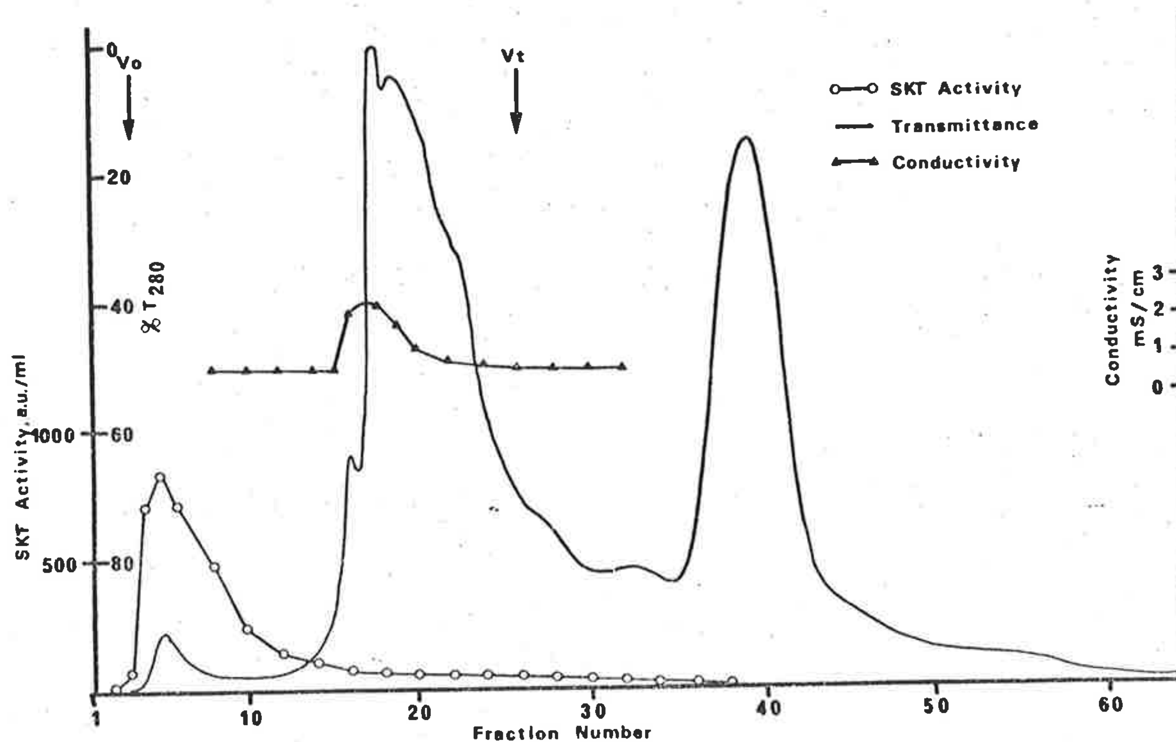


Figure 4.13. Rechromatography of a Bio-Gel P-2 eluted SKT active tailing fraction.
The SKT active fraction, which eluted between K_{av} 0.5-1.7 in the primary chromatography of an acid extract, was concentrated _in vacuo_ at 40°C to 4.0 ml (56000 a.u.) and rechromatographed on Bio-Gel P-2 as described in Figure 4.12. Fraction size was 6.0 ml.

pooled sample, effectively eliminating much of the inactive protein. Later this proteinaceous material was found not to interfere in the subsequent purification of SKT, and these fractions, which contained about 10% of the total SKT activity, were therefore included in the pooled sample.

C. Specific activity of SKT eluted from Bio-Gel P-2

Table 4.12 summarises the yield and specific activity of SKT, eluted from Bio-Gel P-2 with 0.2M-acetic acid, derived from acidic extracts originating from both standard and peptone supplemented cultures. The respective preparations are however not directly comparable because of the different criterion used to pool the CF_s and CF_p derived eluate fractions resulting in 57% of the applied activity being recovered in preparations of the former compared with 66% for the CF_p originating activity. Because of the omission of the first two SKT active fractions which contained much of the eluted protein, from the pooled sample, the specific activity of SKT with respect to protein in CF_s derived eluates showed a small increase over that in the applied sample, while those derived from CF_p actually showed a small decrease in specific activity. This was to be expected on the basis of experiments using culture filtrate concentrates (Table 4.1). The small decrease in specific activity of SKT in the CF_p derived eluate resulted because of the relatively greater recovery of protein compared with SKT activity in the pooled sample. However, from the elution profiles, it was clear that two peaks of u.v. absorbing material low in Lowry reactive content were eliminated. This was reflected by a marginal increase in specific activity based on u.v. absorbance measurements and an increase in the absorbance ratio from about 1.05 to 1.18 (compare Tables 4.11 and 4.12).

Table 4.12. Yield and specific activity of SKT in eluates from Bio-Gel P-2 chromotography of 5% acetic acid extracts derived from standard and peptone supplemented cultures.[a]

Parent culture filtrate of acid extract	Standard (CF_s)	Peptone supplemented (CF_p)	
Bio-Gel P-2 eluate	$P2_s$	$P2_p$ -1	$P2_p$ -2
Number of experiments	2	3	3
Pooled volume (ml)	71.8[b]	75.7	79.1
SKT yield (%)	57.1 (53.5-60.7)	65.3 (58.8-70.0)	67.1 (63.3-72.7)
Specific activities:			
Dry wt. (a.u./mg)	N.D.	6910 (6760-7060)	6020 (5670-6470)
Purification (-fold)		682	604
Protein, Lowry method (a.u./mg)	10820 (10550-11090)	10760 (10300-11240)	9220 (9140-9310)
Purification (-fold)	2.36	1.97	1.69
Protein, Warburg method (a.u./mg)	N.D.	9100 (8330-9820)	7520 (6920-8120)
Absorbance ratio 280 nm/260 nm	N.D.	1.19	1.18

[a] Column loading of SKT ranged 75000 - 175000 a.u.; Preparations $P2_p$-1 and -2 were derived from IR_p-1 and -2 respectively shown in Table 4.11; [b] First two SKT active fractions eluted were omitted (K_{av} 0.0-0.1) from the pooled SKT sample; Values given in parentheses indicate the range of values; N.D., Not determined.

Bio-Gel P-10 chromatography of the Bio-Gel P-2 active-eluate

A. Preliminary findings

Chromatography of a culture filtrate concentrate (CF_s) on either Sephadex G-50 or Bio-Gel P-10 in phosphate buffer resulted in the elution of SKT activity near K_{av}0.8 in association with the culture filtrate salts, but well separated from the excluded u.v. absorbing-Lowry positive material (see Figure 4.14). In two experiments, 86.6 and 90.6% of the applied activity was recovered in eluates from Bio-Gel P-10. For phosphate and other eluants to be described, in the first instance, recovery of SKT was very low and its K_{av} value variable. To obtain reproducible results it was necessary to prime the column with two passages of concentrate before a quantitative estimate was made. Phosphate eluants of pH4.0-8.0 did not affect the elution pattern. However when the eluant was 0.2M-acetic acid, the elution of SKT was greatly retarded with a K_{av} of 1.1 and was therefore eluted well clear of the culture filtrate derived salts but not from other retarded substances (Figure 4.15A). K_{av} values exceeding 1.0 indicate that elution is by a mechanism other than gel filtration. Certain proteins and peptides, which are either highly basic or contain aromatic amino acids are well known to be retarded on Sephadex and Bio-Gel materials (PORATH, 1960; GELOTTE, 1960; PORATH, 1968; DETERMANN, 1968). Nucleic acid derivatives are also retarded on these gels because of high polarity and aromatic nature (HOHN and SCHALLER; 1967; BONILLA, 1969).

Attempts to overcome this type of anomolous chromatographic behaviour have been made by the technique of "aromatic saturation". While PORATH (1960) using a 0.2M-sodium salicylate-containing eluant was unsuccessful

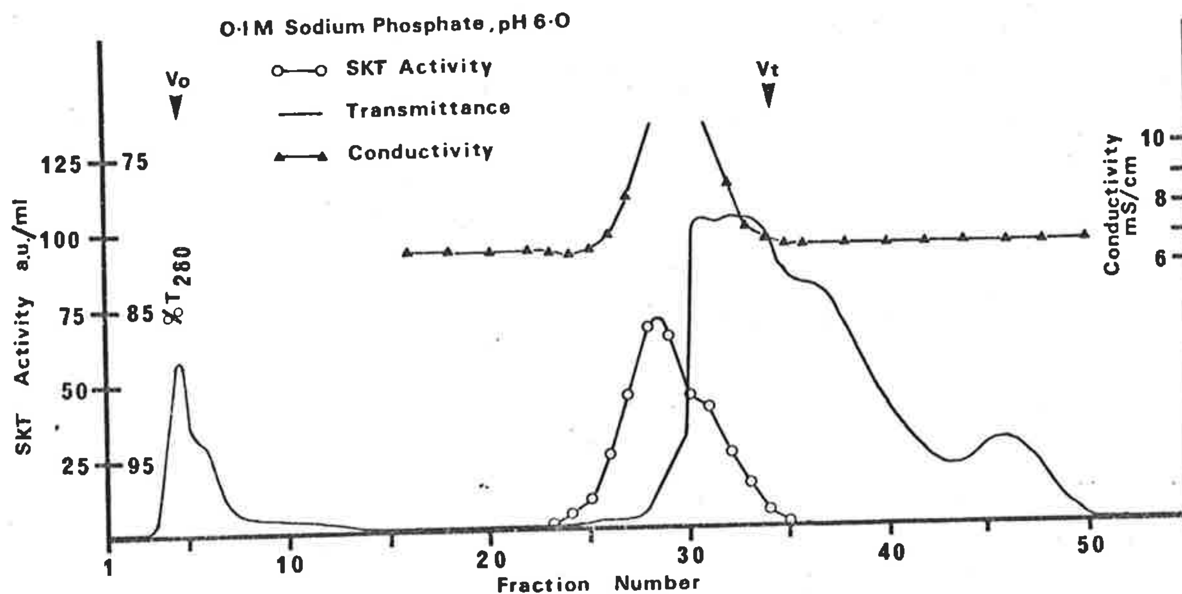


Figure 4.14. Chromatography of a culture filtrate concentrate on Bio-Gel P-10 in sodium phosphate buffer.
2.5 ml of a culture filtrate concentrate (5-fold) was eluted (20 ml/h)from a column (2.1 cm x 38.0cm) of Bio-Gel P-10 (100-200 mesh) with 0.1M-sodium phosphate buffer at pH6.0. Fractions of 3.3 ml were collected and the column Vo and Vt was 30.5 ml and 129.0 ml respectively.

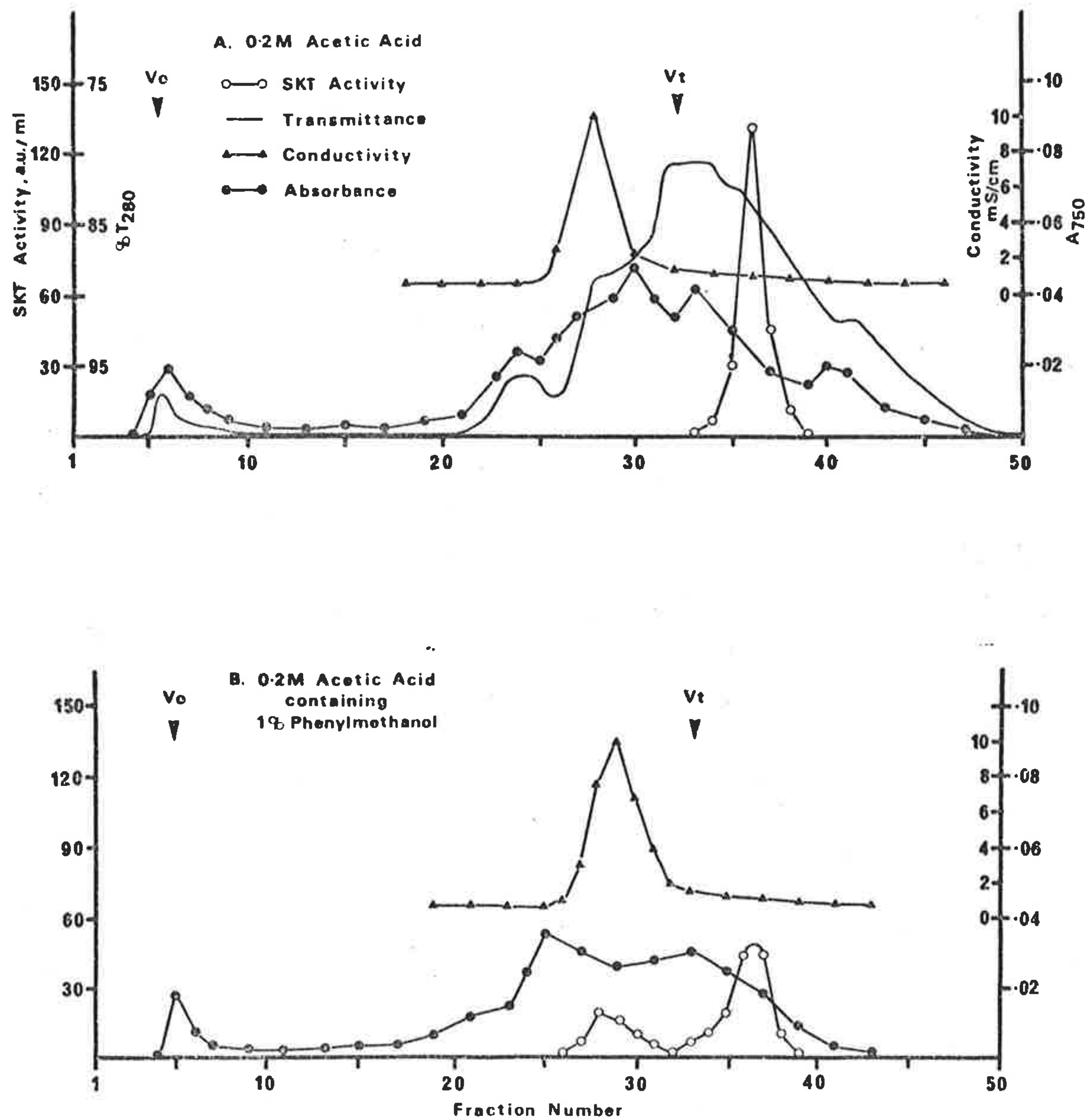


Figure 4.15. Chromatography of a culture filtrate concentrate on Bio-Gel P-10 in (A) 0.2M-acetic acid, and (B) 0.2M-acetic acid containing 1% phenylmethanol.
2.0 ml of concentrate (5-fold, pH3) was applied to a P-10 column of Vo 32.5 ml and Vt 130.0 ml and eluted at 20 ml/h. Fractions of 3.5 ml and 3.4 ml were collected in (A) and (B) respectively.

in reducing the absorption of amino acids on Sephadex gel, MOORE and STEIN (1951) found that the addition of 1% phenylmethanol to the eluant induced both peak sharpening and increased the rate of migration for aromatic amino acids. The two observations are not directly comparable because the latter was made on a column of Dowex 50, a polystyrene sulphonic acid resin.

The addition of phenylmethanol to the 0.2M-acetic acid eluant (prepared by shaking the mixture overnight at room temperature; the culture filtrate concentrate was also made approximately 1% in phenymethanol) only resulted in a partial normalised elution of SKT activity when compared with phosphate eluant (Figure 4.15B). That is, for the experiment shown, 35% of the total SKT recovered (75%) appeared at K_{av}0.8 and 65% remained eluted near K_{av}1.1. This result suggested that SKT contained an aromatic moiety, which in a weakly polar environment interacted strongly with the gel matrix. In these experiments there was no correlation between SKT activity and either u.v. absorbing or Lowry reactive substances. The level of SKT activity was however very low in these experiments.

B. Bio-Gel P-2 eluates

Chromatography of Bio-Gel P-2 eluate SKT activity (derived from batch resin eluates) on Bio-Gel P-10 in aqueous acetic acid however resulted in the bulk of SKT activity eluting near K_{av}0.55, earlier than when culture filtrate concentrates were used. A typical elution profile is shown in Figure 4.16 in which a highly SKT active P-2 eluate, concentrated under reduced pressure by rotary film evaporation at 40°C, was eluted from Bio-Gel P-10 with 0.2M-acetic acid.

Two major peaks of substance were present in the column effluent as revealed by u.v. absorbance measurement at 280 nm and the Lowry method for proteins. A sharp, but skewed peak of proteinaceous material was excluded from the gel while the second, a broad peak, possessing both a leading but more pronounced tailing shoulder, was eluted at K_{av}0.53-0.56 (fraction 22 of Figure 4.16). A trace of SKT activity was usually detected at the excluded peak while most was associated with the broad proteinaceous peak centering on K_{av}0.54. The leading shoulder to this peak did not appear to be SKT active but a tailing shoulder at K_{av}0.75-0.8 of the major SKT active peak appeared to be associated with the tailing proteinaceous peak. This result suggested that in addition to an excluded SKT component, a third active principle was present which was only partially resolved from the major SKT species. Acetic acid, present in the applied sample, eluted near the column Vt at K_{av}0.95.

<u>Specific activities in three fractions eluted from Bio-Gel P-10</u>

The specific activity of SKT present in different fractions eluted from Bio-Gel P-10 with 0.2M-acetic acid was determined to estimate its absolute purity. The fractions selected, as indicated in the elution profile shown in Figure 4.16, were taken as being representative of the three peaks of SKT activity eluted. The results are shown in Table 4.13.

SKT eluted at K_{av}0.54 (fraction number 22) was of greatest activity and purity, showing closely similar specific activities of 22000 a.u./mg whether estimated as total dry wt. or protein by two independent methods. A u.v. absorbance ratio (280 nm/260 nm) of 1.43 indicated that less than 1% nucleic acid was likely to be present.

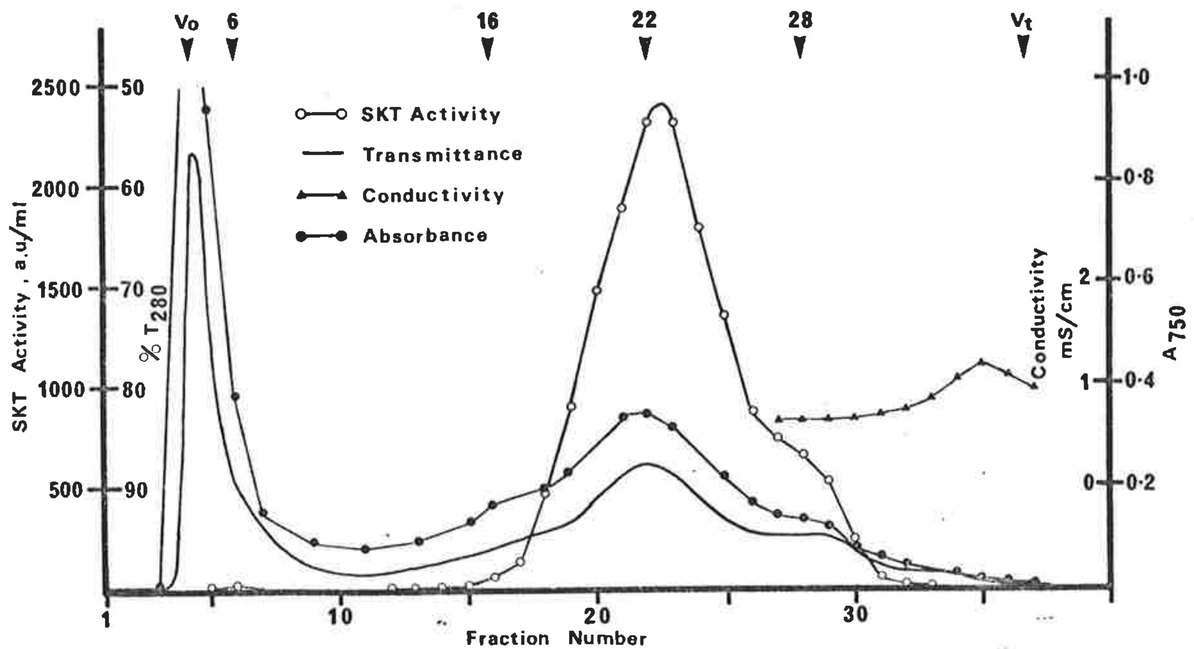


Figure 4.16. Chromatography of Bio-Gel P-2 eluate on Bio-Gel P-10 in 0.20M-acetic acid.
A P-2 eluate in 2.2 ml, containing 58,800 a.u. SKT, was applied to a Bio-Gel P-10 column (2.1 cm x 38 cm) and eluted at 30 ml/h with 0.20M-acetic acid. Fraction size was 3.0 ml and column Vo and Vt was 32.5 ml and 130 ml respectively. Fraction numbers indicated are referred to in the text.

Table 4.13. Specific activity of SKT in different fractions eluted from Bio-Gel P-10.[a]

Fraction number	6	16	22	28
Fraction K_{av}	0.05	0.36	0.54	0.73
SKT activity (a.u./ml)	14.1	61.5	2310	660
Dry wt. (mg/ml)	0.165	0.070	0.105	0.125
Specific activity (a.u./mg)	86	879	22000	5280
Protein, Lowry method (mg/ml)	0.1242	0.0540	0.1068	0.0442
Specific activity (a.u./mg)	114	1140	21630	14900
Protein, Warburg method (mg/ml)	0.4650	0.0315	0.1020	0.0396
Specific activity (a.u./mg)	30.3	1950	22650	16670
Absorbance ratio, 280 nm/260 nm	1.40	1.17	1.43	1.17

[a] Effluent fractions obtained from the experiment shown in Figure 4.16

On the other hand, SKT which eluted at K_{av}0.73 (fraction number 28), while showing a high specific activity on a protein basis, was three-fold less by dry weight. This indicated contamination by non-proteinaceous inert material, possibly polysaccharide or inorganic in nature since the absorbancy ratio (1.17) indicated that less than 2% nucleic acid was present. Specific activities of the K_{av}0.05 and 0.36 fractions of SKT were very low due to the presence of relatively large amounts of inactive proteinaceous material.

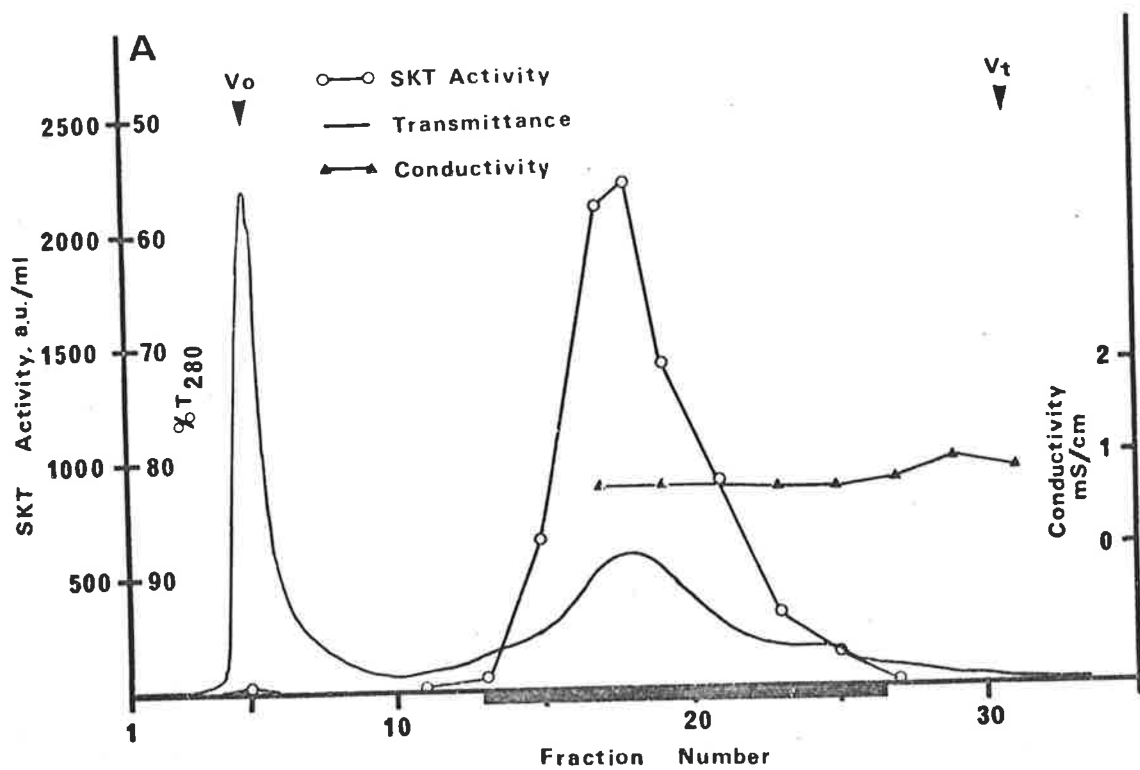
These results are from a single experiment only. Comparison of protein- and gravimetric-based specific activities of different

fractions showed that only K_{av}0.55 SKT activity was eluted from Bio-Gel P-10 in a highly purified state. The remaining fractions contained large proportions of inactive proteinaceous material and possibly other substances.

Chromatographic relationship between Kav 0.55 and 0.8 fractions

In early Bio-Gel P-10 chromatographic experiments, using different preparations of the P-2 acetic acid eluate concentrated under reduced pressure by rotary film evaporation, SKT eluted in two peaks, K_{av}0.55 and K_{av}0.8, the relative proportions of which varied widely. However chromatography of portions of a given concentrate provided reproducibility of the proportion of activity contained in each of the two peaks. Rechromatography of the K_{av}0.55 peak, in some experiments resulted in some of the activity eluting at K_{av}0.8, but rechromatographed K_{av}0.8 material never re-eluted at K_{av}0.55. From a series of controlled experiments it was found that the appearance of SKT activity at K_{av}0.8 was dependent on the temperature at which the eluate was concentrated.

Rechromatography experiments shown in Figure 4.17 (data in Appendix Seven) demonstrate this finding and also show an association between SKT activity and u.v. absorbancy. The total SKT active eluate of Figure 4.17A (fraction numbers 13-26 inclusive, 47.0 ml) reduced to 1.5 ml by rotary film evaporation carried out at 55°C, was reapplied to the Bio-Gel P-10 column and eluted with 0.2M-acetic acid. 32.5% of the applied SKT activity re-eluted at K_{av}0.55 but 46.6% eluted at K_{av}0.81, (Figure 4.17B). The latter peak of SKT also eluted in conjunction with a peak of u.v. absorbing material. The two peaks, pooled as shown by horizontal bars in Figure 4.17B, were concentrated to



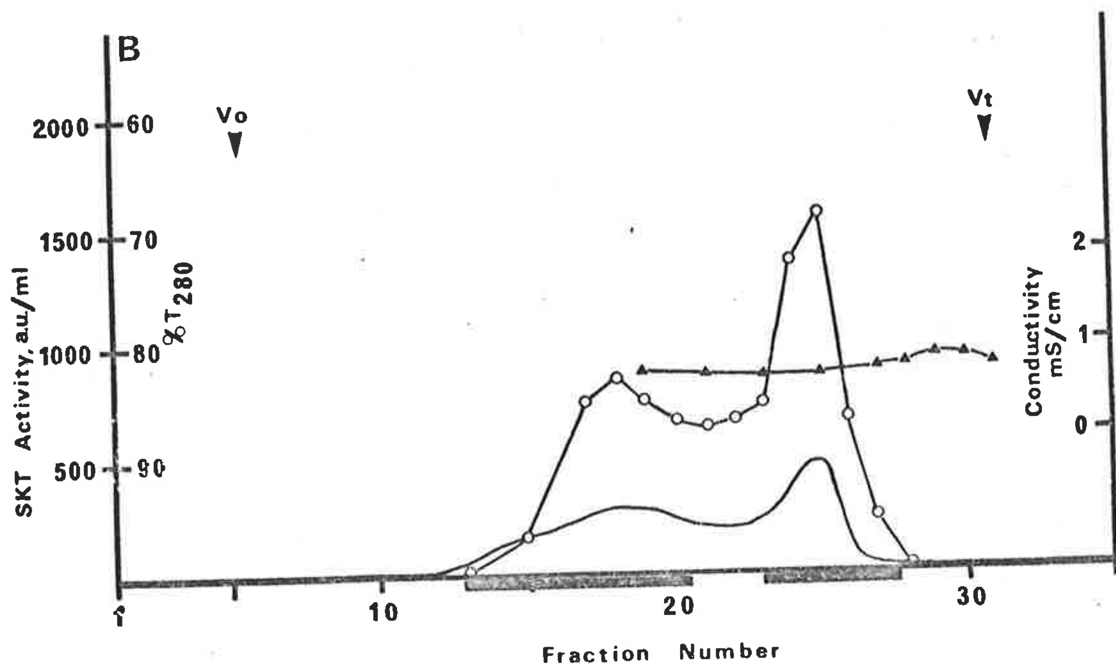
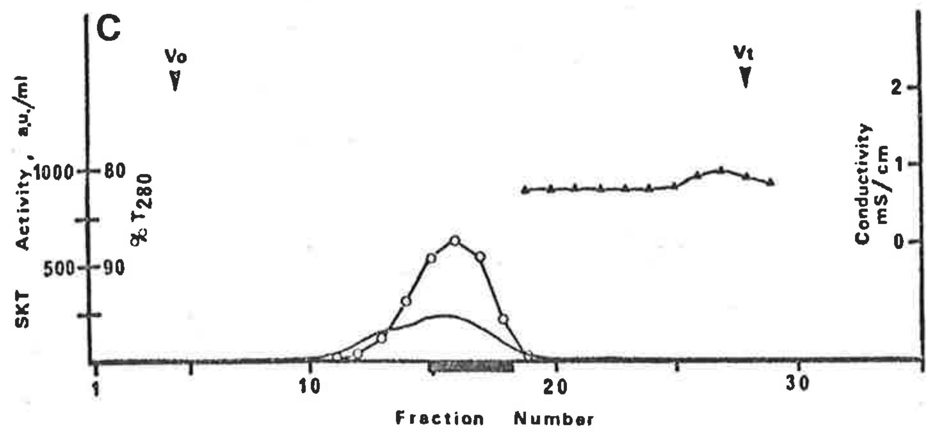
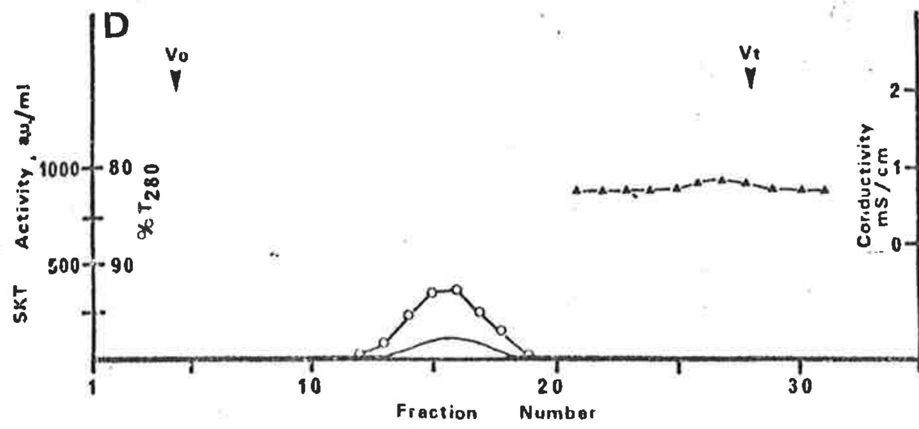


Figure 4.17. Chromatographic relationship between K_{av} 0.55 and K_{av} 0.8 SKT activity.
Selected fractions of SKT, eluted in 0.20M-acetic acid, were concentrated to 1.2 ml by rotary film evaporation under reduced pressure at a bath temperature of either 35°C or 55°C and applied to a Bio-Gel P-10 column (2.1 cm x 38.0 cm) and eluted with 0.20M-acetic acid at 30 ml/h. See the text for further explanation. Appendix Seven summarises experimental conditions.





