# Expression and production of the *Saccharomyces cerevisiae* haze protective factor 2 for sensory studies and further investigation into the role of glycosylation

by

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BE (Chem) Honours/BSc

Thesis submitted for the degree of

**Doctor of Philosophy**

November 2007

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## THESIS SUMMARY

White wine clarity is essential, but it can be marred by the presence of a protein haze. This protein haze is predominantly formed by grape-derived thaumatin-like proteins and chitinases, which can slowly denature and aggregate if left in bottled wines.

Currently bentonite fining is used by the wine industry to prevent protein haze. Bentonite consists of fine clay particles that, when added to wine, bind and remove the haze-forming proteins. However this method is inconvenient, time-consuming, and causes significant losses of wine. It is estimated that this process costs the Australian wine industry $50 m annually in wine losses alone. Alternatives are thus being investigated.

The principal objective of this thesis was to investigate the sensory effects on wine of an alternative method to bentonite fining: addition of haze protective factor 2, known as Hpf2. Hpf2 is a *Saccharomyces cerevisiae* mannoprotein that has been shown to reduce protein haze in wines. It is a highly mannosylated 180 kDa protein, of which approximately 75% by weight is mannose. Previous work has shown that the addition of approximately 200 mg $L^{-1}$ Hpf2 to wines reduces the visible haze in wine by approximately 50%.

Hpf2 is naturally present in wines at concentrations of less than 10 ng $L^{-1}$, much lower than the concentration required for haze protection activity. However, the sensory impacts involved with the addition of such high concentrations of Hpf2 in wine have never been studied. This knowledge is essential for the future commercial prospects of this alternative approach to protein stabilisation of wine.

To undertake sensory studies, over 1 g of Hpf2 would be required. Presently, the laboratory-scale process for the production of a 6-histidine tagged version of the protein, 6xHisHpf2, in a laboratory yeast strain of *S. cerevisiae*, produces only milligram quantities. Consequently, the first challenge of this research was to scale up the existing process to produce sufficient quantities of Hpf2.

The first attempt to increase the production level was by over-expression in the bacteria *Escherichia coli*. Although several approaches were trialled, 6xHisHpf2 was unable to be successfully and consistently expressed in this system. The second method was by improving the original yeast expression system, and the expression level was able to be improved approximately 10-fold. This improved expression method was scaled up to produce and then purify over 1 g of protein. Several quantification methods were assessed to determine the efficiencies of each purification step, with slot blot analysis proving successful.

Sensory trials were conducted to establish the effect of 6xHisHpf2 on wines, with duo-trio studies conducted assessing both aroma and palate of the wines. Invertase, another yeast haze protective factor, was also trialled. It was found that the addition of an active level of 6xHisHpf2 or invertase did not cause a significant difference in the aroma or palate of wines.

In addition to this main study, the role of the glycosylation was studied. 6xHisHpf2, produced in a different yeast, *Pichia pastoris*, was found to be 83 kDa, with only 50% mannose. This protein was compared to the *S. cerevisiae* protein in its ability to reduce protein haze, and it was shown that the *P. pastoris* protein could reduce haze, but not as effectively as the *S. cerevisiae* protein.

The finding that Hpf2 does not affect the sensory properties of wine is essential if Hpf2 is to be used commercially, as winemakers and wine consumers would most likely reject an additive that alters the wine aroma or palate. This work has brought the wine industry a step closer to a new method for protein haze prevention in white wines.

## DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available in all forms of media, now or hereafter known.

Oenone Jean Macintyre
November 2007

## ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors, Liz Waters, Chris Colby, Brian O'Neill and Sakkie Pretorius, for their insights and advice over the course of this project.

I would also like to thank the Bioscience team, both past and present, for their help and enthusiasm over the years, in particular Paul Chambers, Miguel de Barros Lopes and Simon Schmidt. Many thanks to the other members of The Australian Wine Research Institute for their valuable advice and input to the project.

I would like to thanks the project collaborators in Melbourne, Filomena Pettolino, Dave Dunstan and Tony Bacic, for discussions early in the project, and those at Flinders University, Peter Anderson, Ee Leng Tan and Uli Nasution, for their contribution to the *Pichia pastoris* work.

Thanks also to the winemakers who donated unfined wine for the sensory studies and heat test analysis in this study.

Thanks to my family and friends for all their encouragement of my studies over the years – I am grateful to have had such great support through this.

And last but not least, Karloskar, for all your love and support.

## ABBREVIATIONS

| | |
|---|---|
| 6xHis | six consecutive histidine amino acids |
| AWRI | Australian Wine Research Institute |
| BCIP | 5-bromo-4-chloro-3-indolyl-phosphate |
| BSA | bovine serum albumin |
| CDGJM | chemically defined grape juice medium |
| Da | Dalton |
| DNA | deoxyribonucleic acid |
| DDM | Delft defined media |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| GM | genetically modified |
| GPI | glycosylphosphatidylinositol |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HPF | Haze protective factor |
| HPLC | high performance liquid chromatography |
| IMAC | immobilised metal affinity chromatography |
| IPTG | isopropyl β-D-thiogalactopyranoside |
| kDa | kilo Dalton |
| LB | Luria-Bertani |
| LDM | Lund defined media |
| MW | molecular weight |
| NBT | nitro blue tetrazolium |
| Ni-NTA | nickel-nitrilotriacetic acid |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pI | isoelectric point |
| PMI | phosphomannose isomerase |
| PNGase F | peptide-*N*-(acetyl-β-glucosaminyl) asparagine amidase |
| RNA | ribonucleic acid |
| SCM | synthetic complete medium |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |

| | |
|---|---|
| SE | standard error |
| TAE | Tris-acetate-EDTA |
| TBS | Tris buffered saline |
| TBS-T | Tris buffered saline with Tween 20 |
| TCA | trichloroacetic acid |
| TFA | trifluoroacetic acid |
| tRNA | transfer ribonucleic acid |
| Tris | tris(hydroxymethyl)aminoethane |
| V | volts |
| WCE | whole cell extract |
| YPD | yeast peptone extract |

# 1 INTRODUCTION

## 1.1 Background

Clarity of white wine is an important property, but it can be ruined by the presence of grape proteins forming a haze. Pathogenesis-related proteins from grapes can slowly denature in bottles and aggregate, creating a fine haze, which resembles microbial spoilage (Waters et al. 2000).

At present, the most common method of haze prevention is to remove these proteins before bottling using bentonite, a clay that binds proteins and can then be separated from the wine. This method has some disadvantages: it is inconvenient and time-consuming. It also causes losses of wine and the resulting economic loss is estimated at approximately $50 million annually in Australia (Høj et al. 2000). Alternatives such as proteolysis, heat treatment, ultrafiltration and proanthocyanidins have been investigated, but so far they have not found widespread commercial use.

Several yeast mannoproteins have been identified as able to reduce protein haze in white wine (Waters et al. 1991; Ledoux et al. 1992; Moine-Ledoux and Dubourdieu 1999). Addition of these mannoproteins does not remove the wine proteins, but instead prevents the haze particles growing sufficiently large enough to be visible to the naked eye (Waters et al. 1993). Invertase has been identified as one of these protective mannoproteins (Ledoux et al. 1992; Moine-Ledoux and Dubourdieu 1999), while two more have since been named haze protective factor 1, or Hpf1 (Waters et al. 1994a), and haze protective factor 2, Hpf2 (Stockdale 2000). Initial studies of over-expression of Hpf1 and Hpf2 in *S. cerevisiae* indicated that Hpf2 yields were significantly higher than Hpf1 (Brown 2003).

This thesis focuses on the production of Hpf2 and the possible consequences of its application in the wine industry. Hpf2 is a highly mannosylated 180 kDa protein that has been shown to reduce protein haze in wines, and could provide an alternative to bentonite. By way of background information, haze and methods for its prevention, as well as characteristics of mannoproteins, their production, and the ways to assess their impact on wine sensory properties are briefly reviewed.

## 1.2 Haze

### 1.2.1 Types of haze

There are several types of hazes and deposits that can form in white wine. Microbial spoilage at the time of bottling can lead to microbial haze formation (Sponholz 1993). Other deposits can be formed by salt crystallisation, such as potassium bitartrate (Dunsford and Boulton 1981) and calcium tartrate (Clark et al. 1988). Polysaccharides and polyphenols can also contribute to haze formation (Siebert et al. 1996; Vernhet et al. 1996). Flavanol haze has also been observed in white wine, and this results from excessive levels of vine leaves crushed with berries during vinification (Somers and Ziemelis 1985). Protein haze is produced when proteins in the wine denature and aggregate, forming small particles of precipitated protein (Bayly and Berg 1967), with an example of a hazy wine shown in Figure 1-1.

Protein haze can also occur in fruit juices (Klavons and Bennett 1987; Siebert et al. 1996) and beer (Asano et al. 1982; Siebert et al. 1996). Haze formation in wine, beer and fruit juice may be explained by a single mechanism, and polyphenols are apparently involved in these hazes (Klavons and Bennett 1987; Siebert et al. 1996). The proteins involved in beer chill haze come mainly from malt hordein, rather than the yeast (Asano et al. 1982).

### 1.2.2 Wine proteins

Proteins in wine can come from the grapes, the yeast or other microorganisms involved in the fermentation. Typical concentrations of protein in wine vary from 50 to 100 mg $L^{-1}$ (Kock and Sajak 1959), but concentrations of up to several hundred mg $L^{-1}$ have been reported (Bayly and Berg 1967; Ferreira et al. 2002).

Yeast proteins do not contribute to protein haze (Bayly and Berg 1967), in fact, they provide a level of stability, that is they reduce the haze or clouding observed in wines (Moretti and Berg 1965). The unstable proteins found in wine are grape proteins that have survived the winemaking process. This has several implications: they are relatively stable, reasonably resistant to proteolysis, and are not denatured by the low

**Figure 1-1 Protein haze in white wine (left) compared to a clear wine (right)**

pH of wine which is typically around pH 3 - 3.5 (Waters et al. 1996). However, over time or with heat, they can break down and accumulate to form a haze. Hence these proteins are known as heat unstable proteins.

The total protein content of a wine does not appear to correlate with its heat stability (Moretti and Berg 1965; Bayly and Berg 1967). Correlations between the molecular weight (MW) and isoelectric points (pI) of proteins, and the pH of wine with the haze potential of the wine have been investigated. The heat stability of a wine is a measure of the susceptibility of a wine to develop a haze over time. It is generally accepted that low MW, low pI proteins contribute most to instability in wine (Moretti and Berg 1965; Bayly and Berg 1967; Mesrob et al. 1983). However, it has also been observed that high MW proteins (Lamikanra and Inyang 1988), or low MW, high pI proteins (Heatherbell et al. 1984) can lead to instability, indicating that this issue is not entirely understood.

The pH of a wine is related to the heat stability, and those wines with a low pH were more likely to be heat stable (Moretti and Berg 1965; Bayly and Berg 1967). Wines of lower pH are known to have generally lost more soluble protein during the winemaking process (Murphey et al. 1989).

Waters et al. (1992) discovered two major wine proteins, with MWs of 24 and 32 kDa, which caused protein haze in a wine heat test. It was shown that these proteins are resistant to proteolysis at a typical winemaking temperature of 15°C, and also that this is not due to association with phenols or glycosylation (Waters et al. 1995b). These nuisance proteins have been identified by amino acid analysis as the grape pathogenesis-related proteins thaumatin-like proteins and chitinases, which are present in the pulp and skin of grapes (Waters et al. 1996).

The most common method of haze prevention in white wine involves removal of the proteins from either the pre-fermentation grape juice or the wine. It is vital to consider whether the removal of these proteins affects the overall sensory properties of the wine. One of the most abundant proteins in mature berry extracts is the thaumatin-like protein VVTL1 (Tattersall et al. 1997), and this protein significantly contributes to haze (Waters et al. 1996; Peng et al. 1997). Thaumatins are a group of intensely sweet

proteins from the fruit of an African shrub, which is 10 000 times sweeter than sucrose, and the sweetness lingers on the palate even after rinsing the mouth with water (van der Wel and Loeve 1972). A study considered the taste properties of the thaumatin-like protein from wine and found that it is highly unlikely that it contributes to the sweetness of wine (Peng et al. 1997). Other indirect effects the wine proteins may have on wine aroma through interactions with aroma compounds or direct effects on wine mouthfeel have not yet been elucidated.

## 1.3 Methods of protein removal

### 1.3.1 Bentonite fining

Bentonite is used almost exclusively in Australian white wines to remove wine proteins and achieve protein stability of the wine prior to bottling (Rankine 1995). Bentonite is a montmorillonite clay (Rankine 1962), with a net negative charge and so it attracts the proteins in wine, which have positive charges at the low wine pH (Hsu and Heatherbell 1987; Ferreira et al. 2002). It is added to the wine in a process known as fining. The bentonite disperses in the wine, the proteins adsorb onto the bentonite, and then the complex settles (Blade and Boulton 1988).

Bentonite fining has several drawbacks. It is not specific for protein and so it has been postulated that it can remove other compounds from wine, such as esters and alcohols (Miller et al. 1985; Simpson 1986; Leske et al. 1995). This may contribute to the observed loss of aroma and flavour compounds associated with bentonite fining (Miller et al. 1985; Rankine 1995).

Bentonite also has poor settling characteristics and swells considerably when it is dissolved in wine (Rankine and Emerson 1963; Rankine 1995). This leads to long settling times of approximately a week (Leske et al. 1995) and losses of wine volume of up to 10% (Tattersall et al. 2001). Most of this volume can be recovered from the lees in a rotary drum filter, but a loss in quality results if oxidation occurs (Rankine 1995). The Australian wine industry suffers an effective loss of 3% of all white wine, or approximately 15 ML annually. The worldwide financial loss comes to more than

US$300-500 million annually (Høj et al. 2000). A further problem is the disposal of the waste, which contributes to the overall cost of bentonite use (Høj et al. 2000).

### 1.3.2 Possible alternatives to bentonite

Several alternatives are currently the focus of research. Suggested unit operations include ultrafiltration, heat treatment and proteolysis, as well as combinations of these techniques. Removal of proteins using chitin, proanthocyanidins and zinc oxide has also been considered.

Ultrafiltration has been considered as a protein removal method, and approximately 99% of wine or juice protein can be removed using a filter with a MW cut-off of 10 kDa (Flores et al. 1988). Between 3 and 20 mg $L^{-1}$ of protein remains in the wine permeate, but even this low concentration can result in protein instability of the wine (Hsu et al. 1987). This can then be removed with bentonite, reducing the bentonite requirement by 80-95% (Hsu et al. 1987). However ultrafiltration has some disadvantages. Chemical or microbiological contamination of the equipment must be avoided, and high set-up and operating costs are incurred (Miller et al. 1985). In addition, this method is also susceptible to oxidation, and minor losses of aroma compounds have been noted (Miller et al. 1985; Siebert et al. 1996).

Heat treatment of a wine can achieve protein stability, as it causes the denaturation and sedimentation of the potentially unstable wine proteins (Kock and Sajak 1959). The process speeds the aging and allows unstable proteins to be removed before bottling. Several studies have considered the effect of heating on protein stability and sensory properties (Francis et al. 1994; Pocock et al. 2003). Heating of Chardonnay or Semillon wines to 90°C for several minutes, then cooling rapidly, produced no discernible effect on the aroma of the wine, while heating these wines to 45°C for 20 days produced characteristics of bottle-aged wines, an accelerated aging (Francis et al. 1994). However, this study did not consider the stability of the wine. Pocock et al. 2003 heated a wine to 90°C for one minute followed by rapid cooling to achieve a reduction in protein levels of 10 to 50%. The protein levels decreased proportionally with the levels of sulfur dioxide present in the wine. However, heating has been perceived broadly by

the industry to be detrimental to the wine's flavour (Colagrande et al. 1994), and winemakers' attitudes to this practice are unlikely to be easily changed.

Proteolytic enzymes have been considered as a potentially attractive method for protein removal. However several studies demonstrated that they are infeasible in practice (Modra et al. 1989; Waters et al. 1989; Duncan 1992; Waters et al. 1992). Urlaub 1985 found that a mixture of protease, pectin enzyme and other enzymes, when heated in wine to 45-50°C, could be used to reduce the requirement for bentonite. Other studies have found proteases to have a stabilising effect on some types of wine at temperatures between 30 and 37°C (Bakalinsky and Boulton 1985; Lagace and Bisson 1990). Several commercial peptidases were shown to provide no heat stabilising effect on the wine tested (Modra et al. 1989; Duncan 1992). Although proteases are active in wine, they do not alter the protein composition or content significantly, nor do they affect the stability of the wine or the bentonite requirement when used at typical winemaking temperatures (Heatherbell et al. 1984; Waters et al. 1989). It was later discovered that this was due to the nature of the wine proteins. They are inherently resistant to proteolysis and survive through the harsh processing conditions of very low pH and high levels of ethanol (Waters et al. 1992; Ferreira et al. 2002). So while proteases theoretically seem a good candidate for removing proteins from wine, the nature of the wine proteins limits their usefulness. Yet recent observations of wines produced from *Botrytis cinerea*-infected fruit suggests that proteases from this grape vine fungus may be active against grape proteins (Marchal et al. 2006), and this is the focus of parallel work being conducted in this laboratory.

Combining heat treatment with proteases is another alternative. Several studies confirm that protease addition coupled with heating of the wine or juice may eliminate the need for bentonite fining, or may significantly reduce the amount required to stabilise the wine (Heatherbell et al. 1984; Bakalinsky and Boulton 1985; Urlaub 1985; Lagace and Bisson 1990; Duncan 1992; Dizy and Bisson 1999; Jones et al. 2005). Pocock et al. 2003 achieved a reduction of 30 to 60% in bentonite requirements with protein levels in the wine reduced to 40 to 80% of the original levels. However, as heat treatment is involved, winemakers' attitudes may prevent the use of this technology, or limit the uptake.

Vincenzi et al. 2005 found that chitin, a low-cost, abundant polymer, was able to reduce haze by approximately 80% in a white wine on a laboratory scale, removing about 30% of the grape proteins. Preliminary trials with chitin immobilised in a column suggested that this might be suitable for a continual process, with the column able to be regenerated periodically. However, no sensory studies were conducted, so it is unknown if the chitin treatment would affect the organoleptic properties of the wine.

Powers et al. 1988 considered immobilised grape proanthocyanidins as a potential adsorbent for protein removal. Proanthocyanidins were isolated from grape and immobilised on an agarose column, through which the wine was passed. This method stabilised the wine, but was limited by a significant reduction in the protein-binding capacity of the ligand after only a few regeneration cycles of the column. It appears no further work has examined this method in white wines, although some work has been performed with red wines (Ricardo-da-Silvo et al. 1991).

A recent study considered zirconia as an adsorbent material in a packed column (Pashova et al. 2004). The column was shown to be able to stabilise 50 column volumes of wine, but wine treated after this was unstable. The lower MW proteins, between 20 and 30 kDa, were found to be responsible for instability after 50 column volumes were treated. It was also shown that the column could be regenerated successfully, and so could be incorporated as a batch process. Further work on this has improved the process (Salazar et al. 2006), however, current Australian regulations do not allow these immobilised columns for wine stabilisation, as it is an ion exchange column, so the regulations would need to be modified to allow this technology to be used commercially as a wine processing aid.

## 1.4 Haze protective factors

### 1.4.1 Discovery

Haze protection factors were identified more than a decade ago at the Australian Wine Research Institute and independently at the University of Bordeaux (Waters et al. 1991; Ledoux et al. 1992). A carbohydrate-rich component of Muscat Gordo Blanco wine conferred stability on heated wine containing other haze inducing wine proteins (Waters

et al. 1991). This haze protective factor (HPF) was found to be a yeast mannoprotein (Dupin et al. 2000b) and may provide an alternative to protein removal from wine. It could also be employed in conjunction with the previous methods.