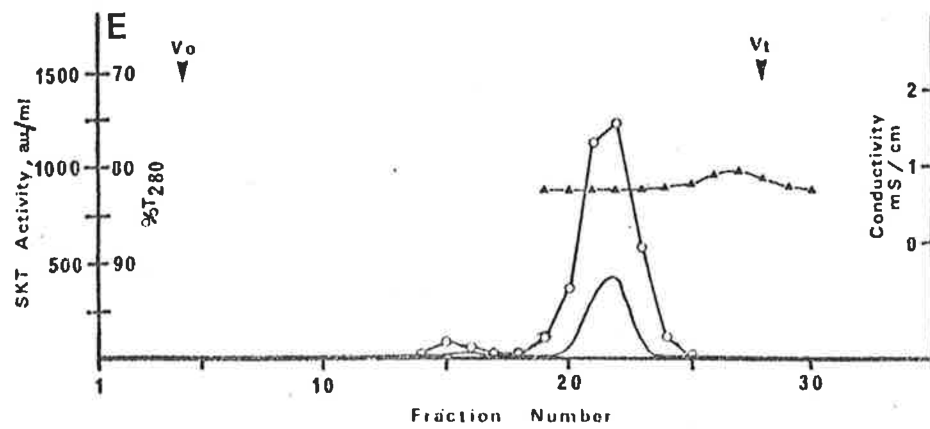


Figure 4.17. Continued.

approximately 2 ml at 35°C instead of at the higher temperature. The retarded fraction re-eluted at K_{av}0.8 and rechromatography of the K_{av} 0.55 fraction also re-eluted at its former value. The u.v. absorbing profile of the K_{av}0.55 material showed a leading shoulder which did not appear to be associated with SKT activity. Pooling of the K_{av}0.55 fraction as shown in Figure 4.17C resulted in removal of that fraction as seen in Figure 4.17D.

Procedure

A column of Bio-Gel P-10 of dimensions 2.0 cm x 39.0 cm (Vt 130 ml) equilibrated in 0.2M-acetic acid was used. A freshly packed column was stabilised by running several zones of a culture filtrate concentrate. Traces of adsorbed SKT activity could be eluted with a zone of 4M-KCl. The sample, Bio-Gel P-2 eluate (65-70 ml), was reduced to 5 ml under reduced pressure at 35°C and clarified by centrifugation. Lyophilised preparations were dissolved in a minimal amount of 5% acetic acid, diluted to 3.5 ml and clarified. Samples size was 0.5-3.0 ml (maximum of 1.5% Vt) containing between 20000 to 70000 a.u. SKT for optimum separation. The rate of elution was 30 ml/h (10.0 ml/cm^2h) and fractions of 3-4 ml were collected.

The central fractions of the SKT peak centering on K_{av}0.55 were pooled (the volume collected totalling 20 ml), concentrated under reduced pressure at 40°C and rechromatographed. The selection of SKT fractions for recycling from preparations containing a large leading shoulder of u.v. absorbing inactive material were chosen to eliminate this material. It may be possible to enhance resolution of the two chromatographic components on a column of greater length.

Specific activity of Kav 0.55 killer fraction

Table 4.14 summarises the specific activity of SKT, derived from both standard and peptone supplemented cultures, eluted near K_{av}0.55 from Bio-Gel P-10 with 0.2M-acetic acid. There was no apparent difference between the specific activities of SKT, with respect to dry wt. or protein, whether derived from CF_s or CF_p. The specific activities based on gravimetric or protein determinations showed a small difference but this may reflect the difference between the standards used to calibrate the methods. Recycle chromatography of the central portions of the K_{av}0.55 SKT did not result in any significant increase of specific activities. The absorbance ratio of recycled SKT was near 1.35, but did not exceed 1.40 (see Figure 4.18).

Table 4.14. Specific activity of SKT eluted with a K_{av} near 0.55 from Bio-Gel P-10[a].

Preparation[b]	$P10_s$	$P10_p$-1	$P10_p$-2
Number of experiments	2	2	3
SKT yield (%)	74.0	72.2	79.1
SKT specific activity:			
Dry wt. (a.u./mg)	21080	20480	22470 ±1090
Purification (-fold)	3400	2020	2220
Protein, Lowry method (a.u./mg)	22170	19970	25010 ±700
Purification (-fold)	4.84	3.65	4.57
Protein, Warburg method (a.u./mg)	25560	24740	26240 ±560
Absorbance ratio, 280 nm/260 nm	1.30	1.26	1.26-1.33[c]

[a] Mean value ± standard error of the mean; [b] Preparations derived from standard and peptone supplemented cultures are denoted by the subscripts s and p respectively; [c] Range.

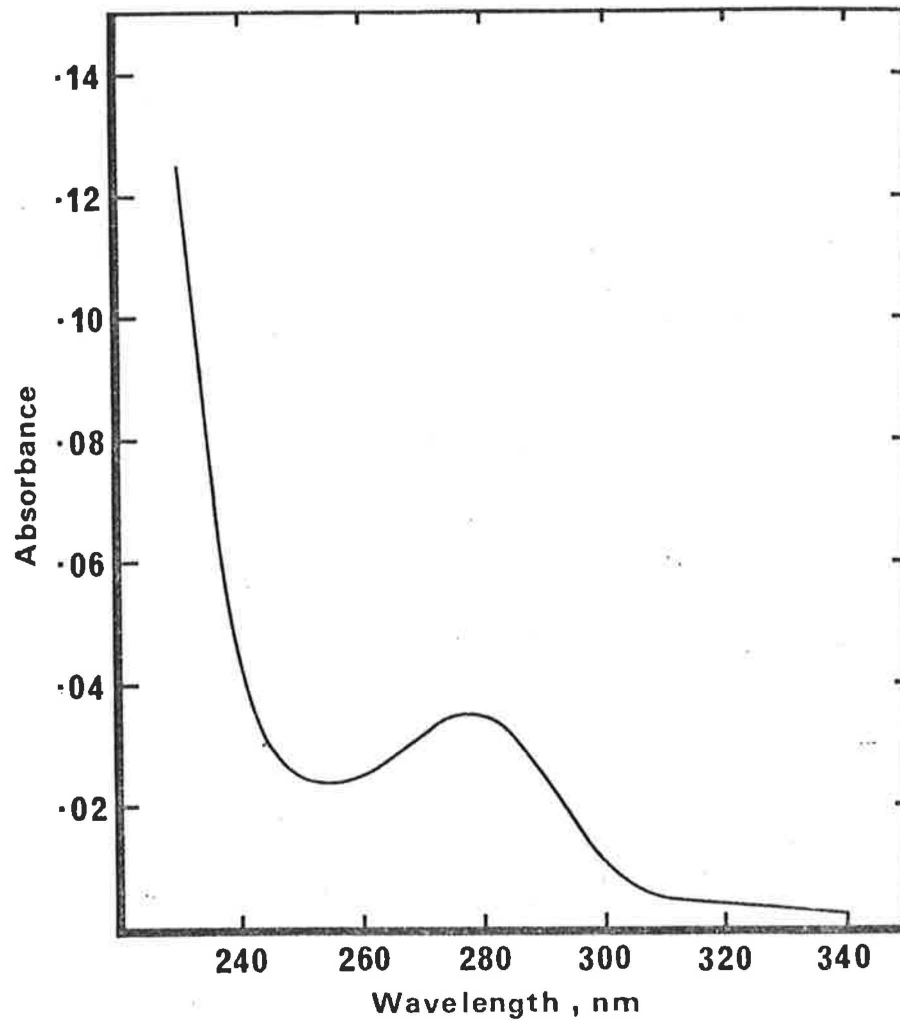


Figure 4.18. Absorption spectrum of purified SKT.
The absorbance of a solution of SKT, specific activity 25250 a.u./mg protein, and activity 870a.u./ml of 0.20M-acetic acid, was measured in 10 mm quartz cells.

Dose-response curve of purified killer activity

The dose-response curves of SKT activity contained in both a culture filtrate and a chromatographically purified preparation were determined on large plates using indicator strain 10. The curve for the assay reference standard (culture filtrate SKT) was prepared by serial two-fold dilution in buffer of pH6.0. The preparation of purified SKT (rechromatographed on Bio-Gel P-10) was kept as a 10000 a.u./ml solution in 0.20M-acetic acid at 4-6°C. It had a specific activity of 25250 a.u./mg protein (Lowry method). The assay curve was prepared by 10-fold dilution in either aqueous ethylenediamine or NaOH of a concentration such that the final pH became 6.0±0.1. Two diluents were compared; 0.1M-sodium potassium phosphate and 0.06I EN-AA buffer, both pH6.0.

The average responses of each sample were determined on large plates of SKT assay agar using a 16 x 4 quasi-Latin square design. The plates were incubated at 22-23°C for 40h. Regression analysis was performed on the averaged responses and the significance examined by the t-test (CHILTON, 1967).

The dose-response curves in which the mean inhibition zone diameter was plotted against the concentration of SKT (log) made in phosphate buffer are shown in Figure 4.19. Straight lines were fitted to the data for values betwen 10 and 100 a.u./ml and extrapolated through a relative SKT concentration of one. The slopes (and their standard errors) for culture filtrate and purified SKT in these experiments were:

SKT preparation	Phosphate buffer	EN-AA buffer
Culture filtrate	7.49±0.09 (5)	7.41±0.07 (5)
Purified	7.06±0.06 (8)	7.09±0.07 (8)

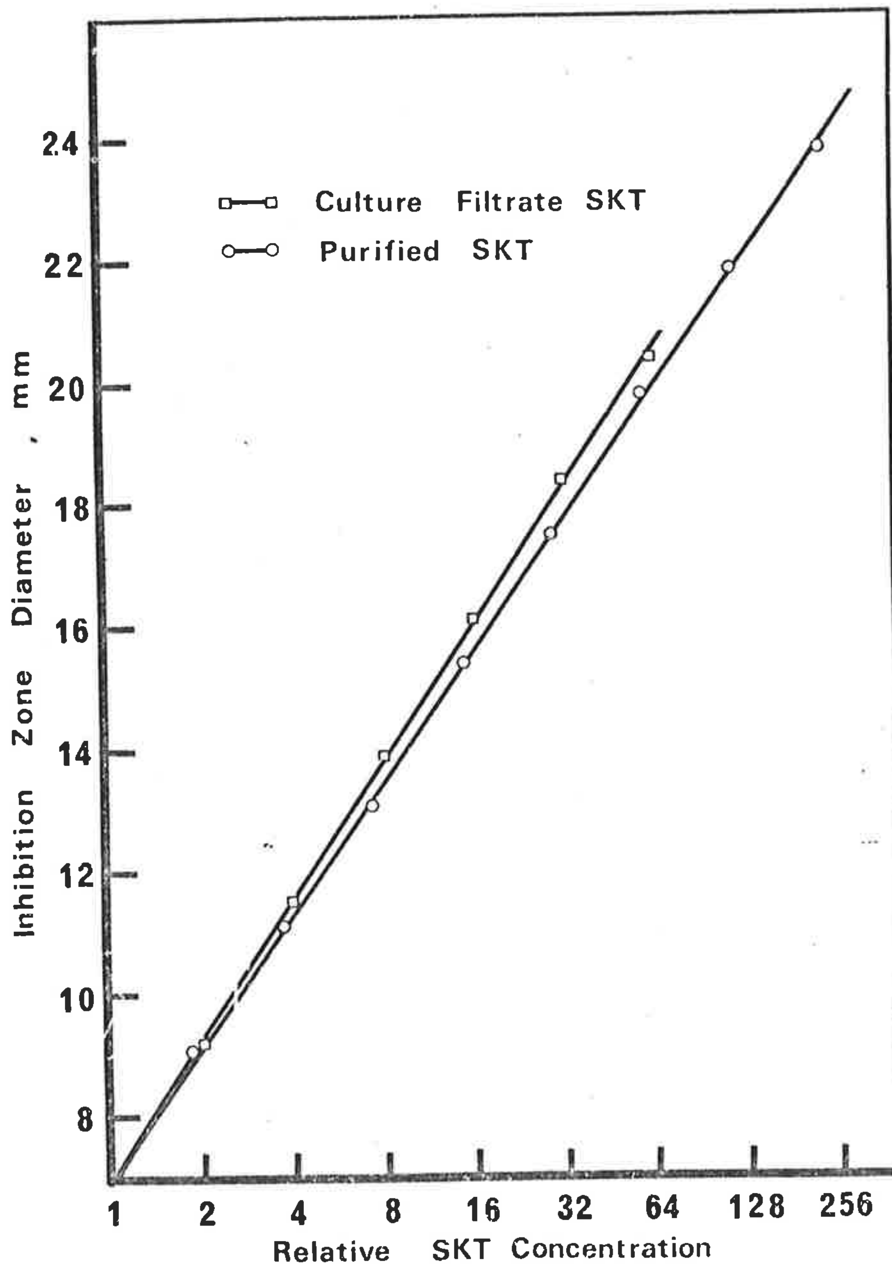


Figure 4.19. Log dose-response curves for reference culture filtrate and purified SKT activity.
The samples, diluted in 0.1M-sodium phosphate buffer, were applied in quadruplicate to a large plate of SKT agar, containing 1 x 10^6 cells/ml H. anomala, and then incubated at 22-23°C for 40h. Mean response is plotted against relative SKT concentration (log scale). Reference response of 20.4 mm corresponds to 100 a.u./ml SKT activity.

The standard errors of the slopes were ±1%, indicating high linearity of the dose-response curves over the range of SKT examined. The type of diluent had no effect on the slope, but at low SKT concentrations the presence of salt (0.5M-NaCl) decreased the response (data not shown), thereby reducing assay sensitivity. The curves for culture filtrate and purified SKT were not parallel ($t > 2.90$ for 9 degrees of freedom), and thus strictly, assay is invalid.

The culture filtrate reference standard resulted in an underestimate of purified SKT activity; the underestimate being a function of SKT concentration. For example, at a relative concentration of 64, 100 a.u./ml, the culture filtrate produced a response of 20.4 mm whereas that of purified SKT was 19.9 mm, equivalent to 85 a.u./ml; an underestimate of 15%. At a relative concentration of 16 (25 a.u./ml) the discrepancy was near 10%.

These results therefore show that use of the culture filtrate as an assay reference lead to an underestimate of SKT potency in purified preparations, but that the type of diluent was unimportant. The nature of the activity potentiating-agent in culture filtrate, either culture medium components or a yeast derived factor, was not investigated.

Molecular weight estimate by gel chromatography

A column of Bio-Gel P-10 was calibrated with standard proteins to estimate the molecular weight of SKT. The column of Bio-Gel P-10 and its use was as described above. The samples were applied to the column in a volume of 0.5-0.8 ml and when necessary adjusted to pH3 with concentrated acetic acid. Solutions containing each protein, singularly

or in admixture, were prepared immediately before application. Insulin and glucagon were solutions of neutral pH kept at 4-6°C.

Examination of the u.v. absorption spectrum of each potein prepared in 0.20M-acetic acid showed, with the exception of bacitracin, either a maximum close to or adequate absorption at 280 nm for detection by this method. Bacitracin showed a maximum at 254 nm. Elution profiles were monitored continuously spectrophotometrically at the appropriate wavelength and automatically recorded as described previously. Ve was measured volumetrically using either a measuring cylinder or fraction collector. Since bacitracin showed two absorption peaks, the active principle was estimated in fractions by a well test assay using Micrococcus lutea (see Appendix Eight). Sucrose was determined by the Phenol-sulphuric acid method of DUBOIS, GILLES, HAMILTON, REBERS and SMITH (1956). Acetic acid provided a measure of total column volume and a fixed reference point and was, in the absence of salts, detected using a Townson flow-cell (0.8 ml capacity) and conductivity meter whose output was recorded on a chart recorder (Rikadenki model B-140) at 80 mm/h. At the completion of the experimental series, the Vt was checked against the weight of distilled water held in the column.

The reference substances were chromatographed singularly to determine their chromatographic purity and then chromatographed in admixture to accurately measure elution volumes. However glucagon proved difficult to detect when applied together with other markers and its Ve was therefore measured separately. Bacitracin produced a series of broad peaks (A_{254} and microbiological-assay) when chromatographed with other proteins and blue dextran, which appeared to interfere with its elution. To obtain a reproducible Ve for bacitracin, the column was primed with a zone of bacitracin (1 mg) followed by an analytical run in

which 0.5 mg bacitracin prepared freshly in 0.5 ml 0.20M-acetic acid was used.

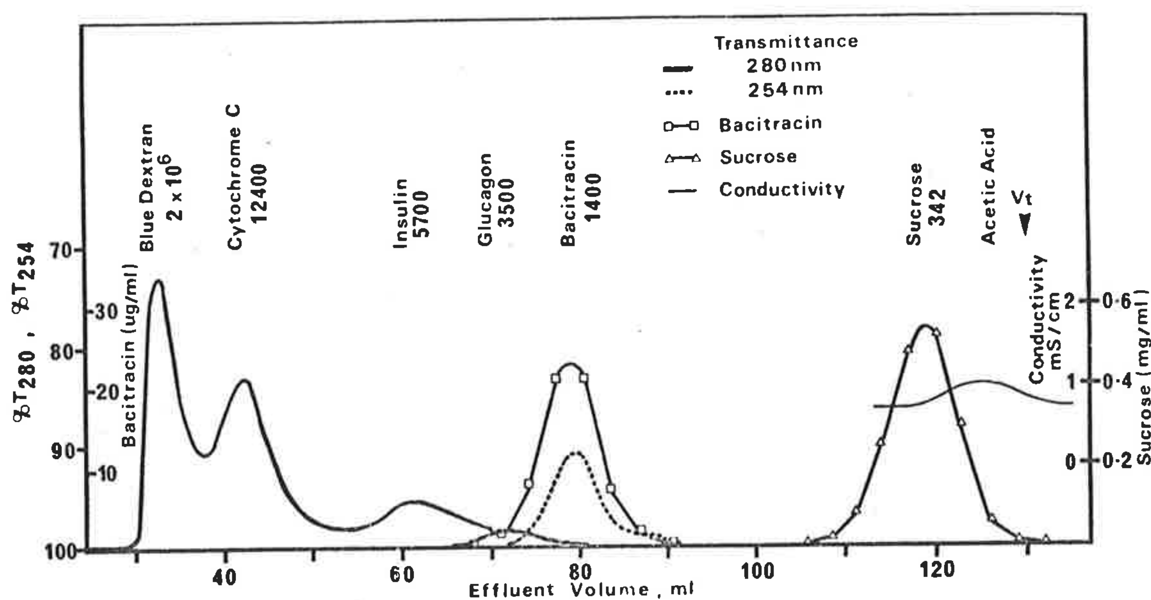
Table 4.15. Substances used in the calibration of the Bio-Gel P-10 column.

Substance	Molecular weight[a]	Quantity	Estimation	Ve[b] (ml)
Blue Dextran 2000	2×10^6	1.0 mg	Absorbance at 280 nm	32.5
Cytochrome *c*	12400	0.2 mg	A_{280}	43.0
Insulin	5730	50.0 i.u.	A_{280}	62.0
Glucagon	3485	0.3 i.u.	A_{280}	72.0
Bacitracin	1400	0.5 mg	1) A_{254} 2) assay with <u>M. lutea</u>	80.0
Sucrose	342.3	5.0 mg		120.0
Acetic Acid		0.5 ml of 10% (v/v)	Conductivi-metric	125.0

[a] Supplied by the manufacturer; [b] Average values (2-5 experiments) determined on a single column, see Figure 4.20.

Chromatrography of the reference substances is shown in Figure 4.20. Average Ve's for the various substances chromatographed are included in Table 4.15 from which values for K_{av} were calculated. A calibration curve, shown in Figure 4.20, was constructed by plotting K_{av} against molecular weight (logarithmic scale). A straight line could be drawn through the three proteins and sucrose as shown, but bacitracin deviated markedly from this line.

Assuming elution of the substances tested to be free of interference effects, and hence a valid calibration curve, the K_{av} for bacitracin suggests that it was eluted as a dimer. A small shoulder curve at a Ve



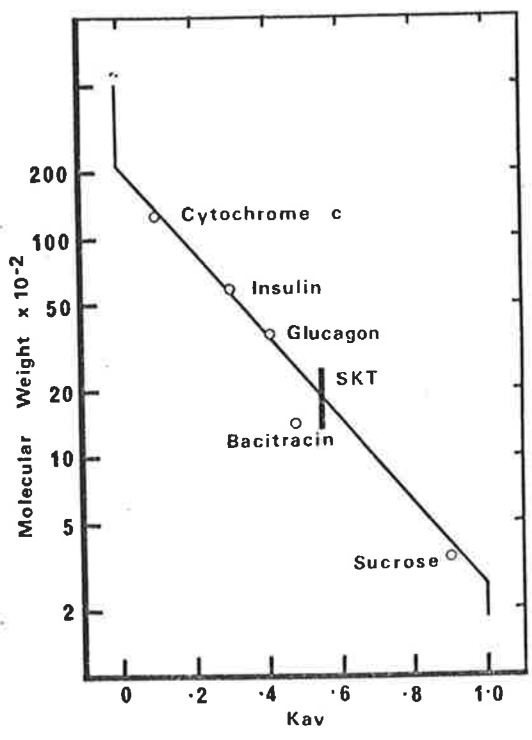


Figure 4.20. Calibration of a Bio-Gel P-10 column with substances of known molecular weight.
Markers, in a volume to 0.8 ml, were chromatographed on a Bio-Gel P-10 column (2.1 cm x 38.0 cm) in 0.20M-acetic acid eluant at a flow rate of 30 ml/h. Fraction size for bacitracin and sucrose was 3.2 ml and 3.6 ml respectively and column Vo and Vt was 32.5 ml and 130 ml respectively. For amount of substance applied, see Table 4.15.

of 90 ml may represent its monomeric form, although its expected Ve from the calibration curve was 95 ml. However, there does not appear to be any evidence in the literature to support exclusive dimerisation of bacitracin in dilute aqueous acetic acid. In fact acetic acid (3%) in combination with other solvents was used in the separation of bacitracin components by counter-current distribution (ABRAHAM, 1957). PORATH (1960) observed bacitracin to be retarted in a series of eluants (pyridine-acetic acid) of increasing acidity, but the gel used was a Sephadex material, denoted B197. The reason for the apparent deviation of bacitracin in this experiment therefore remains obscure.

SKT eluted with a K_{av} of 0.55 (range 0.50-0.60) corresponding to a molecular weight of 2000. The tailing shoulder activity which re-eluted at K_{av} 0.8 therfore has a value of approximately 900.

Homogeneity and molecular weight determination by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Purified SKT was electrophoresed under dissociative conditions (SDS) on polyacrylamide gel to:

(i) demonstrate homogeneity.

(ii) estimate molecular weight.

The samples were denatured in SDS by boiling in either the presence or absence of 2-mercapto-ethanol and electrophoresed in 10% polyacrylamide gel containing 1% SDS. The gels were stained for protein with Coomassie brilliant blue.

Homogeneity of SKT preparations

Purified SKT eluting at K_{av} 0.55 (SKT-1) and two preparations of SKT

eluting at K_{av} 0.8 (SKT-2) were examined by electrophoresis on SDS-containing 10%-polyacrylamide gels. Non-reduced SDS-denatured SKT-1 (purified from CF_s) produced only a single protein-staining disc (see Figure 4.21) with an Rf in the range 0.87-0.89. Reduced SKT-1 had a similar electrophoretic mobility, demonstrating that it was composed of a single polypeptide chain. Gels loaded with up to 100 μg of purified material showed only a single staining band. Samples of reduced SKT-1, purified from CF_p, also showed only one staining band.

Two SKT-2 preparations (derived from CF_p) were examined after reduction, the first designated SKT-2A, obtained from a primary fractionation on Bio-Gel P-10 and the second consisted of pooled K_{av} 0.8 fractions (three experiments) recycled on P-10 (designated SKT-2B). Reduced SKT-2A showed a single band with an Rf of 0.86 whereas SKT-2B, in addition to a band at Rf 0.84, showed two lesser staining bands at Rf 0.75 and 0.71. The latter two proteins may have remained undetected in SKT-2A because of the lesser amount of material available for application to the gels. The major bands of these two preparations probably represent the same protein component, but their mobilities were slightly less than that of SKT-1.

Molecular weight determination

Standard proteins of molecular weights in the range 5000 to 35000 were selected to calibrate the gels for molecular weight determination of SKT active fractions using a semi-logarithmic plot (DUNKER and RUECKERT, 1969) shown as Curve A in Figure 4.22. Since the observed molecular weight of several proteins used (cytochrome c, ribonuclease A) have been shown to deviate from their expected position in SDS polyacrylamide gel, a curve was also drawn using their apparent molecular weight (Curve B)

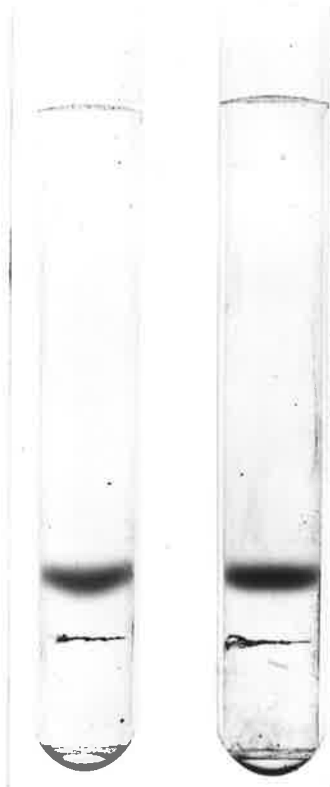


Figure 4.21. SDS-polyacrylamide gel electrophoresis of purified SKT protein.
Rechromatographed SKT activity, after SDS denaturation in the presence or absence of 2-mercapto-ethanol, was applied to 1% SDS-10% polyacrylamide gels (25 μg per gel) and electrophoresed for 3h. The gels were stained with Coomassie brilliant blue.

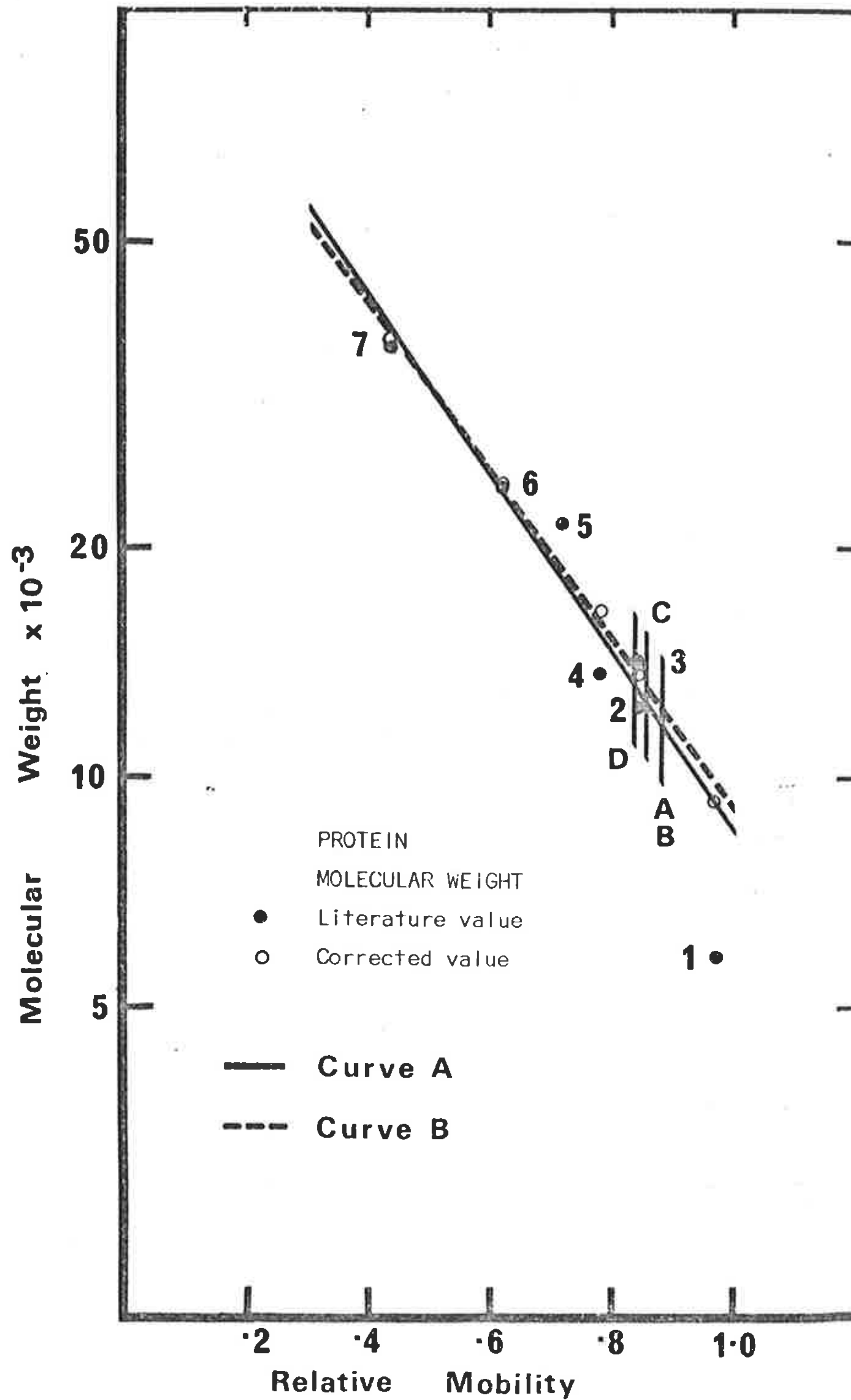


Figure 4.22. Molecular weight determination of SKT protein by SDS-polyacrylamide gel electrophoresis.
Calibration Curve A is fitted to the literature values of molecular weight for the standard proteins and Curve B to the observed values of DUNKER and RUECKERT (1969). See Table 4.16 for explanation of symbols.

values calculated by DUNKER and RUECKERT (1969). Although the experimental conditions used differed slightly from that of DUNKER and colleague, the greater linearity of Curve B, using the apparent molecular weight values observed by these authors confirmed the validity of using their data in these experiments.

Table 4.16. SDS-polycrylamide gel electrophoresis of protein standards used to estimate the molecular weight of SKT-1 and SKT-2 preparations.[1]

Protein	Reduction[a]	Quantity per gel (g)	Rf[b]	Molecular weight	Apparent molecular weight
A. SKT-1	+	25.0	0.88	11500[d]	12500[e]
B. SKT-1	-	25.0	0.885	11250[d]	12250[e]
C. SKT-2A	+	20.0	0.86	12700[d]	13250[e]
D. SKT-2B	+	75.0	0.84	13250[d]	14000[e]
1. Insulin	+	3.35i.u.	0.975	2900	-
2. Insulin	-	3.35i.u.	0.97	5700	9200[c]
3. Cytochrome c	+	50.0	0.84	12400	13600
4. Lysozyme	+	50.0	0.84	14400	13900
5. Ribonuclease A	+	50.0	0.78	13700	16500
6. Trypsin inhibitor	+	83.5	0.72	21500	-
7. Trypsin	+	50.0	0.62	23800	24100
8. Pepsin	+	300.0	0.43	35500	37000

[1] Molecular weight determinations for SKT protein were made from a plot of Rf versus either log molecular weight (Curve A of Figure 4.22) or log apparent molecular weight (Curve B of Figure 4.22) of standard proteins as observed by DUNKER and RUECKERT (1969) on SDS-10% polyacrylamide gel; [a] +, Reduced with 1% 2-mercaptoethanol, -, Not reduced; [b] Mobility relative to bromophenol blue tracker dye; [c] Estimated from Figure 2B of DUNKER and REUCKERT (1969); [d] Determined from curve A of Figure 4.22; [e] Determined from curve B of Figure 4.22.

The Rf of SKT-1, which was not affected by reduction, yielded a molecular weight of 11000-11500 from calibration Curve A and 12000-12500 from Curve B. SKT-2A material was found to be 12500-13000 and 13000-14000 molecular weight from the two curves respectively.

Identity of killer activity

A. Cellulose-acetate membrane electrophoresis

Isolated fractions containing SKT activity were electrophoresed on cellulose acetate membrane strips at pH4.3 to determine, by bio-autography against group 1 and group 2 indicator strains (refer to Figure 2.7, Chapter 2), the identity of the active species.

The method was similar to that described in Methods of Chapter 2. The sample was diluted to 100-500 a.u./ml in 0.025M-sodium tartrate, pH4.3, and 2-5 μl applied per 2.5 cm-width of CAM strip and electrophoresed for 6h at a current of 0.8 mA/cm-width of strip at 4-6°C. SKT activity was visualised bio-autographically against indicator strains 10, 560 and 498 at pH4.3. The presence of acetic acid in samples had only minor effect on electrophoretic mobility.

The electrophoretic mobility of SKT activity present in different fractions isolated from a partially purified concentrate by gel chromatography on Bio-Gel P-10 (experiment described in Figure 4.16) is shown in Figure 4.23. The activity of each of these fractions, detected on plates of strains 10 and 560 but not 498, exhibited similar electrophoretic mobility to that present in the parent culture filtrate. The activity of fraction number 6 could not be detected by this method.

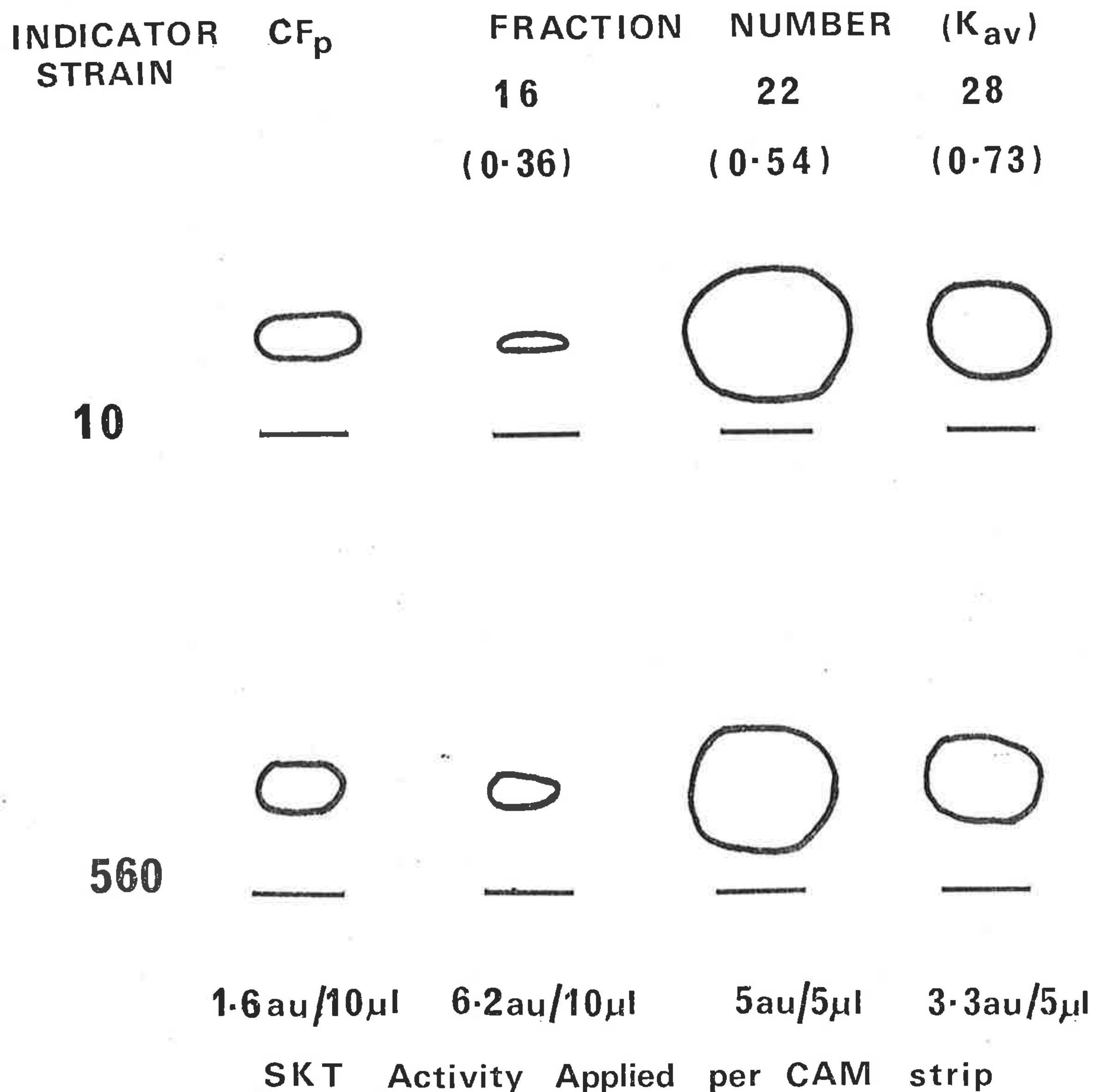


Figure 4.23. Electrophoretic mobility of SKT in different fractions isolated in the Bio-Gel P-10 chromatographic experiment described in Figure 4.16.
Aliquots of each fraction were electrophoresed for 6h at a current of 0.8 mA/cm-width of CAM strip buffered at pH4.3. SKT activity was located on longitudinally sectioned strips by bio-autography, at pH4.3, against indicator yeasts 10, 560 and 498. Horizontal bars represent the sample origon, and migration was towards the cathode.

Preparatively isolated K_{av}0.55 fractions, derived from both CF_s and CF_p, displayed analogous electrophoretic mobilities. The results therefore demonstrated that the K_{av}0.55 and 0.8 fractions contained only a single active species analogous to toxin-A.

B. Killer activity spectrum

The activity spectrum of SKT in different fractions eluted from Bio-Gel P-10 (of Figure 4.16) was determined directly by assay using group 1 and 2 indicator yeasts. The potency of each fraction was determined relative to that of the K_{av}0.54 fraction to indicate any differences which might reflect a dissimilar active component.

The assays were made in Petri dishes containing 12.5 ml of soft-YEPDA buffered at either pH4.3 or 6.0 and seeded with 2 x 10^5 cells/ml of indicator yeast. The fractions tested were made pH4.3 or 6.0 by 10-fold dilution in buffer of appropriate pH (conditions determined using larger volumes) or when of insufficient activity to allow dilution, the samples were adjusted with 0.5M-disodium tartrate containing 2M-NaOH. 0.2M-acetic acid was similarly adjusted to pH4.3 and 6.0 to serve as controls for acetate toxicity. Fraction number 23, obtained from the experiment described in Figure 4.16 was serially diluted in appropriate buffer to provide a dose-response curve for the assay. The assay was performed as described in Methods of Chapter 3.

The results, presented in Table 4.17, reveal that the four fractions, tested at both pH4.3 and 6.0, were active only against group 1 yeasts (strains 10, 605 and 560) and not against group 2 strains. Each fraction exhibited a similar pattern of activity when assayed by different indicator strains confirming that each contained, at least,

Table 4.17. Activity pattern on selected SKT indicator strains of strain 354 culture filtrates and Bio-Gel P-10 eluate fractions.

Test fraction	Assay strains : % activity relative F_{23}						
	10[a]	10	605	560	495	498	499
Assay pH4.3							
CF-YEPD[b]		+	+Z	+	+	+	+
CF_p		5.0	5.5	5.0	-	Tr	-
Buffer control		-	2.0	0.65	x	x	x
F_5		0.6	<2.0	<.65	x	x	x
F_{16}		3.5	4.5	4.0	x	x	x
F_{23}		100.0	100.0	100.0	-	Tr	Tr
F_{28}		20.0	25.0	25.0	-	-	-
Assay pH6.0							
CF-YEPD		+	+	+	-	+Z	+Z
CF_p	4.35	4.0	4.5	4.0	-	Tr	-
Buffer control	-	-	-	-	-	-	-
F_5	0.4	0.55	0.55	0.5	-	-	-
F_{16}	2.67	3.0	2.0	3.0	-	Tr	-
F_{23}	100.0	100.0	100.0	100.0	-	Tr	-
F_{28}	28.7	20.0	20.0	20.0	-	Tr	-

+, Killer activity, derived from Figure 2.1; z, Weak hazy zone activity; Tr, Trace inhibitory activity: hazy zone or inhibition zone size exceeding buffer control zone size; x, Buffer control inhibitory activity; - No activity; [a] Values recalculated from Table 4.13; [b] Filtrate of a YEPD shake culture buffered at pH6.0.

predominantly toxin-A activity. At pH4.3 the buffer control was inhibitory to most of the indicator yeasts, thus obscuring the weak activity of fraction number 5. This problem could be overcome by removal of the solvent by lyophilisation. However, since this fraction was active at pH6.0, it corresponded with toxin-A. At very high concentrations, exceeding 1000a.u./ml (neat F_{23}), toxin-A produced small clear zones of killing on indicator strains 498 and 499. Thus for proper interpretation by direct assay, it is mandatory that the sample contain between 50-250 a.u./ml SKT activity and be tested simultaneously against group 1 and 2 indicator strains.

Carbohydrate estimation

The carbohydrate content of SKT was estimated by reaction with the tryptophan and boric acid-sulphuric acid reagents as described by BADIN, JACKSON and SCHUBERT (1953), standardised with D-glucose. The assays of three separate preparations of SKT recycled on Bio-Gel P-10 were:

3.3 μg	glucose	equivalents	per	138 μg SKT =	2.4% (w/w)
3.5 μg	"	"	"	146 μg SKT =	2.4% "
1.5 μg	"	"	"	103 μg SKT =	1.5% "
				average	2.1% "

Assuming a molecular weight for SKT of 12000, this implied that it contained at most two hexose residues per molecule. The limited quantity of purified SKT prevented a more reliable estimate being made.

Comparison of the absorption spectrum of the reaction products produced by SKT and glucose (see Figure 4.24) suggested that the resultant absorbance of SKT might not be derived from carbohydrate but

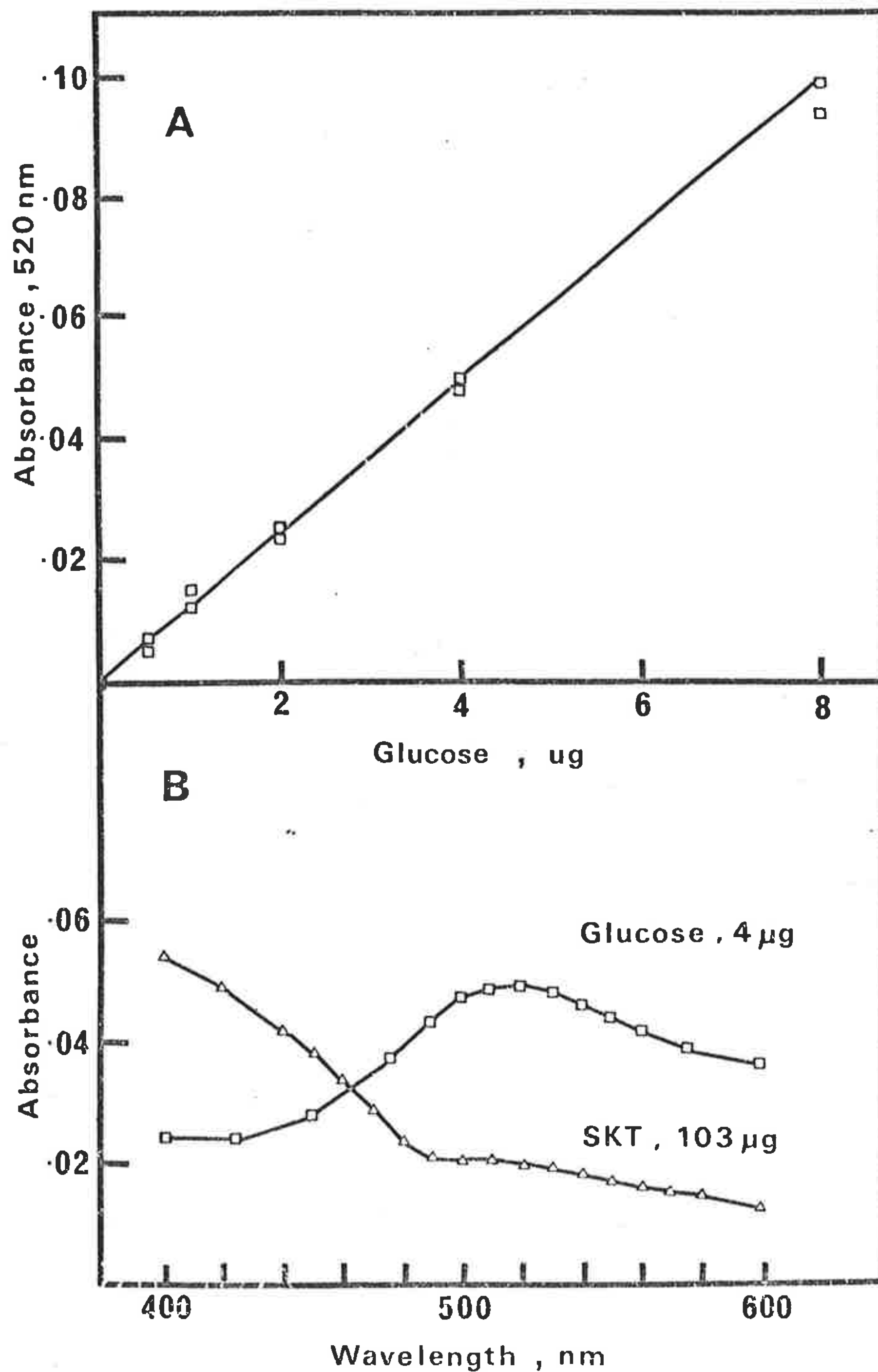


Figure 4.24 Carbohydrate assay of SKT protein.
A. Glucose standard curve by the tryptophan / boro-sulphuric acid method.
B. Spectra of glucose and SKT protein following reaction with tryptophan / boro- sulphuric acid reagent.

from a non-specific reaction, possibly with protein. This possibility gained support by the qualitative similarity between the absorption profile of SKT and that of haemoglobin described by SHETLAR, FORSTER and EVERETT (1948). However this comparison can only be made with due reservation since their method lacked the addition of boric acid employed in the present method of BADIN and colleagues (1952). The boric acid reagent serves to intensify colour yield and shift the absorption maximum to a longer wavelength but does not appreciably alter the absorbance spectrum qualitatively. Although BADIN and colleagues did not report on non-carbohydrate containing proteins, the qualitative similarly of the absorption spectrum of SKT and haemoglobin provided some grounds for comparison in view of the general similarity of both methods in their reaction with carbohydrates. The background absorbance of haemoglobin was estimated from the data of SHETLAR _et al._ to approximate 1% (w/w) hexose at the wavelength for maximum absorption produced by carbohydrate. Therefore it seemed probable that SKT may not contain carbohydrate, but in either case, it contained at most two hexose residues per SKT molecule. This assay difficulty could be overcome by employing a larger amount of SKT and known proteins and glycoproteins as internal standards. It may also be useful to test for hexosamine which is not detected by the present method.

Amino Acid Composition

Three preparations of SKT, two derived from CF_s and the third from CF_p were hydrolysed with either methane sulphonic acid or HCl, and the amino acid composition determined on an automatic amino acid analyser. Since the amount of protein (30-100 μg) available for hydrolysis did not allow an accurate quantitative analysis, the relative proportion of amino acid residues was determined, from which a rough estimate of

molecular weight was determined.

Hydrolysis and analysis

SKT protein, 5-50 μg, was hydrolysed with either 0.5 ml 4N-methane sulphonic acid (MSA) with 0.1% tryptamine or 1.0 ml 6N-HCl in nitrogen flushed sealed tubes at 115°C or 110°C respectively for 22h (INGLIS and colleagues, 1976). Following hydrolysis with MSA, 0.5 ml of pH10.4 citrate/carbonate buffer was added and the pH adjusted to 1.5. The HCl hydrolysate was dried in vacuo and taken up in 0.5-1.0 ml buffer (MOORE and STEIN, 1951). The amino acids in 0.2-0.5 ml of buffered hydrolysate were separated on a Beckman 119 automatic amino-acid analyser by the standard two column procedure.

Results

The result of duplicate amino-acid determinations on three preparations of SKT protein is summarised in Appendix Nine, from which the ratio of amino-acid residues relative to glycine was computed as shown in Table 4.18. No corrections for losses on hydrolysis were made. From the average of these values, the number of residues was calculated to be 84-85, assuming that phenylalanine and histidine were present as single residues. This implied a minimum molecular weight of approximately 10000. Seventeen different amino acids were detected in each of the three preparations. No tryptophan was detected in the methane sulphonic acid hydrolysate.

Generally, the agreement in amino acid composition (ratio of residues) between the two conditions of hydrolysis was reasonable although several apparently anomalous values were found. Apart from the differences

Table 4.18. The relative abundance of amino-acid residues in three preparations of toxin-A protein.[a]

Amino acid	Amino-acid residue ratio relative to glycine							Number of residues	
	Methane sulphonic acid			HCl		Average			
	Prep.1	Prep.2	Prep.3	Prep.1	Prep.2	MSA	HCl	MSA	HCl
ASP	1.08	0.63	0.67	0.93	0.50	0.79	0.72	13	12
THR	0.48	0.23	0.25	0.43	0.19	0.32	0.31	5	5
SER	0.66	0.39	0.87	0.56	0.37	0.64	0.47	11	8
GLU	0.56	0.66	0.37	0.49	0.60	0.53	0.55	9	9
PRO	0.12	0.15	0.03	0.16	0.15	0.10	0.16	2	3
CYS/2	0.26	0.09	Tr	0.39	0.06	0.12	0.23	2	4
GLY	1.00	1.00	1.00	1.00	1.00	1.00	1.00	17	17
ALA	Tr	0.29	0.10	0.19	0.27	0.13	0.23	2	4
VAL	0.32	0.43	0.31	0.41	0.34	0.35	0.38	6	6
MET	0.14	0.04	0.20	0.10	0.01	0.13	0.06	2	1
ILE	0.17	0.20	0.12	0.16	0.14	0.16	0.15	3	3
LEU	0.03	0.20	0.11	0.02	0.17	0.11	0.10	2	2
TYR	0.16	0.10	0.24	0.14	0.08	0.17	0.11	3	2
PHE	0.07	0.05	Tr	0.04	0.04	0.04	0.04	1	1
HIS	0.05	0.09	0.11	0.06	0.06	0.08	0.06	1	1
LYS	0.18	0.41	0.03	0.15	0.38	0.21	0.27	4	5
ARG	0.04	0.21	Tr	0.13	0.15	0.07	0.14	1	2
Total								84	85

[a] The ratio of amino-acid residues in each of three toxin preparations, hydrolysed with either methane sulphonic acid (MSA) or HCl, relative to glycine, was calculated from the amino acid analysis data (average of two analyses) of Appendix Nine. No corrections for loss on hydrolysis were made. The minimum number of residues present in either MSA or HCl hydrolysed preparations was calculated on the basis that phenylalanine and histidine were present as single residues (taken to be 0.06 ratio units). Toxin preparations 1 and 2 were derived from CF_s and preparation 3 from CF_p.

which are attributable to expected losses during hydrolysis (for example, HCl destroys tryptophan and reduces cysteine, cystine, serine and threonine whereas methane sulphonic acid is less destructive to tryptophan), the remainder were thought to be due to machine variation, that is differences in both buffer and ninhydrin age. The compositional differences between each preparation of SKT were however greater. This variation may represent real differences in the composition of each preparation or possibly the presence of a significant contaminant. The latter seems unlikely as all three preparations were homogeneous when electrophoresed on SDS-polyacrylamide gels stained with Coomassie brilliant blue. The most likely explanation is that since the amount of protein available for analysis was very small, the machine was operating at the maximum limit of sensitivity (actual levels were about one fifth of those shown in Appendix Nine, where baseline variations and shifts due to buffer changes become significant. These results can therefore only be considered an indication of the amino acid composition of the SKT protein.

DISCUSSION

The chemical nature of toxin-A

On the basis of its spectrum of activity and biochemical properties, SKT present in and isolated from the chemically defined medium was analogous to toxin-A, produced by strains of *H. saturnus* in complex culture media. Purified toxin-A activity, eluted from Bio-Gel P-10, correlated with and could be accounted for on the basis of protein, estimated by two independent methods. No experiments were made to determine whether purified toxin-A was isolated as an aggregate, but reduction of the electrophoretically homogeneous preparation did not affect electrophoretic mobility on an SDS-gel, demonstrating that it was a single polypeptide. Toxin-A contained at most two hexoses per polypeptide molecule thus providing no evidence for the involvement of a glycoprotein as indicated for other yeast toxins, such as those from *T. galbrata* (BUSSEY and SKIPPER, 1975). The molecular weight was estimated at 11-12 x 10^3 by SDS-gel electrophoresis and 10 x 10^3 from the amino acid composition. The protein isolate contained 20% glycine, was rich in acidic amino acids but low in basic and aromatic residues. The near-neutral pI of toxin-A therefore suggested that some of the acidic residues were present as the amide. The 2-3 tyrosine residues do not account for an ϵ_{280} of approximately 9000 $cm^{-1}M^{-1}$ (calculated from absorbance measured in 0.20M-acetic acid and assuming a molecular weight of 11000) which therefore suggests the presence of one tryptophan residue. None was detected, however, following hydrolysis with methane sulphonic acid which is expected to preserve tryptophan.

Purification of toxin-A

Despite the stable nature of toxin-A, its small molecular size and strongly hydrophilic nature precluded the use of most conventional isolation techniques. Use of the strongly acidic cation exchange resin IR-120 proved most satisfactory in the direct isolation of toxin-A from the culture fluid. The batch technique adopted, though inefficient with respect to the total exchange capacity of the resin, enabled the acquisition of a concentrate rich in toxin-A activity. Stepwise pretreatment of the resin with buffer and the subsequent elution in a small volume of volatile eluant, produced high recovery of toxin free of the bulk culture filtrate components (>500-fold reduction by dry wt.). The cation exchange resin gradient elution experiments indicated a correlation between toxin activity and Lowry positive material, thus establishing the basis of relating toxin activity to protein as an index of toxin-A purity. The subsequent aqueous acetic acid-soluble fraction of the resin eluate showed a 2-fold reduction in total protein compared with a dialysed culture filtrate sample.

The delayed elution of toxin-A, with respect to protein, from Bio-Gel P-10 in aqueous acetic acid was exploited in its purification. Several peaks of inert substances, which interfered with toxin-A elution from P-10, were firstly separated by filtration on the small-pore gel, Bio-Gel P-2, in dilute acetic acid. The bulk of toxin-A activity was then eluted from P-10 between K_{av} 0.5 to 0.6 associated with a peak of protein, but was well separated from the excluded-proteinaceous material. However it usually contained a small leading shoulder of inactive material and a small tailing peak of toxin-A active substance. Appropriate selection of fractions (near K_{av} 0.55) and rechromatography to ensure separation, eliminated these components as demonstrated by

protein homogeneity following gel-electrophoresis.

The steps in purification of toxin-A isolated from both the standard and the peptone supplemented cultures are summarised in Table 4.19. The data shown are the average values of several independent preparations, but in some cases, intermediate isolates were combined to serve as stocks for experimentation. The small addition of peptone to stimulate toxin-A production did not appear to interfere in toxin-A purification. A yield of about 2 mg of purified toxin could be expected from one litre of culture filtrate derived from peptone stimulated culture. On the basis that toxin-A in culture filtrate assayed 115% that of the isolated toxin, standard and peptone supplemented cultures contained approximately 4 and 6 mg/l of toxin respectively.

Gel chromatographic behaviour of toxin-A

It is well established for certain classes of molecules, which elute from a gel column free of interference effects, that their elution volume is a function of their molecular size (ANDREWS, 1964, 1967; BATTLE, 1967). The occurrence of solute-solute or solute-gel matrix interaction however invalidates the method (GELOTTE, 1960; CARNEGIE, 1965; PORATH, 1968; DETERMANN, 1968). On the basis that toxin-A has a minimum molecular weight of $10\text{-}12 \times 10^3$ it would therefore be expected to emerge from a column of Bio-Gel P-10 in aqeous acetic acid with a $K_{av} < 0.2$ (see Figure 4.20) rather than the observed values of 0.55 and greater, depending on the origin of the sample used. Thus the protein molecule was eluted by a mechanism involving a reversible adsorptive interaction with the gel matrix in addition to the molecular sieve action. Under none of the elution conditions examined did the toxin molecule display a value of K_{av} less than 0.5.

Table 4.19. Summary of toxin-A purification from standard and peptone supplemented cultures of _H. saturnus_ AWRI 354.[a]

Purification step	SKT activity (a.u.)	Dry wt. (mg)	Protein (mg)	Specific activity (a.u./mg)	Purification (-fold)	Yield (%)
Standard culture; filtrate	376000	58200	8.2	4210	1.0	100.0
Concentrate;						
IR-120 batch	235800	44.2				62.7
Acetic acid extract	228200		25.9	8800	2.1	60.7
Desalted extract from Bio-Gel P-2 column	130000	N.D.	12.0	10800	2.6	34.6
Fractionation on Bio-Gel P-10 column	96300	4.56	4.34	22200	5.27	25.6
Peptone supplemented culture; filtrate	624000	61600	114.1	5470	1.0	100.0
Concentrate;						
IR-120 batch	423700	74.9				67.9
Acetic acid extract	415000		36.5	11400	2.1	66.5
Desalted extract from Bio-Gel P-2 column	274600	42.4	27.5	10000	1.8	44.0
Fractionation on Bio-Gel P-10 column	207700	9.66	9.23	22500	4.1	33.3

[a] This table was compiled from Tables 4.10, 4.12 and 4.14; N.D. Not determined.

Proteins that either contain aromatic amino acids or are of a strongly acidic or basic nature, are well known to interact with Sephadex and Bio-Gel matrix materials, particularly on small-pore gels (DETERMANN, 1968; see for example PORATH, 1960; RUTTENBURG, KING and CRAIG, 1965; BONILLA, 1969). Toxin-A present in the culture filtrate concentrate eluted at K_{av} 1.1 in acidic eluant but was less retarded in more neutral eluants. Phenylmethanol, a reagent which was expected to reduce gel matrix-aromatic moiety interaction (MOORE and STEIN, 1951) partly reversed the retarding effect of the acidic eluant but did not accelerate the emergence of toxin to K_{av} values of less than 0.8. This finding is consistent with its low content of aromatic amino acids. In view of the near-neutral pI of toxin-A, the molecule may be highly polarised with respect to charge to account for its strong interaction with the gel matrix. Evidence for this was provided by the large proportion of acidic amino acid residues which are present and by the increased retardation in acidic eluant, in which toxin-A carries a nett positive charge.

Recently, in a systematic study using Bio-Gel P-2, it was shown that small anions and certain disaccharides are accelerated ahead of their expected K_{av} values (based on molecular size), an effect not corrected by increasing the ionic strength of the eluant, but was decreased by acidifying the eluant. (BAXTER, FRASER and HOLMES, 1980). This effect was observed in these experiments by the advanced elution of the culture filtrate salts at neutral and alkaline pH compared with acidic pH, but in contrast, toxin-A displayed a reversal of this effect, showing that a different mechanism was operating. It could be expected that agents which reduce or eliminate interaction, such as, phenol-acetic acid-water, $CaCl_2$, guanidine hydrochloride, urea, detergents and

others, would enable a reliable molecular weight estimate when employing suitable polypeptide standards (see for example, PUSZTAI and WATT, 1970; YAZAWA and YAGI, 1972). Another possibility would be to employ Sephadex G-100 or Bio-Gel P-150 in which solute-gel matrix interaction is generally minimal (ANDREWS, 1964, 1967; BATTLE, 1967).

Chromatography of toxin-A active concentrates, prepared by a combination of ion exchange and exclusion from Bio-Gel P-2, showed a different pattern of elution behaviour on Bio-Gel P-10 compared with that present in the culture filtrate concentrate. The bulk of toxin activity eluted at K_{av} 0.55 compared with 1.1 (in acidic eluant) for the culture filtrate preparation (summarised below). However following concentration of the K_{av} 0.55 eluate at moderate temperature, toxin-A co-eluted with protein at a value of 0.8. Under the conditions of the experiment there was no significant reversion of protein or toxin activity to the K_{av} 0.55 fraction.

Concentrate	Eluant	K_{av}
Culture filtrate	Phosphate, pH4-8	0.8
	Acetic acid, pH2.8	1.1
	Acetic acid - phenylmethanol, pH2.8	0.8, 1.1
Ion exchange, Bio-Gel P-2 eluate	Acetic acid, pH2.8	0.55, 0.8
Concn. at 55°C	"	0.8

No experiments were made specifically to fully elucidate the physico-chemical relationship between each of the three observed toxin-A active fractions (K_{av} 0.55, 0.8 and 1.1) obtained from columns of Bio-Gel P-10. All three preparations showed similar electrophoretic

mobilities on CAM strips at pH4.3 (indistinguishable when dissolved in culture filtrate). Since SDS-gel electrophoresis did not divulge any significant difference between the proteins of the K_{av} 0.55 and 0.8 fractions, it is possible that either (i) they differ in molecular conformation or (ii) one species is a specific aggregate form of the other.

The first possibly is suggested by the relatively heat stable nature of toxin-A activity indicating that a particular conformation, although likely to be well defined and possibly constrained by two molecules of cystine, may not be critical to its biological activity. An alteration of its conformation organisation could be expected to alter chromatographic behaviour by a change in molecular shape or exposure of different chemical groups while not affecting its electrophoretic mobility under the conditions employed. The second possibility, regarding aggregation, is however more likely but since acetic acid is known to both precipitate protein as well as dissociate protein aggregates (PORATH, 1963; GINSBURG, BURFORD and THOMAS, 1971) it is not possible to suggest which fraction might represent an aggregate form of the other. It would however seem likely that the K_{av} 0.8 fraction derived from the isolated material corresponded to that present in the culture filtrate, which is probably a monomer by its membrane diffusibility behaviour. This needs to be conclusively demonstrated by rechromatography of the culture filtrate derived (K_{av} 1.1) fraction on Bio-Gel P-10, that is, in the absence of culture filtrate components which may modify the chromatographic behaviour of toxin-A. Accepting this, therefore implies that the isolated K_{av} 0.55 fraction represents an aggregate form of the K_{av} 0.8 fraction which presumably was generated at some stage(s) during the isolation procedure, possibly in the resin eluate concentrate. Demonstration of and conditions affecting

(possible) toxin aggregration would best be studied on sucrose and CsCl gradients by ultracentrifugation.