Several other mannoproteins provide a similar effect in wine. Wine aged on lees requires less bentonite to achieve stability, although the protein concentration is unchanged (Ledoux et al. 1992). Macromolecules from the yeast cell wall, later found to be invertase, provide a protective effect (Ledoux et al. 1992; Moine-Ledoux and Dubourdieu 1999). Interestingly, only a 31.8 kDa fragment of the yeast invertase, an N-glycosylated peptide, is necessary for the haze protection activity (Moine-Ledoux and Dubourdieu 1999). This fragment has been patented as Mannostab (Laffort Oenologie) as a method for tartrate and protein stabilisation of wines (Moine and Dubourdieu 1999).

Other glycoproteins have shown haze protective activity. These include wine arabinogalactan-protein (Waters et al. 1994b), gum arabic and apple arabinogalactan-proteins (Pellerin et al. 1994). One of the similarities between these glycoproteins is that they have all been shown to have a relatively high proportion of carbohydrate to protein (Waters et al. 2000).

### 1.4.2 Mannoproteins

Mannoproteins are a sub-set of glycoproteins – macromolecules consisting of a protein with carbohydrate groups attached as depicted in Figure 1-2. Glycoproteins are generally found in the cell membrane of yeast or are secreted (Elliot and Elliot 1997).

In mannoproteins, the carbohydrate groups are primarily mannose. The long carbohydrate chains are referred to as oligosaccharides. These are attached to either the hydroxyl group of serine or threonine, referred to as O-linked chains or O-glycosylation, or to the amide group of asparagine, called N-linked chains or N-glycosylation (Elliot and Elliot 1997). N-glycosylation occurs in the endoplasmic reticulum where a core oligosaccharide of 14 sugar units is initially attached. Extensions are added to the N-linked core in the Golgi. O-glycosylation also occurs in

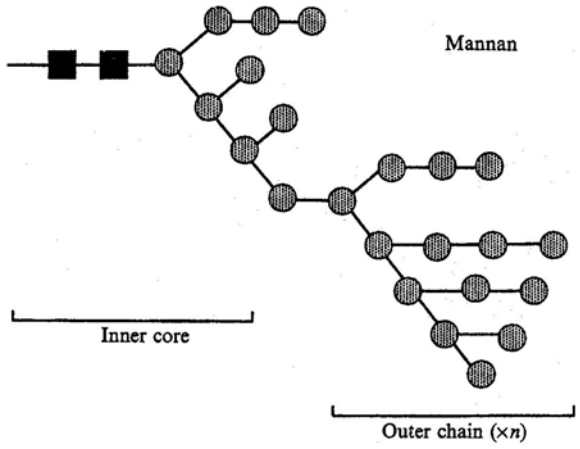


**Figure 1-2 A typical N-linked mannose side chain attached to a protein (Hadfield et al. 1993)**

the Golgi apparatus. The glycosylated protein is then directed to the cell wall. This is done with a signalling sequence, a short amino acid section at the beginning of the protein (Elliot and Elliot 1997).

### 1.4.3 Hpf1 and Hpf2

The mechanism of haze reduction by mannoprotein addition is attributed to lowering the size of the haze particles. Large additions of an HPF reduce the particle size to below 5μm, invisible to the naked eye (Waters et al. 1993). The HPF fraction first isolated was found to contain a large heavily glycosylated protein, with a polysaccharide component of 96% by weight, of which 70% was mannose and 30% glucose, and the protein component was dominated by serine and threonine (Waters et al. 1993).

The active principal of this first fraction, Hpf1, was purified from 600 L of Carignon noir wine (Waters et al. 1994a). Hpf1, with a MW of 420 kDa, consisted of 70% carbohydrate (98% mannose and 2% glucose) and 30% protein and was rich in serine, glycine, threonine and alanine. A second mannoprotein, Hpf2, was later isolated after fermentation of chemically defined grape juice (Stockdale 2000). Its MW was 180 kDa, made up of 75% mannose and 25% protein (Brown 2003). The HPFs were thought to be yeast mannoproteins, derived from the cell membrane, and this was confirmed through immunolocalisation (Dupin et al. 2000b).

Hpf1 contains both N- and O-linked mannose chains (Waters et al. 1994a). Hpf2 appears to also have both N- and O-linked mannose chains. Some of the N-linked chains were removed using PNGase F, an enzyme that cleaves N-linked mannose chains from the protein backbone, and the deglycosylated Hpf2 provided less haze protective activity than the fully glycosylated one, although the separation of the deglycosylated protein from the mannose chains was not complete (Stockdale 2000). Results for invertase were similar, suggesting that the mannose is important for the function (Stockdale 2000). This was repeated using Endoglycosidase H, a similar enzyme to PNGase F, to cleave the N-linked mannose chains from Hpf2, and similar results were obtained (Brown 2003). Removal of the O-linked chains would be possible using mild alkali in the presence of sodium borohydride, a process called β-elimination (Nakajima

and Ballou 1974). Further analysis of invertase has shown that only a fragment of invertase is necessary for activity, and this fragment consists of a short protein section that is N-glycosylated (Moine-Ledoux and Dubourdieu 1999).

As yet, studies have not considered the completely deglycosylated protein. Proteins expressed in *Escherichia coli* systems are synthesised without glycosylation, and may also be incorrectly folded (Elliot and Elliot 1997). If Hpf2 expressed in a bacterial system exhibited haze protection activity, then it could be concluded that glycosylation was unnecessary for the function. In addition, alternative yeast species could be considered, such as *Pichia pastoris*, which has been shown to glycosylate Hpf2 far less than that expressed in *S. cerevisiae*. The Hpf2 produced from over-expression in *P. pastoris* is just under half the MW of the *S. cerevisiae* protein at 83 kDa, and is approximately 50% protein and 50% mannose by weight (Tan 2005).

Further work has been undertaken to elucidate the mode of action of these HPFs but our understanding of the process, and the role of glycosylation, is far from complete. Studies were performed using invertase, as the HPFs are difficult to isolate in large quantities. Invertase reduced the visible haze by protein particle size reduction, and did not precipitate with the proteins, but remained soluble in the wine (Dupin et al. 2000a). A possible explanation for this is that invertase binds some other component of the wine that acts as a catalyst, or is required, for the protein precipitation. To date, similar work has not been conducted on Hpf1 or Hpf2.

## 1.5 Current method of Hpf2 production

A *S. cerevisiae* strain has been developed which over-expresses Hpf2, with an affinity tag of six consecutive histidine residues. The strain is referred to as SB59 and the protein as 6xHisHpf2 (Brown 2003). The over-expressed mannoprotein is secreted into the supernatant and the secreted proteins can be concentrated by ethanol precipitation. The 6xHis tag then allows easy purification of 6xHisHpf2 from the other proteins, by passing the mixture through a nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity column. The Ni-NTA matrix has a high affinity for the 6xHis tag of the mannoprotein (Qiagen 2003b). A block diagram of the process is given in Figure 1-3. This 6xHis tag

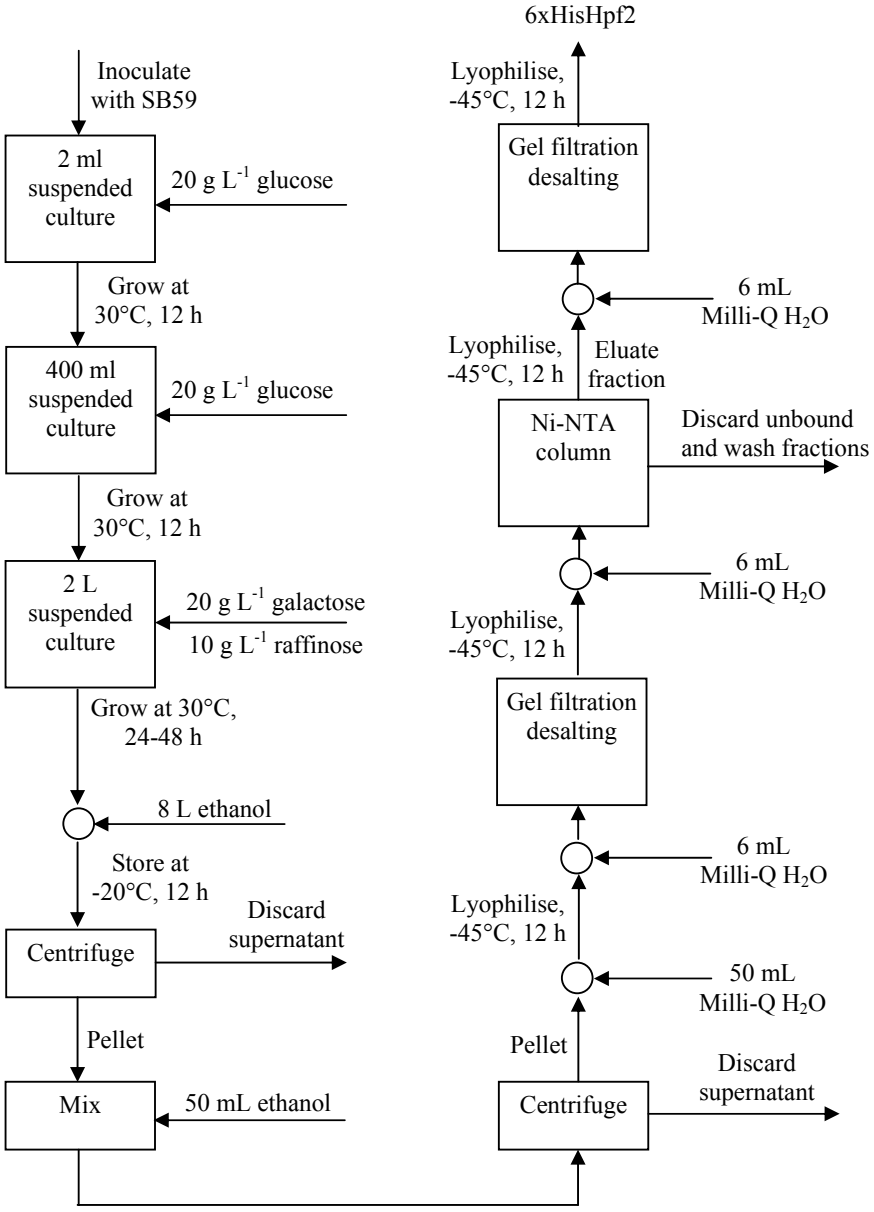


**Figure 1-3 Diagram of the current 6xHisHpf2 expression and purification method**

can be easily and efficiently removed using Factor Xa Protease thereby producing Hpf2 (Qiagen 2003a). However, removal of the tag seems unnecessary because the haze protection activity of the protein appears unaffected by this short tag (Brown 2003).

Expression of Hpf1 has also been attempted in a similar manner. Unfortunately purification on the Ni-NTA column of this protein has been unsuccessful. This is postulated as a consequence of the folding of the protein or the position of the mannose chains, making the 6xHis tag inaccessible to the nickel (Brown 2003).

6xHisHpf2 is currently expressed under the control of the *GAL1* promoter in its native yeast strain, and so the protein is likely to be correctly folded and glycosylated when expressed. The *GAL1* promoter is activated when galactose is present but repressed in the presence of glucose. The current method involves growing the yeast strain containing the 6xHisHpf2 construct in glucose to high cell concentrations, then adding galactose as the inducer and raffinose (a non-repressive sugar) as the carbohydrate source for the yeast (Brown 2003). Both galactose and raffinose are expensive, and this poses difficulties, as raw materials will incur a significant cost for the production of the large amount of 6xHisHpf2 required for sensory analysis.

These high cost raw materials could be avoided by changing the promoter for 6xHisHpf2. There are two types of promoters: constitutive and regulated. A gene with a constitutive promoter is expressed continuously and is used for the growth-associated production of industrially important proteins that are not toxic to the host cell and that are not susceptible to proteolysis. A gene with a regulated promoter is only expressed at certain times and under certain growth conditions. The *GAL1* promoter is an example of a regulated promoter, and is activated when galactose is present and glucose as absent. The strengths of promoters, that is, how much of their corresponding protein is produced, have been studied extensively (Cantwell et al. 1986; Park and Ramirez 1990; Hadfield et al. 1993; Nacken et al. 1996; Monfort et al. 1999; Park et al. 2000; Lim et al. 2002). The strongest constitutive promoters appear to be *ADH1*, *PGK1*, *TEF1*, *TDH* and *GPD*, and the strongest regulated promoters are *CUP1* and the *GAL* promoters. Regulated promoters are often chosen for expression of heterologous proteins for several reasons: yeast can be grown to high biomass with an inexpensive carbohydrate source, then switched to protein production; the continuous production of a protein may

retard the growth of plasmid-carrying cells and limit the maximum protein levels; and the protein may be toxic or detrimental to the cells (Mellor 1989; Marino 1991).

## 1.6 Process scale-up

A major aim of this project is to assess the sensory impact of Hpf2 on wine. To produce sufficient material for sensory analysis, the current process for production of 6xHisHpf2 requires significant improvement at both the expression and purification stages.

### 1.6.1 Fermentation and expression

#### *1.6.1.1 Fermentation of* E. coli

Microorganisms are commonly used for large-scale production of proteins in the biotechnology industry. *E. coli* is probably the most used organism, as it is quick and simple to genetically modify and grow rapidly and cheaply. However, it is yet to be determined whether Hpf2 expressed in *E. coli* will have haze protective activity.

To express Hpf2 in a bacterial host, the gene must be ligated into a bacterial expression vector, such as pET or pGEX. As in yeast, the promoter can be either regulated or constitutive, and a regulated promoter is generally chosen for stability of the clone. The recombinant vector is transformed into *E. coli* and then the protein can be expressed. The protein will be either secreted from the cell or aggregate within the cell to form an inclusion body. Protein purification is simpler when the protein has been secreted. This can be controlled by adding secretion signals to the protein, although this is not always advisable as it may affect the protein activity. If the protein forms an inclusion body, this can be isolated fairly cleanly from the other cell material after cell disruption, solubilised and then refolded (Scopes 1974). These methods are discussed later in further detail.

Optimisation of the growth conditions for the microorganism is the next step to large-scale production of a protein. This will include maximising cell growth and product expression. Physical and environmental variables need to be considered. Physical variables will include bioreactor design, while environmental factors consist of temperature, media selection, oxygen concentration, pH and concentration of the

inductor for the promoter (Hubbard 1987). This should lead to the optimal processing conditions for high recovery of the product, with adequate quality and minimum cost and effort.

#### *1.6.1.2 Fermentation of* S. cerevisiae

There are some problems producing eukaryote proteins in a prokaryote such as *E. coli*. The transcription, translation and post-translational processing differ in prokaryotes (Kingsman et al. 1988). Consequently *E. coli* is unable to incorporate disulfide bonds into proteins or to glycosylate them (Elliot and Elliot 1997). *E. coli* also contain toxic and pyrogenic cell-wall compounds, which may contaminate the final product, a problem for food or pharmaceutical products (Kingsman et al. 1988).

The use of *S. cerevisiae* can avoid these problems, as it is not pathogenic and is already widely used in the biotechnology, food and wine industries. This yeast has a secretion system similar to higher eukaryotes and there are now well-established methods for large-scale production of *Saccharomyces* species and their products (Kingsman et al. 1988; Park and Ramirez 1990). One problem associated with the fermentation of *S. cerevisiae* is that ethanol is produced in the presence of excess carbohydrate, even in the presence of oxygen (Collins 1990). This can be avoided by limiting the carbohydrate, and yields can be up to 0.5 g cells g carbohydrate$^{-1}$ (Collins 1990).

The cloning and optimisation issues discussed with respect to *E. coli* are generally also applicable to *S. cerevisiae*. The major difference is that Hpf2 is secreted when expressed in *S. cerevisiae*, whereas if it is expressed in *E. coli*, inclusion bodies are likely to form, which require solubilisation and refolding.

### 1.6.2 Downstream processing

There are four main steps in purification of a protein: recovery, separation, purification, and formulation. Each of these steps will involve at least one separation process. The first step involves removing the cells from the supernatant, generally using either filtration or centrifugation. If the protein is secreted by the cells, further processing of the supernatant is required. If the protein aggregates as inclusion bodies, the cells must be ruptured to release the inclusion bodies.

Disruption of the microorganisms can be done by either mechanical or non-mechanical means. Non-mechanical disruption can be divided into physical, chemical and enzymatic methods. For laboratory-scale purification, non-mechanical means are often employed, such as osmotic shock (physical), detergents or solvents (chemical) or lytic enzymes (enzymatic) (Middelberg 1992). Due to the cost of chemicals or enzymes, mechanical methods are usually applied to large-scale protein production, with the most common techniques being bead milling or homogenisation (Middelberg 1992).

Once the inclusion bodies have been released from the cells, they must be separated from the cell debris. This is typically done using differential sedimentation, a separation method based on density, or cross-flow filtration (Kotlarski 1998). The inclusion bodies are dissolved with a denaturant, either a mild detergent such as Triton or sodium dodecyl sulfate (SDS), or more commonly a chaotropic agent such as urea or guanidine hydrochloride. This step also allows further purification of the protein as contaminants associated with the inclusion bodies can then be separated with centrifugation. Refolding of the protein is undertaken by changing the solution to favour the adoption of the native state, which is generally the lowest energy configuration of the protein, such as reducing the concentration of urea. This has traditionally been done using empirical scale-up of laboratory-scale processes, although recently modelling and cost analysis have been used in an attempt to improve this process (Kotlarski 1998). Chaperonin proteins can also be added after removing the denaturant to help with the protein refolding (Scopes 1993).

The second step is the removal of materials with vastly different properties compared to the desired product, typically using filtration, adsorption or solvent extraction. Filtration is commonly used for large scale operations. It is a solid-liquid separation, where the liquid is forced through a solid support or a filter medium. Filtration can be performed batch-wise or on a continual basis, with the accumulating filter cake removed constantly (Belter et al. 1988).

The third step is a highly selective process for removing impurities with similar chemical and physical properties, using chromatography, electrophoresis or precipitation. Chromatography typically uses a packed column of adsorbent particles,

which are either solid, porous solid or gel (Belter et al. 1988). Methods of chromatography vary according to application, with the relevant differences being cost and selectivity. Selectivity refers to the product purity obtained from the column. For moderate scale operations, affinity chromatography is attractive due to the excellent selectivity, although the cost is excessive for large volumes. For large scale processes, ion exchange chromatography has good selectivity without being too expensive (Belter et al. 1988). This technique relies upon the different net charge on proteins at a given pH and the adsorbent interacts with the protein through electrostatic charge (Scopes 1993).

The final step is determined by the end use of the desired product. If the final product is to be a liquid, then filtration is often used. For a solid end product, crystallisation is the most common technique, often supported by drying, either lyophilisation or spray-drying (Belter et al. 1988). Crystallisation is often chosen due to the exceptional purity achieved, the uniform crystal shape that facilitates subsequent processing, and the appearance of the crystal, which has high customer approval (Belter et al. 1988).

### 1.6.3 Scale-up of the process

The economics of the process will strongly influence the synthesis of an optimal downstream process. The value of the product and acceptable product quality are paramount. The yield and purity of the desired product must be quantified at each stage. If alternative separation techniques are available for a stage, the costs should be compared (Belter et al. 1988).

Once the process for growth of the microorganism has been established, the method of purification of the protein and scale-up of this approach can begin as an iterative process. Scale-up is essentially an economic problem, with the 'best' method taken to be the cheapest one for an acceptable product quality. Scale-up involves the design of a large system based on knowledge of a smaller one, using a combination of experience, heuristics and modelling (Bjurstrom 1985).

## 1.7 Sensory analysis

To become a commercially acceptable product, it must first be shown that the addition of an active concentration of Hpf2 to wines do not alter the wine's sensory properties. Sensory analysis is defined as the identification, scientific measurement, analysis and interpretation of the properties or attributes of a product as they are perceived through the five senses of sight, smell, taste, touch and hearing (Carpenter et al. 2000).

Sensory analysis consists of three broad areas: discrimination, description and preference. Discrimination is concerned with whether a difference exists between products, and if so, how great the difference is. Description analysis is used to describe and measure any differences between products, such as whether one product is more bitter than another. Preference or acceptance analysis is applied to identify liking or acceptability of products, and asks whether one product is as good as another, or which product is preferable (Carpenter et al. 2000).