Molecular weight determination

Limitations on the estimation of molecular weight by SDS-polyacrylamide gel electrophoresis have been summarised by GORDON (1975, pp. 153s-159s) and relate to the recognition of anomalous electrophoretic behaviour which stems from either SDS-binding anomalies or atypical conformation of the complex with SDS. In the former, glycoproteins, in particular, bind lower amounts of SDS, thus reducing mobility and hence a consequential molecular weight over-estimate. In the latter, atypical conformation results in altered mobility which again yields an incorrect estimate. Recognition of such anomalies is usually by determination of free solution mobilities by means of a Ferguson plot (GORDON 1975) where mobility is measured in gels of a different acrylamide concentration and extrapolated to zero concentration. Anomalous behaviour is then recognised by a considerable deviation from the average value for 'normal' proteins. Since toxin-A contained little or no carbohydrate (tryptophan boro-sulphuric acid assay) and mobility was not affected by reduction, there seemed little reason to suspect anomalous electrophoretic behaviour, so this aspect was not investigated. However it must be pointed out that SDS-denatured proteins with a molecular weight below 10000 no longer fit the semi-logarithmic relationship between mobility and molecular weight (WILLIAMS and GRATZER, 1971). Since the SKT proteins appeared at the fringe of this relationship, the values obtained may represent an over-estimate. Mobilities of SDS-proteins of low molecular weight are largely normalised in the presence of 8M-urea (SWANK and MUNKRES, 1971) and this technique could therefore be used to confirm the accuracy of

the above estimate (see for example SANO, NOZU and INOUE, 1978). The molecular weight estimate based on the amino acid composition of three independent preparations of toxin-A indicated reasonable agreement with the present estimate.

Possible improvements to the isolation procedure

Several factors, not investigated, which may be expected to improve efficiency of toxin-A adsorption to resin include (i) reduction of culture filtrate ionic strength and (ii) use of a resin-type of greater surface area. The high ionic of the culture filtrate was due to citrate, and could be reduced either by dilution, requiring toxin isolation from the larger volume by a column method, or by removal of the salt, possible by precipitation of citrate as the calcium salt or by anion exchange with Dowex 2(OH). The latter method could be used to advantage by eliminating the requirment for NaOH neeeded to adjust the resin -filtrate mixture pH for optimum adsorption of toxin. In view of the molecular size of toxin-A, no penetration of the ion exchange resin used (8% DVB) could be expected (HIRS, MOORE and STEIN, 1952) and it is doubtful whether resin of low DVB content, such as AG50W-X1 or -X2 as used in isolation of peptides would also provide increased capacity (JONES, 1964; SCHROEDER, 1967a). Resin of increased surface area, such as provided by the macroporous cation exchanger AG MP-50 (WALL, 1970) or by resin of small particle size, for example CG-120 types 1 or 11, may be useful. Toxin-A isolated from a column of Bio-Gel P-2 adsorbed to CM-cellulose and SP-sephadex A-25 at pH4.0, indicating that possibly any type of cation exchanger could be used, providing their mechanical properties were appropriate to the batch or column method employed.

The batch step-wise elution procedure and subsequent recovery of

toxin-A in a small volume of eluate resulted in removal of some 99.9% of the culture filtrate solids. This was greater than expected from the gradient elution experiments in which only about 95% of culture filtrate material was removed. The greater recovery of dry weight material in the latter experiments may have been the result of the much greater contact time between eluant and resin allowing culture filtrate salts adequate time to diffuse from the resin particles (14-52 mesh). This effect would be particularly enhanced in eluate of low ionic strength in which complete equilibrium between resin and eluant could be expected to be of the order of several hours (HELFFERICH, 1962). Two observations lend support to this notion (i) Washing resin with eluate (0.0075I, pH8.0) for more than a few seconds did not increase the yield of toxin whereas the pH slowly decreased over a period of 30 min. (ii) An eluate (0.0075I, pH8.0) left in contact with the resin overnight (pH maintained at 6.5 to 7.5) had a dry weight of 1.8-fold greater than expected. These experiments also indicate that the toxin molecule only adsorbed to the surface of IR-120 cation exchange resin. Furthermore, in the batch elution method, only sufficient titrant was added to elute toxin activity, whereas in the column method, the effective ratio of eluant to resin was much greater, thus promoting exchange with other non-volatile components leading to increased dry weight of the eluate.

A criticism of the experiments made in the development of the step-wise elution procedure of toxin-A concerns the evaluation of the toxin's specific activity. Although dry weight determination was not interfered with by eluant solutes, the exchanged culture filtrate salts however did interfere, but this was minimised in low ionic strength eluant. It would have however, in addition, been more instructive to follow toxin specific activity with respect to total protein, using the ninhydrin procedure following alkaline hydrolysis to avoid interference

by ethylenediamine and the ammonium cation.

Because of the developmental aspects of the purification procedure carried out, only small quantities of purified toxin were prepared which therefore limited the extent of analysis made. In particular this problem hampered amino acid composition analysis, and hence these results can only be considered preliminary. Although, toxin-A isolated from Bio-Gel P-10 was shown to be homogeneous, its state of aggregation is not yet known. It may be possible to replace the Bio-Gel P-10 step by chromatography on glyceryl-controlled pore glass under conditions similar to those described by PALFREE and BUSSEY (1979). Application of the Bio-Gel P-2 concentrate dissolved in buffered urea may allow recovery of toxin-A in a non-aggregate form.

CHAPTER 5

OBSERVATIONS ON THE KILLER ACTION OF TOXIN-A

INTRODUCTION

In Chapters Two and Three it was shown that filtrates obtained from cultures of H. saturnus killer strains which contained toxin-A were lethal for sensitive yeasts. Experiments were therefore made to study the toxic effects, in greater detail, using a highly purified preparation of toxin-A protein. The purpose of these experiments was to determine the sensitivity of a highly susceptible strain to toxin-A, and to examine ultrastructural changes in a treated population of cells by electron microscopy. The samples of cells were fixed by either a chemical or a physical method to reduce the problem of fixation artefacts.

MATERIALS AND METHODS

Toxin-A preparations

Except in preliminary electron microscope experiments, toxin-A was a highly purified sample prepared by rechromatography on Bio-Gel P-10 as described in Chapter 4. The sample was a 10000 a.u./ml solution in 0.20M-acetic acid and contained 420 μg/ml protein (Lowry method). This solution was stored at 4-6°C, at which no loss of activity was detected over one month when assayed against a frozen sample. The crude sample of toxin-A was an Amberlite IR-120 resin eluate concentrate (derived from a standard culture filtrate) containing 40000 a.u./ml and was also kept at 4-6°C (pH6.0).

Minimal inhibitory concentration

Plates of 0.081M-phosphate buffered YEPDA (10 ml, pH6.0) containing various concentrations of crude toxin-A (resin-eluate concentrate) were surface inoculated with 50 μl of buffered yeast suspension containing 0.5-1x10^6 cells/ml (total cell count). The plates were incubated at 28°C for 30h and examined for growth.

Sensitive strains

The highly sensitive yeast, H. anomala strain 10, was employed in both killing and electron microscopic studies. Work cultures were clones derived from a mass inoculated stock slope culture, and typically displayed a minimal inhibitory concentration of 5-10 a.u./ml. The MIC of other sensitive yeasts, including strains of Sacch. cerevisiae were at least 10-fold greater.

Survival experiments

Exponentially growing cultures were prepared by diluting (10-100-fold) an overnight shake culture into fresh YEPD (100 ml, 1000 ml flask) containing 0.081M-phosphate buffer (pH6.0) and incubating at 28°C with vigorous shaking (120 strokes/min), for at least two generations (generation time approximately 100 min). Cells were harvested by centrifugation and either resuspended in buffered YEPD or washed 3x in phosphate buffer followed by resuspension in this buffer. 5.0-10.0 ml portions, at the required cell density (usually 1.0-1.5x 10^7/ml), were dispensed into 100 ml conical flasks to which was added toxin-A diluted in phosphate buffer (final volume 0.5 ml). Controls received 0.5 ml buffer containing the maximum level of acetic acid added to the

treatments. pH was unaffected. Incubation was recommenced and the mixtures sampled at various time intervals.

Turbidity was measured at 600 nm (10 mm cell) by reading against sterile medium in a Hitachi Perkin-Elmer UV-VIS spectrophotometer. Viability was determined, following appropriate dilution in the respective buffered medium, by spreading 0.1 ml portions (in triplicate) on plates of buffered YEPDA. Plates were incubated at 28°C for 2d, and the number of colonies were counted. Total cell count (cells and their visible buds scored separately) was determined in a haemocytometer (BUSSEY, 1972). Survival is expressed as the percentage of cells able to produce colonies on YEPDA at the end of an experiment where 100% is the number of colony forming units per millilitre, immediately before the addition of toxin. Multiplicity, m, was calculated from the survival ratio: $N/No = e^{-m}$ (REEVES, 1965).

Electron microscope studies

Cells growing in buffered YEPD (50 ml, shake culture) were treated with toxin (100 a.u./ml final concentration) similarly as described above. The nomenclature of cell components is as adopted by MATILE, MOOR and ROBINOW (1969) except that a vacuole is referred to as a large vesicle as suggested by CARTLEDGE, ROSE, BELK and GOODALL (1977).

A. Chemical fixation - thin section electron microscopy

Cells from control and toxin-A treated cultures of *H. anomala* strain 10 were gently collected by low speed centrifugation. The pellet was fixed in an aqueous 2% solution of potassium permanganate for 30 min at room temperature (22-24°C) and then thoroughly washed with distilled

water, dehydrated with an ethanol series and embedded in Spurrs resin (see Appendix Ten). Silver sections were cut, stained firstly with saturated aqueous uranyl acetate for 10 min and then with Reynolds' basic lead citrate for 8 min, and subsequently examined in a Siemens Elmiskop I electron microscope at 80 kV.

B. Freeze-etch technique

Cells were collected as described in A. and either suspended in 20% glycerol and kept at 4-6°C for 20h before freezing or transferred directly to gold specimen mounts and freeze-etched as described by SWIFT and MUKHERJEE (1978). The replicas were additionally cleaned with 50% chromic acid, and examined as described in A.

RESULTS

Sensitivity to toxin-A

Time-course effects of toxin-A on culture turbidity and cell viability were examined in populations of both growing (in buffered YEPD medium) and resting cells (in phosphate buffer).

At low toxin multiplicities (less than 4), turbidity of YEPD cultures increased at lesser rates than did the control whereas at higher mutiplicities (for example 6.0, Figure 5.1B) growth was completely arrested by 100 min and thereafter turbidity decreased to a second level which stabilised by 300 min. Turbidity eventually began to increase again after 20h of incubation. At the higher toxin multiplicities, the turbidity decrease was not due to cell lysis (none evident by 20h of

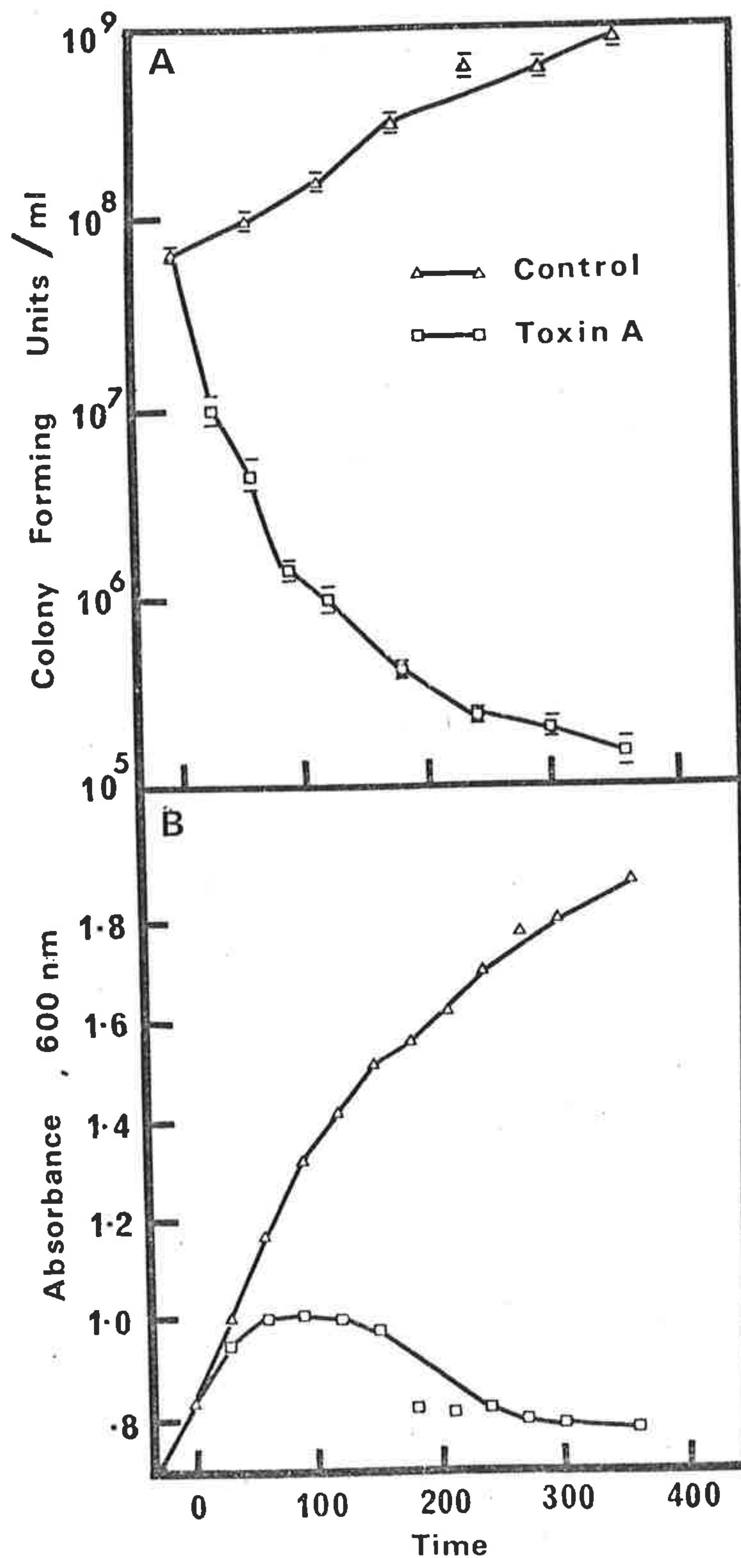


Figure 5.1. Sensitivity of H. anomala strain 10 to toxin-A protein: A. Colony forming ability, B. culture turbidity. Toxin-A (protein 4.2 µg/ml [100 a.u./ml], final concentration) or buffer (0.1 ml of 0.2M-acetic acid) was added at time zero to shake cultures of cells growing in phosphate buffered YEPD (pH6.0, 10.0 ml) at 28°C. Bars represent the standard error of mean colony counts.

incubation) and the cell count was similar at both 100 and 300 min. Both samples contained budding cells which were at different stages of development. However, cell clumping became pronounced after 100 min of toxin treatment and by 300 min 35% of cells were present in clumps composed of between 5 and 20 cells. At lower concentrations of toxin, clumping was both less frequent and composed of lesser numbers of cells. The control culture contained comparatively few clumps composed of more than three cells.

The behaviour of control cells in buffer interfered with turbidity measurement and cell counting. Both of these decreased with respect to time, the latter by 30% in the first 30 min of incubation, as the result of rudimentary pellicle formation (accumulation of cells on the surface of the liquid). The actual decrease observed was dependent on the degree of shaking prior to sampling; vigorous shaking temporarily restored turbidity to near its initial value but on standing the submerged lipophilic cells rapidly rose to the surface to reform a pellicle. Thus it was difficult to obtain reproducible values both within and between duplicates. However, microscopically the toxin-induced cell clumping phenomenon was clearly observed. After 4.5h of shake incubation, a field of 1000 control cells had on average 32 clumps containing 18% of cells whereas a field of 1000 treated cells (100 a.u./ml toxin-A) had 47 clumps containing 42% of cells. No attempt was made to prevent or disperse either pellicle formation or clumping.

The addition of toxin to growing cells at high multiplicity (for example approximately 6.0, see Figure 5.1A) resulted in an initial sharp decline of colony forming units after which the rate of decline steadily decreased. At progressively lower concentrations of toxin, the rate of killing was reduced. The subsequent plateau period, before cell

viability began to increase, also became progressively reduced.

The extent of sample dilution prior to plating on agar medium did not significantly affect the viable count indicating that the cell-toxin complex was not readily dissociated. Furthermore, samples of toxin-treated cells diluted in either buffered YEPD or buffer and held for 30 min before plating did not affect the viable count. No attempt was made to disperse toxin-induced cell clumps prior to viable counting. If it is assumed that cell clumping is a specific effect of toxin-A (that is only cells having received a lethal dose of toxin are able to clump), then plate counts would reflect true viable counts; however, this needs to be demonstrated. The toxin dose-clumping response was not quantified but it was evident that the proportion of cells involved in clumping was considerably smaller than those having received a lethal dose of toxin. This therefore suggests that only those cells having adsorbed a large excess of toxin may adhere together.

Killing of immobilised cells demonstrated that cell contact was not a necessary stage in the killing process. Cells (50-100) were either spread on YEPD plates containing toxin (0 or 100 a.u./ml) or collected on membrane filters (0.45 μm, Gelman) placed firstly on toxin-agar plates (5h, 28°C), then briefly on toxin-free plates and finally incubated on fresh agar plates. None of the treated cells survived.

The survival of resting cells on the other hand exceeded 50% at the highest level of toxin tested (100 a.u. per millilitre of buffer containing initially 2×10^7 cells/ml, see below). Survival, however, was probably underestimated: reduction in the numbers of colony forming units due to cell clumping, and it was likely that the pellicle which formed was composed mainly from viable cells which would therefore

enrich the sampled population with respect to non-viable cells. The addition of tween 80 markedly reduced pellicle formation but was not employed in these experiments since it could be expected to introduce unknown effects. The problem may best be dealt with by choice of a non-pellicle forming yeast.

Effect of toxin (100 a.u./ml) on resting cells, added at time zero.

Time (min)	-30	0	60	270
Total count	2.7×10^7	1.9×10^7	1.2×10^7	1.04×10^7
Viable count	1.7×10^7	9.3×10^7	6.0×10^6	3.8×10^6

Estimation of the lethal unit

The survival of cells grown in buffered YEPD was studied as a function of the concentration of toxin-A as described by BUSSEY and SKIPPER (1976). Survival was determined at 420min following toxin addition by which time killing was more than 99.5% complete. Assay of the supernatant fluid showed that 2.0 μg/ml protein produced saturating levels of toxin (2×10^7 cells/ml). The survival curve (see Figure 5.2) approximated that of a straight line, which when fitted by the method of least squares passed near to the origin, and was therefore suggestive of a single-hit killing mechanism. From two such curves, using a toxin preparation of 24000 a.u./mg, 1.0 mg of toxin protein corresponded to $0.5\text{-}1 \times 10^{11}$ lethal units and hence 1 a.u. of toxin contained about 3×10^6 lethal units. Assuming a molecular weight of 11000, one lethal unit of this toxin preparation contained 5×10^5 molecules.

Electron microscopy

A. Chemical fixation

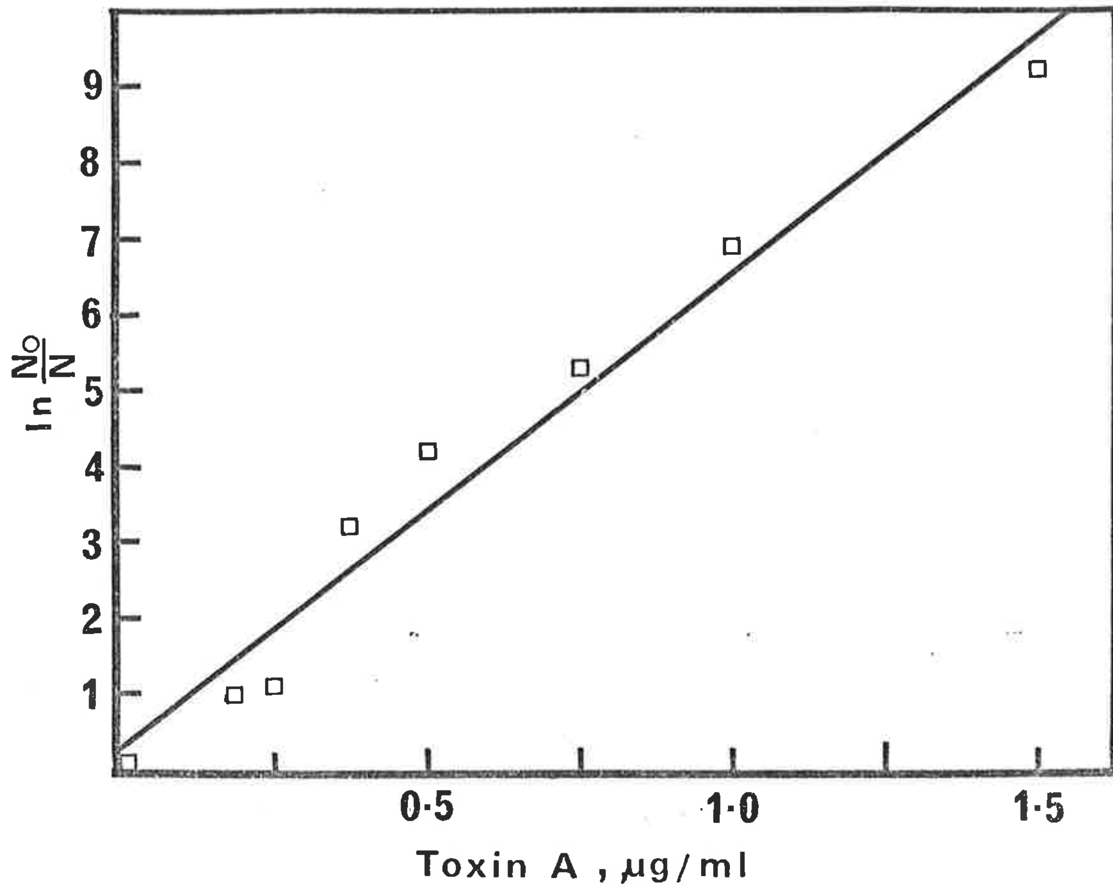


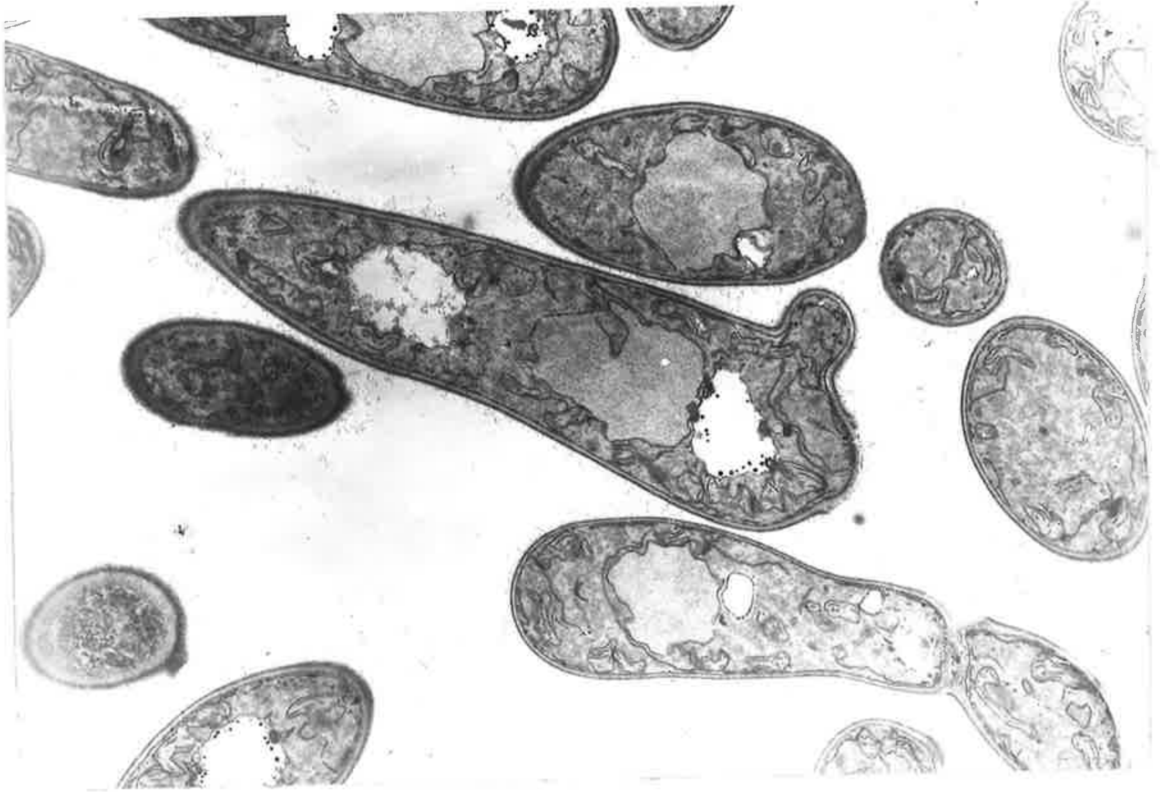
Figure 5.2. Survival curve for *H. anomala* 10 treated with toxin-A. Cells were grown in buffered YEPD (pH6.0, 28°C) to about 1x10^{7}/ml and then treated with various concentrations of toxin-A protein. Initial cell number, No, was determined before toxin-A addition and survival, N, was determined 420 min following the addition of toxin. The line, y=0.23+6.37x, was fitted by the least-squares method in which standard error of the slope, x, was ±0.47.

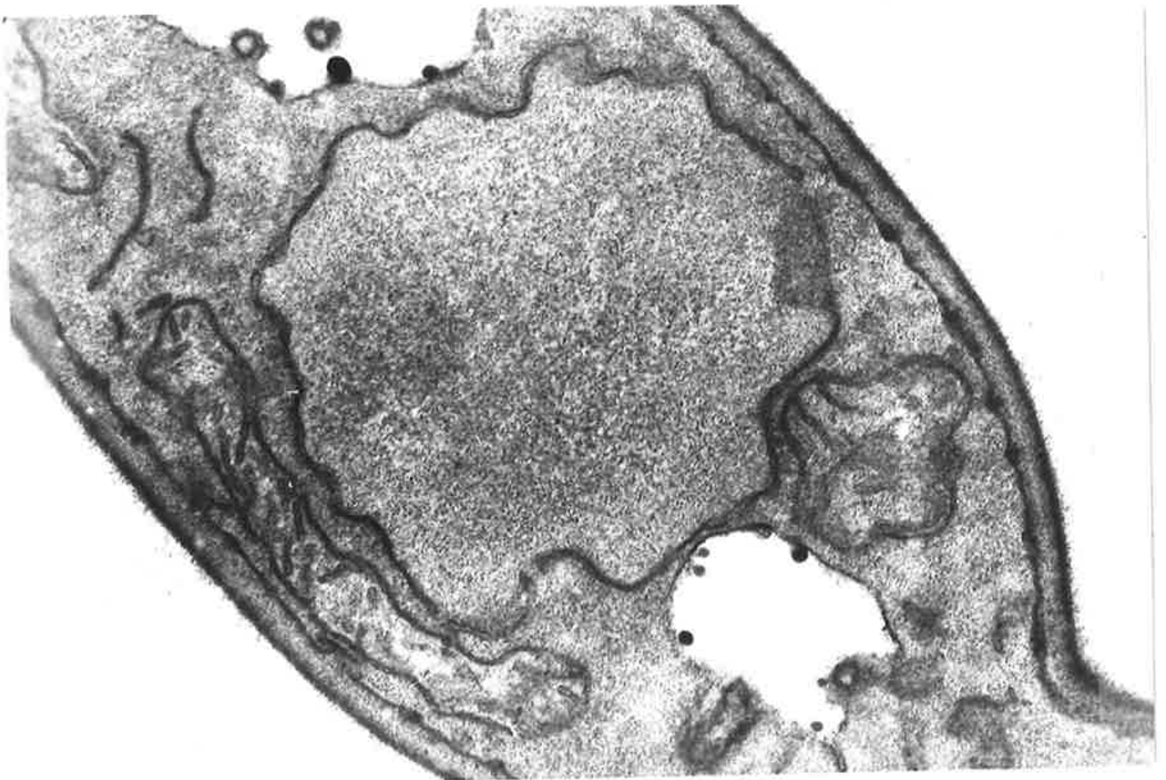
Control cells

Strains of H. anomala occur as either a more highly fermentative or a more highly oxidative type (WICKERHAM, 1970). In the former, cells are predominantly spheroidal to ellipsoidal and form glistening colonies on malt extract agar whereas more highly oxidative types are characterised by filamentous-like cells of widely variable size which produce matt or rugose colonies. Strain 10 belonged to the highly oxidative type. Cells grown by shake culture in YEPD ranged in length 1.5-4 times their diameter (2-6 μm) of which first generation cells were more ellipsoidal to ovoid in shape. Although the culture was therefore heterogeneous with respect to cell size and their distribution, cell wall thickness was seen to be relatively constant, being about 900 A (see Figure 5.3A).

The permanganate fixative provided adequate preservation of H. anomala ultrastructure, which was comparable to that published by BANDONI, BISALPUTRA and BISALPUTRA (1967) using a similar method (see Appendix Eleven). A representative field of cells shown in Figure 5.3A provides an indication of cell-size variability (note different cell orientations). The absence of clarity observed at the poles of each cell is due mainly to the greater curvature of the cell wall at that point. An appearance of shrinkage or distorted outline of the larger organelles (nucleus, large vesicle and to a lesser extent mitochondria) is evident, however the fine structure of the various membranes remains discernable (Figure 5.3B). The plasma membrane where clearly resolved was continuous and contained invaginations at irregular intervals, but invaginations were less frequently observed in buds. The invaginations contained dense electron-scattering material which is also apparent in the photomicrographs of both BANDONI, BISALPUTRA and BISALPUTRA (1967)

Figure 5.3. A. Electron micrograph of a thin section of pelleted, permanganate fixed control cells of *H. anomala.* Magnification, x 10900
B. Electron micrograph of a control cell at greater magnification showing membrane structure.
Magnification, x 45000





for H. anomala and CONTI and BROCK (1965) for H. wingei, but this material is clearly different to the membrane aggregate-like material associated with certain invaginations of Sacch. cerevisiae (SENTANDREU and NORTHCOTE, 1969). The double-layered membrane structure of the nuclear envelope, mitochondria and endoplasmic reticulum was satisfactorily resolved. Some two to six nuclear pores per thin section were apparent by gaps in the nuclear envelope at which point the inner and outer membranes were fused (continuous) but it was not possible by this method to observe the pore contents (fine-grained matter of high density) as described by ROBINOW and MARAK (1966). The endoplasmic reticulum frequently occurred as a series of long structures lying closely opposed to the plasma membrane and to a lesser extent randomly orientated within the central area of the cytoplasm. One to two large vesicles and occasionally smaller vesicles were present in the cytoplasm, but the latter were more common at the neck of a bud and also in the bud during its early development.

Toxin treated cells

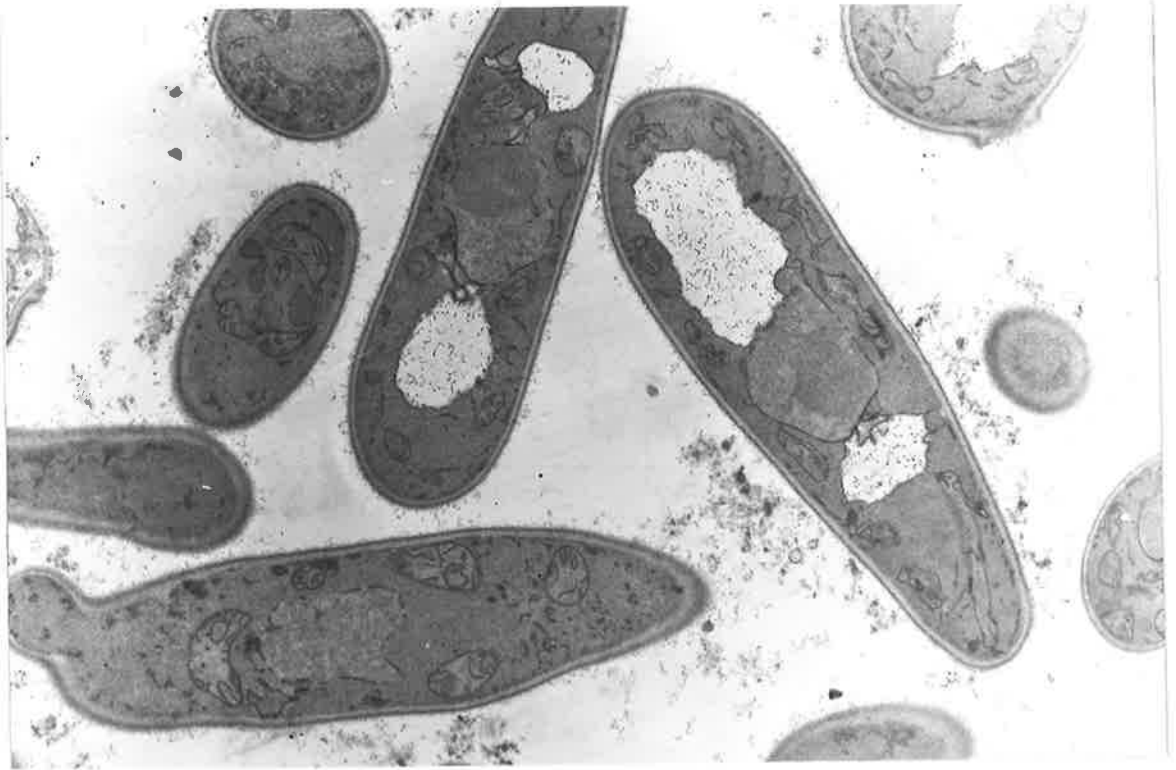
Cells growing aerobically in buffered YEPD at 28°C were treated with 4 μg/ml (final concentration) of purified toxin-A protein; cell survival was approximately 1.4% at 1h, 0.6% at 2h and 0.1% at 5h. The first observable ultrastructural alteration was the "flattening" of the invaginated plasma membrane structure followed by a striking increase in the numbers of gaps in the nuclear envelope(see Figures 5.4 and 5.5). Although by 1h more than 98% of cells had received a lethal dose of toxin, less than 20% of cells showed significant ultrastructural change, but by 2h more than 80% of cells were damaged and at 3h cells were present which showed extensive loss of structural organisation.

The loss of the invaginated plasma membrane structure appeared to be synchronised and relatively rapid, that is, 1-hour treated cells either possessed invaginations or showed no evidence of them. However, the plasma membrane remained intact with no consistent perforations being apparent. The membrane of vastly altered cells at 5h also appeared to be intact.

The initial alteration of the nuclear envelope involved an increase in the number of gaps which simultaneously occurred in adjacent areas of both membranes (Figure 5.6). The gaps were of a similar size to that of the nuclear pore but the structure of many of these differed in that they did not show a definite continuity (merging) of the inner and outer membrane. With respect to time, the gaps enlargened and frequently a considerable section of the membrane disintegrated. The nucleoplasm of nuclei that contained numerous membrane-gaps (initial stage) retained the staining properties of control cells, but the nucleus appeared more rounded suggesting a freer mobility between the cytoplasm and

Figure 5.4. Electron micrograph of cells following contact with toxin for 2 hours.
Magnification, x 10600

Figure 5.5. Electron micrograph of cells in contact with toxin for 5 hours.
Magnification, x 10400



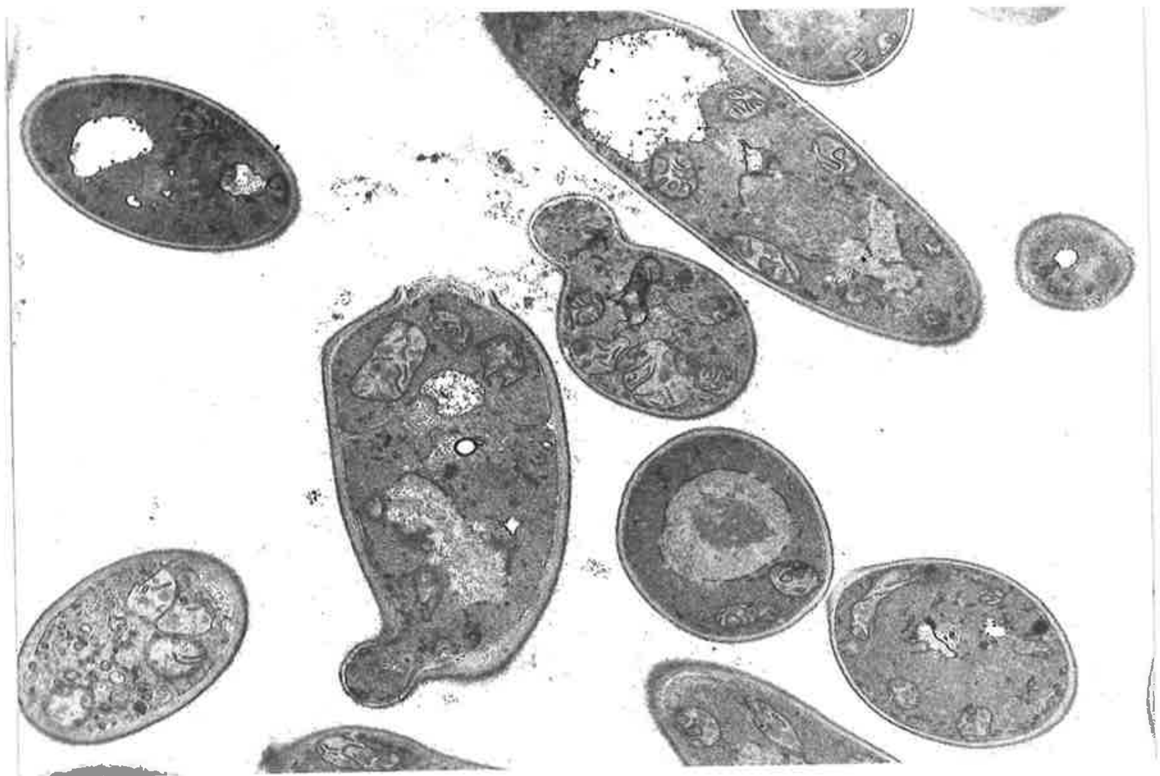
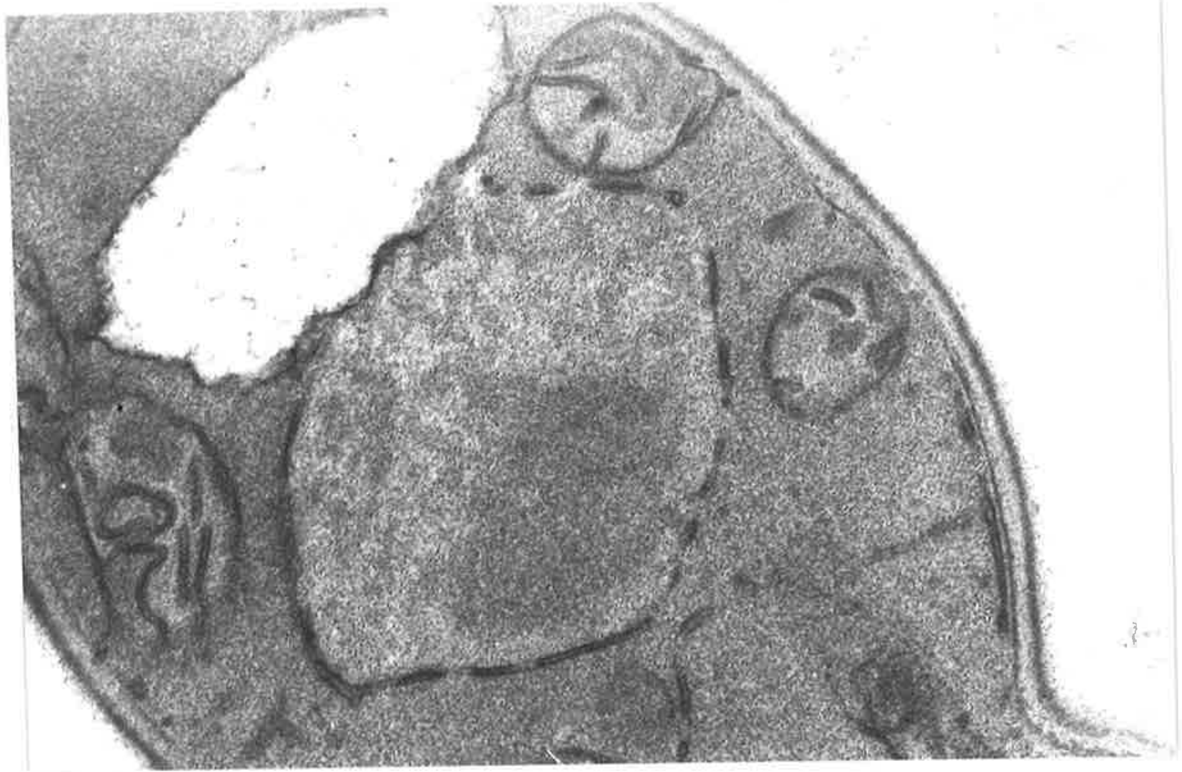
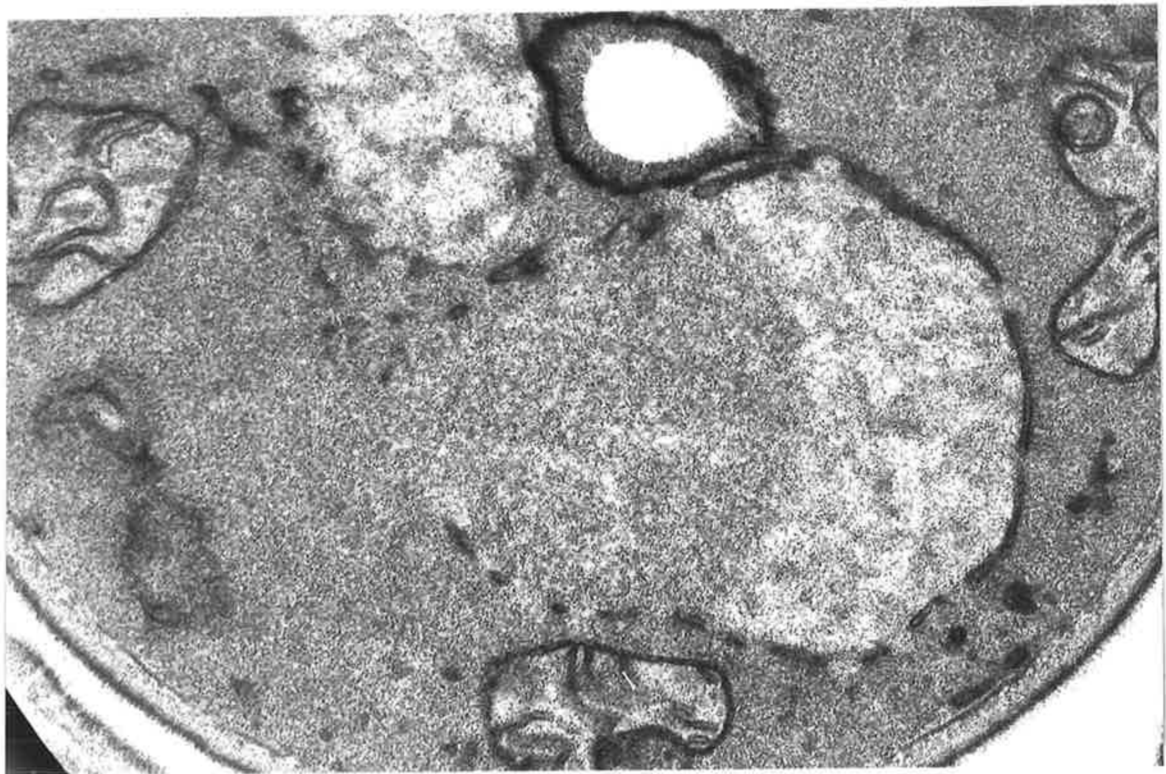


Figure 5.6. Electron micrograph of toxin-treated cells showing loss of plasma membrane invaginations and disintegration of the nuclear envelope and endoplasmic reticulum.
Magnification x 53000 (Upper) x55000 (Lower)





nucleoplasm, thus reducing the shrinkage artefact. More intensely stained regions of the nucleoplasm were present in nuclei showing extensive membrane disintegration indicating a merging of the cytoplasmic ground substance and the nucleoplasm.

Although less readily ascertained, the endoplasmic reticulum also appeared to fragment in cells containing damaged nuclei (Figure 5.6). This was evident by a less frequent occurrence of extensive dual-layered membrane profiles which in control cells were most abundant below the plasma membrane. Endoplasmic reticulum disintegration appeared to occur in a similar way as for the nuclear envelope. The large vesicle in cells at 5h retained a similar appearance to those in control cells suggesting that its membrane (tonoplast) was unaffected. Although the mitochondria displayed a more rounded (less-distorted) appearance and were less elongate, they were otherwise unaffected.

From about 3h following contact with toxin, cells showing complete loss of structural integrity became evident. The cell wall retained its staining properties and the underlying plasma membrane also appeared to retain continuity without apparent perforation. Large gaps were occasionally encountered but similar gaps were also seen in the controls. There was no evidence of a nucleus or endoplasmic reticulum in the cytoplasm of cells which contained both numerous rounded vesicularised structures of variable diameter and rounded but otherwise apparently unaltered mitochondria. Several vastly degenerated cells were observed which showed a single point of lysis in the cell wall and partial expulsion of contents. This was probably an artefact of preparation since no empty cells were observed.

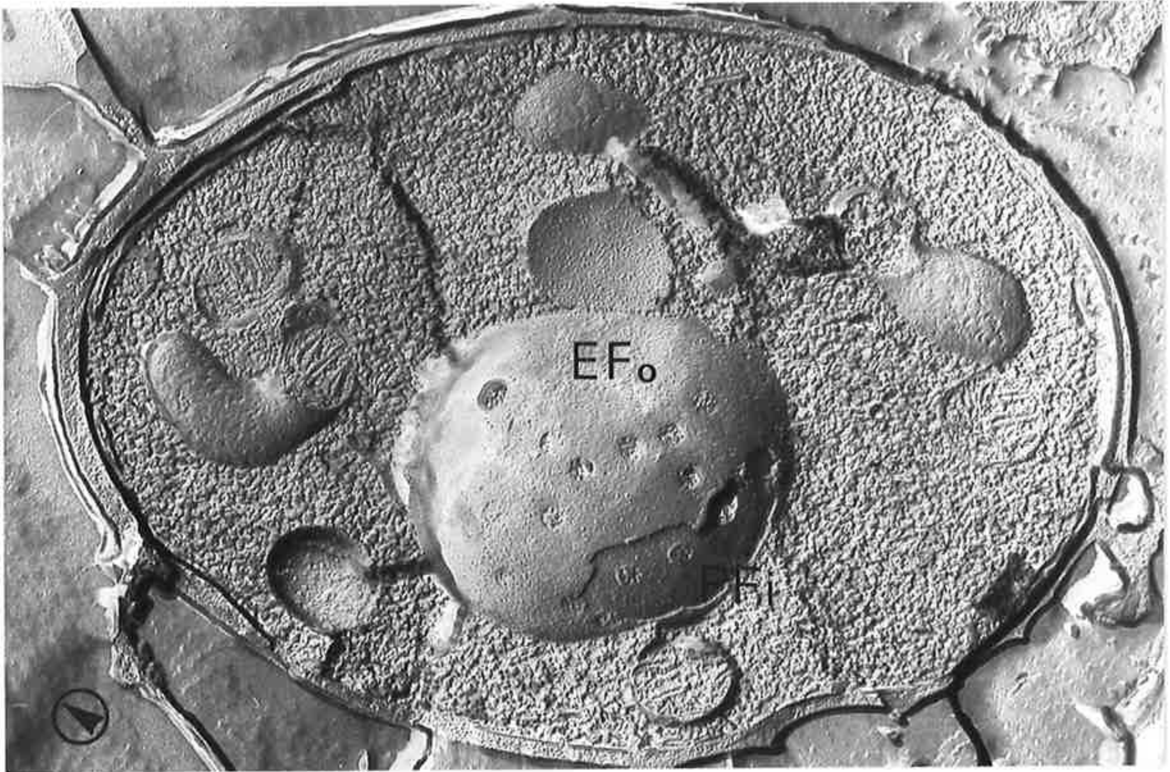
Freeze-etch technique

Control cells

In a study such as this, cells are normally pre-fixed chemically and then treated with a cryoprotective agent (glycerol) before the freeze-etching process. But to avoid the problems associated with chemical fixation, cells were frozen directly in the medium, and control cells were in addition pretreated with glycerol to control ice-crystal damage. The membrane fracture nomenclature introduced by BRANTON and colleagues (1975) and extended by WILLISON and JOHNSTON (1978) has been adopted in this study.

The ultrastructure of a control cell is depicted in Figure 5.7, revealing the rounded appearance of the nucleus, large vesicle and mitochondria (compare with Figure 5.3). The exoplasmic fracture face (EF face) and protoplasmic fracture face (PF face) of the plasma membrane is shown in Figure 5.8, which reveals a random pattern of elongate invaginations, of similar appearance to those described for Sacch. cerevisiae (MOOR and MULETHALER, 1963, KRAMER, KOPP, NIEDERMEYER and FUHRMANN, 1978). The exposed plasma membrane-bound particles are clearly apparent on both the EF and PF faces as described for other yeasts (NOZAWA and colleagues, 1974; KRAMER et al., 1978; SLEYTR and MESSNER, 1978). A surface view of the nucleus, shown in Figure 5.7 depicts the EF face of the outer membrane and PF face of the inner membrane revealing the associated nuclear pore, some of which show pore contents (SEVERS and JORDAN, 1975; WILLISON and JOHNSTON, 1978). Variability of pore size and structure remains in doubt (HARRIS, 1978): WILLISON and JOHNSTON (1978) have documented likely artefacts of the freeze-etch technique which result in the enlargement of the pore diameter. No attempt was made to quantitate nuclear pore size and

Figure 5.7. Freeze-etch replicas of a control (upper) and toxin-treated cell (lower).
The samples, collected two hours after the addition of toxin, were fixed by freezing directly in the medium. Magnification, x31000 (upper) x26000 (lower)
PF, protoplasmic fracture face; EF, endoplasmic facture face; i or o, inner or outer membrane.


EFo
PFi

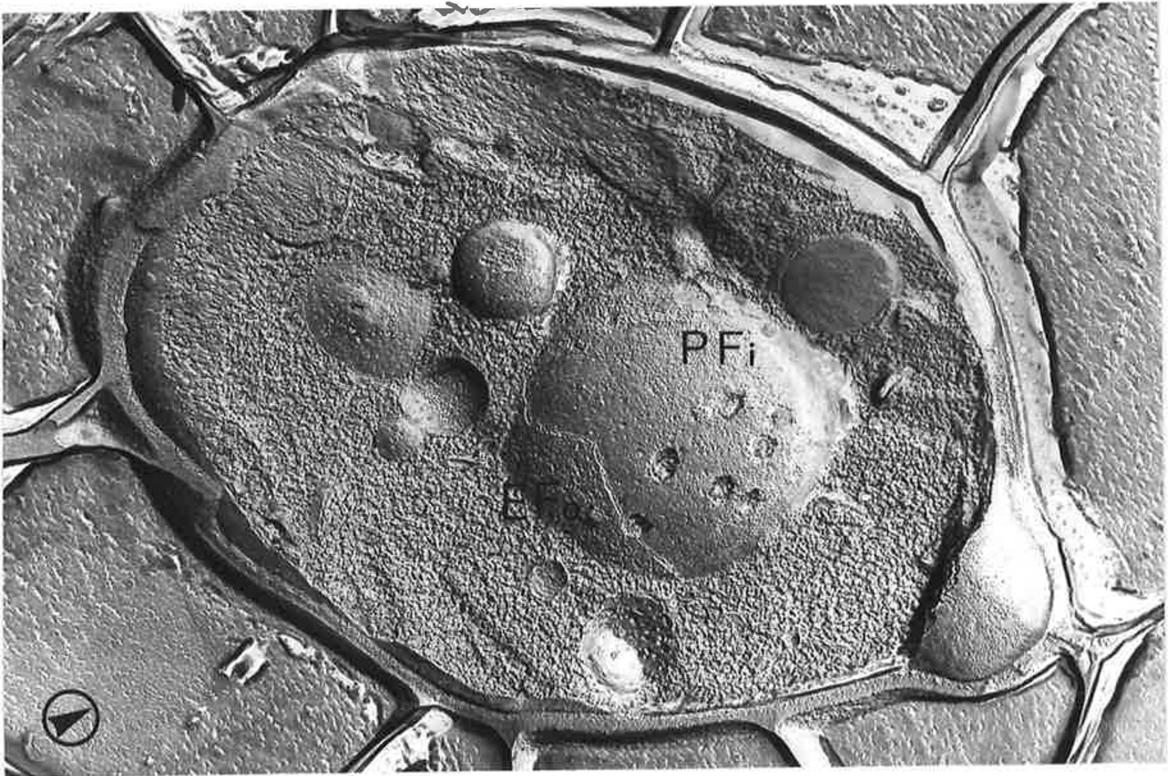
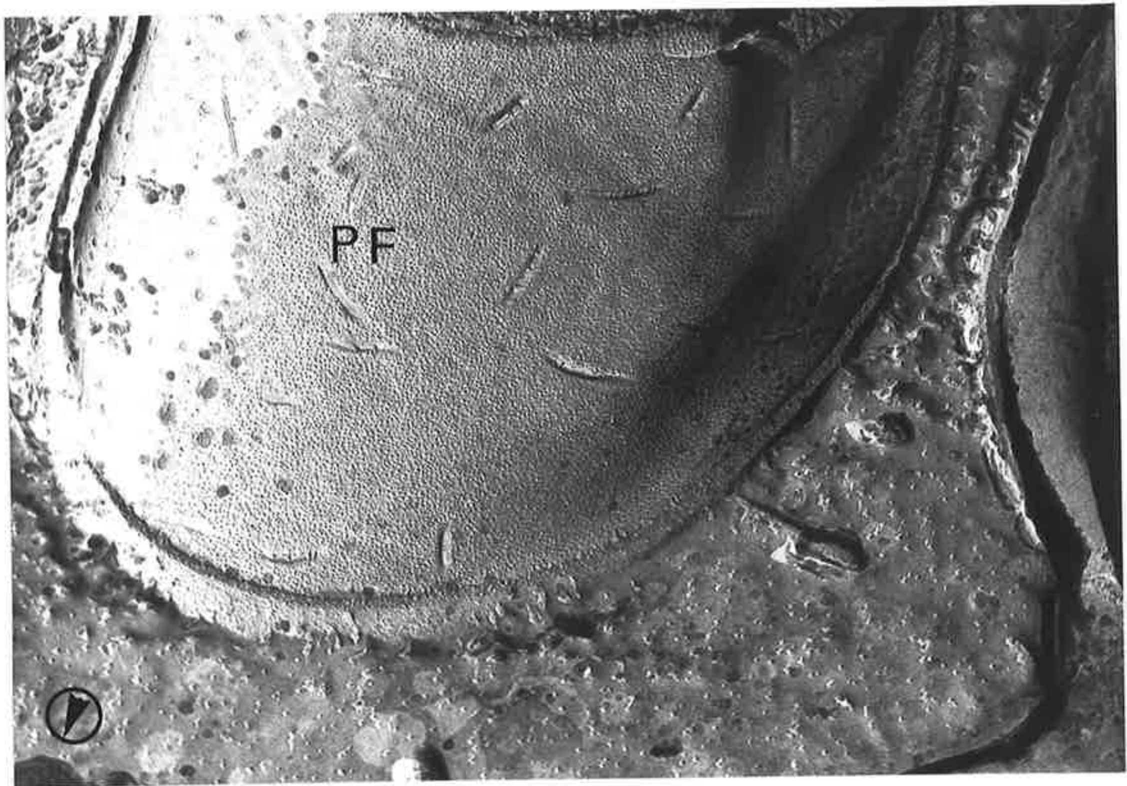

PFi

Figure 5.8. Freeze-etch replicas showing the PF and EF fracture faces of the plasma membrane of control (upper) and toxin treated cells (lower).
Cells were frozen in growth medium, except for the EF face control which was suspended in glycerol prior to freezing.
Magnification, x38000 (upper) x54000 (lower)


PF

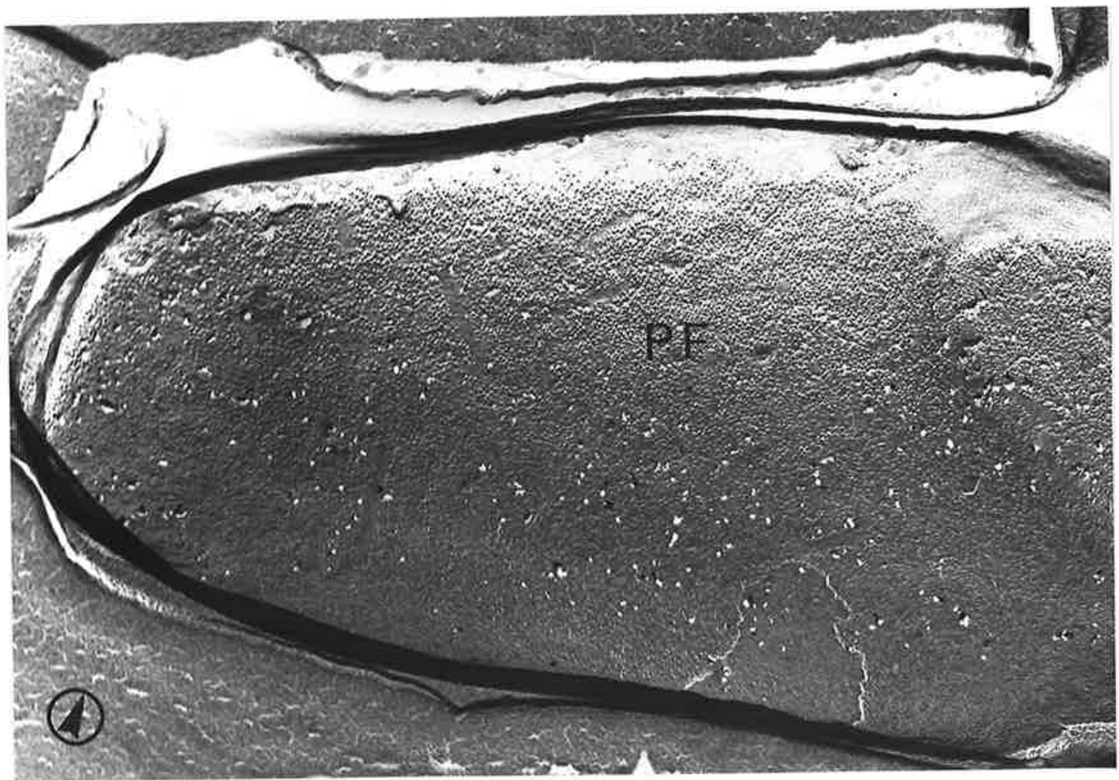
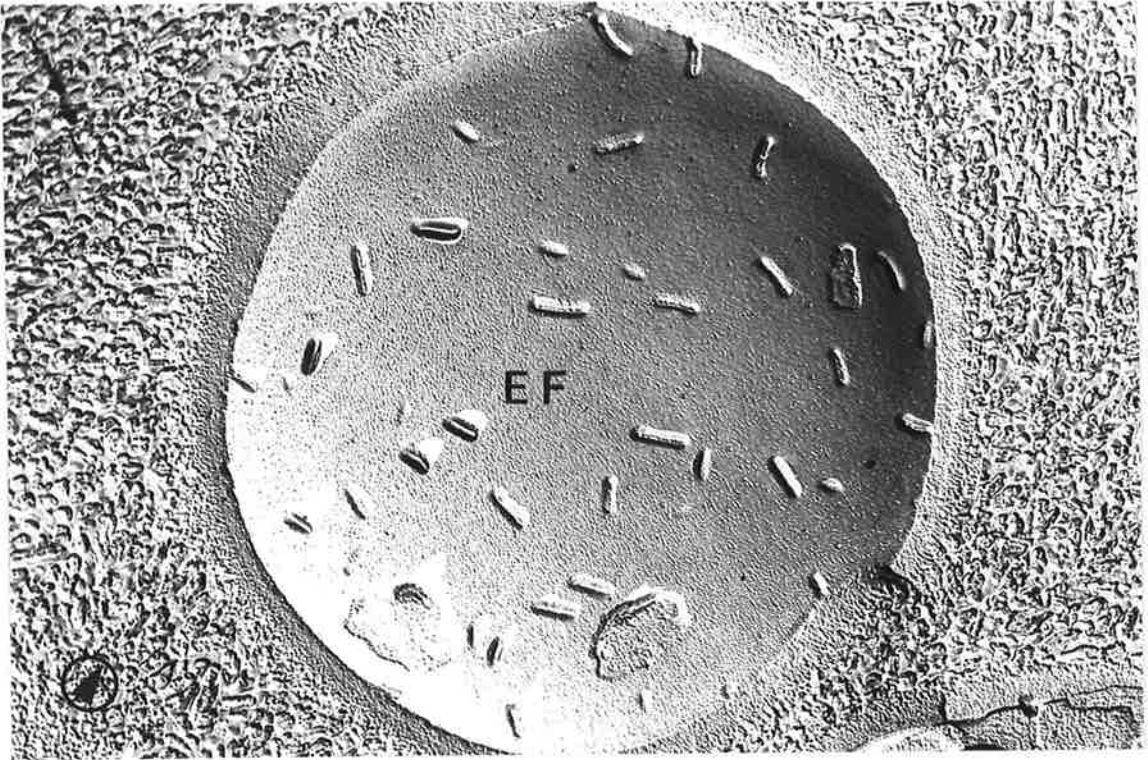
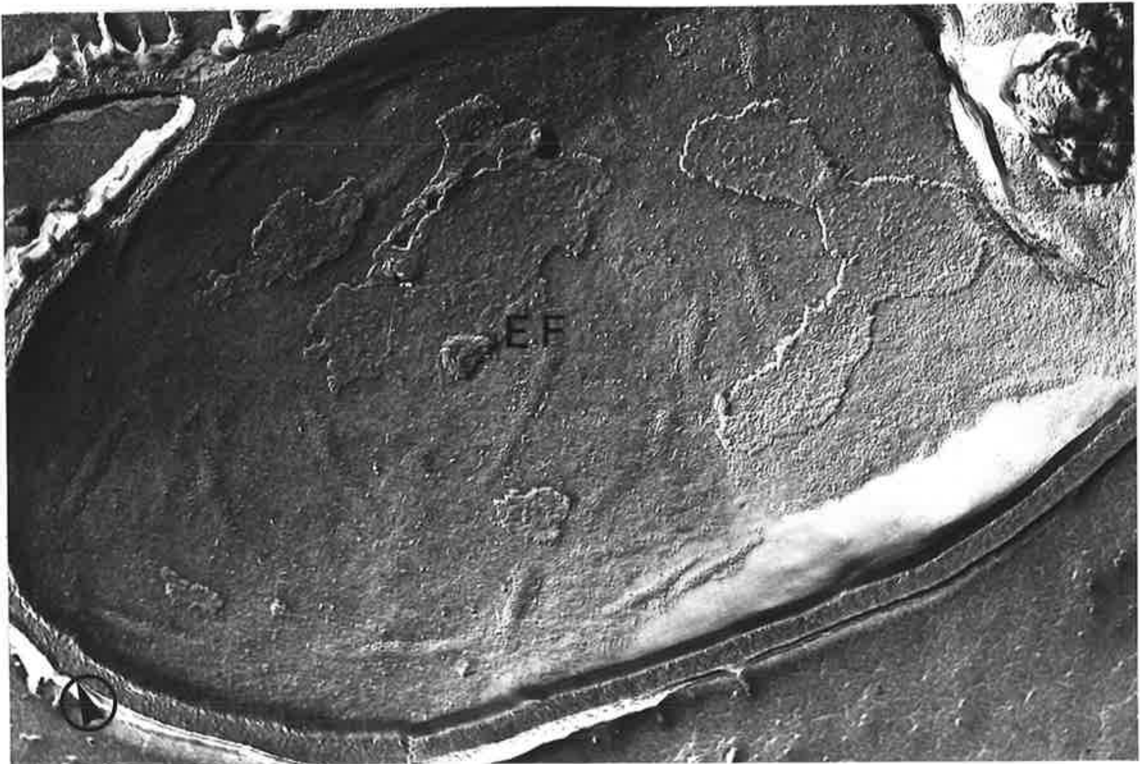

PF

Figure 5.8. Continued.
Magnification, x31000 (upper) x49000 (lower)


EF


EF

frequency of occurrence.