### 1.7.1 Sensory tests

Each area of sensory analysis has specific types of tests. Common discrimination tests include the duo-trio, the triangle, the paired comparison (difference) and the ranking tests. The duo-trio test is used to measure an unspecified difference between two products. A reference sample A and a pair of samples X and Y are provided, with either X or Y being the same product as A and the remainder being the other product. The assessor must decide whether X or Y is the same as A. The triangle test is similar, with three samples given to the assessor. Two of the samples will be one product and the third one will be the other product. The assessor must choose which sample is the odd or different one. The paired comparison (difference) test is used to determine if two samples differ in a specified characteristic. The samples are given in pairs and the assessor must choose which is, for example, more bitter (Lawless and Heymann 1998; Carpenter et al. 2000). For the ranking test, the assessor must rank between 3 and 5 samples in order of a specific characteristic, such as bitterness (Carpenter et al. 2000).

There are two main phases of descriptive tests: qualitative and quantitative. Attributes can initially be identified in the first phase and then assigned ratings in the second

phase. These two phases can be conducted by the same panel, and generally a trained panel is used. The first phase can be undertaken either collectively (consensus and descriptive profiling) or individually (free-choice profiling). The panel find words to describe the characteristics of the product, often referred to as free description. The second phase is conducted either collectively (consensus profiling) or individually (descriptive and free-choice profiling). The panel rates the product on the chosen characteristics, on an agreed scale (Carpenter et al. 2000).

Acceptance tests can be

- monadic – samples are presented one at a time;
- sequential monadic – samples are presented sequentially and assessed one at a time; and
- paired (preference) – samples are presented two at a time.

In the paired (preference) test, direct comparison is made by the assessor who asked whether there is a preference between the samples. It is considered good practice to repeat this test 24 hours later with the same assessor panel to establish the consistency of the assessors. This is called a repeat paired comparison (preference) test (Lawless and Heymann 1998; Carpenter et al. 2000).

### 1.7.2 Expected effects of Hpf2 on taste and aroma

The sensory effects of adding mannoproteins to wine have been studied, and it appears that mannoproteins do not make a significant difference. Will et al. 1991 considered that addition of 0, 0.6 and 1.5 g $L^{-1}$ of purified yeast mannoprotein to an ultrafiltered Riesling, representing extreme low, normal and extreme high concentrations in wine. No statistical difference was observed in sensory quality, using triangle and ranking tests. Also, there was no increase in the experimentally determined viscosity of the treated wine (Will et al. 1991).

The effect of Mannostab (Laffort Oenologie), the invertase fragment with haze protective activity, has also been studied (Rowe 2002). Sensory analysis of red and white wine showed no statistical significant difference between the treated and untreated wines, although the concentration used is not disclosed. As this fragment is

assumed to behave in a similar nature to Hpf2, it is expected that Hpf2 will have little or no effect on the sensory properties of wine.

Recent studies have examined how yeast derived mannoproteins interact with other wine compounds, particularly aroma compounds (Comuzzo et al. 2006; Chalier et al. 2007). Mannoproteins have been shown to change the volatility of wine aroma compounds, with low levels increasing the volatility of some esters, with the wine described as more flowery and fruity in descriptive analysis, while higher levels increased the fatty acid volatility, giving a more yeast, herbaceous and cheese-like aroma (Comuzzo et al. 2006). Chalier et al. 2007 considered four individual aroma compounds, with up to 150 mg $L^{-1}$ mannoprotein extract from two wine yeasts added. They found that isoamyl acetate was unaffected by the mannoprotein addition, while the volatilities of hexanol, ethyl hexanoate and β-ionone were reduced by up to 80%. These studies suggest that the addition of 200 mg $L^{-1}$ Hpf2 (an active concentration for haze reduction) to wine may affect the aroma volatility. However, only sensory studies with Hpf2 will be able to address this issue completely.

## 1.8 Attitudes to genetically modified organisms

A commonly used method to produce large quantities of proteins is to use genetically modified organisms (GMO). Pharmaceuticals and vaccines are already produced in this way and are generally accepted worldwide (Braun 2002). This is most likely due to the obvious benefit to the consumer from these products. However, attitudes to food vary in Europe and the US, Australia's two biggest wine export markets (Australian Wine and Brandy Corporation 2007). GM food is largely accepted in the US and this may be due to the public being unconcerned or unaware of the issue (Harlander 2002). Despite the generally positive view of science and technology in Europe, there is a negative view of GM food, with the public particularly concerned about choice and consequently labelling (Frewer 2003).

The situation in Australia is complex. Attitudes in Australia are changing, with more than half of the population now willing to buy GM food, compared to only one fifth six years ago, and up to 90% willing to eat it in processed foods (ACNielsen 2000; AAP 2006). Further studies predict that the GM foods most likely to be accepted in Australia

will need to have direct consumer benefits, have a gene modification within the organism, be seen to be safe to people and the environment, and be developed with some perceived consultation and regulation (Cormick 2005).

Globally, the main concern is seen as regulation of GM products, with an international regulator preferred, such as the World Health Organisation or the United Nations (Frewer 2003). The public perception of this new method of food production is not helped by other problems associated with modern food technology, such as the recent outbreaks of BSE and foot and mouth disease (Braun 2002). To overcome these negative perceptions of GM food, open and well-informed dialogue between scientists and the public will be necessary, with greater public involvement than at present (Braun 2002; Frewer 2003).

If large-scale production of Hpf2 is to occur, it is clear that the public are more likely to accept a 'natural' product, rather than a GM one. Several options could be considered, including:

- optimisation of the conditions for a wild type strain of *S. cerevisiae* so production of Hpf2 is maximised;
- screening all winemaking and brewing strains of yeast for a high expresser of Hpf2;
- investigation into mating and hybrids to find a strain that produces more Hpf2;
- use of lees to extract Hpf2; and
- chemical mutagenesis, then screen for a high expresser of Hpf2.

Previous work has shown that Hpf2 can be purified from yeast lees (Dupin et al. 2000b; Stockdale 2000), and other work has found that yeast cell wall mannoproteins are released from long storage periods on yeast lees after fermentation, particularly with mixing (Llaubères et al. 1987). Hpf1 has been shown to be involved in mating and expression is increased at this stage of the life cycle (Brown 2003).

However, all these methods have the disadvantage of being time-consuming and expensive. A GM production method would be more likely to be economically viable. Australian law allows GM foods to be sold, after thorough safety testing. Food must be labelled as GM food if there is more than 1% GM material in the product, or 0.1% if the

additive is a flavour (Food Standards Australia and New Zealand 2007). Recent developments in the European Union (EU) indicate that this method may be acceptable. Since the EU lifted its 5 year moratorium on GM foods, all food will have to be labelled as a GM product if it contains 0.9% or more GM material (Evans-Pritchard 2003). The amount of Hpf2 that will reduce the size of haze to acceptable levels is likely to be below 1 g $L^{-1}$, or 0.1% (Brown 2003). This would imply that although a GM protein was used in the wine processing, it would not have to be labelled as a GM product in either Australia or Europe.

There are already promising outcomes in the cheese industry. Cheese making requires rennet, a protein found in the stomach lining of calves. Approximately 60% of all cheese produced is made from chymosin, the active component of rennet produced from recombinant expression in *E. coli* (Flamm 1991). The US Food and Drug Administration first approved chymosin produced from *E. coli* in 1990, and this was followed by *Kluyveromyces marxianus* in 1992 and *Aspergillus niger* in 1993.

## 1.9 Summary

At present, there is no cheap, fast or efficient method to produce Hpf2 in sufficient quantities for primary research. Development of a technique to produce large quantities of Hpf2 can lead to sensory studies to determine its effect on wine. Whether this technique will use a yeast or bacterial expression system needs to be ascertained. If these studies show that Hpf2 is a commercially viable product, design of a commercial process for protein production would need be conducted in conjunction with feasibility studies.

The aims of this thesis are as follows:

- to establish a fermentation method to express large quantities of Hpf2 (Chapters 2 and 3);
- to find a robust quantification method to determine the levels of Hpf2 produced (Chapter 4);
- to establish a purification method to produce sufficient Hpf2 for sensory analysis (Chapter 4);

- to determine the sensory effect of active levels of Hpf2 in wine (Chapter 5); and
- to study the role of glycosylation on the haze prevention activity of Hpf2 (Chapter 6).

# 2 EXPRESSION OF 6XHIS-TAGGED HPF2 FROM *ESCHERICHIA COLI*

## 2.1 Introduction

A major aim of this project was to establish whether Hpf2 could be an acceptable alternative to bentonite as a means of preventing visible protein haze in white wine. An important aspect of this was to determine the sensory impact of Hpf2 at an active concentration in wine, which initial studies have shown to be approximately 200 mg of Hpf2 per litre of wine (Brown 2003). To undertake sensory studies, several litres of wine are required, and to treat this quantity of wine, several grams of Hpf2 are required. The initial studies used the *S. cerevisiae* strain S288c over-expressing 6xHisHpf2 under the control of the *GAL1* promoter on the pYES2/GS plasmid. The yield of 6xHisHpf2 from *S. cerevisiae* using the previously established method is approximately 5 mg $L^{-1}$ of culture, so approximately 1000 L would be required to produce sufficient 6xHisHpf2 for sensory studies. This is an unreasonable volume for a preliminary study, considering the yeast is a GMO and work would have to be performed in a strictly controlled environment. Also a large amount of raffinose would be required as the carbon source during the expression of the protein: if the previously established method was used, approximately 10 kg of raffinose would be needed, costing approximately $20 000 (Sigma), and this cost could not be justified for a preliminary study.

Two options were studied: expression of 6xHisHpf2 from *E. coli*, an organism that is well established for its ease of genetic modification and high expression levels of recombinant protein, as discussed in this chapter, or improvement of the fermentation and processing conditions to obtain higher expression levels of 6xHisHpf2 from *S. cerevisiae*, as discussed later in the thesis.

*E. coli* strains are often chosen for initial protein expression studies, as they have a well understood physiology and relatively simple transcription and translation system. *E. coli* systems are also efficient and cost-effective, and high-level production is well-established. High yields of grams per litre of heterologous protein have been reported (Kannan et al. 1995; Peng et al. 2004), and manipulation of the host-plasmid system and

expression conditions can lead to soluble, functional protein (Cabilly 1989; Georgiou and Valax 1996; Ghosh et al. 2004).

It is important to consider that Hpf2 will not be glycosylated when expressed in a prokaryote system and it has not yet been established whether glycosylation is required for its activity. Expression of Hpf2 in a prokaryote has both the advantage that it allows further research into the role of the glycosylation on the activity, but also a disadvantage that this route may ultimately lead to the expression of a non-functional protein that cannot be used for sensory studies or commercialisation.

Other methods of Hpf2 production that could be considered included using *S. cerevisiae* with a different promoter for the recombinant expression, such as *CUP1*, as this eliminates the high cost associated with raffinose in the *GAL1* system, or using a different yeast species, such as *Pichia pastoris* which is well-documented as expressing high levels of recombinant protein. Expression of 6xHisHpf2 in *P. pastoris* would result in a different glycosylation pattern, leading to further information about the function of glycosylation in the activity of Hpf2.

## 2.2 Materials and methods

### 2.2.1 Molecular biology techniques

#### *2.2.1.1 Preparation of chromosomal DNA from yeast*

Yeast chromosomal DNA was isolated following the method of Ausubel et al. (1994), using breaking buffer (Appendix 1) and glass beads to break open the cells, except cells were shaken using a Mini-Beadbeater 8 (Biospec Products), instead of vortexing.

#### *2.2.1.2 Enzyme treatment of DNA*

Restriction enzymes were sourced from Roche Biochemicals and were used according to the manufacturer's instructions. Briefly, this involved diluting 10x restriction enzyme buffer stock (supplied with the enzyme) (Ausubel et al. 1994) to a final concentration of 1x in the DNA sample to be digested. The enzyme was added and the digest was incubated at 37$^{o}$C for between 1 and 2 h (Ausubel et al. 1994).

Plasmid and insert DNA (1:3) were ligated using T4 DNA ligase (Roche Biochemicals). A 10x ligase buffer stock was diluted to 1x in a mixture of the DNA samples and water. The enzyme (20 U $\mu g^{-1}$ DNA) was added to a final volume of 10 µL. The ligation mix was incubated at 16°C for 16 h (Ausubel et al. 1994).

#### *2.2.1.3 Competent* E. coli

Competent DH5α, BL21(DE3) and BL21(DE3)pLysS *E. coli* cells were prepared following the method of (Inoue et al. 1990). Competent *E. coli* DH5α were used for propagation of plasmids. Transformation of *E. coli* was performed following the calcium chloride method of Inoue et al. (1990).

#### *2.2.1.4 Isolation of plasmid DNA from* E. coli

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Sambrook and Russell (2001) or using the QiaPrep Spin Miniprep Kit (Qiagen).

### 2.2.2 Amplification of the *HPF2* gene and cloning into pETBlue-1

The *HPF2* gene was amplified from the genome of a haploid strain of S288c with the primers JM1 and JM2 (Appendix 2). Polymerase chain reaction (PCR) mixes (20 µL) contained 1x strength Expand High Fidelity amplification buffer (supplied with Expand High Fidelity DNA polymerase), deoxyribonucleotide triphosphate (dNTPs) (300 µM of each), primers (0.3 µM of each), $MgCl_2$ (2 mM), Expand High Fidelity DNA polymerase (Roche) (1.0 U) and template DNA (10 ng). Expand High Fidelity DNA polymerase possesses proofreading 3' to 5' exonuclease activity and provides high fidelity amplification. The thermal cycler was programmed as follows: denaturation of DNA at 94°C for 3 min followed by 30 cycles of denaturation of DNA at 94°C for 1 min, annealing of primers at 54°C for 1 min and extension of DNA at 72°C for 1.5 min, followed by a final extension time of 10 min at 72°C.

To confirm that the PCR had amplified the correct region containing the *HPF2* gene, 1 µL of the mix was run on a 1.0% agarose gel (Appendix 1) whereafter fragments were visualised and their sizes determined with reference to standards. The remainder of the PCR product was run on a 0.8% agarose gel and the fragment was gel purified using a QIAquick Gel Extraction Kit (Qiagen).

The plasmid pETBlue-1 (Novagen) was supplied pre-linearised and was used directly. The plasmid and insert DNA were mixed at a 1:3 ratio and ligated using T4 DNA ligase (Roche) at 16$^{o}$C for 16 h. The ligation product was transformed into competent *E. coli* and transformants were selected on ampicillin. Plasmid DNA was prepared from the transformants by the method described in Section 2.2.1.4 and this DNA was digested with restriction enzymes to identify correct clones. The resultant plasmid was named pETBlue6xHisHpf2.

To ensure that no amplification artefacts had been introduced by the Expand High Fidelity DNA polymerase, the plasmid DNA was sequenced at the Australian Genome Research Facility (Brisbane, Australia) using ABI BigDye Terminator chemistry. The primers used for sequencing are listed in Appendix 2. Purified plasmid DNA was transformed into BL21(DE3) and BL21(DE3)pLysS.

### 2.2.3 Fermentation in LB, M9 and 2xYT media

Culturing and expression of *E. coli* were done in Luria-Bertani (LB), M9 or 2xYT media (Appendix 1). The media were supplemented with 50 mg $L^{-1}$ ampicillin (Sigma) for transformants harbouring the pETBlue6xHisHpf2 plasmid, and 32 mg $L^{-1}$ chloramphenicol (Sigma) for those harbouring the pLysS plasmid.

### 2.2.4 Over-expression of 6xHisHpf2 in BL21(DE3) and BL21(DE3)pLysS

Transformed cultures were grown overnight in approximately 1 mL of LB supplemented with ampicillin at 37°C. The pellet from this culture was resuspended in fresh LB supplemented with ampicillin and grown at either 30°C or 37°C to an optical density at 600 nm ($OD_{600}$) of between 0.4 and 0.8. Samples were taken prior to induction as negative uninduced controls. Induction was performed by the addition of between 0.05 and 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma). Expression was carried out at temperatures between 4°C and 37°C, with samples generally taken at 3 h, but up to 24 h. Samples were centrifuged (16 100 g, 2 min, 22°C), the supernatant removed, and the pellet stored at -20°C until analysed by Western blot for protein expression.

### 2.2.5 Preparation of soluble fractions from whole cell extracts

Two solubilisation methods were used. The major one was using B-PER Bacterial Protein Extraction Reagent (Pierce) according to the manufacturer's instructions, with the addition of protease inhibitor cocktail and DNase. Briefly, the cell pellet from 10 mL of culture was freeze-thawed then solubilised by the addition of 1 mL B-PER (Pierce), 1% protein inhibitor cocktail (Sigma) and 2 μL DNase (Sigma). The mix was incubated at 10 min at room temperature with agitation, then soluble protein was separated from insoluble protein by centrifugation (16 100 g, 15 min, 4°C).

The second method was sonication. The cell pellets were freeze-thawed then dissolved in 500 μL of 20 mM Tris–HCl, pH 7.9, and lysed by ultrasonication in a UP400S instrument (Dr. Hielscher GmbH) at 60% amplitude, cycle 0.5 for 90 s. Soluble protein was separated from insoluble protein by centrifugation (16 100 g, 15 min, 4°C).

### 2.2.6 Purification of 6xHisHpf using immobilised metal affinity chromatography under denaturing conditions

The Nickel-Nitrilotriacetic acid (Ni-NTA) immobilised metal affinity chromatography (IMAC) resin (Qiagen) was used for denaturing purification. The cell samples were prepared according to Qiagen 2003b. The cell pellets were thawed on ice for 15 min then resuspended in lysis buffer (Appendix 1) at 5 mL $g^{-1}$ wet weight. The cells were incubated at room temperature for 1 h with shaking to lyse the cells, then soluble protein was separated from insoluble material by centrifugation (16 100 g, 30 min, 22°C). Lysate (4 mL) was added to 50% Ni-NTA slurry (1 mL), which had been equilibrated with equilibrium buffer (Appendix 1), and mixed gently at room temperature for 1 h. The resin-lysate mix was added to an empty column and the flow-through collected. The column was washed twice with wash buffer (4 mL) (Appendix 1) and each fraction was collected. The 6xHis protein was eluted with 3x 1 mL elution buffer (Appendix 1).

### 2.2.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were made up to 1x SDS-PAGE sample buffer using 5x stock (Appendix 1), mixed briefly and boiled for 5 min. Whole cell extracts were prepared by resuspending the cell pellet in an equal volume of 2x SDS-PAGE sample buffer. Equivalent culture volume samples were prepared in order to compare yields of protein per volume. After boiling the samples were vortexed at high speed followed by briefly centrifuging. Samples were allowed to cool to room temperature before loading on the gel. SDS-PAGE was performed as described by Ausubel et al. (1994). Tris-glycine polyacrylamide gels (4-20% gradient) were sourced from Gradipore or Bio-Rad. Gels were run in SDS-glycine running buffer (Appendix 1) at 100 V (constant voltage), until the dye front had run to the end of the gel. Gels were either Coomassie blue stained or prepared for transfer on to nitrocellulose for Western blotting.

### 2.2.8 Coomassie blue staining for detection of proteins on SDS-PAGE gels

The gels were incubated in Coomassie blue stain (Appendix 1) for approximately 1 h. The stain was discarded and the gel was destained (Appendix 1). The destain was changed several times until the background gel colour was reduced sufficiently to detect bands.

### 2.2.9 Transfer of proteins to nitrocellulose membrane

Wet transfer of proteins on to nitrocellulose membrane (BA85, Schleicher and Schuell) was done using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell following the manufacturer's instructions. Immediately after the SDS-PAGE gel had finished running, the gel was equilibrated in cold transfer buffer (Appendix 1) for 5 min. The fibre pads (Bio-Rad), filter paper (GB002, Schleicher and Schuell) and nitrocellulose were also soaked in transfer buffer for 15 min. The transfer cassette was assembled as described by the manufacturer. Transfer was conducted at 100 V (constant voltage) for 1 h in transfer buffer.

### 2.2.10 Western blot analysis

After transfer, the membrane was blocked in blocking buffer (Appendix 1) either for 1 h at room temperature with gentle rocking, or overnight at 4°C. This was followed by a rinse and three 10 min washes in Tris-buffered saline with Tween 20 (TBS-T) (Appendix 1). The blot was probed with the primary antibody, mouse anti-6xHis (Sigma), diluted 1:3 000 in blocking buffer for at least 1 h at room temperature. The membrane was briefly rinsed and washed for 10 min in TBS-T three times. The secondary antibody, goat anti-mouse conjugated to alkaline phosphatase (Promega), was diluted 1:7 500 in blocking buffer and the membrane was incubated for at least 1 h at room temperature. The membrane was rinsed in Milli-Q $H_2O$. To develop the blot, it was incubated in the presence of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (prepared as suggested by Promega) in alkaline phosphate buffer (Appendix 1) until bands appeared. The membrane was washed in Milli-Q $H_2O$ and gently blotted with filter paper and allowed to dry.

## 2.3 Results

### 2.3.1 Plasmid construction

The yeast Hpf2 has a putative N-terminal signal sequence and a C-terminal GPI anchor, which are assumed to be removed in the mature protein. To compare directly with this protein, the bacterial protein was constructed based on the putative mature amino acid sequence, without the signal sequence or GPI anchor.

The expression vector pETBlue6xHisHpf2 was constructed successfully as determined by sequencing, and is shown in Figure 2-1. The fusion protein will be 408 amino acids with a theoretical MW of 42.4 kDa and pI of 9.25.

### 2.3.2 Transformation efficiency and expression in BL21(DE3) and BL21(DE3)pLysS

The pETBlue6xHisHpf2 plasmid was transformed into the BL21(DE3) strain. The plasmid constructed with the insert orientated in the incorrect direction

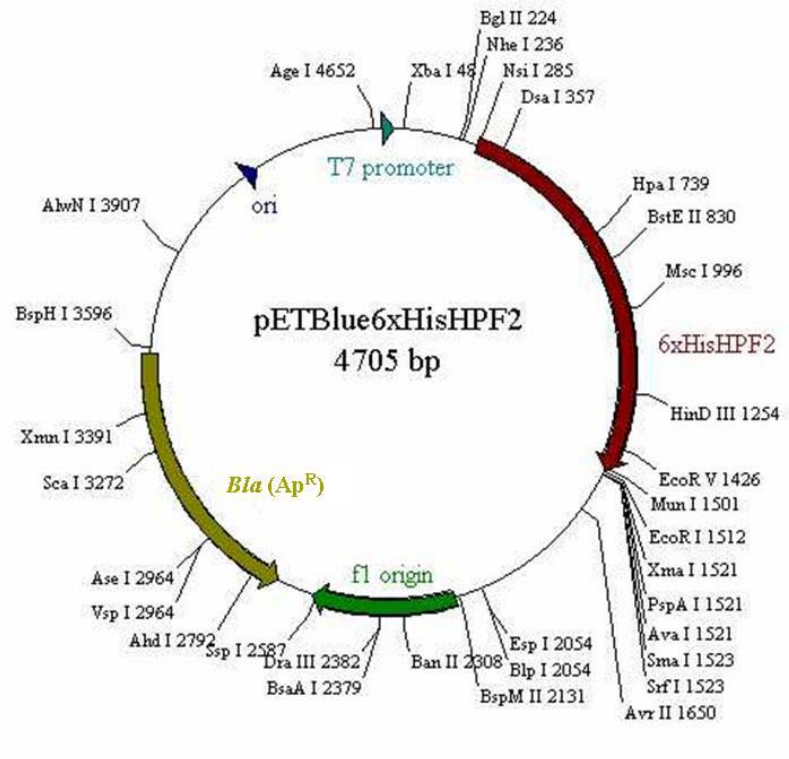


**Figure 2-1 Plasmid map of pETBlue6xHisHpf2, with T7 promoter and ampicillin resistance (ApR)**

(pETBlue6xHisHpf2back) was used as a control and a non-expressing plasmid conferring ampicillin resistance was used as a positive control. This was attempted a number of times with equal concentrations of plasmid, with the efficiency of transformation of the pETBlue6xHisHpf2 plasmid very low: in a typical experiment, 28 transformants from the pETBlue6xHisHpf2back plasmid were obtained compared with none for the pETBlue6xHisHpf2 plasmid. In another experiment, over 100 transformants were generated for the non-expressing plasmid compared to none for the pETBlue6xHisHpf2 plasmid. Figure 2-2 shows an agarose gel of both the uncut plasmids, which was used to confirm the orientation of the 6xHisHpf2 insert. The expected sizes for digestion with HindIII and XbaI of pETBlue-1 are 1206 and 3499 base pairs (bp) with 6xHisHpf2 in the correct orientation, and 484 and 4221 bp with 6xHisHpf2 in the incorrect orientation.

The pETBlue6xHisHpf2 plasmid was transformed into the BL21(DE3)pLysS strain. Several transformants were picked and expression was tested at 30°C for 1 h in LB medium supplemented with 50 mg $L^{-1}$ ampicillin. Expression levels from the equivalent of 300 μL of whole cell extract were analysed by Western blot and expression was not detected in any of the transformants tested (data not shown).

Increasing the time of incubation at 37°C with LB after the heat shock to 3 h resulted in several BL21(DE3)pETBlue6xHisHpf2 transformants. These were analysed for expression by Western blot as described previously, and shown in lanes 3 to 10 of Figure 2-3.

Transformant B had the highest expression level after 3 h, transformant C had slightly lower expression, while transformant A had negligible expression. The expression level after 6 h was lower for both transformant B and C, suggesting that the heterologous protein is being degraded by the host cell. One of the BL21(DE3)pLysS transformants is shown in lane 11 of Figure 2-3, with negligible 6xHisHpf2 expressed. Yin et al. (2003) reported similar differences in expression levels of particular proteins in these two strains.

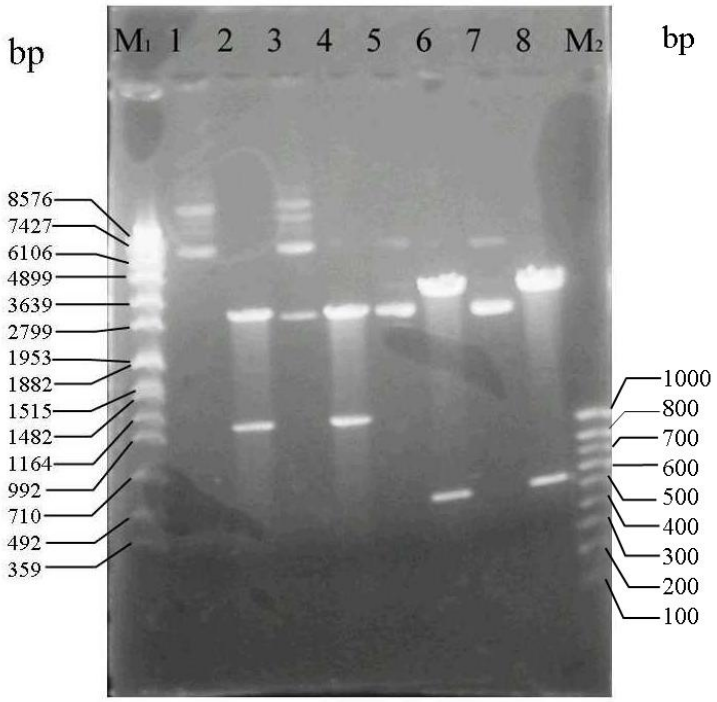


**Figure 2-2 Plasmid DNA and restriction enzyme digest of two pETBlue6xHisHpf2 plasmids and two pETBlue6xHisHpf2back plasmids. M1, MW marker VII, 4 µl (Roche); 1, uncut pETBlue6xHisHpf2 plasmid; 2, pETBlue6xHisHpf2 digested with HindIII and XbaI; 3, uncut pETBlue6xHisHpf2 plasmid; 4, pETBlue6xHisHpf2 digested with HindIII and XbaI; 5, uncut pETBlue6xHisHpf2back plasmid; 6, pETBlue6xHisHpf2back digested with HindIII and XbaI; 7, uncut pETBlue6xHisHpf2back plasmid; 8, pETBlue6xHisHpf2back digested with HindIII and XbaI; M2, MW marker XIV, 4 µL (Roche). 1% agarose was used.**

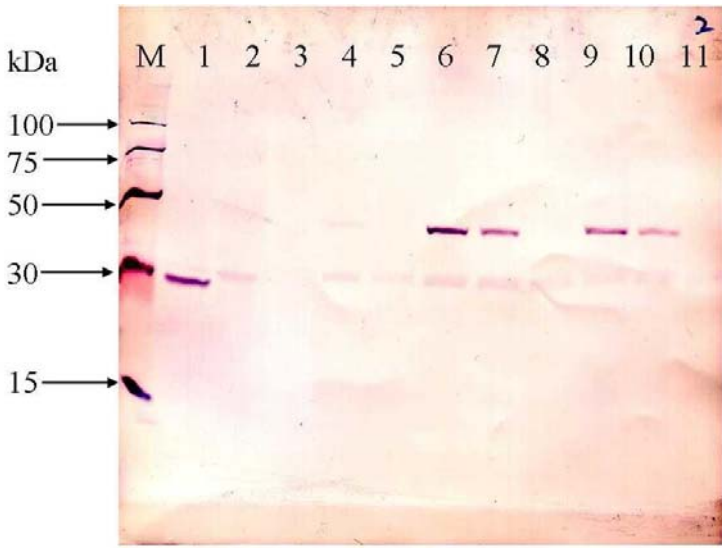


**Figure 2-3 Comparison of expression levels in transformants of BL21(DE3) and BL21(DE3)pLysS. M, Colour marker, 5 µL (Sigma) and 6xHis ladder, 2.5 µL (Qiagen); 1, 3 µg of ~28 kDa 6xHis-tagged protein (positive control); 2, untransformed BL21(DE3), 6 h expression, whole cell extract (WCE); 3, BL21(DE3) transformant A uninduced, WCE; 4, BL21(DE3) transformant B uninduced, WCE; 5, BL21(DE3) transformant A, 3 h expression, WCE; 6, BL21(DE3) transformant B, 3 h expression, WCE; 7, BL21(DE3) transformant C, 3 h expression, WCE; 8, BL21(DE3) transformant A, 6 h expression, WCE; 9, BL21(DE3) transformant B, 6 h expression, WCE; 10, BL21(DE3) transformant C, 6 h expression, WCE; 11, BL21(DE3)pLysS transformant, 6 h expression, WCE. Lanes 2-11 - equivalent of 300 µL culture, all grown to $OD_{600}$~0.6 then induced with 0.1 mM IPTG. Growth and expression were at 30°C.**

### 2.3.3 Expression using standard conditions for maximum protein levels

BL21(DE3) transformant B was chosen for further study, as it had the highest expression level. This is now referred to as BL21(DE3)p6xHisHpf2. A time course of expression was done at 37°C, 0.1 mM IPTG in LB and 50 mg $L^{-1}$ ampicillin. The results of this are shown in a Western blot in Figure 2-4. This suggests degradation of the heterologous protein with time. If the newly synthesised polypeptide is recognised by the proteolytic machinery of the cell, the net or steady-state accumulation of the protein will be very low and can decrease with time.

There was not a large difference in expressed protein between the uninduced control and the highest level of 6xHisHpf2 expression as determined by Western blot, in lanes 4 and 5 of Figure 2-4. This lack of differentiation was more clearly seen in the Coomassie stain comparing the whole cell extract from an uninduced and induced culture of BL21(DE3)6xHisHpf2, shown in Figure 2-5. The T7 promoter generally leads to very high expression level, so the heterologous protein can be detected easily in the induced whole cell extract compared to an uninduced or untransformed control by Coomassie stain (Kannan et al. 1995; Urban et al. 2003; Peng et al. 2004). In this case, the expression level was very low, as there were no obvious bands seen in the induced whole cell extract (lane 2) that were not present in the untransformed whole cell extract (lane 1).

### 2.3.4 Purification of insoluble material using Ni-NTA

Purification of 6xHisHpf2 was attempted using a denaturing method of IMAC, as described in Section 2.2.6 and the results are shown in Figure 2-6. Most of the protein was extracted from the cell pellet using the lysis buffer (lane 3), which contains 8 M urea, although most of the protein did not bind the Ni-NTA resin and is seen in the flow-through, lane 4. The first wash fraction contained the rest of the 6xHis protein. This suggests that 6xHisHpf2 cannot be purified with this method.

A second IMAC, TALON (BD Biosciences Clontech) which is a cobalt-based resin, was assessed for its ability to bind the unglycosylated 6xHisHpf2 expressed by *E. coli*

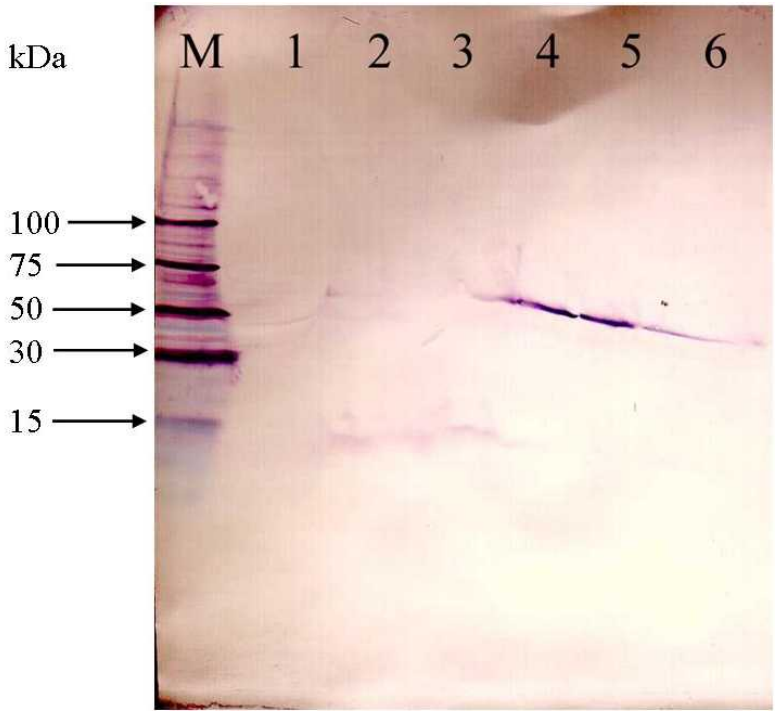


**Figure 2-4 Time course of expression of 6xHisHpf2 from BL21(DE3)p6xHisHpf2. M, Colour marker, 5 μL (Sigma) and 6xHis ladder, 2.5 μL (Qiagen); 1, BL21(DE3)p6xHisHpf2 uninduced, WCE; 2, BL21(DE3)p6xHisHpf2, induced, 1 h expression, WCE; 3, BL21(DE3)p6xHisHpf2, induced, 2 h expression, WCE; 4, BL21(DE3)p6xHisHpf2, induced, 3 h expression, WCE; 5, BL21(DE3)p6xHisHpf2, induced, 4 h expression, WCE; 6, BL21(DE3)p6xHisHpf2, induced, 5 h expression, WCE. Lanes 2-6 - equivalent of 400 μL culture, all grown to $OD_{600}$ of ~0.6 then induced with 0.1 mM IPTG.**

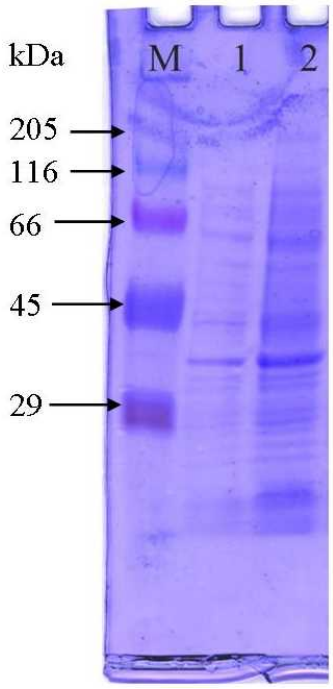


**Figure 2-5 Coomassie stain of expression of 6xHisHpf2 from BL21(DE3)p6xHisHpf2. M, Colour marker, 5 μL (Sigma); 1, uninduced BL21(DE3)p6xHisHpf2, WCE; 2, BL21(DE3)p6xHisHpf2 induced with 0.1 mM IPTG, 3 h of expression, WCE. Both lanes have the equivilant of 300 μL of culture loaded.**

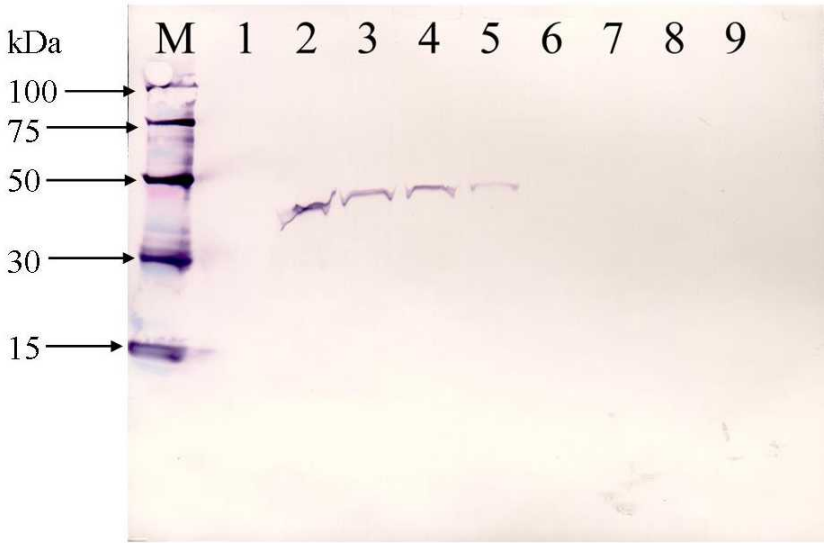


**Figure 2-6 Purification of 6xHisHpf2 using a denaturing method. M, Colour marker, 5 μL (Sigma) and 6xHis ladder, 2.5 μL (Qiagen); 1, untransformed BL21(DE3), WCE; 2 BL21(DE3)p6xHisHpf2, induced with 0.1 mM IPTG, expressed at 30°C for 3 h, WCE; 3, lysate; 4, unbound fraction; 5, Wash fraction 1; 6, Wash fraction 2; 7, Eluate fraction 1; 8, Eluate fraction 2; 9, Eluate fraction 3. All fractions are equivalent to 250 μL of culture.**

during this study, according to the manufacturer's purification method. 6xHisHpf2 did not bind to TALON (data not shown), suggesting that the 6xHis tag may be inaccessible in the *E. coli*-expresed protein. This is consistent with previous observations that neither the glycosylated nor the partially deglycosylated yeast 6xHisHpf2 bind TALON resin (Brown 2003).

### 2.3.5 Investigation into increasing the solubility of 6xHisHpf2

#### *2.3.5.1 Reduction in temperature or IPTG concentration do not increase soluble expression*

Prior attempts to express 6xHisHpf2 had resulted in insoluble material which could not easily be purified by Ni-NTA, so an attempt was made to express 6xHisHpf2 in a soluble form. Proteins expressed in a soluble form are more likely to have activity than insoluble, refolded proteins after purification (Georgiou and Valax 1996; Ghosh et al. 2004). A commonly used method to increase the solubility of an over-expressed heterologous protein is to decrease the growth temperature (Schein and Noteborn 1988; Moore et al. 1993; Fang and Ewald 2004). This leads to a slower growth and thus expression rate, allowing the cellular machinery a longer time to fold the proteins correctly.

BL21(DE3)p6xHisHpf2 was grown at 30°C to an $OD_{600}$ of approximately 0.5. IPTG was added to a final concentration of 0.1 mM, and the culture was separated and placed at a range of temperatures. Temperatures from 4°C to 37°C were considered and the results are shown in a Western blot in Figure 2-7. After 3 h of expression, the cell pellets were prepared using the B-PER method. The soluble fractions from all temperatures are all negligible. Similar results were observed using sonication to obtain the soluble fraction (data not shown).