Toxin-treated cells

Examination of replicas of toxin-treated cells (1-5h exposure to toxin-A) frozen directly in the medium largely confirmed the observations made from chemically fixed cells.

Surface views of the plasma membrane revealed "flattened" invaginated structures which however remained discernable, particularly in the EF face of the membrane by a more pronounced appearance of the membrane particles than present in the surrounding face of the membrane (see Figure 5.8). Cryoprotected toxin-treated cells similarly exhibited no evidence of plasma membrane invaginations, which however were present in the cryoprotected controls. The plasma membrane PF and EF faces of treated cells appeared to show a less regular array of membrane-bound particles than control cells.

The infrequent occurrence of fractures through the nuclear envelope of toxin-treated cells exposing surface views prevented confirmation of the events leading to gross nuclear disintegration observed in chemically fixed cells. Cells in which surface fractures of the nucleus resulted were either comparable with control cells, that is, the plasma membrane and nuclear envelope sculpturing was similar to that of control cells (hence these cells were presumambly as yet undamaged), or the fracture face of both the inner and outer membranes of the nuclear envelope showed a more densely granular appearance (see Figure 5.7). Small pits were also seen on the facture face of both membranes of toxin-treated cells. It is possible therefore that the nuclear envelope was in some way altered prior to the appearance of gaps, such that the membranes did

not provide an adequate fracture path during freeze-fracturing to produce surface views. The nuclear pores of cells in which surface fractures of the nucleus were obtained and had evidence of membrane alteration (lacked definite plasma membrane invaginations) exhibited irregular margins suggestive of the initial stage of envelope damage (see Figure 5.7). Insufficient numbers of crossfractured nuclei were examined to rule out the possibility of nuclei containing large gaps. After 3-4h of contact with toxin, numerous cells were observed containing no evidence of a nucleus which suggests that the transition from the initial stage of damage to complete disintegration may be relatively rapid. It is possible therefore that the permanganate fixative exaggerated the appearance of initial envelope damage.

The endoplasmic reticulum was not readily observed in medium-frozen cells because of the greater granularity of the cytoplasmic ground material. When identifiable in degenerated cells, it was present in only relatively short sections compared with that in control cells. The mitochondria were more spherical than the more elongate structures in control cells but their cristae remained evident. The large vesicle appeared to be unaltered in degenerating cells observed up to 5h following treatment with toxin. Vastly altered cells, which contained no evidence of a nucleus and were packed with vesicles of variable size, usually contained a large vesicle but mitochondria were difficult to identify. Toxin treated cells (1-3h) kept in glycerol for 20h were similar to the vastly degenerated cells observed above and in chemically fixed preparations. The particulate structure of the plasma membrane was highly irregular and numerous vesicular-like structures were present between the cell wall inner surface and the plasma membrane.

DISCUSSION

Toxin-A killed metabolically active but not resting cells of H. anomala strain 10, and induced a cell clumping response within both populations of cells. The latter phenomenon involved a lesser proportion of cells than bound a lethal dose of toxin. This was not quantitated but must be considered in the interpretation of cell survival data. Killing and clumping were independent as demonstrated by (i) clumping of viable resting cells and (ii) cells immobilised on membranes or agar plates were killed. The two reponses are therefore independent. Clumping was roughly dose-dependent and though it needs conclusive demonstration, it is reasonable to assume that clumping was a specific toxin-induced response, whereas survival as determined probably reflects true survival. Thus with due caution, the data suggests that for subsaturating levels of toxin, cells were killed by a single-hit process, that is, a single adsorbed toxin unit (lethal unit) causes within a certain probability, the death of a sensitive cell (JACOB, SIMINOVITCH and WOLLMAN, 1952; REEVES, 1965). The killer factor and T. glabrata - produced PEST also kill sensitive cells by a single-hit process (BUSSEY, 1972; PALFREE and BUSSEY, 1979; BUSSEY and SKIPPER, 1975).

The lethal unit for purified toxin-A was determined as $5x10^5$ molecules for killing of H. anomala. This figure is some 10-fold greater than that determined for freshly prepared pure killer toxin isolated from Sacch. cerevisiae T158C (PALFREE and BUSSEY, 1979). As the proportion of inactive molecules in a preparation is unknown, the values represent upper limits. Nevertheless, it remains difficult to reconcile a single-hit mechanism involving a lethal unit of this magnitude. Inactivation of toxin-A did not exceed 50% during purification and was

probably much less as it was shown to be fully stable under the conditions employed. A remote possibility might be that the killer strain produces inactive as well as active toxin, which co-purify under the conditions employed. Other possibilities which have been advanced to account for a low killing probability displayed under certain conditions by some colicins might also be applicable to the toxin-A assay system and include (i) genetic aspects of the test organism and (ii) the environment of the test organism (physico-chemical aspects) (REEVES, 1972; HARDY, 1975). In the former, colicin sensitivity is under genetic control and this has also been demonstrated for the *Saccharomyces* killer system (AL-AIDROOS and BUSSEY, 1978). The lethal unit of killer toxin varied 4-fold depending on the test strain, which were of different origins. The hypothesised "nonlethal" receptor may account for such a difference (HARDY, 1975). Growth conditions of the organism can affect by an unknown mechanism subsequent colicin sensitivity (see for example MAYR-HARTING and SHIMELD, 1965; cloacin DF13, DE GRAAF, SPECKMAN and STOUTHAMER, 1969) and cultural conditions following colicin adsorption may also affect survival (see for example BEPPU and ARIMA, 1967; KOPECKY, COPELAND and LUSK, 1975). The fate of sensitive cells having adsorbed a sake yeast killer toxin was shown to be dependent on the plating medium used to determine survival: minimal medium or calcium ion supplemented YEPD plates rescued cells which did not recover on YEPD plates (KOTANI and colleagues, 1977). Any of these possibilities or their combinations may be operating in the toxin-A *H. anomala* killing system.

As yet information on the structure and specificity of toxin binding sites in yeast is limited, but may hold an explanation as to the magnitude of the toxin-A lethal unit. BUSSEY and colleagues have demonstrated that sphaeroplasts of sensitive cells are similarly

susceptible to killing as are whole cells but bind only a fraction of the toxin adsorbed to whole cells (BUSSEY, SHERMAN and SOMERS, 1973). The physiological response is similar to whole cells suggesting that the wall site is not essential to the killing process and is not responsible for the observed delay between toxin binding and toxin-induced membrane damage (BUSSEY, 1974). However, the cell wall was important in the mediation of resistance, by an inability to bind toxin (BUSSEY, SHERMAN, and SOMERS, 1973; AL-AIDROOS and BUSSEY, 1978). This situation therefore resembles that for colicins E1 and K in which recent evidence suggests that the receptors for these colicins function merely to overcome the natural barrier functions of the cell envelope and presents the toxin to the inner membrane target site. These specific receptors are known to possess other physiological functions (reviewed by KONISKY, 1978, pp. 104-107). The wall binding sites in yeast may have a similar function in providing passage of the toxin to an inner reactive site. Preliminary evidence indicates that the wall site(s) in Sacch. cerevisiae is carbohydrate although protein was not excluded (AL-AIDROOS and BUSSEY, 1978). In H. anomala, toxin binding experiments are required to determine specificity and the number of sites present. The wide spectrum of activity of toxin-A seems to resemble more the situation in bacterial species which react to gram-positive bacteria produced bacteriocins. Here there seems to be a general lesser specificity in binding of toxin, that is, in several reported instances both resistant and sensitive strains bind bacteriocin and in other instances sensitive strains do not bind detectable amounts of bacteriocin (reviewed by KONISKY, 1978). Toxin-A may bind to a large number of possible cell wall sites which are functionally "nonlethal" but only toxin which reaches an inner site is effective.

The colony-forming ability of resting cells was essentially unaffected

by toxin-A, but no experiments have yet been made to demonstrate conclusively irreversible binding of toxin to the cell. This may best be made with radio- labelled toxin (see BUSSEY, SHERMAN and SOMERS, 1973). Thus interpretation of this result can only be conjecture for the present. Possible explanations which might account for the resistance of resting cells include: (i) binding is an energy-dependent process, (ii) toxin bound exclusively and irreversibly to non-lethal cell wall sites, (iii) cell-toxin complex rapidly dissociated on diluting prior to plating for viable counting, (iv) resting cells are able to inhibit or inactivate the toxin or (v) bound toxin is unable to gain access to the target once cells recommence metabolic activity. Circumstantial evidence indicated cell binding of toxin: (i) small decrease in activity of the incubation medium after 420 min of contact with cells and (ii) induction of cell clumping. The first method does not eliminate loss of activity by toxin inactivation and the second assumes a common binding site for both the clumping and lethal effects. Accepting that these observations indicate adsorption of toxin, binding is thus an energy-independent process which has been observed for other killer toxins (BUSSEY, SHERMAN and SOMERS, 1973; IMAMURA, KAWAMOTO and TAKAOKA, 1975; SKIPPER and BUSSEY, 1977). The resistance of resting cells to toxin-A therefore resembles the action of killer factor which is irreversibly blocked in ethanol grown (respiring) cells but not glucose grown cells (dependent on glycolysis) by cyanide or antimycin which interfere with mitochondrial function (SKIPPER and BUSSEY, 1977). On the other hand, the action of PEST (killing and potassium efflux) was unaffected by the presence of energy blocks. It would therefore appear instructive to study the effects of blocked energy yielding metabolism on the action of toxin-A.

It is of interest to note that an antifungal fatty acid-containing

oligopeptide (proposed composition) aculeacin, from *Aspergillus aculeatus* was also ineffective against resting cells, but no indications were given concerning adsorption of the toxin (MIZOGUCHI, SAITO, MIZUNO and HAYANO, 1977). Evidence was presented that cells were killed by lysis resulting from inhibition of glucan synthesis. As for toxin-A, it is essential to demonstrate toxin adsorption before one can hypothesise and seek a tolerance mechanism, although this has proved difficult with several bacteriocin systems (MAHONY and BUTLER, 1971; HALE AND HINSDIL, 1975; DAJANI, TOM AND LAW, 1976).

Correlation between the lethal action and cell clumping response needs to be confirmed as these experiments do not exclude the remote possibility of a second component which escaped detection biochemically. One procedure would be to examine for the clumping response induced by the preparation in which the toxin was inactivated with respect to killing (heat or alkali treated, pronase digestion). It would be interesting to discover the nature of the clumping phenomenon in which toxin-A may be (i) acting as an agglutinin such as a sexual agglutinating factor (CRANDALL and BROCK, 1968; SHIMODA and YANAGISHIMA, 1975) or (ii) mimicking a mating pheromone by induction of a cell surface change which results in adhesion of cells (TKACZ and MacKAY, 1979; FINKELSTEIN and STRAUSBERG, 1979). The latter possibility would seem unlikely in view of clumping occurring between resting-cells which therefore suggests a simple agglutinative reaction. The weak response in relation to killing activity of the toxin may reflect the quantity of toxin required to form a stable bond.

Electron microscopy of both chemically fixed and freeze-etched cells presented a generally similar picture of the sequence of events following toxin induction, which culminated in complete disarray of

cellular organisation. The first observable event involved the plasma membrane as evidenced by "collapse" of the invaginated structure in thin sections, although in freeze-etched samples the sites of the former invaginations remained discernable, and the membrane fracture faces showed an apparent altered distribution of membrane-bound particles. Protoplasts retain invaginations (MATILE, MOOR and ROBINOW, 1969; KOPP, 1975; KRAMER, KOPP, NIEDERMEYER and FUHRMANN, 1978) and although there seems to be no evidence that they are necessarily fixed structures, toxin-induced collapse suggests that the membrane had in some way been altered such that the structure was no longer stabilised. Loss of invaginations from the plasma membrane may therefore be an indicator of membrane damage.

The less uniform distribution of membrane particles observed in both the exoplasmic (EF) and protoplasmic fracture face (PF) of the plasma membrane following toxin treatment seemed to be concomitant with flattening of membrane invaginations. The apparent redistribution of these particles, which are thought to be proteinaceous (SEGREST, GULIK-KRZYWICK and SARDET, 1974) may have resulted either (i) directly by interaction of several membrane particles with one or more molecules of toxin, or (ii) indirectly as a consequence of an unknown toxin-induced change to the membrane fluidity. The first possibility would seem to be more favourable in view of the strongly hydrophilic nature of toxin-A and the evidence that some particles which are seen in the fracture face are also exposed at the true membrane outer surface (SEGREST *et al.* 1974; KOPP, Figure 7, p.41, 1975). Alternatively the redistribution of membrane particles superficially resembles, though redistributed to a much lesser extent, that observed when fungal cells are treated with the polyene drug Amphotericin B (NOZAWA, KITAJIMA, SEKIYA and ITO, 1974). Here it was suggested that the aggregation of

particles was a consequence of altered membrane fluidity resulting from limited stabilising interaction of ergosterol, which had bound the polyene, with membrane phospholipids (FINKELSTEIN and HOLZ, 1973; De KRUIJFF and DEMEL, 1974). Although whole cells adsorbed toxin-A, no experiments have yet been made to show whether sphaeroplasts bind toxin and indeed whether cell bound toxin is "transported" to the membrane. It must be pointed out that the cells in this study were treated at high multiplicity (8 to 9) which would provide conditions for the adsorption by a cell of a large number of molecules (much greater than $5x10^5$), some of which presumably reach the outer surface of the membrane to induce the observed cytological effects.

Structural alterations of the nuclear envelope and endoplasmic reticulum appeared to be coordinate but were preceded by plasma membrane alterations. Examination of freeze-etched cells did not provide evidence for the latter stages of nuclear envelope disintegration, so caution is required in the interpretation of nuclear damage observed in chemically fixed cells. Initially the appearance of the nuclear envelope membrane fracture faces showed a marked change in granularity. Whether the altered granularity and the presence of pits were actually present or are a freeze-etch artefact as a consequence of a toxin-induced membrane alteration is not known. Whichever is the case, the membrane clearly had in some way been affected. The altered membrane pore structure similarly may have been susceptible to fracturing artefact but none the less reflects altered membrane properties. The method of preservation has been demonstrated to influence nuclear pore size (WILLISON and RAJARAMAN, 1977) and artefactial enlargement of the pore may result during the facturing process (WILLISON and JOHNSTON, 1978).

In chemically fixed cells, the nuclear envelope showed a striking increase in the numbers of gaps but many of these differed from the nuclear pore in that the inner and outer membranes were not fused at the margins of these gaps. Interestingly, the gaps appeared simultaneously in adjacent sections of the two membranes suggesting that the gap originated from a nuclear pore. The absence of shrinkage sensitivity during chemical fixation or dehydration processing (MOOR and MULETHALER, 1963) indicated that the gaps introduced no restriction on communication between the cytoplasm and nucleoplasm as do nuclear pores of intact nuclei. The nuclear pore appears to be a complex structure which is though to regulate the passage of smaller molecules across the envelope (reviewed by HARRIS, 1978). Following the appearance of gaps in the nuclear envelope, large sections appeared to disintegrate. The concurrent increase in electron density of the nucleoplasm, giving the appearance of mixing with the comparatively more electron-dense cytoplasmic ground substance, strongly suggested that envelope degeneration was not a fixation artefact but had occurred prior to chemical fixation. It would seem logical that once the membranes of the envelope had weakened, they might not provide an adequate fracture path as in control cells (Da SILVA and BRANTON, 1970). Insufficient numbers of cross-fractured nuclear envelopes were examined to rule out the dramatic increase in numbers of gaps observed in chemically fixed cells.

The endoplasmic reticulum exhibited disintegration in an apparently similar manner to the nuclear envelope, that is, in chemically fixed cells gaps appeared in adjacent sections of the double membrane system and the absence of continuous sections of endoplasmic reticulum was confirmed in freeze-etched samples. The absence of a similar disintegration of the remaining cell membrane structures highlights the known chemical, fine structure and certain functional similarities

between the nuclear envelope and the endoplasmic reticulum (MOOR and MUHLETHALER, 1963; MATILE, MOOR and ROBINOW, 1969). This membrane system is particularly active during cytokinesis, being involved in formation and extension of the bud envelope and in nuclear division. The apparent sensitivity of this membrane to toxin-A may therefore provide information on control of proliferation and degeneration during the cell cycle. Degeneration appeared to commence at existing pore structures and further studies with toxin-A may provide an understanding on the maintenance of this ephemeral structure.

The mitochondrial membrane system appeared to remain principally resistant to toxin-induced degenerative effects and may owe its indifference at least in part, to a unique membrane composition and function (MATILE, MOOR and ROBINOW, 1969). Furthermore, it is likely that mitochondrial function was repressed by growth in 2% glucose (HENSON, WEBER and MAHLER, 1968) and hence the mitochondria may be less susceptible to possible gross damage. Similarly the tonoplast (membrane limiting the large vesicle) remained essentially indifferent to degenerative changes occurring within the cytoplasm. As this organelle is osmotically sensitive, damage to the tonoplast presumably would have been reflected by a significant change in dimensions. It is an accumulative organ, which although differs biochmically from the plasma membrane (KRAMER, KOPP, NIEDERMEYER and FUHRMANN, 1978) is implicated in cell envelope growth (MATILE, MOOR and ROBINOW, 1969; CARTLEDGE, ROSE, BELK and GOODALL, 1977). The numerous vesicularised structures which ultimately appear in degenerated cells were presumably derived from the fragmented endoplasmic reticulum and nuclear envelope.

The time-course of cell survival showed that 95% of cells had adsorbed a lethal dose of toxin within one generation (approximately 110 min) by

which time growth had nearly ceased and more than 80% of cells showed ultrastructural damage. Growth (culture turbidity) was rapidly arrested with respect to toxin adsorption but ultrastructural damage was delayed (less than 2h). No biochemical processes have yet been monitored in an attempt to defined the primary event which would appear to become apparent by 60 min following contact with toxin. The extensive nuclear damage apparent by 2-3h suggests that cell damage was irreversible and colony forming ability was probably lost by this time. There was no evidence for a dependency on the cell cycle for initiation of toxin action, since cells displayed the whole range of growth phases after growth had completely abated (7h). The extensive and rapid ultrastuctural damage seen in these experiments were for toxin multiplicities of 8-9 (7h) but damage may be initially less extensive at lower levels of toxin though of course cells having absorbed a single lethal unit will still lose colony-forming ability.

The specific nature of the initial cytological changes which ensued shortly after adsorption of a lethal dose of toxin appeared to be toxin-specific and possibly associated with the primary target rather than being secondary events which might occur in dying cells, such as when cells are induced to undergo autolysis (GALE, 1963; GALE and McLAIN, 1964). Until the biochemical aspects of toxin-induced killing are established, such a view remains conjecture. Numerous drugs and proteins are known to interfere with the plasma membrane structure and function and also with nuclear processes, but few are known to affect principally the nuclear envelope or endoplasmic reticulum. A prerequisite for such agents is their ability to cross the plasma membrane. Thiobenzoate, which was thought to inhibit microbial sulphate reduction system, following a perceptible change in the nucleoplasm staining properties induced gaps in the nuclear envelope (GALE and

McLAIN, 1964). That this was a secondary effect was not excluded. Amanitan, a peptide toxin, induced ultrastructural lesions in mouse liver cell nuclei 15 min following administration (WIELAND and WIELAND, 1972). This toxin inhibits RNA sythesis. Phalloidin, another toxic peptide produced by certain species of *Amanita* affected structure of the endoplasmic reticulum in liver cells by an unknown mechanism (WIELAND and WIELAND, 1972). There are no doubt further examples in the literature. However, for toxin-A, the intracellular damage can only be considered a secondary event in the absence of demonstration that toxin-A or a mediating agent is associated with the nuclear envelope or endoplasmic reticulum.

CHAPTER 6

GENERAL DISCUSSION

The purpose of this thesis was two-fold: (i) to survey yeasts related to H. saturnus for killer ability and to relate their toxins to those of H. saturnus and (ii) to characterise the widely active, stable killer factor produced by the yeast, Hansenula saturnus, which on preliminary indications differed in many important respects from the killer factor of Saccharomyces cerevisiae.

This chapter summarises the findings of Chapters Two to Five with relation to the general literature. Specific discussions of particular experiments have been dealt with in the appropriate chapters and are therefore only summarised in this chapter.

Following the discovery of a widely active toxin in the yeast H. saturnus NA 9, additional strains were examined for similar activity on standard yeast culture media. Amongst the 51 strains examined from the collections of Dr. Nancy Atkinson and The Australian Wine Research Institute, only an additional strain of H. saturnus (AWRI 354) was active. Such a result was not unexpected in view of a similar survey made by MacWILLIAM (1959) in which yeasts were demonstrated to possess little antifungal activity. The wide activity spectrum of H. saturnus included a large proportion of Saccharomyces (22 of 27 strains) and Hansenula strains (17 of 26 strains), but insufficient numbers of yeasts belonging to other genera were tested to draw conclusions about their sensitivities. Thus, in direct contrast to the killer factor of Sacch. cerevisiae, which, with one exception, is only active against

Saccharomyces strains (BUSSEY and SKIPPER, 1976; YOUNG and YAGIU, 1978) that of H. saturnus is broad spectrum and therefore resembles more closely the killer factors of non-Saccharomycete yeasts (YOUNG and YAGIU, 1978). This indicates that the toxin may be acting as a general fungal antibiotic and not have the need for any exacting requirements for activity, such as a unique receptor site or site of attack.

Five of the six strains of H. saturnus studied were killers on methylene blue-containing buffered agar plates, their spectra of activity being pH and strain dependent. But as the strains were cross-resistant they were likely to possess analogous killer toxin and resistance systems. The non-killer strain was also resistant, suggesting that it too might possess an analogous resistance mechanism, but this needs to be demonstrated by, for example, genetic manipulation.

The reduced activity spectrum of strain 9 relative to the other H. saturnus strains and the pH-dependency of the activity spectra, indicated that several killer factors were involved. A study of the killer factor activity present in the filtrates of strains 9 and 354 grown on complex media under several different conditions demonstrated three distinct species. Initially two types of activities were revealed by the two distinct patterns of responses of a variety of sensitive strains: group 1 strains were sensitive to killer activity at both low and neutral pH, whereas group 2 strains were active only at low pH. Furthermore, the filtrates of killer strains grown under static conditions, in contrast to shake conditions, showed only traces of activity against group 2 strains. That the two groups of indicator strains under certain conditions (group 1 at pH6.0 and group 2 strains at pH4.3) responded exclusively to two different principles was confirmed by the abolition of responses by group 2 strains following

heating of the killer solution. Thus, group 1 strains at both low and neutral pHs were sensitive to the thermostable component while both group 1 and 2 strains were sensitive only to the thermolabile component at low pH.

The effect of pH on the activity of filtrates from cultures of strains 9 and 354 against the two groups of indicator strains was interpreted as resulting from three different killer factors. The heat stable component, where activity was little affected by pH (pH3.5-7.0), was designated toxin-A. Strain 498 (group 2) was insensitive to toxin-A but responded in two different ways to the heat labile activity, which was also active against strains 10 and 560 (group 1). The component which exhibited maximum activity at pH4.3-4.7 and became undetectable at pH5.5 was designated toxin-B. This activity was present in the filtrates of all strains except the pH4.3 shake filtrate of strain 9. The component which was maximally active at or below pH4.3 and was inactive at pH5.0 was designated toxin-C (the major component of the pH4.3 shake filtrate of strain 9),

Three components were also demonstrable following their partial electrophoretic separation on cellulose acetate membranes, but further experimentation is required to correlate two of the three electrophoretic components with toxin-B and toxin-C. The most rapidly mobile component was identified as toxin-A by its activity at pH6.0 and resistance to thermal denaturation. The component having least electrophoretic mobility correlated with toxin-B since all solutions known to contain this activity were active against both indicator strains 495 and 498 while toxin-C, which was of intermediate mobility to that of toxin-A and -B, was active only against strain 498 and not 495. The interpretation needs to be confirmed by comparing the activity of

the two partially separated electrophoretic components against all group 2 indicator strains at both pH4.7 and 4.3 and correlating this with their pH-activity profiles against the same indicator strains.

Toxin-B and toxin-C activities further differed from toxin-A by being less readily diffusible through dialysis membrane. Toxin-B was inactivated by other proteases in addition to pronase, suggesting therefore that it was a proteinaceous macromolecule resembling the more stable PEST glycoproteins (BUSSEY and SKIPPER, 1975). The chemical nature of toxin-C is unknown, but a protein component was not eliminated under the conditions of the experiment. Toxin-A, the only killer factor produced in chemically defined culture medium, had properties analogous to that in filtrates derived from complex media. It was found to be a stable protein of approximately 11000 molecular weight.

Toxin-A was produced in both chemically complex and defined media by either static or shake culture (pH independent) by both strain 9 and 354, but with the exception that strain 9 under shake culture conditions produced only reduced amounts of activity in complex media. The toxin produced by strain 9 has not yet been purified to enable comparison with purified strain 354 toxin, but as four biochemical tests indicated that it did not differ from this toxin, it is more likely that its biosynthesis or excretion is affected. This could be examined by comparing toxin activity in cultures of strain 9 and 354 which are shifted from static to shake conditions in both chemically defined and complex medium. Another possibility is that strain 9 elaborates a toxin inhibitory or inactivating agent. Toxin-B and toxin-C activities were produced only in shake culture on complex media at both low and neutral pHs, but toxin-C predominated at pH4.3 and toxin-B at pH6.0. However, strain 9 differed from strain 354 in that only toxin-C was produced at

pH4.3. The absence of these activities in static culture is presumably related to oxygen tension of the culture which is well known to control synthesis of a variety of metabolic products, for example sterols (HOSSACK, BELK and ROSE, 1977). However these activities were not apparent in chemically defined culture media even when supplemented with peptone and yeast extract. Presumably either toxin-B and -C were inactivated in chemically defined media or their synthesis was repressed by certain components of the growth media.

The finding of a stable, widely active killer factor in H. saturnus prompted a survey of related yeasts. A survey of 12 strains, obtained from The National Collection of Yeast Cultures, England, and 9 strains from The Centraalbureau voor Schimmelcultures, The Netherlands, for killer activity at either neutral (pH6.0) or low pH (pH4.7 and 4.3) revealed that 11 strains were active at low pH, three being strongly active at neutral pH. This result therefore further extends the findings of PHILLISKIRK and YOUNG (1975) that Hansenula yeasts show a high incidence of killer ability, but also demonstrates that some strains (four different species) possess broad killer activity with respect to pH. The majority of killers are broad spectrum like H. saturnus killer activity, suggesting that they are relatively non-specific fungal antibiotics, in contrast to the plasmid determined killer factors of Saccharomyces strains which show high specificity (BUSSEY and SKIPPER, 1976; YOUNG and YAGIU 1978).

Killer activity of the filtrates of the strains active at neutral pH (with the exception of H. saturnus) were however weaker relative to the activity observed at pH6.0 by plate culture. Two strains not showing killer activity on plates at neutral pH did however display some activity in their filtrates produced by shake culture. Of 10 non- H.

saturnus killer strains active at low pH on agar medium, killer activity of filtrates from all but four was confirmed by monitoring the viability of sensitive strains. For the remaining, inhibitory activity was apparent but killing was not ruled out. This needs to be re-examined at their pH optima by determining the proportion of cells surviving treatment. Three of these strains were hazy zone killers and their culture filtrate activity was only weak or undetectable.

Each of the 16 killer strains studied was resistant to the toxin(s) that it produced. On the basis of cross-reaction and the reaction of non-killer strains , the killer strains fell into eight groups of killer type and their respective eight groups of resistance phenotypes, thus yielding eight classes of killer yeasts. Three groups (HK_1 to HK_3) contained the eight strains which were active at near-neutral as well as at low pH. HK_1, which contained the five strains of H. saturnus, was subdivided because of differences in the activity spectrum of strain 9 and the remaining strains. The evidence is that the strains of these subgroups show only quantitative differences in the toxins involved. The three hazy zone killers, two of which possess unproven killer action against other killer strains, occupy groups HK_7 and HK_8. Strains which had a wide spectrum of activity against killer strains also generally behaved similarly against non-killers. The HK_2 strain killed strains belonging to all but one killer group. In general, the extensive killer strains were widely resistant to other killer types, but three groups of narrow spectrum killers were also resistant to the majority of killers. Only the two groups HK_3 and HK_7 contained strains of more than one species and their killer factors had similar if not identical properties. Strains from five groups (HK_1 to HK_5) elaborated at least two different toxins and hence these strains are likely to possess several distinct killer and resistance systems which

are not apparent from their killer phenotypes. Thus it is possible that several of these strains could possess killer factors in common, or indeed with any of the other groups. The properties of the toxins make this possibility unlikely, but the apparent absence of relationships needs to be confirmed by, for example, the reaction of resistant mutants.

The activities of killer yeast culture filtrates assayed against two sensitive strains, with respect to pH, showed six broadly different profiles, four of which are similar to those described by PHILLISKIRK and YOUNG (1975). Hence these killer activities, in this respect, resemble those of yeasts belonging to other genera. The predominant pattern, type B, was similar to Group 2 of PHILLISKIRK and YOUNG (1975) in showing optimum activity at pH4.3-4.7, reduced activity at pH3.9 and no activity at pH5.5. It contained all of the Hansenula killer activities studied by these workers and was displayed by killer factors from five different killer groups. The broad activity of toxin-A with respect to pH is clearly unique and appears to owe this property in part to the fact that it is stable over a wide range of pH. The killer activity of H. anomala NA 10 (HK_6) also has not been previously described. This activity progressively increased with decreasing pH suggesting that a proton gradient might be necessary for its lethal action (compare with PEST: BUSSEY and SKIPPER, 1975).

The killer factors showed four patterns of stability with respect to pH, hence providing in part an explanation for the differing killer activities in relation to pH. Toxins from five killer groups were partly or wholly stable at pH6.0 contrasting with those studied by YOUNG and YAGIU (1978). Also, toxins from two killer groups were unstable at pH3.0. Inactivation at pH6.0 was rapid (less than 5 min), indicating a

direct effect of hydrogen ion concentration rather than indirect inactivation by, for example a protease. Therefore, inactivation may result from, for example: molecular conformation alteration; irreversible binding of an inhibitor; or loss of an activating component.

Inactivation of killer activity from eight strains, belonging to six killer groups, by proteolysis (principally papain and pronase) confirms the conclusion of YOUNG and YAGIU (1978) that a proteinaceous component is needed for killer activity. Thus there is also a striking similarity with the bacteriocins which are mainly proteins or are protease sensitive (KONISKY, 1978).