Increasing the time of induction at lower temperatures lead to a decrease in the expression level, which is consistent with the likely degradation of the 6xHisHpf2 seen previously. Also, lowering the temperature did not lead to any soluble 6xHisHpf2 (data not shown).


**Figure 2-7 Decrease in temperature does not increase the soluble fraction of 6xHisHpf2 expressed. M, Colour marker, 5 μL (Sigma) and 6xHis ladder, 2.5 μL (Qiagen); 1, uninduced BL21(DE3)p6xHisHpf2; 2, WCE of BL21(DE3)p6xHisHpf2, expression at 4°C; 3, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 4°C; 4, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C; 5, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 16°C; 6, WCE of BL21(DE3)p6xHisHpf2, expression at 22°C; 7, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 22°C; 8, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C; 9, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 30°C; 10, WCE of BL21(DE3)p6xHisHpf2, expression at 37°C; 11, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 37°C. All induced fractions were induced with 0.1 mM IPTG with 3 h expression time, and the equivalent of 300 μL of culture was loaded.**

This experiment was repeated several times, and another example of the results is shown in Figure 2-8, and no expression was seen at 30°C or 37°C. This demonstrates the variability of expression from this transformant, which was seen through the course of this project.

Both Figure 2-7 and Figure 2-8 show additional protein bands with slightly lower MW than 6xHisHpf2 in some lanes. This agrees with the observation of degradation of the heterologous protein by the *E. coli*, seen in Figure 2-4.

Figure 2-9 shows an example of an experiment that resulted in soluble expression of 6xHisHpf2. The culture was grown and expressed at 30°C, lower than the optimum 37°C for *E. coli*, induced with 0.1 mM IPTG and samples taken after 3 h of induction. The cell pellet was solubilised by sonication. However, this result could not be replicated, as the next time this was attempted, no expression was seen, even in the whole cell extract. This again indicates the instability and variability of this transformant.

Reducing the IPTG concentration has also been shown to increase the soluble yield of recombinant protein from *E. coli* (Urban et al. 2003; Turner et al. 2005). IPTG was varied from 0.05 to 1 mM, and negligible soluble heterologous protein was detected by Western blot, indicating that this method will not yield soluble protein (data not shown).

*2.3.5.2 Minimal and rich media do not lead to an increase in soluble 6xHisHpf2*

Another method that has been shown to influence the solubility of the over-expressed protein is modifying the growth media. Different media and expression times were considered. M9 media is a minimal mineral media, which has been shown to increase the soluble expression of some heterologous proteins (Cabilly 1989; Galindo et al. 1990; Georgiou and Valax 1996). 6xHisHpf2 was expressed in M9 media at 4, 22 and 30°C. Samples were taken at various times after induction with 0.1 mM IPTG. Figure 2-10 shows a Western blot of the expression levels of the whole cell extracts as well as the soluble fraction obtained by the B-PER method. Although some expression was seen in the whole cell extract for most of these growth conditions, there was negligible

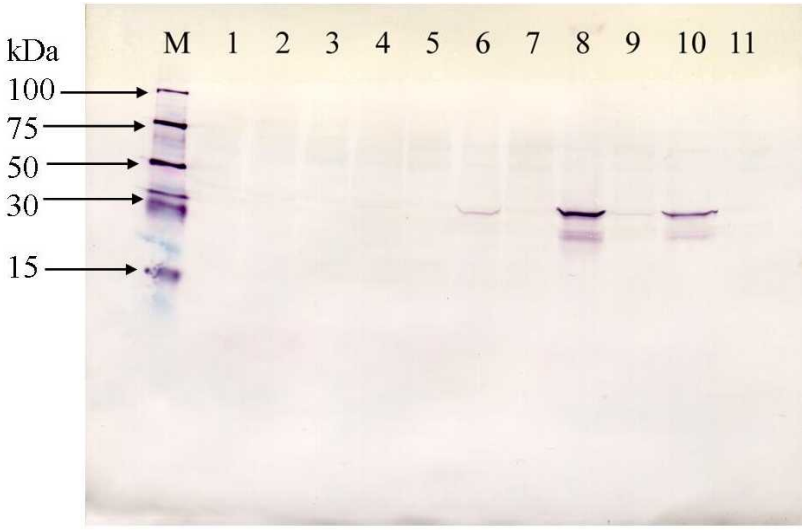


**Figure 2-8 Variability of expression levels. M, Colour marker, 5 μL (Sigma) and 6xHis ladder, 2.5 μL (Qiagen); 1, uninduced BL21(DE3)p6xHisHpf2; 2, WCE of BL21(DE3)p6xHisHpf2, expression at 37°C; 3, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 37°C; 4, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C; 5, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 30°C; 6, WCE of BL21(DE3)p6xHisHpf2, expression at 22°C; 7, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 22°C; 8, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C; 9, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 16°C; 10, WCE of BL21(DE3)p6xHisHpf2, expression at 4°C; 11, soluble fraction of BL21(DE3)p6xHisHpf2, expression at 4°C. All induced fractions were induced with 0.1 mM IPTG with 3 h expression time, and the equivalent of 300 μL of culture was loaded.**

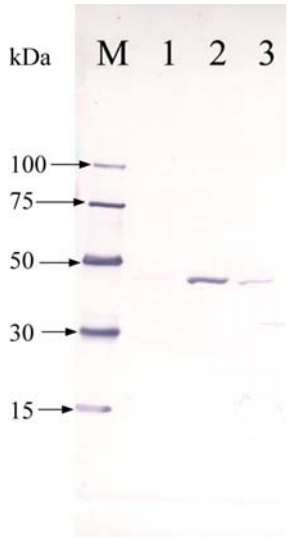


**Figure 2-9 Solubility of 6xHisHpf2 at 30°C. M, 6xHis ladder, 5 μL (Qiagen); 1, uninduced BL21(DE3)p6xHisHpf2; 2, WCE of BL21(DE3)p6xHisHpf2; 3, soluble fraction of BL21(DE3)p6xHisHpf2. Both samples were induced with 0.1 mM IPTG and expressed at 30°C with 3 h expression. Equivalent of 300 μL culture loaded per lane.**

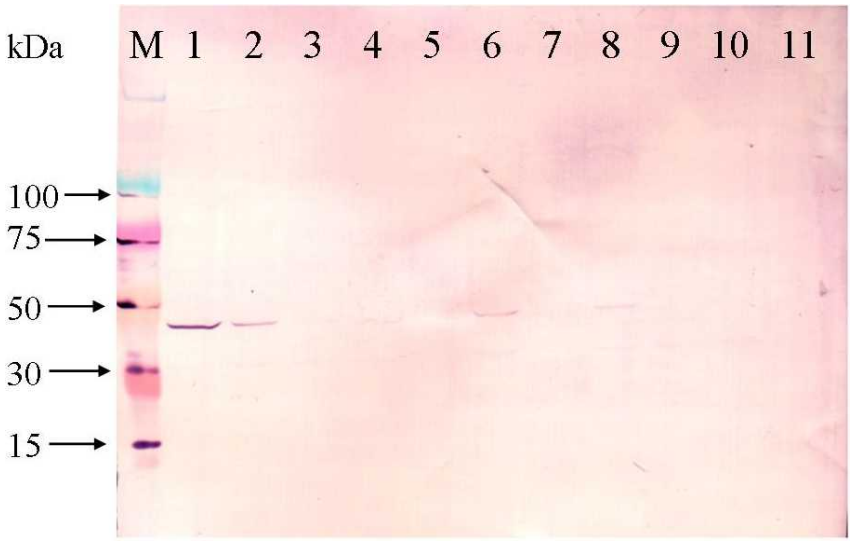


**Figure 2-10 Comparison of expression levels in M9 media at different growth temperatures and induction times. M, Colour marker, 5 μL (Sigma) and 6xHis ladder, 2.5 μL (Qiagen); 1, WCE, 30°C, 3 h expression; 2, WCE, 22°C, 3 h expression; 3, soluble fraction, 22°C, 3 h expression; 4, WCE, 22°C, 20 h expression; 5, soluble fraction, 22°C, 20 h expression; 6, WCE, 4°C, 3 h expression; 7, soluble fraction, 4°C, 3 h expression; 8, WCE, 4°C, 20 h expression; 9, soluble fraction, 4°C, 20 h expression; 10, WCE, 4°C, 26 h expression; 11, soluble fraction, 4°C, 26 h expression. Lanes 2-11 - equivalent of 300 μL culture, all grown to $OD_{600}$ of ~0.6 then induced with 0.1 mM IPTG.**

soluble 6xHisHpf2 seen. At both 4 and 22°C, there was more 6xHisHpf2 in the first sample than in subsequent time samples, again indicating degradation of the heterologous protein.

Rich media has also been shown to increase the soluble expression level for some proteins (Moore et al. 1993; Peng et al. 2004). 6xHisHpf2 was expressed in 2xYT media at 16°C. The whole cell extract and the soluble fraction were examined by Western blot. The expression level of 6xHisHpf2 in the whole cell extract was very low and negligible in the soluble fraction (data not shown).

#### *2.3.5.3 Additives to increase the expression of soluble material*

Varying other factors during growth and expression has been shown to increase the soluble fraction of heterologous protein expression in *E. coli*. One method is to heat shock the culture prior to induction, as this causes the expression of heat shock proteins and chaperone proteins that help repair and correctly fold proteins (Baneyx 1999). BL21(DE3)p6xHisHpf2 was grown in LB and 50 mg $L^{-1}$ ampicillin at 30°C to an $OD_{600}$ of approximately 0.8, then incubated at 42°C for 10 min. IPTG was added to a final concentration of 0.1 mM and 6xHisHpf2 was expressed at 25°C for 3 h. The whole cell extract and soluble fractions were analysed by Western blot and negligible soluble 6xHisHpf2 was observed (data not shown).

The addition of glyclglycine to the media has been reported to increase the soluble fraction of heterologous protein from the T7 promoter (Ghosh et al. 2004). In the presence of glyclglycine, the bacteria spend considerable energy transporting the additive, which leads to a reduction in the rate of protein synthesis (Ghosh et al. 2004). BL21(DE3)p6xHisHpf2 was grown in LB, 50 mg $L^{-1}$ ampicillin and 0.5 M glyclglycine (Sigma) at 30°C to an $OD_{600}$ of approximately 0.6. IPTG was added to a final concentration of 0.1 mM and 6xHisHpf2 was expressed at 25°C for 3 h. The whole cell extract and soluble fractions were analysed by Western blot and very little 6xHisHpf2 was observed in the whole cell extract and negligible 6xHisHpf2 in the soluble fraction (data not shown).

*2.3.5.4 Variability of expression*

Figure 2-11 illustrates expression of 6xHisHpf2 from several colonies, which were picked from plating out the frozen stock of BL21(DE3)p6xHisHpf2 on LB agar plates supplemented with 50 mg $L^{-1}$ ampicillin. This was done after the expression became inconsistent, and the frozen stock was used to obtain single colonies for expression analysis. This again shows the variability of expression from BL21(DE3)p6xHisHpf2. The single colonies were grown at 30°C, then induced with 0.1 mM IPTG, with samples taken after 3 h of expression. Each individual colony gave similar expression at both 16°C and 30°C. Lane 1 also shows the leaky expression without induction of colony 1, which may affect the culture if the protein is at all toxic. Samples of equivalent culture volume were loaded, in order to compare yields of protein per volume, which is the most relevant property to consider for scale-up of the process. This variability was seen over the course of the project, with expression occurring in one experiment, but not in the subsequent experiment.

## 2.4 Discussion

The *HPF2* gene was cloned into the pETBlue-1 plasmid to express the 6xHis-tagged protein. The pETBlue-1 plasmid employs a T7 promoter, so the gene product is only expressed in the presence of the phage T7 RNA polymerase. The BL21(DE3) strain carries a chromosomal copy of the gene for T7 RNA polymerase, and its promoter is strongly activated by the inducer IPTG. Expression can be further controlled by expressing in the presence of the T7 lysozyme. This is expressed constitutively at a low level from the pLysS plasmid, and is a dual function protein: it inhibits the action of the T7 RNA polymerase prior to induction and it helps lyse the cells on freeze-thawing to extract the over-expressed protein. When the T7 polymerase is expressed, it is at such high levels due to the strength of the T7 promoter, that its action should only be slightly inhibited by the T7 lysozyme, allowing expression of the heterologous protein.

The efficiency of transformation of the pETBlu6xHisHpf2 varied significantly depending on the *E. coli* strain being transformed. Transformation into DH5α was very efficient, into BL21(DE3)pLysS was less efficient, and into BL21(DE3) had a very low efficiency. This may be explained by the levels of expression of 6xHisHpf2 in these

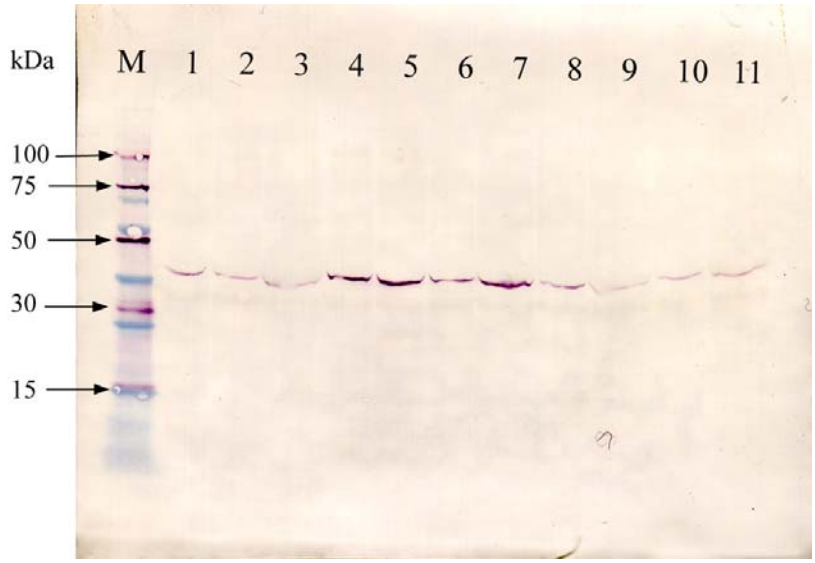


**Figure 2-11 Expression at 16°C and 30°C. M, Colour marker, 5 μL (Sigma) and 6xHis ladder, 2.5 μL (Qiagen); 1, uninduced BL21(DE3)p6xHisHpf2, colony 1; 2, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C, colony 1; 3, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C, colony 1; 4, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C, colony 2; 5, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C, colony 2; 6, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C, colony 3; 7, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C, colony 3; 8, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C, colony 4; 9, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C, colony 4; 10, WCE of BL21(DE3)p6xHisHpf2, expression at 16°C, colony 5; 11, WCE of BL21(DE3)p6xHisHpf2, expression at 30°C, colony 5. All induced samples were induced with 0.1 mM IPTG and expressed for 3 h. Equivalent of 400 μL culture loaded per lane.**

different strains. The T7 promoter cannot be induced in DH5α, as this strain lacks the T7 RNA polymerase. A very low level of expression would be possible in the BL21(DE3)pLysS strain, but this will be limited by the presence of the T7 lysozyme, as it inhibits the T7 RNA polymerase. Greater basal expression will be observed in the BL21(DE3) strain, as there is no inhibition of the T7 RNA polymerase, and low levels of this will be produced even in the absence of the inducer IPTG. The differences in efficiency levels indicate that any expression of 6xHisHpf2 is detrimental to the bacteria, so transformants with even low levels of expression are selected against.

No expression was observed in the BL21(DE3)pLysS transformants upon induction with IPTG. It appears that the T7 lysozyme's inhibitory action prevents any transcription by the T7 RNA polymerase. The time course of expression with 0.1 mM IPTG induction at 37°C in Figure 2-4 shows intracellular degradation of 6xHisHpf2. This is consistent with the product being disadvantageous to the cell – if it is harmful, the proteolytic machinery will break it down to eliminate it from the cell.

Although it was also observed that the highest expression level during the time course was quite low, an attempt was made to purify some 6xHisHpf2. This would enable a heat test to be conducted and determine the haze protective activity of the bacterially expressed 6xHisHpf2. A 6 M urea buffer was used to solubilise any 6xHisHpf2 and most of this was extracted from the cell pellet, as seen by comparing the whole cell extract and the lysate in lanes 2 and 3 of Figure 2-6, respectively. The unbound fraction contains a similar amount to the lysate, with some more protein coming through in the first wash fraction. This indicates that 6xHisHpf2 does not bind Ni-NTA under these conditions. This could be because the 6xHis tag is inaccessible and so cannot physically come into contact with the bound nickel. The high urea concentration should not interfere with the binding, as previous studies have used the Qiagen method employed in this study to purify 6xHis-tagged proteins in the presence of 6 M urea (Chang et al. 2001; Zimmermann and Pfeifer 2003; Singh et al. 2004).

β-Mercaptoethanol can also interfere with the Ni-NTA, however Qiagen 2003b indicates that concentrations of up to 20 mM can be used without affecting binding. Further work on denaturing purification could focus on several matters: using guanine rather than urea as the denaturant; adjusting other compounds in the lysis buffer; or by

changing the 6xHis tag, either extending it by adding histidines or by changing the location to the C-terminal of the protein.

Most methods employed to increase the solubility of heterologous proteins aim to reduce the rate of protein synthesis to allow the cell machinery time to fold the protein correctly as it is synthesised. Several methods that followed this theory were tried in an attempt to increase the soluble expression level of 6xHisHpf2. The first was to decrease the temperature of the expression phase. This has previously been shown to lead to an increase in soluble protein formation, as it slows the metabolism of the cells, and so the rate of protein formation is lowered. This allows a greater time for the protein to be correctly folded after translation (Schein and Noteborn 1988; Cabilly 1989; Georgiou and Valax 1996; Urban et al. 2003). A range of temperatures from 4 to 37°C and longer expression times were considered. Results varied between experiments, with some soluble protein seen at 16 and 30°C, although this was inconsistent and could not be replicated.

Lowering the amount of IPTG used for induction has also been shown to increase the soluble expression of heterologous protein in *E. coli*, in a similar manner as the decrease in temperature, by slowing the rate of expression and allowing the cell machinery to correctly fold the protein as it is produced (Turner et al. 2005). This method was attempted without any success for 6xHisHpf2.

The media composition was investigated as a way to increase soluble 6xHisHpf2. Minimal media should again slow down the expression rate, allowing time for the protein to fold correctly (Galindo et al. 1990). This was not observed for this system, with low expression and negligible soluble protein. Rich media have been used to increase expression also, possibly as it allows a higher biomass, so although the yield of protein per cell is not changed, the yield of protein per unit culture volume is increased (Moore et al. 1993). This was not observed in the case of 6xHisHpf2, with lower expression seen in 2xYT than in LB medium.

Several other methods have been reported to increase the soluble yield of some heterologous proteins. Two of these were evaluated: heat shock of the culture (Santoro et al. 1998; Baneyx 1999) and glyclglycine addition (Ghosh et al. 2004). Heat shocking

the culture before induction should cause heat shock proteins to be expressed, which can then fold the over-expressed protein as it is translated. The addition of glyclglycine to the culture during expression also slows down the translation rate, as it uses most of the cell's energy for its transport into the cell. Neither of these methods increased soluble 6xHisHpf2 expression.

There are many possible ways that could be trialled in an attempt to increase the solubility of 6xHisHpf2 using the pETBlue6xHisHpf2 vector *in vivo* that have not been included in this study. These include using BL21(DE3) Origami strain, which enhances disulfide bond formation (Novagen) (Prinz et al. 1997; Cassland et al. 2004; Medina-Godoy et al. 2004). Other additives such as sorbitol, betaine and sucrose, to change the osmotic pressure on the cells, have been shown to yield more soluble heterologous protein (Blackwell and Horgan 1991; Yu et al. 1995; Thomas and Baneyx 1997; Ghosh et al. 2004) in a similar method to the glyclglycine method used. Adding ethanol to the media at inoculation can induce heat shock proteins and chaperones to help protein folding (Thomas and Baneyx 1997). Co-expression of heat shock proteins or other co-factors have also been successful methods (Thomas and Baneyx 1996; Weickert et al. 1996; Thomas and Baneyx 1997).

In order to pursue the expression of 6xHisHpf2 in *E. coli*, expression levels would need to be increased. This would best be achieved by changing the plasmid construct, including investigating different fusion partners and codon optimisation. However it may not be possible to increase the yield as the protein appears to be toxic to the host – it is degraded with time, there is a low efficiency of transformation into expression strains, and transformants lost the ability to express with time. A fusion partner may prevent or mask the toxicity of the protein and allow higher yield.

A fusion partner could be very beneficial for further work, as they can be used to achieve many objectives, such as protein immobilisation, ease of purification, and increases in solubility and yield (Nilsson et al. 1997; Stevens 2000). Using a highly soluble fusion partner, such as thioredoxin, NusA or glutathione S-transferase, can increase the both the overall and the soluble yield of the heterologous protein (Kim and Lee 1996; Davis 1999; Perrin et al. 2003; Fang and Ewald 2004; Turner et al. 2005). Maltose binding protein has also been reported to increase the solubility of heterologous

protein expressed in *E. coli* (Kapust and Waugh 1999). Alternatively, the hydrophobic protein ketosteroid isomerase can be used as a fusion partner, leading to increases in yield but causing inclusion bodies (Majerle et al. 2000; Morreale et al. 2003). This may be advantageous, as inclusion bodies are easily purified, but refolding can lead to inactive protein. In the current study, a 6xHis tag was used as the fusion partner, mainly to enable simple purification. Other tags are often employed for this purpose, such as cellulose binding protein (Shpigel et al. 1999) and glutathione S-transferase (Fasshauer et al. 1999), sometimes in conjunction with a 6xHis tag to enable cleaner purification.

Codon optimisation may also lead to increased heterologous protein yield. There are 61 codons that code for the 20 amino acids used in proteins, so there are redundancies. Each organism uses the different codons in different frequencies, so when expressing a foreign protein, the host organism may not produce many of the tRNAs required for the codons (Wu et al. 2004). Codon optimisation was not attempted in this study, so rare codons may have prevented the host cells producing high quantities of the heterologous protein. Alternatively, a strain encoding rare codon tRNAs could be employed, such as BL21(DE3) Rosetta (Novagen) (Choi et al. 2004).

## 2.5 Conclusions

Yields of 6xHisHpf2 from *E. coli* were very low. Expression levels were inconsistent over the course of the project, and many results could not be replicated. Purification of the insoluble material could not be achieved and soluble expression experiments were not able to be replicated.

If this work was to be continued, the first step would be to increase the yield of 6xHisHpf2 from *E. coli*. Establishing a method of purification is also required, and lastly, increasing the solubility is important, as the protein is most likely to be active if it is expressed correctly folded and does not need to be denatured for purification then refolded. Careful choice of a fusion partner could give all of these requirements.

A decision was made to not consider these options further, as none of the work in *E. coli* had indicated that high levels of expression were possible, and manipulation of soluble protein levels was not achieved using several techniques. Most importantly,

simultaneous work on increasing the yield of 6xHisHpf2 from *S. cerevisiae* suggested this system could be used for producing sufficient quantities for sensory analysis, so it was decided to leave the *E. coli* research and focus on optimising the existing *S. cerevisiae* system.

# 3 OPTIMISATION OF 6XHISHPF2 EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

## 3.1 Introduction

Previous studies on over-expression of 6xHisHpf2 led to the construction of SB59, which is the *S. cerevisiae* laboratory strain S288c harbouring the gene for a 6xHis-tagged Hpf2 protein in an over-expression plasmid, p6xHisHpf2 (Brown 2003). This pYES2/GS plasmid has a uracil gene (*URA3*) for selection pressure and the gene of interest is under control of the *GAL1* promoter. Expression is heavily repressed in the presence of glucose. Induction is initiated by the addition of galactose, and a secondary non-repressing carbon source can be present. The over-expression system developed for SB59 resulted in yields of purified 6xHisHpf2 of 5 mg $L^{-1}$. This was sufficient for initial assessment of the haze protective activity of the tagged protein, although higher expression levels are required for sensory assessment of 6xHisHpf2, a major aim of this study. For initial sensory trials, up to four litres of wine are required, indicating that up to 1 g of 6xHisHpf2 will be needed. With the current over-expression system, this would require fermentations in the order of 100 litres.

SB59 contains several auxotrophies, being Δ*his*, Δ*leu*, Δ*lys* and Δ*trp*, as well as Δ*ura* for selection of the *URA3* marker on the plasmid. Several studies have found auxotrophies to be deleterious to the growth rate of yeast and expression levels of heterologous proteins (Çakar et al. 1999; Pronk 2002; Görgens et al. 2004). Transformation of the plasmid into a S288c strain with only the uracil auxotrophy may result in a fitter transformant.

The growth of SB59 was previously investigated in both synthetic complete media (SCM) and chemically defined grape juice media (CDGJM), and expression in SCM (Brown 2003), both of which are defined media. The use of complex media would prevent the selective pressure on the strain to maintain the plasmid, but this might be offset by higher growth rates and expression levels. Expression was only examined in SCM previously, in the absence of glucose, with induction by galactose and addition of raffinose as a secondary non-repressing carbon source. Other non-repressing carbon sources that are more economical than raffinose and can be utilised by *S. cerevisiae*

include ethanol, glycerol and lactate. Ethanol may be a useful secondary carbon source, as it is cheap, readily available and does not interfere with the pH of the media. However high levels of ethanol can inhibit yeast growth and, consequently, protein expression, so the optimal level of ethanol is likely to be less than 20 g $L^{-1}$ (Walker 1998).

The pH of CDGJM is 3.2 and SCM is 5.0. The optimal pH range for yeast growth is reported to be 3.5-5.0 (Pelczar et al. 1986). Changing the media pH may increase yeast growth rates and 6xHisHpf2 expression levels. In a similar manner, only one galactose concentration has formerly been considered. As galactose is used by the yeast as both a carbon source and as an inducer for the *HPF2* gene, the concentration of galactose in the expression media will therefore affect the expression levels of 6xHisHpf2.

The expression time, from when the yeast culture is moved from a glucose-containing growth media to a fresh galactose-containing media, is also important. The optimal expression time can vary from several hours to several days (Li et al. 2000; Lim et al. 2002; Lee and DaSilva 2005). The initial method of 6xHisHpf2 production involved 24 h of expression, however the optimum may be more or less than this.

Other defined yeast media have been developed specifically for high yeast growth and protein expression. These include the media referred to as Lund defined media (LDM) (Verduyn et al. 1992), and Delft defined media (DDM) (van Hoek et al. 2000). Both media consist of similar components to CDGJM, but in different proportions. In particular, the concentration of several of the main components, such as ammonia and phosphate, are about 10-fold higher in DDM. This increase in nutrients may lead to both higher growth rates of the yeast and expression levels of 6xHisHpf2.

Work by others has shown that the yield of over-expressed secreted yeast proteins increases approximately proportionally with increases in the biomass concentration (Fieschko et al. 1987; Mendoza-Vega et al. 1994; Yang et al. 1997). Initial yields achieved with SB59 in SCM of 5 mg $L^{-1}$ correspond to the order of 5 mg $g^{-1}$ biomass in cultures with approximately 1 g $L^{-1}$ biomass. The biomass of expression cultures can be increased to more than 10 g $L^{-1}$, which could lead to a 10-fold increase in 6xHisHpf2 expression. Yields of up to 5 g $L^{-1}$ recombinant protein have been reported in

*S. cerevisiae* (Fieschko et al. 1987; Park et al. 2000), so there is potential for improvement on the current method of 6xHisHpf2 production.

A major aim of the current study is to assess whether 6xHisHpf2 has an impact on wine sensory properties. Approximately 1 g of 6xHisHpf2 is required to treat 4 L of wine for initial sensory studies. This would require fermentations in the order of 100 L with the current over-expression system, and thus optimisation of the expression system was undertaken to develop a higher yielding system for the production of 6xHisHpf2.

## 3.2 Materials and methods

### 3.2.1 Transformation of S288c*Δura* with p6xHisHpf2

S288c*Δura* was transformed by the lithium acetate/polyethylene glycol method described by Ausubel et al. (1994).

### 3.2.2 Media

Culturing of *S. cerevisiae* was performed in the following: yeast-peptone-dextrose media, YPD (Sherman and Roman 1963); synthetic complete media, SCM (Ausubel et al. 1994); chemically defined grape juice media, CDGJM (Jiranek et al. 1995); Lund defined media, LDM (Verduyn et al. 1992); or Delft defined media, DDM (van Hoek et al. 2000). The pH of the media was 5.5, unless otherwise mentioned. All media are described in Appendix 1.

Glucose was present at 20 g $L^{-1}$ for the growth phase, and was replaced with galactose plus a non-repressing carbon source for expression from the *GAL1* promoter. The SCM and CDGJM were deficient in uracil for transformants harbouring the p6xHisHpf2 plasmid.

### 3.2.3 Over-expression of 6xHisHpf2

Expression was induced by transferring the yeast to a media containing 20 or 50 g $L^{-1}$ galactose and a non-repressing carbon source. The non-repressing carbon source was absent or added at 10 or 20 g $L^{-1}$. All experiments were in a volume of 100 mL in

250 mL Erlenmeyer baffled flasks, shaken at 30°C, with two replicates, induced at 1 g $L^{-1}$ biomass from overnight cultures grown in the same media with 20 g $L^{-1}$ glucose, unless otherwise indicated.

### 3.2.4 SDS-PAGE

SDS-PAGE was performed as described in Section 2.2.7, except the supernatant samples were desalted using Econo-pac columns according to the manufacturer's instructions (Bio-Rad) and lyophilised prior to adding the SDS-PAGE sample buffer.

### 3.2.5 Coomassie blue staining for detection of proteins on SDS-PAGE gels

Coomassie blue staining was performed as described in Section 2.2.8.

### 3.2.6 Transfer of proteins to nitrocellulose membrane

Transfer was performed essentially as described in Section 2.2.9, with transfer conducted at 100 V for 1 h or 40 V for 4 h in transfer buffer (constant voltage).

### 3.2.7 Western blot analysis

Western blot analysis was performed as described in Section 2.2.10.

### 3.2.8 PNGase F treatment

200 µL supernatant was concentrated to 40 µL using Microcon centrifugal filter units (Millipore). Protein samples were deglycosylated using PNGase F according to the manufacturer's instructions (Sigma). Milli-Q $H_2O$ was added to the protein sample to 90 µL, and 0.2% SDS in 100 mM β-mercaptoethanol (10 µL) was added. The sample was heated to 100°C for 10 min, then deglycosylation buffer (10 µL, 500 mM sodium phosphate), 15% TRITON X-100 (10 µL) and PNGase F (0.1 µL) were added. The sample was incubated at 37°C overnight and heated to 100°C for 5 min.

### 3.2.9 Purification of 6xHisHpf2

The purification process is displayed in Figure 3-1.

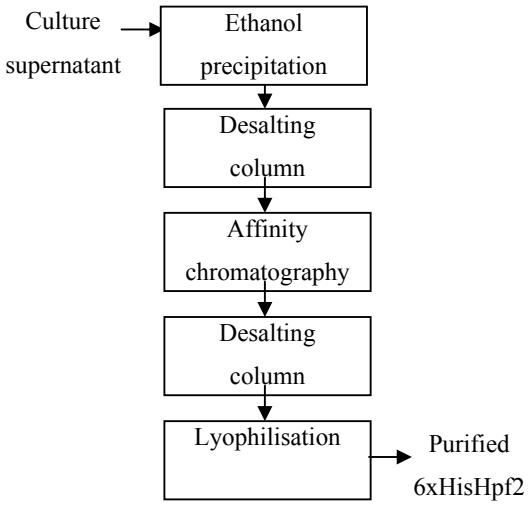


**Figure 3-1 Block diagram of 6xHisHpf2 purification process**

Proteins from the supernatant samples were concentrated by ethanol precipitation. Briefly, four volumes of 95% ethanol was acidified with 0.6% 10 M hydrochloric acid and added to one volume of supernatant, stored at -20°C overnight, then centrifuged (16 000 g, 20 min, -15°C). The pellet was resuspended in 3 mL water and then desalted using a 3 mL desalting column (Bio-Rad) into water. 6xHisHpf2 was purified by affinity chromatography using Ni-NTA (Qiagen) according to the manufacturer's instructions. Samples were desalted into water using a 3 mL desalting column, lyophilised, and the dry weight measured.

## 3.3 Results

### 3.3.1 Initial media and strain selection

SB59 was grown for 24 h at 30°C in the complex rich media YPD and defined minimal media SCM. The supernatant samples (equivalent of 2 mL per lane) were analysed by Coomassie staining, shown in Figure 3-2.

The p6xHisHpf2 expression plasmid based on the pYES2/GS plasmid described previously (Brown 2003) was transformed into S288c*Δura* strain, with only the uracil auxotrophy. Ten colonies were picked from the transformation, grown for 24 h in SCM with 20 g $L^{-1}$ galactose and 10 g $L^{-1}$ ethanol. Expression levels were compared by Coomassie staining (data not shown) and the colony displaying the highest expression of 6xHisHpf2 was named JM59 and used for further study.

SB59 (containing 4 additional auxotrophies) and JM59 were grown in 200 mL SCM containing 20 g $L^{-1}$ galactose and 10 g $L^{-1}$ raffinose for 24 h at 30°C. 6xHisHpf2 was purified on an affinity column and lyophilised. JM59 was grown in 200 mL CDGJM containing 20 g $L^{-1}$ galactose and 10 g $L^{-1}$ raffinose for 24 h at 30°C. 6xHisHpf2 was purified and the dry weight obtained. The highest yields were obtained growing JM59 on CDGJM and the lowest with SB59 on SM (Table 3-1).

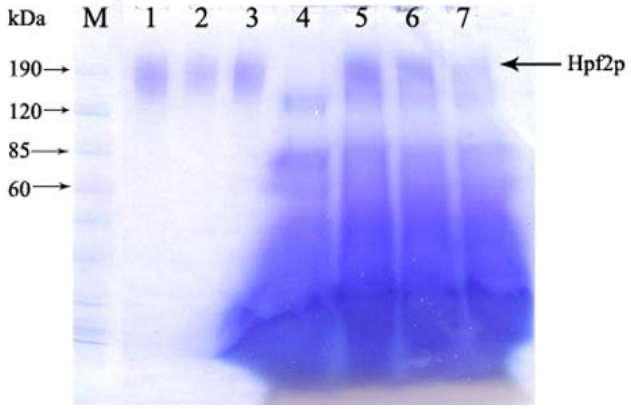


**Figure 3-2 Coomassie stain of SDS-PAGE separation of SCM and YPD supernatant samples. Lane M: Colour ladder (Invitrogen); Lane 1-3: SB59 grown in SCM; Lane 4: S288c grown in YPD; Lane 5-7: SB59 grown in YPD.**

**Table 3-1 Yields of 6xHisHpf2 obtained from SB59 and JM59 grown in SCM and CDGJM**

| Strain | Media | Yield (mg $L^{-1}$) |
|---|---|---|
| SB59 | SCM | 5 |
| JM59 | SCM | 20 |
| JM59 | CDGJM | 30 |

### 3.3.2 Secondary carbon source

The effect of the secondary non-repressing carbon source on 6xHisHpf2 expression was considered, by growing JM59 in 100 mL CDGJM containing 20 g $L^{-1}$ galactose and 10 g $L^{-1}$ of either raffinose, glycerol, ethanol or lactate. 6xHisHpf2 was expressed for 24 h at 30°C, and 4 mL of supernatant was analysed by Western blot. The yield was similar for raffinose, glycerol and ethanol, with lower expression with lactate, shown in Figure 3-3.

### 3.3.3 Effect of media pH

JM59 was grown in CDGJM at pH 3.2 and 5.5, with significantly higher expression detected by Western blot at pH 5.5 (Figure 3-4).

In further studies, JM59 was grown for 48 h in CDGJM at pH 4.5, 5.0 or 5.5. Supernatant samples were deglycosylated with PNGase F to increase transfer efficiency for Western blot analysis. The MW of the deglycosylated 6xHisHpf2 was approximately 52 kDa. Figure 3-5 shows expression levels of 6xHisHpf2 at 24 and 48 h, and expression is highest at pH 5.5 after 24 h. All subsequent media optimisation was performed at pH 5.5.

### 3.3.4 Effect of galactose concentration

Two levels of galactose concentration were considered, the original concentration of 20 g $L^{-1}$ and a high level of 50 g $L^{-1}$. Samples of the supernatant were taken at 24 and 48 h and treated with PNGase F, then analysed by Western blot, as shown in Figure 3-6. The highest 6xHisHpf2 level occurred with 50 g $L^{-1}$ galactose after 48 h expression.

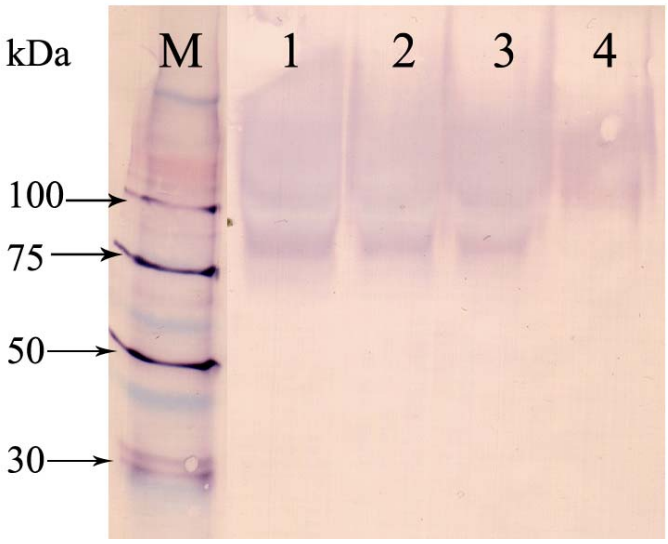


**Figure 3-3 Western blot of desalted supernatants after expression with different carbon sources. Lane M: Colour and 6xHis ladder; Lane1: JM59 with raffinose; Lane 2: JM59 with glycerol; Lane 3: JM59 with ethanol; Lane 4: JM59 with lactate.**

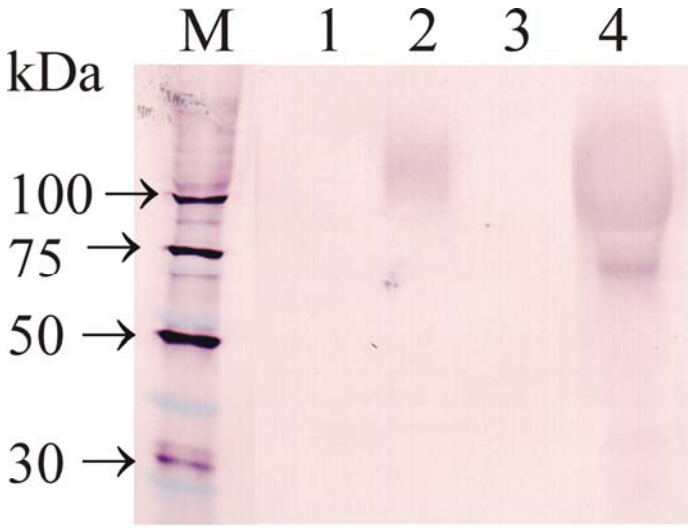


**Figure 3-4 Western blot of desalted supernatant after expression in CDGJM at pH 3.2 and 5.5. Lane M: Colour and 6xHis ladder; Lane 1: S288c at pH 3.2; Lane 2: SB59 at pH 3.2; Lane 3: S288c at pH 5.5; Lane 4: SB59 at pH 5.5.**