The killer activity elaborated by strains from five killer groups was to some degree diffusible through dialysis membrane indicating that at least one of the killing components of these strains is of small molecular size and not likely to exceed about 20000 molecular weight. This does not exclude the possibility that the toxin exists in solution largely as aggregates or in association with polysaccharide as is the case for killer factor and PEST (BUSSEY and SKIPPER, 1975, 1976; PALFREE and BUSSEY, 1979). There was no conclusive evidence that any of the killer factors were unable to penetrate cellophane and hence they were unlikely to be viral in nature.

Killer activities broadly occupied two groups with respect to thermal inactivation: stable at 25°C but inactivated at 37°C (thermolabile); stable at 37°C but inactivated at 80°C (thermostable). Thermostable activity was present in the culture filtrates of four groups of killer strains and in two of these that component was conclusively demonstrated to exhibit killer activity at pH6.0 (HK_1 and HK6). Thus, toxins more

stable to changing pH generally may also be more stable to heat. Apart from the killer factor of Sacch. cerevisiae reported by WOODS, ROSS and HENDRY (1974), no heat stable killer toxins have been reported. The killer activities from 15 strains of various genera, including four strains of Hansenula, studied by YOUNG and YAGIU (1978) were inactivated at 35°C and were irreversibly inactivated at pH6.0

The electrophoretic separation of killer factors on cellulose acetate membranes proved particularly useful in the demonstration of several toxins in a killer solution, enabling correlation between various biochemical properties. All killer factors detected by this procedure were neutrally or positively charged at approximately pH4.3. Toxins from strains 10 and 16 were relatively highly charged molecules as judged by their rapid mobility compared with the other positively charged toxins.

In confirmation of the findings of YOUNG and YAGIU (1978), the toxins of different killer classes were biochemically distinct but all had in common the requirement of a proteinaceous component for killer activity. This work further shows the existence of toxins of broad activity with respect to pH, several of which were also thermostable small molecules. Apart from the report of T. glabrata, this study demonstrates that some six species of Hansenula also elaborate several distinct toxins, of which many could readily be separated by cellulose acetate membrane electrophoresis.

YOUNG and YAGIU (1978) made the important discovery that the genetic determinant for the killer character in non-Saccharomycete yeasts is not cytoplasmic since it was not cured by methods known to eliminate cytoplasmic genetic elements. Moreover no detectable dsRNA was isolated

from the cytoplasm of these yeasts, but the involvement of chromosomal genes needs to be demonstrated. It is possible that the killer character of the strains of the present study is also determined by chromosomal genes. If this proves to be the case, the high incidence of killer character in these yeasts provides a unique opportunity to consider this characteristic in relation to the phylogenetic relationships between these yeasts as proposed by WICKERHAM (Reviewed by WICKERHAM and BURTON, 1962; WICKERHAM, 1970). The finding that the toxins are proteinaceous could make such an approach particularly rewarding, and may provide an indication of the significance of the killer character in the ecology of these yeasts.

A phylogenetic scheme indicates the supposed evolution of species based on a variety of criteria. Briefly, various species are positioned on different branches of a phylogenetic scheme according to thallism and general habitat to indicate postulated evolutionary pathways from primitive ancestors. The distance from the origin represents their apparent state of evolvement which is based principally on biochemical complexity. Thus more recently evolved species, for example, ferment a greater range of sugars, show a greater intensity of fermentation, produce esters, show a loss of dependency on vitamins and more predominantly exist vegetatively as diploids. No discussion will be made on the validity of the scheme proposed by WICKERHAM except to note that it is largely supported by other data, such as, DNA base ratio and serology (NAKASE and KOMAGATA, 1969), proton magnetic resonance spectra of mannans (GORIN and SPENCER, 1970) and numerical analysis (CAMPBELL, 1973). The validity of criteria used in the separation of species remains in doubt (FUSON, PRICE and PHAFF, 1979; reviewed by PRICE, FUSON and PHAFF, 1978).

It is interesting to look at the distribution of the killer character among the different species in relation to the scheme of WICKERHAM (1970), but as yet the numbers of strains and species examined is extremely small and therefore cannot allow any conclusions to be made (see Figure 6.1). Several indications are however evident: (i) only the more recently evolved species show killer ability, (ii) the more primitive species show greater sensitivity to killing and (iii) the species of line 1a (homothallic) when compared with those of line 2 (heterothallic) show a wider range of activity with respect to pH and generally a wider spectrum of activity. It must be reiterated that the sample size is very small and its distribution is limited, but the strains were selected without any prior knowledge of possible killer ability or sensitivity.

It is of interest to note that H. dimennae (5762) and H. californica (496), which produce similar killer factors, are closely related, and H. wingei (2431) and H. candensis (497), which also produce similar toxins, although placed on different lines in the scheme of WICKERHAM, show substantial DNA sequence relatedness (FUSION, PRICE and PHAFF, 1979).

One of the aims of this thesis was to characterise the stable killer toxin produced by H. saturnus in a chemically defined medium. This work was commenced prior to the finding of heat labile toxins produced in chemically complex medium under shake culture conditions. The killer activity present in filtrates of H. saturnus grown in chemically defined medium was denoted SKT, but is analogous to toxin-A present in filtrates of cultures grown on complex medium. Preliminary to the development of a purification procedure was the definition of an assay procedure and the acquisition of a high titre solution of the toxin.

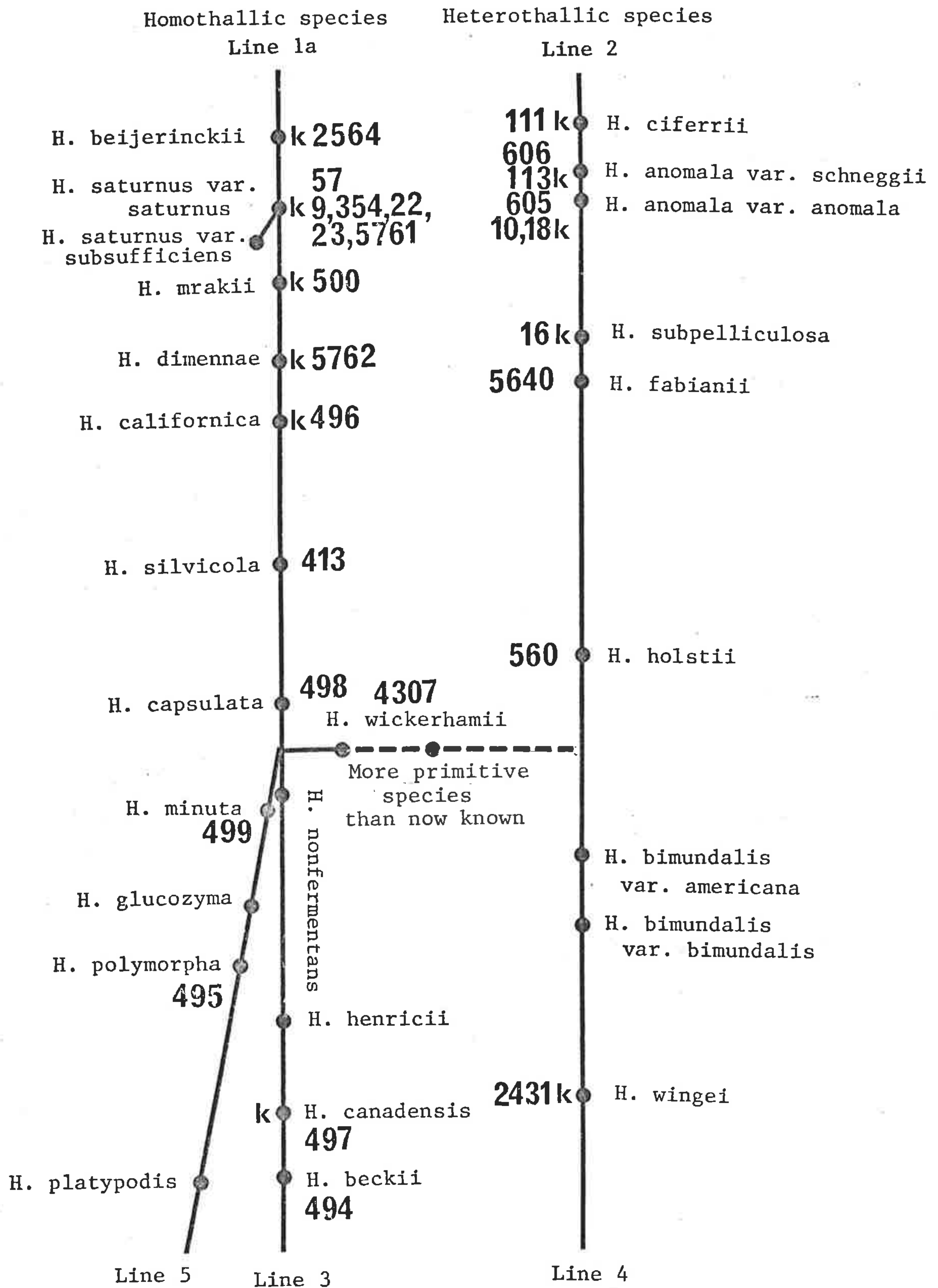


Figure 6.1. Killer ability of species placed in the phylogenetic scheme of WICKERHAM (1970).
Strains of species examined for both killer ability, denoted by k, and sensitivity to killing (see Table 2.3) are indicated by the strain numbers of Table 2.1. Note that line 1b, for which no strains were obtained, is omitted.

Of the various assay procedures available for the comparison of antibiotic activity of a solution, the large plate format of LEES and TOOTILL (1955a,b) was adopted as it was suited to multiple comparisons against a single standard and provided adequate precision. A computer programme was written for the Youden square method to facilitate determination of assay. The indicator yeast used was H. anomala NA 10 and conditions were established under which sharply defined inhibition zones were obtained, allowing a reproducibility of assay of better than ±5% (standard error). The dose-response curve for toxin-A in the culture filtrate was linear over a wide range (exceeding 10 fold) when the assay response (inhibition zone diameter) was plotted against relative concentration of toxin, at high concentrations. Solutions containing toxin-A of greater purity produce similar dose-response curves but their slopes varied. Purified toxin-A of about 100 a.u./ml under-assayed by approximately 15% against the crude standard indicating that a substance(s) potentiated its activity in the crude filtrate preparation. This is a problem often encountered in purification work (HEWITT, 1977) and may to some extent be overcome by adopting as standard, progressively purified solutions. Since the various preparations of toxin-A showed different slopes to both crude and purified toxin, this was not done since it would produce further uncertainties. The activities of all preparations were therefore evaluated in terms of the crude standard near its potency of 100 a.u./ml and therefore under-assayed by up to 20%. No attempt has yet been made to assay the various preparations against purified toxin-A to correct for assay errors. Thus the yield of toxin activity and calculated specific activity in most experiments are underestimated.

The culture conditions of H. saturnus were broadly examined with the prime purpose being the attainment of a highly active solution of toxin

activity. Preliminary experiments made using a chemically defined medium (LEDERBERG, 1956) showed that yield was greatest in aerated medium and that pH had little effect in the range pH4.0-6.5 where it was optimal. Toxin activity accumulated in the medium during the active phase of cell growth: the bulk of toxin activity was liberated during the transition of log-phase to stationary phase of growth but no significant amounts were released during stationary phase, during which time the accumulated toxin was fully stable. Thus toxin-A is probably an extracellular toxin which does not require cell autolysis for its release (RAYAUD and ALOUF, 1970). Its biosynthesis and release from the cell may therefore be similar to that of other yeast extracellular proteins and could be expected to be blocked by drugs which interfere with production and excretion of exoproteins (KUO and LAMPEN, 1974). Cycloheximide inhibited killer production in Saccharomyces cerevisiae (BUSSEY, SHERMAN and SOMERS, 1973). The accumulation, in moderately agitated culture medium, of the killer toxin produced by Sacch. cerevisiae was not proportional to growth and it was suggested that the toxin was either inactivated or excreted at a rate not proportional to toxin concentration or cell titre respectively (PALFREE and BUSSEY, 1979). The killer activity produced by a strain of Sacch. cerevisiae, studied by KOTANI, SHINMYO and ENATSU (1977), was reported to increase in parallel with cell growth under static culture conditions in liquid medium.

A chemically defined medium for toxin production was determined by a systematic modification of the formulation due to WICKERHAM (VAN DER WALT, 1970). The basal medium was extremely simple showing that the minimal requirement for cell growth also supported toxin production. This suggests that the toxin-A protein, or a derivative or precursor may be essential to cell growth, possibly to the cell envelope since it is

released from the cell. The toxin's rich content of acidic amino acids, and lesser amounts of serine and threonine is not inconsistent with this notion (PHAFF, 1971). It may be possible to obtain information on this aspect by monitoring the patterns of proteins in different cellular fractions of mutant killer strains (ELLIOTT and McLAUGHLIN, 1979; TREW, FRIESEN and MOENS, 1979).

Supplementation of the basal medium with complex biochemical substances and growth factors did not stimulate toxin production greatly. Peptone, but not yeast extract, stimulated toxin production by 30-60% (depending on batch size of culture) but it has not yet been shown whether this is due to its nitrogen content or to free amino acids or peptides. In this respect toxin-A production in minimal medium differs to that of killer factor where its yield was greatly stimulated by both yeast extract and peptone (WOODS and BEVAN, 1968; PALFREE and BUSSEY, 1979).

The strongly hydrophilic and low molecular weight nature of toxin-A activity limited the choice of routine methods for the preparative isolation and concentration of this substance. An isoelectric point of 5-6 and the high stability of activity at acidic pH allowed the use of the strongly acidic cation exchange resin, Amberlite IR-120(H) in its primary isolation. Although the method was inefficient under these conditions in terms of the total exchange capacity of the exchanger, it was nonetheless highly effective. Adsorption and elution conditions were examined in detail. A batch procedure was developed in which more than 65% of activity present in the clarified neat culture fluid was recovered in a reduced volume (about 10-fold) free of the bulk of culture medium components (100-fold reduction by weight). The use of a volatile buffer system, ethylenediamine-acetic acid, enabled development

of the procedure by following toxin specific activity on a dry weight basis and also facilitated isolation of a salt free toxin preparation. pH-gradient elution of toxin activity on a column of cation exchange resin, under different conditions, established a correlation with reactivity to Folin's reagent in the Lowry method for proteins.

There are several probable improvements to the toxin isolation procedure, not yet pursued, which could be expected to improve efficiency of the process, including a reduction in ionic strength of the medium prior to adsorption with resin and use of a macroporous resin.

The ion exchange resin eluate concentrate or its lyophilised residue was not fully soluble in aqueous solution, except in alkali which inactivated the toxin. More than 95% of the activity was soluble in 5-10% aqueous acetic acid in which it was stable. The clarified extract was passed through a bed of Bio-Gel P-2, equilibrated in 0.2M aqueous acetic acid, in which the larger proportion of activity eluted near the excluded proteinaceous fraction and was free of several retarded peaks of u.v. absorbing substances. The activity exhibited pronounced tailing in this eluant, and although tailing could be reduced at higher pH, elution of the delayed inactive peaks was accelerated resulting in little overall improvement. Furthermore the use of aqueous acetic acid did not interfere in the subsequent Bio-Gel P-10 gel chromatographic step.

Chromatography of the Bio-Gel P-2 eluate concentrate on a column of Bio-Gel P-10 equilibrated in aqueous acetic acid resulted in the fractionation of activity into three peaks. All were identified as toxin-A by their activity spectra and electrophoretic behaviour on

cellulose acetate membrane. The bulk of activity eluted near K_{av} 0.55 and could be totally accounted for as protein: specific activities by weight and protein, estimated both colourimetrically and spectrophotometrically, were similar. Some activity eluted near K_{av} 0.8 in conjunction with protein and other substances, while a trace of activity, probably adsorbed to protein, was excluded from the gel together with most of the applied proteinaceous material.

Toxin-A, which eluted from Bio-Gel P-10 at K_{av} 0.55, when heated to about 55°C in the eluate concentrate, re-eluted primarily near K_{av} 0.8. The mechanism of transformation is not yet known but it may involve disaggregation, possibly from that of a dimer to one of a monomer. However, toxin-A, in the culture fluid concentrate was retarded and eluted at K_{av} 1.1, but in the presence of phenylmethanol some of the activity eluted near K_{av} 0.8. This indicated that the adsorptive interaction between toxin-A and Bio-Gel was not primarily due to a high content of aromatic residues (MOOR and STEIN, 1951); amino acid analysis revealed only 4 aromatic residues. The K_{av} 0.8 fraction may represent the native molecule liberated into the culture medium. Toxin-A from all fractions showed similar CAM electrophoretic mobility.

Calibration of the Bio-Gel P-10 column with standard proteins indicated that purified toxin-A had a molecular weight of near 2000. However, dialysis, SDS-polyacrylamide gel electrophoresis and amino acid analysis indicated a molecular weight of approximately 11000, suggesting that toxin-A was strongly retarded under a wide range of protein concentrations and eluant conditions. The mild denaturant, urea, may prove useful in this respect, as PALFREE and BUSSEY (1979) were able to disaggregate the killer toxin in urea and eliminate its retardation on a Sepharose column. It may be possible to establish the molecular

relationships between each of the toxin-A fractions by polyacrylamide gel electrophoresis and by the ultracentrifuge as for other small proteins, such as insulin or the neurophysin-hormone complex (GINSBURG, BURFORD and THOMAS, 1971).

Toxin-A, which eluted at K_{av} 0.55, was purified 5-fold with respect to protein and 3500-fold by dry weight over that present in the filtrate of culture in the minimal medium. The toxin was judged to be pure by (i) similarity in specific activity based on total weight of substance and on protein, (ii) a single band of killer activity following cellulose acetate membrane electrophoresis and (iii) a single symmetrical band of protein after electrophoresis on SDS-polyacrylamide gel. In the latter, mobility was unaltered by reduction with 2-mercapto-ethanol revealing that the toxin was composed only of a single polypeptide. The molecule was estimated to contain at most two hexose residues and was therefore not associated with polysaccharide, as is the case with native killer factor from Sacch. cerevisiae and PEST (BUSSEY and SKIPPER, 1975, 1976; PALFREE and BUSSEY, 1979). Seventeen standard amino acids were present (approximately 85 residues) of which glycine constituted 20% of the residues. The molecule was also rich in the acidic amino acids, aspartic acid and glutamic acid, and serine, but was low in basic and aromatic amino acids.

It is of interest to compare the composition of toxin-A to that of the killer toxin of Sacch. cerevisiae which was recently shown by PALFREE and BUSSEY (1979), to be a polypeptide of molecular weight 11500 containing at most two hexose residues per molecule. Both toxins were rich in glycine and acidic amino acids but the Sacch. cerevisiae toxin contained substantially more aromatic amino acid residues. Sequence homology of the two toxins is certainly warranted, particularly in view

of several apparent molecular similarities.

The preliminary experiments on interaction between the sensitive strain *H. anomala* NA 10 and purified toxin-A provide some indication on the killing action of toxin-A, but as yet no investigations into the primary site of action have been made. Toxin-A induced two apparently independent effects in sensitive cells: killing of growing but not resting cells and cell clumping in both populations of cells. Cells were killed under conditions which prevented cell to cell contact, and resting cells (washed and suspended in buffer) showed the clumping response even though few cells were killed. In a population of growing cells, the toxin-induced cell clumping effect, measured turbidimetrically, was delayed by about 100 min with respect to toxin binding as was indicated by cell survival. Since the clumping response was weak in relation to the proportion of cells having bound a lethal dose of toxin, it seems reasonable to assume that only cells having adsorbed toxin, possibly in much larger amounts than required to kill the cell, are able to adhere together. It is therefore unlikely that cell clumping interfered in the determination of viable counting and subsequent calculation of cell survival. This is subject to further testing.

Accepting that cell-clumping did not interefere in the calculation of survival, the survival curve for *H. anomala* at subsaturating levels of toxin was approximately linear indicating that killing followed a single-hit mechanism, that is the adsorption of a single lethal dose of toxin resulted in cell death without the requirement of a co-operative action, which would be evident by a shoulder in the curve (REEVES, 1965, 1972). The cell toxin complex was not readily dissociated and hence the time-course of cell survival reflected the binding of a lethal dose of

toxin. By 2h, more than 95% of cells able to bind a lethal dose did so and by 6-7h adsorption was near to completion. At low toxin multiplicities, one milligram of purified toxin-A was calculated to possess $0.5-1.0\times10^{11}$ lethal units, and assuming a molecular weight of 11000, a lethal unit was calculated as 5×10^{5} molecules, for killing of the sensitive yeast, H. anomala NA 10. The proportion of inactive toxin is unknown. This value presumably reflects an equal but low probability of killing displayed by each adsorbed molecule of toxin-A in this system (REEVES, 1972). A possible explanation is that the cell wall contains a large number of toxin binding sites of which only a small proportion may initiate the lethal result (HARDY, 1975). A study of the binding site(s) could be expected to provide an explanation for this result.

Toxin-A therefore resembles the Saccharomyces killer factor and PEST in that both also kill sensitive cells by a single-hit process (BUSSEY, 1972; BUSSEY and SKIPPER, 1975). A lethal unit of freshly prepared killer toxin protein contained either 10000 or 43000 molecules, depending on the sensitivity of the strain, but it was pointed out that the values be considered upper limits since it was not possible to measure the proportion of active toxin (PALFREE and BUSSEY, 1979).

BUSSEY and colleagues have elucidated some interesting aspects regarding the toxin binding site in Sacch. cerevisiae, which may provide an explanation as to the magnitude of the lethal unit observed for yeast killer toxins. They demonstrated that while whole cells of a sensitive strain bound toxin, their sphaeroplasts which only bound a small fraction of the toxin were also killed in a similar manner to that of whole cells, indicating that the cell wall was not necessary in the killing process (BUSSEY, SHERMAN and SOMERS, 1973; BUSSEY, 1974). Furthermore, sphaeroplasts showed a similar delay before the onset of

toxic effects indicating that the lag did not represent transport of the toxin from a wall site to one at the membrane. It must however be noted that significant rescue from killing was invoked by incubating cells in buffer known to inactivate the toxin or by digestion of the cell wall suggesting that the toxin may remain at the cell surface for a period of time (BUSSEY, 1972). Also, the cell wall plays an important role in resistance, where mutants unable to bind toxin were resistant to killing while their sphaeroplasts often remained sensitive (BUSSEY, SHERMAN and SOMERS, 1973; AL-AIDROOS and BUSSEY, 1978). It seems possible that the cell wall of H. anomala may non-specifically adsorb toxin which then may no longer participate in the lethal process. Thus, only toxin which penetrates to an inner site, perhaps the plasma membrane is effective. Such a situation could be investigated through toxin adsorption experiments as employed by BUSSEY and colleagues. The wide spectrum of activity of toxin-A tends to support the notion that toxin-A does not require a specific binding site in the cell wall. However, this would create a difficulty in explaining the mechanism of resistance. The toxin-A situation may be similar to some bacteriocins produced by gram positive bacteria which show an apparent lack of adsorption specificity (reviewed by KONISKY, 1978).

Resting cells were not killed by toxin-A. However the fate of the adsorbed toxin is unknown and hence it is not possible to distinguish whether the cell-toxin complex was unstable or whether the cells were tolerant to the adsorbed toxin. The cell clumping response however suggested that the cell had bound toxin irreversibly, but possibly to non-lethal sites at the cell surface. Should bound toxin be conclusively demonstrated, it would be of interest to find whether resting sphaeroplasts are similarly tolerant to killing. A possible mechanism of action might involve an energy dependent transport of toxin

across the plasma membrane to a toxin sensitive intracellular site. Electron microscopy suggests that toxin-A has an early effect on membrane integrity of the nuclear envelope and endoplasmic reticulum.

It may be most rewarding to initially investigate the action of toxin-A on plasma membrane integrity since electron microscopy revealed early ultrastructural changes. The resistance of resting cells to toxin-A resembles the energy dependent action of the *Saccharomyces* killer toxin but differs from this system in that killer action, toxin-induced altered membrane permeability, is reinvoked on removal of the energy-poison, resulting ultimately in cell death (SKIPPER and BUSSEY, 1977). The action of toxin-A against resting cells clearly differs from that of the *Saccharomyces* killer toxin studied by KOTANI, SHINMYO and ENATSU (1977) in that treated cells remained viable on rich plating medium, whereas in the latter, cell death resulted on plating medium unless calcium ions were included.

It would be of interest to investigate the cell clumping aspect of toxin-A, which in a broad sense resembles the action of a mating pheromone. However, since cell adhesion resulted in resting cells, toxin-A is presumably simply acting as an agglutinin, perhaps like the sexual agglutination polypeptide in yeast (CRANDALL and BROCK, 1968; SHIMODA and YANAGISHIMA, 1975). The weak agglutination response in relation to the killing response suggests that a large dose of toxin is required to form a stable cell-cell complex. Toxin-A activity is destroyed by heat, alkali and pronase, and it would therefore be instructive to examine the clumping ability of denatured toxin.

Cell survival experiments provide no information on the time of cell death following the adsorption of a lethal dose of toxin. However,

electron microscopy of toxin-treated cells fixed either by a chemical or physical method revealed discernible ultrastructural alterations within an hour of toxin adsorption and that by 2-3h, cells showed gross damage to the nuclear envelope and endoplasmic reticulum, at which stage these cells were probably no longer viable. By 4-5h a large proportion of cells exhibited complete loss of intracellular structural integrity, although the plasma membrane and mitochondria remained apparently physically intact. There was no evidence of early cell lysis.

Initial toxin-induced changes involved the plasma membrane, in which invaginations were no longer present and the fracture faces of frozen cells appeared to show an altered distribution of the membrane-bound particles. Both of these effects suggest that the membrane was in some unknown way markedly affected and it therefore would be instructive to monitor membrane function as a possible early site of toxin action. It is unlikely that the hydrophilic toxin-A protein would interact directly with the membrane lipids, as for example does the polyene antibiotic Amphotericin B, which binds to sterols and, by an as yet unclear mechanism, alters membrane permeability (BITTMAN, 1978). However, it is more likely that toxin-A may bind to surface proteins associated with the membrane to induce structural alterations.

The effects of toxin-A on the integrity of the nuclear envelope and endoplasmic reticulum but not the tonoplast and mitochondrial membrane, suggests a specificity of action, but these effects may only be secondary. Evidence gained from both chemically and physically fixed cells suggested that membrane degeneration commenced by destabilisation of the nuclear pore structure, since the outer and inner membranes showed a co-ordinated degeneration in adjacent sections of the envelope. The endoplasmic reticulum, which is also known to possess pores,

appeared to fragment in a similar manner (MATILE, MOOR and ROBINOW, 1969). The structure of the nuclear pore is not yet well understood (HARRIS, 1978). Toxin-A may in some way interfere with either the mechanism that stabilises the pore structure or the control of proliferation and degeneration of the nuclear envelope and endoplasmic reticulum which occurs during the cell cycle (MATILE, MOOR and ROBINOW, 1969). The mitochondrial membrane, which is structurally different to other membranes (PARKS, McLEAN-BOWEN, TAYLOR and HOUGH, 1978), may have resisted structural disintegration as it is likely that mitochondrial function was repressed under the conditions of growth and the membrane may therefore be less vulnerable to degenerative effects.

Thus, toxin-A completely inhibits cell growth with little delay (at most 60 min) following adsorption which, at the ultrastructural level, is accompanied by structural alterations to the plasma membrane and is closely followed by degeneration of the nuclear envelope and endoplasmic reticulum. At this stage the cell is presumably no longer viable. Although further experimentation is needed, the results of this study indicate both similarities and differences in action with those of other yeast toxins.

Toxin-A, produced by killer strains of H. saturnus under a wide range of cultural conditions, is a stable toxin which is lethal for a wide spectrum of yeasts. The toxin therefore differs in these respects to all other known yeast toxins but shares in common a protein basis for its killer activity. Its primary site of action remains to be determined.

APPENDIX ONE - EFFECT OF pH ON KILLER ACTIVITY

The effect of pH on killer activity of culture filtrates obtained from *H. saturnus* strains 9 and 354[a].

Indicator yeast	pH	Killer strain 9				Killer strain 354			
		pH 4.3		pH 6.0		pH 4.3		pH 6.0	
		Static	Shake	Static	Shake	Static	Shake	Static	Shake
Strain 10	3.5	1.00	1.00	.97	.73	1.00	1.00	1.00	1.00
	3.9	.97	.94	.93	.88	.97	.96	.96	.96
	4.3	.83	.90	.83	1.00	.87	.85	.85	.85
	4.7	.77	.79	.76	.98	.76	.74	.76	.76
	5.1	.72	.36	.74	.53	.69	.64	.69	.61
	5.5	.97	.67	.97	.46	.90	.86	.96	.86
	6.0	.90	.67	1.00	.47	.90	.86	.91	.82
	6.5	.74	.35	.82	.19	.82	.79	.86	.67
	7.0	.64	.19	.71	.12	.65	.61	.69	.55
	Relative potency[b]	.99	.50	.96	.62	.89	1.00	1.00	1.00
Strain 560	3.5	.88	.96	.95	1.00	1.00	1.00	1.00	1.00
	3.9	1.00	1.00	1.00	.99	.97	.99	1.00	.98
	4.3	.81	.93	.89	.89	.93	.85	.89	.88
	4.7	.66	.67	.77	.85	.77	.73	.73	.66
	5.1	.61	.28	.61	.29	.58	.50	.61	.43
	5.5	.82	.48	.85	.32	.88	.71	.86	.62
	6.0	.74	.43	.77	.29	.77	.63	.80	.55
	6.5	.64	.28	.71	.14	.67	.54	.69	.45
	7.0	.47	.09	.61	.04	.56	.46	.64	.40
	Relative potency	.84	.58	.78	.83	.70	.94	.77	1.00
Strain 498	3.5[c]	Not determined							
	3.9[c]	Not determined							
	4.3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	4.7	.21	.56	.61	1.00	.49	.94	.59	.88
	5.1	.00	.00	.00	.24	.00	.08	.00	.13
	5.5	Z[d]	.00	Z	.00	Z	Z	Z	Z
	6.0	Z	.00	Z	.00	Z	Z	Z	Z
	6.5	Not determined							
	7.0	Not determined							
	Relative potency	.15	.48	.08	.83	.27	.80	.07	1.00

[a] Killer activity is expressed as (inhibition zone width)^2/(maximum zone width)^2; [b] Relative activity of those culture filtrates assigned a value of 1.00 (ie. relative activity at their optimum-pH for activity; [c] Yeast growth much reduced; [d] Indefinite hazy zone <4mm width.