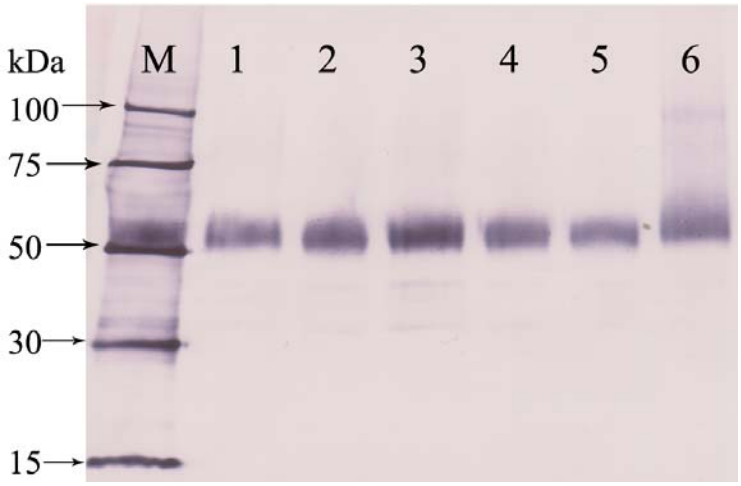


**Figure 3-5 Western blot of PNGase F treated supernatant samples at varying pH. Lane M: 6xHis ladder (Qiagen); Lane 1: pH 5.0 at 24 h; Lane 2: pH 5.0 at 48 h; Lane 3: pH 5.5 at 24 h; Lane 4: pH 5.5 at 48 h; Lane 5: pH 4.5 at 24 h; Lane 6: pH 4.5 at 48 h.**

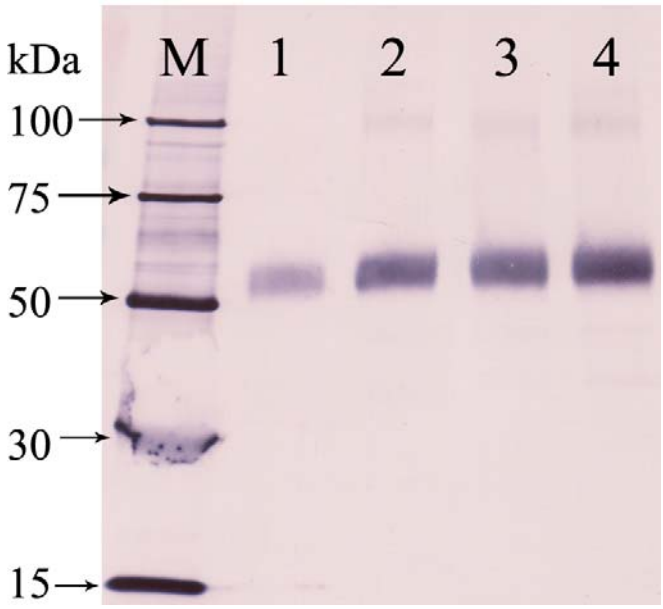


**Figure 3-6 Western blot of PNGase F treated supernatant samples with low and high galactose concentration. Lane M: 6xHis ladder; Lane 1: low galactose at 24 h; Lane 2: low galactose at 48 h; Lane 3: high galactose at 24 h; Lane 4: high galactose at 48 h.**

### 3.3.5 Effect of ethanol concentration

The expression levels of 6xHisHpf2 were examined from cultures with 50 g $L^{-1}$ galactose and ethanol concentrations from 0 to 20 g $L^{-1}$, shown in Figure 3-7. 6xHisHpf2 was highest when expressed with 10 g $L^{-1}$ ethanol for 48 h.

### 3.3.6 Comparison with other defined media

JM59 was grown in LDM and DDM and the expression levels of 6xHisHpf2 were compared with those from CDGJM. All media were at pH 5.5 with 50 g $L^{-1}$ galactose and 10 g $L^{-1}$ ethanol. The highest yield was seen for expression for 48 h in CDGJM, shown in Figure 3-8.

### 3.3.7 Time optimisation

It was observed that the expression level of 6xHisHpf2 varied with time in several previous results. A time course of expression in the optimised media was conducted to determine the best time to harvest the supernatant for maximum 6xHisHpf2 production. Samples were taken periodically for 48 h and the Western blot analysis is shown in Figure 3-9. The optimal expression time was found to be 32 h.

### 3.3.8 Effect of biomass

The time course was repeated for both lower and higher biomass concentrations of 0.5 and 2.0 g $L^{-1}$, and the peak productions occurred at 24 and 32 h, respectively. The peak expression time for 0.5 g $L^{-1}$ is shown in Figure 3-10, and that of 2.0 g $L^{-1}$ in Figure 3-11. The optimal expression time was found to be 24 h for an initial biomass concentration of 0.5 g $L^{-1}$, and 32 h for 2.0 g $L^{-1}$.

The peak expression time points of each biomass were compared and the expression of 6xHisHpf2 was very similar, shown in Figure 3-12, indicating that the biomass concentration is not directly proportional to 6xHisHpf2 expression in this system.

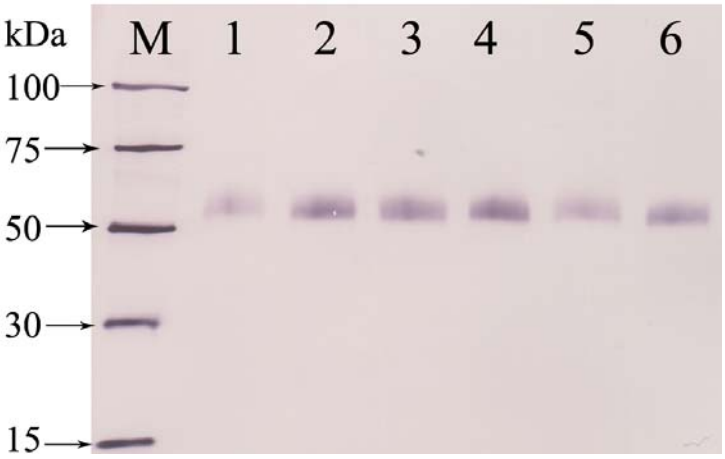


**Figure 3-7 Western blot of PNGase F treated supernatant samples with varying ethanol concentration. Lane M: 6xHis ladder; Lane 1: 0 g $L^{-1}$ ethanol at 24 h; Lane 2: 0 g $L^{-1}$ ethanol at 48 h; Lane 3: 10 g $L^{-1}$ ethanol at 24 h; Lane 4: 10 g $L^{-1}$ ethanol at 48 h; Lane 5: 20 g $L^{-1}$ ethanol at 24 h; Lane 6: 20 g $L^{-1}$ ethanol at 48 h.**

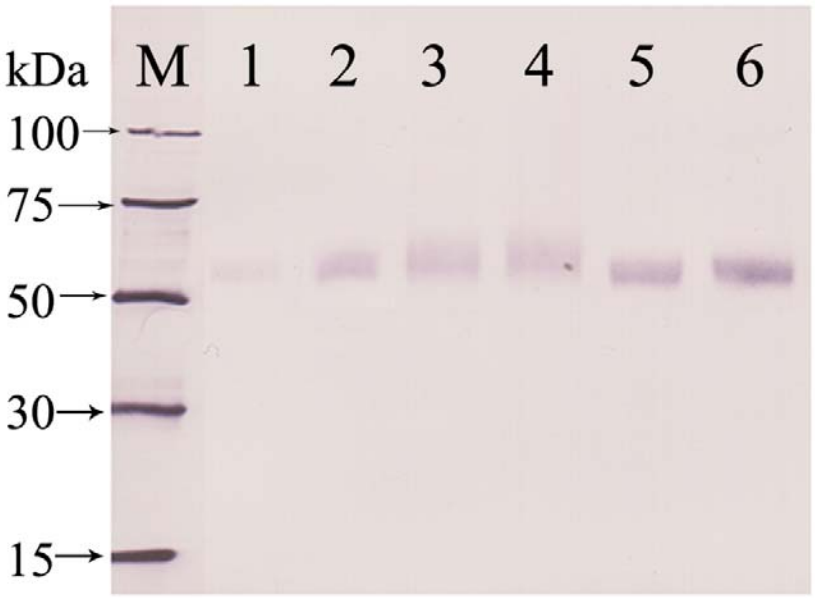


**Figure 3-8 Western blot of PNGase F treated supernatant samples from 3 defined media. Lane M: 6xHis ladder; Lane 1: LDM at 24 h; Lane 2: LDM at 48 h; Lane 3: DDM at 24 h; Lane 4: DDM at 48 h; Lane 5: CDGJM at 24 h; Lane 6: CDGJM at 48 h.**

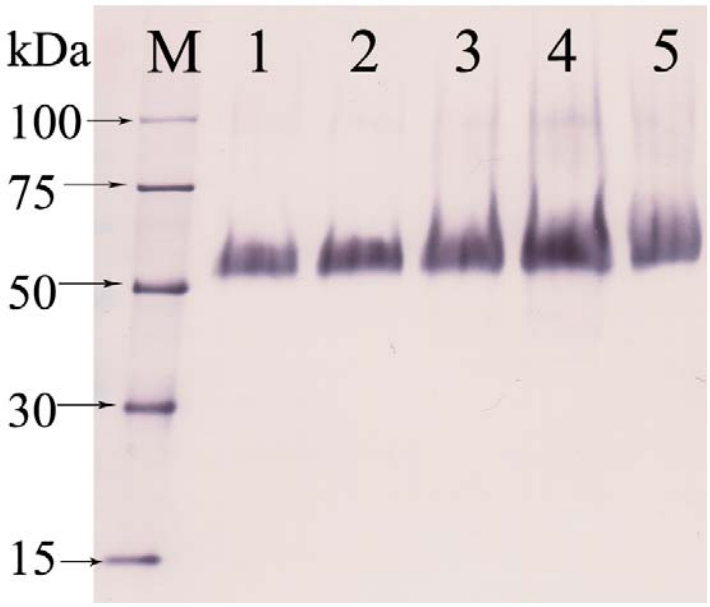


**Figure 3-9 Western blot of PNGase F treated supernatant samples over 48 h. Lane M: 6xHis ladder; Lane 1: 8 h; Lane 2: 16 h; Lane 3: 24 h; Lane 4: 32 h; Lane 5: 48 h.**

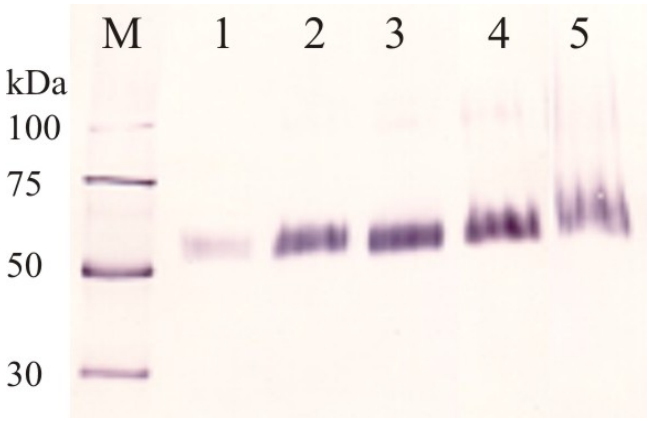


**Figure 3-10 Western blot of PNGase F treated supernatant samples with a starting biomass of 0.5 g $L^{-1}$. Lane M: 6xHis ladder; Lane 1: 8 h; Lane 2: 16 h; Lane 3: 24 h; Lane 4: 32 h; Lane 5: 48 h.**

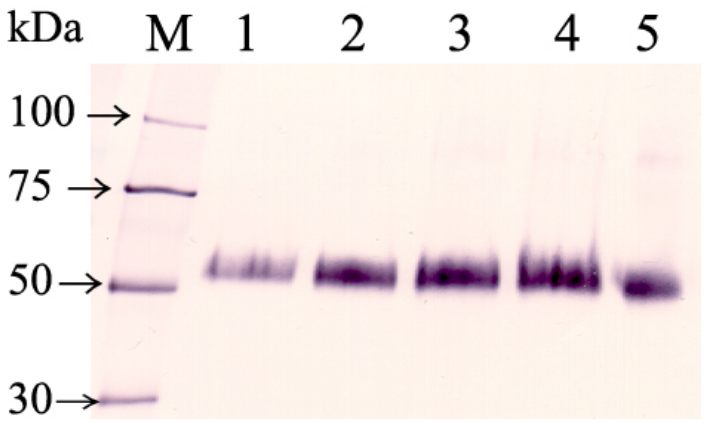


**Figure 3-11 Western blot of PNGase F treated supernatant samples with a starting biomass of 2 g $L^{-1}$. Lane M: 6xHis ladder; Lane 1: 8 h; Lane 2: 16 h; Lane 3: 24 h; Lane 4: 32 h; Lane 5: 48 h.**

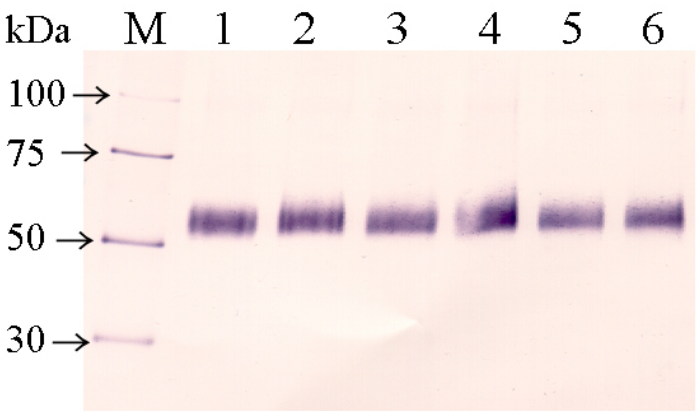


**Figure 3-12 Western blot of PNGase F treated supernatant samples at optimal expression time for varying initial biomass concentrations. Lane M: 6xHis ladder; Lane 1: 0.5 g $L^{-1}$ for 24 h, replicate 1; Lane 2: 0.5 g $L^{-1}$ for 24 h, replicate 2; Lane 3: 1.0 g $L^{-1}$ for 32 h, replicate 1; Lane 4: 1.0 g $L^{-1}$ for 32 h, replicate 2; Lane 5: 2.0 g $L^{-1}$ for 32 h, replicate 1; Lane 6: 2.0 g $L^{-1}$ for 32 h, replicate 2.**

## 3.4 Discussion

The main results obtained for the optimisation of 6xHisHpf2 expression in *S. cerevisiae* are SDS-PAGE separations represented as either Coomassie stains or Western blots of either the whole or deglycosylated protein. Coomassie staining displays all the proteins loaded onto the gel, so 6xHisHpf2 is detected by size, while for Western blot analysis, 6xHisHpf2 is recognised by the 6xHis tag. Western transfers gave variable results from experiment to experiment, most likely due to the difficulty of transferring such a large mannosylated protein (Brul et al. 1997). Due to the large mannose chains, 6xHisHpf2 tends to smear in the separation gel, resulting in a large band in both Coomassie stains and Western blots, as seen in Figure 3-2 and Figure 3-3. As a result, later comparisons were conducted by deglycosylating the protein with the enzyme PNGase F prior to SDS-PAGE followed by Western transfer. This gave results suitable for qualitative analysis, determining which sample has higher or lower levels of 6xHisHpf2, but it was not possible to quantitatively analyse the samples in order to determine 6xHisHpf2 concentrations in samples. A quantification method was being developed in conjunction with this work, described in more detail further, but was not available at the time. Qualitative analysis was considered sufficient for preliminary optimisation work. One exception was for the initial work on strains and media, shown in Table 3-1, where a full purification was conducted and dry weights of purified protein were obtained for comparison.

Expression levels of 6xHisHpf2 were similar in SCM and YPD, as shown in Figure 3-2. However, the YPD samples contained large amounts of contaminating proteins compared to the relatively clean SCM samples, indicating that purification would be much simpler from the defined media. This agrees with reports that defined media is significantly better for product isolation (Zhang and Greasham 1999).

The strain JM59 was constructed with no superfluous auxotrophies and compared to SB59. When SB59 was grown in SCM with galactose, the yield of purified 6xHisHpf2 was found to be 5 mg $L^{-1}$, while JM59 gave a yield of 20 mg $L^{-1}$. This is consistent with previous work that showed enhanced growth rates and recombinant protein expression levels in strains with fewer auxotrophies (Çakar et al. 1999; Pronk 2002; Görgens et al. 2004). For each auxotrophy, the yeast must take up the corresponding essential nutrient

from the media, as it is not able to make it itself, and it is hypothesised that this can limit the growth rates and recombinant protein expression levels.

The yield of purified 6xHisHpf2 expressed from JM59 grown in CDGJM was found to be 30 mg $L^{-1}$, an increase over the original system involving SCM. CDGJM has trace levels of cobalt, approximately 30 μg $L^{-1}$, while SCM lacks cobalt, a nutrient that has been suggested as being important to growth (Hahn-Hägerdal et al. 2005). This may explain why expression levels are higher in CDGJM.

SCM was originally developed as a selective media, while CDGJM was designed for use in wine research as equivalent to grape juice. Neither was developed to be rich in nutrients nor promote recombinant protein expression. Therefore, two other media, LDM and DDM, were considered, both of which have been developed to be high in nutrients to lead to high growth rates and protein levels (Verduyn et al. 1992; van Hoek et al. 2000). Figure 3-8 show a comparison of 6xHisHpf2 expression levels in these two media as well as CDGJM. Unexpectedly, the highest expression occurs in CDGJM, indicating this is a better medium for 6xHisHpf2 production. The only components that are notably higher in CDGJM are calcium chloride, folic acid and amino acids. Calcium chloride is approximately 10-fold higher in CDGJM. No folic acid is added to the LDM or DDM, while it is present at 0.2 mg $L^{-1}$ in CDGJM. Similarly, LDM and DDM have no amino acids present, while CDGJM has 1 g $L^{-1}$ amino acids added. These differences may account for the higher 6xHisHpf2 expression observed in CDGJM, although no mechanism was investigated due to time constraints. Consequently, CDGJM was selected for further optimisation.

The secondary carbon source for expression was investigated. Expression of 6xHisHpf2 was similar with raffinose, glycerol or ethanol as the secondary carbon source, with little expression with lactate, as seen in Figure 3-3. Raffinose is approximately 20-fold more expensive than ethanol and glycerol, at roughly \$2 000 $kg^{-1}$. Glycerol is a very viscous solution, making it difficult to measure small volumes consistently. Due to its low cost compared to raffinose and its ease of use compared with glycerol, ethanol was considered further, however high levels of ethanol have been shown to inhibit yeast growth and, consequently, protein expression (Walker 1998). Therefore the effect of ethanol concentration on the expression levels of

6xHisHpf2 was considered. Figure 3-7 shows that expression is higher with 10 g $L^{-1}$ ethanol than with no ethanol present. Expression drops at an increased ethanol level of 20 g $L^{-1}$, supporting the previous work regarding inhibition of growth or expression by ethanol. Further optimisation was done with 10 g $L^{-1}$ ethanol.

CDGJM was designed to represent grape juice, and as such, it has a low pH of 3.2. Previous work has suggested that a higher pH of 3.5 – 5.0 is more appropriate for *S. cerevisiae* growth (Pelczar et al. 1986). Figure 3-4 shows that when JM59 is grown at pH 5.5, higher expression levels of 6xHisHpf2 are achieved that for pH 3.2. This increase did not appear to be related to biomass, as the biomasses for the two cultures were approximately equal through the experiment. The pH was further optimised around 5.0, with the highest expression of 6xHisHpf2 seen for a pH of 5.5, shown in Figure 3-5.

The effect of galactose concentration on 6xHisHpf2 expression levels was considered. Figure 3-6 compares the expression levels of 6xHisHpf2 with induction by 20 or 50 g $L^{-1}$ galactose. Expression is higher with 50 g $L^{-1}$ galactose with 48 h of induction. This suggests that galactose is limiting the production of the recombinant protein at the lower concentration. Measuring galactose levels in the 'high' galactose samples at 24 and 48 h indicated that galactose was still present at both time points (data not shown). This suggested that galactose was not limiting at these levels, so further increases in galactose levels were not investigated. All further optimisation was performed with the galactose of 50 g $L^{-1}$.