APPENDIX TWO-EXPRESSIONS RELATING TO YIELD IN ION EXCHANGE EXPERIMENTS

ADSORPTION EFFICIENCY

The amount of SKT (a.u.) adsorbed to an ion exchange resin (g swollen wt.) under a given set of conditions is given by:

$$\text{Resin adsorbed SKT (a.u./g)} = \frac{\text{adsorbed SKT (a.u.)}}{\text{wt. resin (g)}}$$

The amount of resin adsorbed SKT is taken to be the difference in culture filtrate (CF) activity before and after (designated supernatant fluid, SF) adsorption with ion exchange resin.

$$\text{Adsorbed SKT (a.u.)} = \text{CF SKT (a.u.)} - \text{SF SKT (a.u.)}$$

$$= \text{Vol. CF(ml)} \times [\text{CF SKT (a.u./ml)} - \text{SF SKT (a.u./ml)}]$$

Therefore the effective capacity of the resin for SKT (resin adsorbed SKT) is given by:

Effective resin capacity (a.u./g)

$$= \frac{\text{Vol. CF (ml)} \times [\text{CF SKT (a.u./ml)} - \text{SF SKT (a.u./ml)}]}{\text{wt. resin (g)}} \quad ....1)$$

Efficiency of adsorption may be given by the ratio of adsorbed SKT to the total culture filtrate SKT,

$$\text{Adsorption efficiency (\%)} = \frac{\text{Adsorbed SKT}}{\text{total SKT}} \times \frac{100}{1}$$

$$= \frac{\text{Vol. CF (ml)} \times [\text{CF SKT (a.u./ml)} - \text{SF SKT (a.u./ml)}]}{\text{Vol. CF (ml)} \times \text{CF SKT (a.u./ml)}} \times \frac{100}{1} \quad ......2)$$

For small ratios of resin wt. to CF vol. (<0.1), increase of CF vol. by resin adherent liquid is small and may be ignored, therefore equation 2 reduces to:

$$= \frac{\text{CF SKT (a.u./ml)} - \text{SF SKT (a.u./ml)}}{\text{CF SKT (a.u./ml)}} \times \frac{100}{1} \quad ......3)$$

ELUTION EFFICIENCY

The amount of SKT activity eluted from a given quantity of ion exchange resin (g swollen wt.) under a given set of conditions is:

$$\text{Eluted SKT (a.u./g)} = \frac{\text{Total Eluate SKT (a.u.)}}{\text{wt. resin (g)}}$$

$$= \frac{\text{Vol. Eluate (ml)} \times \text{Eluate SKT (a.u./ml)}}{\text{wt. resin (g)}}$$

$$\text{Elution efficiency (\%)} = \frac{\text{Eluted SKT (a.u.)}}{\text{Resin adsorbed SKT (a.u.)}} \times \frac{100}{1}$$

APPENDIX TWO, continued

$$= \frac{\dfrac{\text{Vol. (Eluate (ml) x Eluate SKT (a.u./ml)}}{\text{eluted wt. resin (g)}}}{\dfrac{\text{Vol. CF (ml) x [CF SKT (a.u./ml) - SF SKT (a.u./ml)]}}{\text{adsorbed wt. resin (g)}}} \times \frac{100}{1} \quad \text{......4)}$$

EXTRACTION EFFICIENCY

The efficiency of the SKT extraction (adsorption and recovery) from the CF by a given quantity of ion exchange resin is given by:

$$\text{Extraction efficiency (\%)} = \frac{\text{Eluted SKT (a.u.)}}{\text{CF SKT (a.u.)}} \times \frac{100}{1}$$

$$= \frac{\text{Vol. Eluate (ml) x Eluate SKT (a.u./ml)}}{\text{CF SKT (a.u.)}} \times \frac{100}{1} \quad \text{.....5)}$$

For the case where a different quantity of resin is used for adsorption and elution, the equation becomes:

$$\text{Extraction efficiency (\%)} = \frac{\dfrac{\text{Vol. Eluant (ml) x Eluate SKT (a.u./ml)}}{\text{eluted wt. resin (g)}}}{\dfrac{\text{Vol. CF (ml) x CF SKT (a.u./ml)}}{\text{adsorbed wt. resin (g)}}} \times \frac{100}{1} \quad \text{......6)}$$

APPENDIX THREE - FORMULATION OF CONSTANT IONIC STRENGTH ETHYLENEDIAMINE-ACETIC ACID BUFFER.

Procedure

Composition of constant ionic strength buffer mixtures of ethylenediamine and acetic acid were determined by an empirical method described by PERRIN and DEMPSEY (1974). An outline of the procedure is as follows:

1. Measure pH on a solution of acetic acid and ethylenediamine.
2. Estimate ionic equilibrium compositions using thermodynamic pKa values.
3. Compute first estimate of ionic strength (I).
4. Convert pKa to a practical constant, pKa', using Davies' equation,

$$pKa' = pKa-(2n-1)\ \{0.5I^{\frac{1}{2}}/(1+I^{\frac{1}{2}})-0.1I\}$$

   where n=1 for acetic acid, and n = 0 or -1 for pKa_1 or pKa_2 respectively for ethylenediamine (EN).

5. Compute ionic compositions using pKa' values.
6. Compute ionic strength.
7. Repeat steps 4, 5 and 6 until no further (significant) change in pKa' results.

A series of constant ionic strength buffers spanning the effective buffer range of ethylenediamine-acetic acid (pH4-8) was determined by the dilution approach. Since dilution results in a change of pH (as a consequence of altered ionic strength), it is necessary to remeasure pH and calculate the true ionic strength and hence recalculate the necessary degree of dilution to achieve required ionic strength. This

procedure is repeated until the required value of ionic strength is obtained.

In the present work a series of low ionic strength buffers were required, of less than 0.1I. A series of mixtures with pH intervals of about 0.2 units were therefore made up from stock 0.20M solutions (standardised, see Methods of Chapter 4) with the composition, xml acid + (10 - x) ml base. Mixtures of the composition 9:1 to 5.5:4.5 of acid to base fell within the range pH4-8 and ranged 0.05-0.2I.

Mixture preparation and pH measurement

This work was carried out at 23°C. Standardised stock solutions of 0.200M $\pm$ 0.002 acetic acid and ethylenediamine were freshly prepared with the mixture compositions (total volume of 10.0 ml) as shown in Table A using A-grade burettes. pH was measured with a combination electrode (type N-65, Schott Jena, Mainz) digital pH meter (Model No.1852, Townson, Australia). Occasionally the pH measurements were checked against a dual electrode pH meter (Radiometer M-26) and found to agree within 0.03 pH units. Both pH meters were two point calibrated at 23°C for greatest accuracy as recommended by the manufacturers. The primary calibration buffers were phosphate pH6.50 (Radiometer) and phthalate pH4.00 (Beckman, 14044, U.S.A.) and three further buffers, acetate pH4.65 (Townson and Mercer, Australia), phosphate pH6.87 (Beckman, 14268, U.S.A.) and borate pH9.20 (Beckman, 14049, U.S.A.), were used as secondary calibration standards to check electrode linearity and response. These buffers were prepared in freshly boiled deionised distilled water and stored in airtight containers at 4°C, a portion being prewarmed to 23°C before use. The electrodes were rinsed with deionised water between samples, and the solution being measured

was gently shaken to hasten the electrode response, while the reading was taken on a statically positioned solution.

It was found that on measuring alternately calibration standards and acetic acid ethylenediamine solutions that the meter response, particularly when using the combination electode, took an inordinately long period to stabilise. Experience showed that the long term meter stability was such that reference to the standards was only necessary hourly and then no recalibration was usually necessary. To achieve this stability, however, it was essential that the meter be switched on for an hour before calibration was undertaken.

Results

A computer programme was written to facilitate computations. The programme was set up for pH measurements made at 23°C, where the pKa^{s} for acetic acid was 4.76, and ethylenediamine, 6.904 and 9.988. Programme input comprised: measured pH, volume of acid, volume of base and total solution volume. Programme iteration to final pKa' was arbitrarily set so that ionic strength was accurate to 10^{-6} units. Programme output tabulated input data plus ionic strength, buffer capacity (calculated according to PERRIN and DEMPSEY, 1974; note corrections to this text) and final volume of solution to produce an estimated 0.06I.

Two series of dilutions were required to obtain mixtures of ionic strength to better than 0.060. Mixtures of approximately 0.03, 0.015 and 0.0075 ionic strength were derived by two-fold dilution of stock 0.06I mixtures. No attempt was made to futher refine these values since the present demands did not warrant it. Also no attempt was made to

obtain a continuous series of pH values of say exactly 0.2 pH intervals, which could be done by interpolation of the data. The compositions for 0.0075, 0.015, 0.03 and 0.06I mixtures in the range pH4.5-7.5 are given in Table A.

Accuracy of the data

A computer programme was subsequently obtained from Professor D. Perrin for computing directly equilibrium compositions for a buffer composed of a monobasic acid and diacidic base.

A portion of the values computed by this programme for 0.06I is shown in Table B and is compared with those calculated from Table A. It can be seen that the values determined experimentally agree well with those computed from ionic equilibrium theory.

Table A.

IONIC STRENGTH CALCULATIONS FROM PH MEASURED ON MIXTURES OF ACETIC ACID AND ETHELENE DIAMINE

TEMP = 23C
PKA HOAC = 4.76
PK1 EN = 6.904
PK2 EN = 9.988
ACETIC ACID = .20M
ETH. DIAMINE = .20M

****

ML HOAC	ML EN	TOT. VOL.	PH	I	BUFFER CAPACITY	FINAL VOL TO I=0.06
8.5	1.5	14.74	4.37	.0600	.0594	
8.5	1.5	29.48	4.40	.0301	.0300	
8.5	1.5	58.96	4.42	.0151	.0151	
8.5	1.5	117.92	4.44	.0076	.0077	
8.4	1.6	15.71	4.42	.0600	.0571	
8.4	1.6	31.42	4.45	.0301	.0288	
8.4	1.6	62.84	4.47	.0151	.0145	
8.4	1.6	125.68	4.49	.0076	.0073	
8.2	1.8	17.67	4.52	.0600	.0522	
8.2	1.8	35.34	4.55	.0301	.0262	
8.2	1.8	70.68	4.57	.0151	.0132	
8.2	1.8	141.36	4.59	.0076	.0067	
8.0	2.0	19.76	4.64	.0600	.0468	
8.0	2.0	39.52	4.67	.0301	.0235	
8.0	2.0	79.04	4.69	.0151	.0118	
8.0	2.0	158.08	4.71	.0076	.0059	
7.8	2.2	21.74	4.75	.0600	.0412	
7.8	2.2	43.48	4.78	.0301	.0206	
7.8	2.2	86.96	4.80	.0151	.0103	
7.8	2.2	173.92	4.82	.0075	.0052	
7.5	2.5	24.68	4.93	.0600	.0323	
7.5	2.5	49.36	4.96	.0301	.0161	
7.5	2.5	98.72	4.98	.0150	.0081	
7.5	2.5	197.44	5.00	.0075	.0041	
7.2	2.8	27.66	5.17	.0600	.0223	
7.2	2.8	55.32	5.19	.0300	.0112	
7.2	2.8	110.64	5.21	.0150	.0056	
7.2	2.8	221.28	5.23	.0075	.0028	
7.0	3.0	29.54	5.38	.0600	.0156	
7.0	3.0	59.08	5.40	.0299	.0079	
7.0	3.0	118.16	5.42	.0150	.0040	
7.0	3.0	236.32	5.44	.0075	.0020	
6.9	3.1	30.44	5.53	.0600	.0121	
6.9	3.1	60.88	5.55	.0299	.0062	
6.9	3.1	121.76	5.56	.0149	.0032	
6.9	3.1	243.52	5.57	.0074	.0016	
6.8	3.2	31.21	5.72	.0600	.0090	
6.8	3.2	62.42	5.73	.0299	.0047	
6.8	3.2	124.84	5.74	.0149	.0025	
6.8	3.2	249.68	5.74	.0074	.0013	

Table A. Continued.

6.7	3.3	31.68	5.97	.0600	.0070
6.7	3.3	63.36	5.97	.0298	.0038
6.7	3.3	126.72	5.96	.0149	.0020
6.7	3.3	253.44	5.96	.0074	.0011
6.6	3.4	31.63	6.24	.0600	.0070
6.6	3.4	63.26	6.21	.0298	.0038
6.6	3.4	126.52	6.19	.0148	.0020
6.6	3.4	253.04	6.17	.0074	.0011
6.5	3.5	30.88	6.52	.0600	.0090
6.5	3.5	61.76	6.48	.0298	.0047
6.5	3.5	123.52	6.43	.0149	.0024
6.5	3.5	247.04	6.39	.0074	.0013
6.4	3.6	30.02	6.71	.0600	.0113
6.4	3.6	60.04	6.65	.0299	.0058
6.4	3.6	120.08	6.60	.0150	.0029
6.4	3.6	240.16	6.56	.0075	.0015
6.3	3.7	29.03	6.87	.0600	.0136
6.3	3.7	58.06	6.81	.0299	.0069
6.3	3.7	116.12	6.75	.0150	.0035
6.3	3.7	232.24	6.70	.0075	.0017
6.2	3.8	28.07	7.00	.0600	.0154
6.2	3.8	56.14	6.93	.0301	.0078
6.2	3.8	112.28	6.87	.0151	.0039
6.2	3.8	224.56	6.82	.0075	.0020
6.1	3.9	27.07	7.12	.0600	.0169
6.1	3.9	54.14	7.04	.0302	.0085
6.1	3.9	108.28	6.98	.0151	.0043
6.1	3.9	216.56	6.93	.0076	.0021
6.0	4.0	26.08	7.23	.0600	.0180
6.0	4.0	52.16	7.15	.0302	.0090
6.0	4.0	104.32	7.08	.0152	.0045
6.0	4.0	208.64	7.03	.0076	.0023
5.9	4.1	25.15	7.33	.0600	.0186
5.9	4.1	50.30	7.25	.0302	.0094
5.9	4.1	100.60	7.19	.0152	.0047
5.9	4.1	201.20	7.13	.0076	.0024
5.8	4.2	24.22	7.43	.0600	.0188
5.8	4.2	48.44	7.35	.0302	.0095
5.8	4.2	96.88	7.28	.0152	.0048
5.8	4.2	193.76	7.22	.0077	.0024
5.7	4.3	23.30	7.53	.0600	.0186
5.7	4.3	46.60	7.45	.0302	.0094
5.7	4.3	93.20	7.38	.0152	.0048
5.7	4.3	186.40	7.32	.0077	.0024
5.6	4.4	22.33	7.64	.0600	.0178
5.6	4.4	44.66	7.55	.0303	.0092
5.6	4.4	89.32	7.49	.0152	.0046
5.6	4.4	178.64	7.43	.0077	.0024
5.5	4.5	21.53	7.74	.0600	.0169
5.5	4.5	43.06	7.65	.0303	.0087
5.5	4.5	86.12	7.59	.0152	.0044
5.5	4.5	172.24	7.53	.0077	.0022

Table B. Comparison of 0.06I buffer pH-composition (total molar concentration) data determined experimentally (calculated from Table A.) and computed from equilibrium theory.

pH	EXPERIMENTAL		THEORETICAL	
	M-HOAc	M-EN	M-HOAc	M-EN
4.37	0.1153	0.0204	0.1184	0.0200
4.42	0.1069	0.0204	0.1099	0.0200
4.52	0.0928	0.0204	0.0955	0.0200
4.64	0.0810	0.0202	0.0821	0.0200
4.76	0.0718	0.0202	0.0727	0.0201
4.93	0.0608	0.0203	0.0616	0.0201
5.17	0.0521	0.0202	0.0525	0.0202
5.38	0.0474	0.0203	0.0478	0.0203
5.53	0.0453	0.0204	0.0456	0.0204
5.72	0.0436	0.0205	0.0438	0.0206
5.97	0.0423	0.0208	0.0425	0.0211
6.24	0.0417	0.0215	0.0421	0.0220
6.52	0.0421	0.0227	0.0424	0.0236
6.71	0.0426	0.0240	0.0430	0.0253
6.87	0.0434	0.0255	0.0439	0.0272
7.00	0.0442	0.0271	0.0448	0.0291
7.12	0.0451	0.0288	0.0458	0.0312
7.23	0.0460	0.0307	0.0468	0.0334
7.33	0.0469	0.0326	0.0478	0.0355
7.43	0.0479	0.0347	0.0489	0.0378
7.53	0.0489	0.0369	0.0501	0.0401
7.64	0.0502	0.0394	0.0513	0.0426
7.74	0.0511	0.0418	0.0524	0.0449

APPENDIX FOUR - DATA AND CALCULATIONS FOR GRADIENT ELUTION CHROMATOGRAPHY EXPERIMENTS

Recovery of SKT activity in selected active fractions obtained under three conditions of gradient elution of a column of IR-120 resin. Total activity in the computed total pooled volume was calculated on the basis of fraction size. Residual activity was determined by assay of the adsorbed culture filtrate and pre-gradient eluates made at pH4.64 (0.06I). SKT yield in the eluate (elution efficiency) is therefore the total pooled-activity expressed as a percentage of the total activity originally present in the culture filtrate (40000 a.u.) less the residual activity.

Gradient	Expt.	Fract. size (ml)	Fract. number pooled	Volume pooled (ml)	SKT activity (a.u./ml)	Total volume (ml)	Total SKT activity (a.u.)	Residual activity (a.u.)	SKT yield (elut. effic.) (%)
0.0075I	A1 a)	11.2	48-80	247.5	48.5	369.6	17926	840	45.8
pH4.71-7.03	b)	11.2	81-102	166.0	7.0	246.4	1725		4.4
	A2 a)	11.2	50-80	248.0	45.1	347.2	15659	820	40.0
0.06I	B1 a)	10.8	12-52	336.2	44.0	442.8	19483	780	49.7
pH4.64-7.23	B2 a)	11.0	14-46	310.2	56.0	363.0	20328	950	52.1
	b)	11.0	47-60	130.0	11.0	154.0	1694		4.3
0.0075I-0.06I	C1	10.8	14-25	54.0	198.0	118.8	23522	1120	60.5
pH4.71-7.23	C2	11.2	15-30	134.4	146.0	179.2	26163	780	66.7
	C3	11.2	16-31	145.6	130.0	179.2	23296	920	59.6

a) Major SKT active peak; b) Tailing active peak.

APPENDIX FOUR, continued.

Specific activity of SKT based on dry wt. and protein content in pooled eluate fractions.

Expt.	Volume lyophilised	Dry wt. (mg)	Total dry wt. (mg)	Specific activity (a.u./mg)	Protein, B.S.A. equiv. (mg)	Total protein (mg)	Specific activity (a.u./mg)
A1 a)	245.0	110.1	166.1	107.9	1.936	2.920	6138
b)	N.D.						
A2 a)	245.0	105.7	149.8	104.5	1.495	2.118	7391
B1 a)	N.D.						
B2 a)	307.0	301.3	356.3	57.1	1.903	2.251	9034
b)	N.D.						
C1	N.D.						
C2	132.0	177.7	241.2	108.5	2.297	3.118	8390
C3	143.0	165.7	207.6	112.2	1.988	2.491	9351

N.D. Not determined.

APPENDIX FIVE - THE EFFECT OF RESIN TO ELUATE RATIO ON PURITY

Experimental conditions to study the effect of resin to eluate ratio on the recovery and purity of SKT. One litre of culture filtrate (100 a.u./ml) was adsorbed with 100 g swollen IR-120(H) resin at pH3.5, washed with deionised water and equilibrated in 1 litre of 0.06I EN-AA, pH4.52. The pH was returned to 4.52 with 0.2M-EN. The resin was collected on a sintered glass filter, washed with 1 litre of deionised water over a period of 30 min and the excess moisture removed by drawing air through the resin bed for 30 min. The resin adherent liquid content was determined on three poritions of this resin. 0.50-5.0 g portions of this resin was then eluted with 2.50 ml of either 0.0075I EN-AA buffer, pH7.03 or 0.06I EN-AA buffer, pH6.71. The mixtures were titrated to either pH7 or 8 with 0.20M-EN using a 50 μl graduated syringe. Experimental data and calculations are given in the following table.

EXPERIMENTAL DATA AND CALCULATIONS

Adsorption conditions

1000 ml of culture filtrate (CF) was adsorbed with 100 g swollen IR-120(H) Culture filtrate SKT activity was 100 a.u./ml.

Elution volume	Expt.1	Expt.2
Resin adherent liquid (ml (g)/g wet wt.)	0.044	0.040
Vol. of eluate buffer (ml)	2.50	2.50

Total elution vol. = 2.50 ml + (adherent liquid x wt. swollen resin) + ml titrant.

APPENDIX FIVE, continued.

Elution efficiency	Expt.1	Expt.2
Adsorbed CF SKT (a.u./ml)	4.3	5.0
Pre-elution @ pH4.52 (a.u./ml) (activity corrected to a vol. of 1 l)	4.2	3.9
Total pre-eluted SKT (TPSKT, a.u./ml)	8.5	8.9

$$\text{Elution efficiency (\%)} = \frac{\text{Tot. elut. vol. x eluant SKT x 100.0 g}}{\text{1000 ml x (100.0 - TPSKT) x wt. eluted resin}} \times \frac{100}{1}$$

$$\text{Extraction efficiency (\%)} = \frac{\text{Tot. elut. vol. x eluant SKT x 100.0 g}}{\text{1000 ml x 100.0 x wt. eluted resin}} \times \frac{100}{1}$$

$$\text{Volume concentration ratio (VCR)} = \frac{\text{adsorption vol. per wt. swollen resin}}{\text{elution vol. per wt. swollen resin}}$$

$$\text{SKT concentration ratio} = \frac{\text{VCR x extraction efficiency (\%)}}{100}$$

APPENDIX SIX - EFFECT OF RESIN PRETREATMENT AT 0.015I ON PURITY

Adsorption and pre-elution conditions

Wt. swollen resin (g) per vol. CF (ml)	20.0/200.0
Culture filtrate SKT activity (a.u./ml)	100.0
Non-Adsorbed CF at pH3.50, SKT (a.u./ml)	3.2
Pre-eluted SKT at pH4.55 (0.06I) (a.u./ml) (activity corrected for a vol. of 200 ml)	4.0

Pre-elution with 0.015I buffers, SKT (a.u./ml) (corrected for a volume and resin wt. of 20.0 g/200 ml)

$$= SKT \times \frac{20.0}{0.91} \times \frac{10.046}{200.0}$$

$$= SKT\ (a.u./ml) \times 1.10$$

Total SKT activity eluted from resin by pretreatments (a.u./ml) $= 3.2 + 4.0 + (SKT \times 1.10)$

Elution conditions

Pre-elution with 0.015I buffer:

resin wet wt. (g)	0.954
adherent liquid (g, ml)	0.046
eluant volume (ml)	10.000
total eluate volume (ml)	10.046

Elution at 0.0075I, pH7.0:

adherent content, estimated (g, ml)	0.09
eluant volume (ml)	2.50
total eluate volume (ml)	= 2.59 + ml titrant

Elution efficiency (%) (Equation 4, Appendix Two)

$$= \frac{(2.59 + ml\ titrant) \times 0.0075I\ eluate\ SKT \times 20.0}{200.0 \times [100.0 - 3.2 - 4.0 - (SKT \times 1.10)] \times 0.91} \times \frac{100}{1}$$

Extraction efficiency (%) (Equation 6, Appendix Two)

$$= \frac{(2.59 + ml\ titrant) \times 0.0075I\ eluate\ SKT \times 10.0}{200.0 \times 100.0 \times 0.91} \times \frac{100}{1}$$

APPENDIX SIX, Continued

The effect on SKT specific activity and elution efficiency of IR-120 resin pre-treament with 0.015I EN-AA buffer at various pHs. SKT activity was eluted from the resin with 0.0075I EN-AA buffer of pH7.03 titrated to pH7.2-7.3.

Pre-treatment with 0.015I EN-AA buffer: 0.91 g (swollen wt.) resin/10.0 ml buffer					
Eluant pH	5.56	5.96	6.19	6.43	deionised[a] water
Equlibrium pH	5.20	5.33	5.46	5.72	N.D.
Eluate conductivity (mS/cm)	0.80	0.80	0.83	0.90	N.D.
SKT activity (a.u./ml)	<1.8	<1.8	4.2	22.0	<1.8
SKT elution with 0.0075I EN-AA buffer of pH7.05: 0.91 g (swollen wt.) resin/2.50 ml buffer					
Equilibrium pH	6.39	6.66	7.02	7.28	4.85
Titration pH	7.23	7.31	7.28	7.28	7.21
Titrant, 0.20M-EN (ml)	0.007	0.003	0.001	0.0	0.042
Total eluate volume (ml)	2.597	2.593	2.591	2.590	2.632
SKT activity (a.u./ml)	188	188	178	144	188
Dry wt. (mg/ml)	0.23	0.24	0.26	0.22	0.30
Specific activity (a.u./mg)	817	783	685	655	627
Elution efficiency (%)	<59.1	<59.0	57.5	59.7	59.9
Extraction efficiency (%)	53.7	53.6	50.7	41.0	54.4

[a] Control resin treated with deionised water; N.D., Not determined.

APPENDIX SEVEN - BIO-GEL P-10 RECHROMATOGRAPHIC EXPERIMENTS. [a]

Experimental conditions	Chromat. profiles of Fig. 4.17.	Sample volume (ml)	reduced vol. (ml)	SKT activity (a.u.)	Effluent Fractions pooled no.	volume (ml)	SKT activity (a.u./ml)	Total vol. calculated (ml)	total SKT activity (a.u.)	Yield %
Primary elution	A	73.0[b]	2.3	55480	13-26	49.0	780	51.8	40400	72.8
Fractions 13-26 concentrated by RFE @ 55°C	B	47.0	2.1	36660	B-1 13-20	28.2	402	29.6	11900	32.5
					B-2 23-27	17.4	924	18.5	17090	46.6
Recycle of B-1 concentrated by RFE @ 35°C	C	27.5	1.8	11060	15-18	15.6	480	16.4	7870	71.2
Recycle of fractions 15-18 concentrated by RFE @ 35°C.	D	15.0	2.0	7200	N.D.					
Recycle B-2 concentrated by RFE @ 35°C.	E	17.0	2.2	15710	19-24	23.4	585	24.6	14390	91.6

[a] Chromatographic profiles are shown in Figure 4.17: this table summarises experimental conditions and activities of the eluates; [b] The starting sample was a Bio-Gel P-2 0.2M-acetic acid eluate derived from the peptone supplemented large batch culture; N.D., Not determined; RFE, Rotary film evaporation under reduced pressure (water venturi pump).

APPENDIX EIGHT - ASSAY OF BACITRACIN

Bacitracin was assayed by a well test based on an assay described by GROVE and RANDALL (1955, pp. 76-81). *Micrococcus lutea* was the indicator and preliminary tests showed that EN-AA buffers did not interfere in the assay.

Aliquots of column effluent fractions (in 0.20M-AA) were neutralised to pH6.0 by diluting 100 µl with 0.90 ml diluent consisting of 1 part 0.20M-EN to 17 parts distilled water. A standard curve for Bacitracin, (potency 55.3 units/mg) was set up in the range 0.20-100 µg/ml by serial dilution in EN-AA buffer of the same composition (pH6.0). Seeded agar plates were prepared by inoculating 15 ml Nutrient agar (Oxoid) with one drop of a 24h culture (at 22-23°C) of *M. lutea* in Nutrient broth (Oxoid). Six 7 mm wells were cut per plate and 70 µl sample added to each well. After incubation at 22-23°C for 48h, the zones of inhibition were measured to 0.1 mm with vernier calipers.

Two samples and a reference (25 µg/ml bacitracin) in duplicate were accommodated per plate. The sample inhibition zone diameters of each plate were averaged and corrected according to the internal assay reference (GROVE and RANDALL, 1955). The assay standard curve was plotted on semi-logarithmic graph paper by joining the points of the corrected average zone diameter versus bacitracin concentration in µg/ml from which potency of the samples were determined.

APPENDIX NINE - AMINO ACID COMPOSITION

Amino acids in three preparations of SKT protein.[a]

Amino acid	Sample amino-acid content (nmol/ml)				
	Preparation 1		Preparation 2		Preparation 3
	MSA	HCl	MSA	HCl	MSA
Aspartic acid	18.7	34.8	35.1	36.4	12.8
Threonine	8.3	16.1	12.8	13.5	4.8
Serine	11.4	20.8	21.6	27.1	16.5
Glutamic acid	9.7	18.3	37.1	43.5	7.0
Proline	2.2	6.1	8.2	11.2	0.5
Cystine/2	4.5	14.7	5.0	4.6	Trace
Glycine	17.3	37.4	55.6	72.7	19.0
Alanine	Trace	7.2	16.3	19.4	1.8
Valine	5.5	15.5	23.7	25.0	5.8
Methionine	2.5	3.6	2.4	0.8	3.8
Isoleucine	3.0	5.8	11.1	10.0	2.3
Leucine	0.5	0.8	11.2	12.3	2.0
Tyrosine	2.8	5.3	5.4	5.9	4.5
Phenylalanine	1.2	1.4	2.5	3.0	Trace
Histidine	0.8	2.1	5.0	4.6	2.0
Lysine	3.1	5.6	22.5	27.7	0.5
Arginine	0.8	4.7	11.5	11.0	Trace

[a] Results are the mean of duplicate determinations and are expressed as nanomoles per ml hydrolysed (methane sulphonic acid or HCl) sample which contained 30-100 μg/ml protein. Preparations 1 and 2 were derived from CF_s and preparation 3 from CF_p.

APPENDIX TEN - ELECTRON MICROSCOPY: DEHYDRATION AND EMBEDDING

Chemically fixed pellets, following thorough washing with either distilled water or phosphate buffer as appropriate, were embedded according to the following schedule:

1. 10 min in 30% ethanol (aqueous)
   15 min in 50% ethanol
   25 min in 75% ethanol
   30 min in 95% ethanol
   30 min in 100% ethanol
   30 min in 100% ethanol

2. 1.5h in a mixture of 50% Spurrs resin.
3. Left overnight in the first lot of Spurrs resin.
4. 5h in a second lot of resin.
5. Encapsulated in fresh Spurrs resin, and cured at 60°C for 6h.

APPENDIX ELEVEN - ELECTRON MICROSCOPY: CHEMICAL FIXATION

Chemical fixatives

The commonly employed chemical fixatives, permanganate, glutaraldehyde and osmium tetroxide, yield variable results for different yeasts (KOPP, 1975). Apart from their different mechanisms of action, the nature of the cell wall has an important bearing on their efficacy. The ability of these fixatives to preserve the cellular structures of *H. anomala* was therefore compared : the results are tabulated below.

The effect of chemical fixatives on the preservation of the ultra-structure of *H. anomala* 10.

Fixative Conditions[a]	Ultrastructural features
Osmium tetroxide (1h)	Completely unsatisfactory; no structures observed within the cell wall.
Glutaraldehyde (30 min) with/without osmium tetroxide (2h) post-fixation	Unsatisfactory preservation; membrane structure unresolvable.
Glutaraldehyde (1h) followed by potassium permanganage (30 min) post-fixation.	All membranous structures preserved, but plasma membrane not sharply defined. Preserved the contents of the large vesicle (vacuole) and produced little distortion (invaginisation) of the nuclear and plasma membranes.
Potassium permanganate (30 min)	Preservation of all membranous organelles showing good structural definition. Nucleus and large vesicle were distorted.
Potassium permanganate (30 min) (unstained)	Low contrast, but no apparent ultrastructural differences compared with stained preparations.

[a] All were 2% solutions in 0.1M-potassium phosphate buffer (pH7.2) except for potassium permanganate which was an aqueous 2% solution.

As reported for other yeasts, osmium tetroxide was a completely ineffective fixative, evidently being unable to penetrate the yeast cell wall since protoplasts were readily amenable to fixation (ROBINOW and MARAK, 1966; OSUMI, MIWA, TERANISHI, TANAKA and FUKUI, 1974). The penetration of glutaraidehyde was also found to be poor, a result which has been improved by post-fixing with either permanganate or osmium tetroxide (see for example SENTANDREU and NORTHCOTE, 1969; FUKUI, TANAKA, KAWAMOTO, YASUHARA, TERANISHI and OSUMI, 1975). However, for H. anomala post-fixation by permanganage did not yield a perceptively better result than obtained with aqueous potassium permanganate alone. The ultrastructural detail of permanganate fixed cells was comparable with that for H. anomala obtained by BANDONI, BISALPUTRA and BISALPUTRA (1967) and also with that for H. wingei in which permanganate fixed cells were post-fixed with osmium tetroxide (CONTI and BROCK, 1965). The rapid penetration and action of permanaganate results in high insensitivity to conditions of pH, temperature, type of buffer and other factors (HAYAT, 1970). Despite the well known disadvantages associated with permanganate (oxidative degradation, dissolution of plasma components, marked grainy appearance and absence of ribosomes) its rapid action and distinct staining or membranes was considered of paramount importance for the present study.

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