The effect of expression time on 6xHisHpf2 levels was investigated. 6xHisHpf2 levels in the supernatant were observed to increase from 0 to 32 h, then decrease to 48 h, indicating that there is a peak in production around 32 h. This could be due to protein degradation or loss in the supernatant, although this was not investigated further.

The final factor investigated was the effect of biomass on 6xHisHpf2 expression. Previous experiments were conducted with an initial biomass of 1.0 g $L^{-1}$ at the time of induction with galactose. Two other biomass concentrations were considered – 0.5 and 2.0 g $L^{-1}$. The 6xHisHpf2 levels were very similar in all samples, seen in Figure 3-12. Previous studies have suggested that biomass and recombinant protein levels are

proportional (Fieschko et al. 1987; Mendoza-Vega et al. 1994; Yang et al. 1997), which was not observed in this case. One reason for this may be that some component of the medium is limiting the level of 6xHisHpf2 produced, meaning that increasing the biomass will have little effect on protein production.

## 3.5 Conclusions

It was shown that the *S. cerevisiae* strain JM59 produces higher levels of 6xHisHpf2 than SB59, a strain with several unnecessary auxotrophies. Of the five media considered, CDGJM was found to be best for expression of 6xHisHpf2. The highest levels of 6xHisHpf2 occurred when expressed from JM59 in CDGJM containing 50 g $L^{-1}$ galactose, 10 g $L^{-1}$ ethanol, pH 5.5, expressed for 32 h, with an initial biomass concentration of approximately 1 g $L^{-1}$. Further optimisation could consider a multi-factorial approach, although a method to quantify 6xHisHpf2 would need to be established.

# 4 PRODUCTION OF 6XHISHPF2: QUANTIFICATION AND EFFICIENCY

## 4.1 Introduction

Once the method for expression of 6xHisHpf2 had been optimised in chemically defined grape juice media, approximately 1 g of the protein was needed for sensory analysis. To achieve this quantity, batches of four 1 L cultures were grown and 6xHisHpf2 was purified. The purification method varied from batch to batch to determine the simplest and most effective method.

The purification consisted of several stages. The first step was to recover the supernatant from the yeast culture, and this was achieved using centrifugation followed by filtration. The supernatant was then pre-treated to concentrate the proteins and reduce the volume, either solely by ultrafiltration or in conjunction with ethanol precipitation. The 6xHis-tagged protein was then separated from the concentrate using metal affinity chromatography, known as Ni-NTA, and eluted using histidine. Imidazole is commonly used for this elution step, but for this work, histidine was used to elute 6xHisHpf2 as the protein was being prepared for sensory analysis. Histidine is a naturally occurring amino acid and is safe for consumption, so trace levels in the final product could be tolerated. Imidazole is toxic, so avoiding its use was preferable. The 6xHisHpf2 was cleaned up by desalting, either by dialysis alone or in combination with size exclusion chromatography. Finally, the sample was lyophilised to produce a solid dry product. This production process was approved by the University of Adelaide Human Ethics Committee, allowing the resulting 6xHisHpf2 to be used in sensory studies.

A suitable quantification method was required to establish the efficiency of each purification step and several commonly-used protein quantification techniques were investigated. The major challenge with 6xHisHpf2 is the extent of glycosylation, which has been shown to interfere with protein estimations such as the Bradford assay and the BCA assay (Waterborg and Matthews 1998). Other quantification methods include the two non-specific techniques: the micro-biuret and the Lowry methods. The extinction coefficient, $E_{280}$, can be used for purified protein, although none of these would be

suitable for crude samples containing other proteins. High performance liquid chromatography (HPLC) is a method that could be used to quantify a specific protein in a mixed sample, and two column types are commonly used, C8 and C4. Two methods that make use of the 6xHis tag are the ELISA method and the slot blot assay.

The micro-biuret method uses a colour reaction between the proteins in solution and alkaline copper sulfate to give a rapid and sensitive estimate of total protein concentration (Itzhaki and Gill 1964). It has been used in a wide range of applications, see for example Starger et al. 1978, Kariya et al. 1981 and Page and Ferguson 1989. The Lowry assay is also based on the reaction between the proteins and an alkaline copper solution. The complexes that form react with the Folin reagent creating a blue compound that can be measured at 750 nm (Lowry et al. 1951; Waterborg and Matthews 1998).

HPLC is an analytical technique which allows proteins (or other samples) to be separated based on their interactions with the stationary phase. Alexandre et al. (2000) used HPLC for the partial purification of a 49 kDa mannoprotein based on its hydrophobicity. For this work, C4 and C8 columns were considered, with the stationary phase consisting of either four or eight carbon chains, repetitively. The proteins in the sample should be eluted by increasing the solvent concentration and then can be detected by absorbance at 280 nm (O'Gara et al. 1995). Earlier work on highly glycosylated proteins, including Hpf1 and Hpf2, had shown that they were not able to be purified using the C8 column (McKinnon 1996), although detection with the deglycosylated proteins had not been attempted.

Enzyme-linked immunosorbent assay (ELISA) allows quantification of a specific protein based on its interaction with an antibody. It has been shown to be sensitive and accurate when used for a range of proteins (O'Callaghan 1991; Kramer et al. 1995). In this case, the protein is 6xHis-tagged, and the anti-polyhistidine antibody is commercially available. Many samples can be measured simultaneously using 96-well plates. First, the protein sample to be analysed is bound to the inner surface of the well, then it is probed with the primary antibody, followed by the secondary antibody, then the detection substrate, such as BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) or pNPP (*p*-nitrophenyl phosphate), is

added. The absorbance at a specific wavelength can then be measured and the protein concentration determined (Qiagen 2001).

Slot blots are commonly used to quantify DNA, RNA or protein (Billingsley et al. 1985; Sahm et al. 1999; Nicklas and Buel 2003). The samples are bound to a nitrocellulose membrane and then can be probed and detected by an antibody specific to the protein of interest. The apparatus consists of 48 wells uniformly spaced over a nitrocellulose membrane connected to a vacuum flask. The samples are loaded into the wells and drawn through by vacuum. Once the proteins are bound, the membrane can be probed with the appropriate antibody, using the same system as a Western blot.

The production of 6xHisHpf2 for sensory analysis was conducted in conjunction with the establishment of a method for determination, so the quantification could only take place after the sample was prepared. Unfortunately this did not allow for significant improvement of the purification method during the process, yet it allowed the inefficiencies in the process to be highlighted in retrospect.

## 4.2 Materials and methods

### 4.2.1 Quantification

#### *4.2.1.1 SDS-PAGE silver stain*

The SDS-PAGE was performed as described in Section 2.2.7. The gel was fixed in 50% methanol/10% acetic acid for 30 min on a rocking platform. It was incubated for 15 min in 5% methanol, followed by three 5 min washes in Milli-Q $H_2O$. The gel was incubated for 120 s in freshly prepared 0.2 g $L^{-1}$ sodium thiosulfate, followed by three 30 s washes. It was incubated for 25 min in 2 g $L^{-1}$ silver nitrate, then washed three times for 60 s in Milli-Q $H_2O$. The gel was developed in development solution (Appendix 1) for up to 10 min until the proteins became visible. The development was stopped by incubating the gel in 14 g $L^{-1}$ sodium EDTA for 10 min, then the gel was washed in Milli-Q $H_2O$.

*4.2.1.2 Micro-biuret method*

The micro-biuret method was performed as described by Itzhaki and Gill 1964. Briefly, proteins were precipitated using either the chloroform/methanol or the trichloroacetic acid (TCA) method, as described below. The standards were prepared as follows:

$A_1$ –Milli-Q $H_2O$ (2 mL) and 0.21% (w/v) $CuSO_4.5H_2O$ (in 30% NaOH, 1 mL)

$A_2$ –Protein sample (2 mL) and 0.21% (w/v) $CuSO_4.5H_2O$ (in 30% NaOH, 1 mL)

$B_1$ –Milli-Q $H_2O$ (2 mL) and 30% NaOH (1 mL)

$B_2$ –Protein sample (2 mL) and 30% NaOH (1 mL)

The mixtures were vortexed, incubated for 5 min at room temperature, and absorbances were read at 310 nm. The adjusted optical density, $OD_{sample}$, of the protein sample was calculated as:

$$OD_{sample} = (A_2 - A_1) – (B_2 - B_1) \qquad \textbf{4-1}$$

All samples were prepared in duplicate.

For the methanol/chloroform precipitation, protein solution (100 µL) was added to methanol (400 µL). Chloroform (100 µL) and Milli-Q $H_2O$ (300 µL) was added. The sample was vortexed well and centrifuged (16 100 g, 3 min, 20°C). The aqueous layer was removed and methanol (300 µL) was added. The sample was vortexed well and centrifuged (16 100 g, 3 min, 20°C). The supernatant was removed and the pellet washed with methanol, and the sample was spun briefly. The supernatant was removed and the pellet allowed to air dry (Wessel and Flügge 1984).

For the TCA precipitation, protein solution (100 µL) was added to 20% TCA (43 µL) to make a 6% TCA solution. The sample was stored on ice for 1 h, then centrifuged (16 100 g, 5 min, 20°C). The supernatant was removed and the pellet washed with acetone, and the sample was centrifuged (16 100 g, 5 min, 20°C). The acetone wash was repeated, and the supernatant was removed and the pellet allowed to air dry (Ozols 1990).

#### *4.2.1.3 Lowry method*

The Lowry method was performed as described by Waterborg and Matthews 1998, based on the method of Lowry et al. 1951. Briefly, proteins were precipitated using either the chloroform/methanol or the TCA method, as described in Section 4.2.1.2. The pellet was resuspended in 1 M NaOH (50 µL) and incubated at 100ºC for 10 min. After cooling to room temperature, complex forming reagent (Appendix 1) (250 µL) was added, mixed well, and incubated for 10 min at room temperature. Folin reagent (25 µL, prepared as 50% stock solution in Milli-Q $H_2O$) was added and the sample was vortexed and incubated at room temperature for 30 min. The absorbance was read at 750 nm. All samples were prepared in duplicate.

#### *4.2.1.4 Extinction coefficient*

The extinction coefficients ($E_{280}$ and $E_{205}$) were calculated based on the absorbance at 280 nm ($A_{280}$) and 205 nm ($A_{205}$), using the method of Scopes 1974 and Pace et al. 1995. Briefly, for a protein solution of unknown concentration:

$$E_{205} = 27 + 120\frac{A_{280}}{A_{205}} \qquad \textbf{4-2}$$

$$E_{280} = \frac{A_{205}}{A_{280}E_{205}} \qquad \textbf{4-3}$$

And for a known protein concentration:

$$E_{280} = \frac{[6xHisHpf2]}{A_{280}} \qquad \textbf{4-4}$$

#### *4.2.1.5 HPLC*

Protein samples were initially deglycosylated using PNGase F as described in Section 3.2.8. The method was later adapted to eliminate the detergents. Milli-Q $H_2O$ was added to the protein sample to 90 µL, and 200 mM β-mercaptoethanol (10 µL) was added. The sample was heated to 100°C for 10 min, then deglycosylation buffer (10 µL) and PNGase F (0.1 µL) were added. The sample was incubated at 37°C overnight and the reaction was stopped by heating to 100°C for 5 min.

The samples were diluted in Milli-Q $H_2O$ appropriately and injected into a Vydac C8 column (The Sep/a/ra/tions Group) previously equilibrated in solvent A (Appendix 1).

Components were eluted by a gradient of 0 to 80% of solvent B (Appendix 1) over 30 min. The Vydac C4 column (The Sep/a/ra/tions Group) was equilibrated with solvent A, and components were eluted by a gradient of solvent C (Appendix 1) from 0 to 95% over 10 min, held at 95% for 10 min, and reduced back to 0% for 5 min.

#### *4.2.1.6 ELISA*

Two ELISA methods were attempted, based on the method described in Qiagen 2001. For the standard method, protein samples were diluted into 50 mM $NaCO_3$, pH 10.6, and immobilised onto the inner surface of 96-well plates (Greiner) overnight at 4ºC. The wells were washed four times with phosphate buffered saline (PBS) (Appendix 1), and blocked with 250 µL blocking buffer for 2 h at room temperature on a shaking platform. The wells were washed four times with PBS and probed with anti-polyhistidine antibody (Sigma) diluted 1:7 500 in PBS/BSA (0.2% BSA in PBS) for 1 h at room temperature. The wells were washed four times with PBS, and probed with anti-mouse antibody, conjugated to alkaline phosphatase (Sigma), diluted 1:7 500 in PBS/BSA for 1 h at room temperature. The wells were washed four times with PBS, and incubated with 200 µL of NBT/BCIP in alkaline phosphatase buffer (prepared as in Section 2.2.10) for 45 min, and the absorbance at 590 nm was read. All samples were run in quadruplicate.

In an attempt to improve the method, two variations were introduced: high protein binding plates (Sigma) were used and 1 mg $mL^{-1}$ pNPP (Sigma) in PBS was used as the detection agent instead of NBT/BCIP.

#### *4.2.1.7 Slot blot*

Samples were deglycosylated, either with or without detergent, as described in Section 4.2.1.5. Samples were diluted into Milli-Q $H_2O$ to an appropriate concentration and 200 µL was used for each well. A standard curve was prepared for each slot blot using a 6xHis ladder (Qiagen), loading 0 to 250 ng per well, and all samples were run in quadruplicate. The membrane (BA85, Schleicher and Schuell) and filter papers (Bio-Rad) were prepared by incubating in TBS for 10 min. The apparatus was assembled according to the manufacturer's instructions (Bio-Rad) and 100 µL TBS was loaded into each well and drawn through with the vacuum. The samples were loaded and allowed to flow through, followed by 200 µL TBS to wash the wells. The

membrane was blotted as in a Western blot, described in Section 2.2.10. The membrane was scanned and the density of each band was determined using ImageJ (NIH) software and quantification analysis was performed using Microsoft Excel.

### 4.2.2 Purification

#### *4.2.2.1 Supernatant recovery*

The JM59 culture, grown in CDGJM, was centrifuged (16 000 g, 20 min, 20°C) in 400 mL buckets in a Beckman J2-21M/E centrifuge using a JLA-10,500 rotor. The supernatant was gently poured off the yeast pellet. The supernatant was passed through a two-stage vacuum filter of 0.8 µm to 0.2 µm pore size (Pall).

#### *4.2.2.2 Volume reduction*

The filtered supernatant was recirculated through a 10 kDa cut-off ultrafiltration cartridge (Amersham Biosciences) at 15 psi until the retentate volume was reduced to approximately 10%. The filtrate and the retentate were collected and 6xHisHpf2 levels were qualitatively assessed by Western blot to ensure that it remained in the retentate (data not shown).

In addition, for some purification batches, the protein fraction was further concentrated by ethanol precipitation. Acidified ethanol (4 L, 95% ethanol, 0.6% 10 M HCl) were added to the retentate (1 L) and stored at -20°C overnight. The precipitate was recovered by centrifugation (16 000 g, 20 min, -20°C) in a Beckman J2-21M/E centrifuge using a JLA-10,500 rotor.

#### *4.2.2.3 Metal affinity chromatography*

The retentate or ethanol precipitate was diluted 1 in 5 with 5x Ni-NTA binding buffer (Appendix 1) and the pH was adjusted to 8.0. The Ni-NTA Superflow column (25 mL, Qiagen) was equilibrated with binding buffer and the sample was applied. The column was washed with three column volumes of binding buffer and the 6xHisHpf2 was eluted with five column volumes of elution buffer (Appendix 1). All samples were collected for later analysis.

#### *4.2.2.4 Desalting and finishing*

The eluate fractions from the Ni-NTA purification were desalted by either size exclusion chromatography, using Econo-Pac columns, following the manufacturer's instructions (Bio-Rad), followed by dialysis, using dialysis tubing (12 kDa cut-off, Sigma), or dialysis alone. The tubing was prepared according to the manufacturer's instructions, to remove sulfur compounds, and samples were dialysed against Milli-Q $H_2O$ overnight. After dialysis, the purified protein samples were lyophilised until the protein was dry.

### 4.2.3 Purity assessment

A sample of the final 6xHisHpf2 produced was prepared at 2 mg $mL^{-1}$ based on dry weight after lyophilisation. This was checked for purity by amino acid analysis, a mannose assay and SDS-PAGE silver stain. A 10 µL sample was sent for amino acid analysis, performed by the Australian Proteome Analysis Facility (Sydney). The SDS-PAGE silver stain was performed as described in Section 4.2.1.1.

The mannose assay was performed as described by Dupin et al. (2000a). Briefly, the sample was weighed out and resuspended at 2 mg $mL^{-1}$. Polymeric forms of mannose and glucose present in the samples were hydrolyzed into monomeric sugars by the addition of sulfuric acid (final concentration 1.5 M) to the sample. The solution was heated for 90 min at 100°C in sealed glass tubes. Cooled hydrolyzed samples (60 µL) were transferred to microplate wells and neutralized with NaOH (90 µL, 2 M) and triethanolamine buffer (75 µL, 25 mM, pH 7.6). The total amounts of monomeric glucose were determined enzymatically using the D-glucose/D-fructose UV method determination kit (set of enzymes E2 and E3, Boehringer Mannheim) followed by the determination of the monomeric mannose content with the enzyme phosphomannose isomerase (PMI, Sigma). The absorbance ($A_{340}$) was read prior to the addition of enzymes E2 and E3 (7.5 µL each) and after 1 h of incubation at 25°C. As yeast cells do not release any fructose or polymers containing fructose (Usseglio-Tomasset 1978), the enzymes E2 and E3 were used simultaneously and the corresponding $A_{340}$ reading after incubation was taken as the measure of the glucose content only. PMI (8 µL) was added and after 1 h at 25°C the $A_{340}$ was again taken. The difference between $A_{340}$

before and after PMI addition was a measure of the concentration of mannose in the sample.

## 4.3 Results

### 4.3.1 Establishing a method for quantification

The filtered supernatant from a JM59 culture was analysed by SDS-PAGE silver stain, shown in Figure 4-1. A large smear at approximately 180 kDa, the expected MW for 6xHisHpf2, was observed. Very few contaminating proteins were observed.

#### *4.3.1.1 Micro-biuret and Lowry methods*

The micro-biuret and Lowry methods were investigated by considering samples in two of the buffers used during the purification and prepared by precipitation with either methanol/chloroform or TCA. Purified 6xHisHpf2 was prepared at concentrations from 10 µg to 250 µg in CDGJM and Ni-NTA elute buffer and these samples were precipitated using either the methanol/chloroform or the TCA precipitation method. Controls containing no protein were prepared for each buffer and precipitation method. Figure 4-2 shows the absorbance readings for these samples using the micro-biuret method and Figure 4-3 shows the results for the Lowry assay.

Figure 4-2 shows that for low levels of protein, the absorbance readings given by the micro-biuret method were negative with respect to the blank samples, indicating that this method would not be suitable for quantification of 6xHisHpf2. The Lowry method gave a stronger linear relationship between the absorbance and the protein concentrations, however even the best fitting relationship of the elution buffer sample precipitated with methanol/chloroform overestimates concentrations by up to 50%. Also, all four curves should be coincidental, as they represent equal concentrations of 6xHisHpf2, however the confidence intervals do not overlap, while the slopes, given in Table 4-1, are significantly different by a Student t-test.

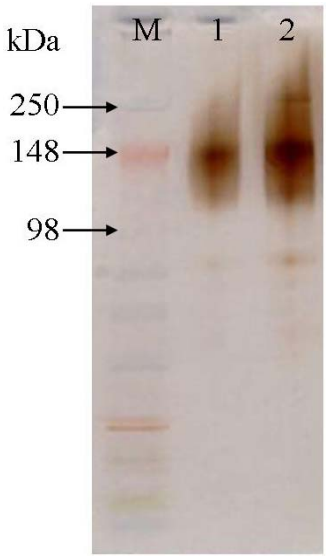


**Figure 4-1 SDSPAGE silver stain of filtered supernatant fractions from *S. cerevisiae* JM59 grown in CDGJM. M: Colour marker (Invitrogen); 1: 0.2 µL supernatant; 2: 0.5 µL supernatant.**

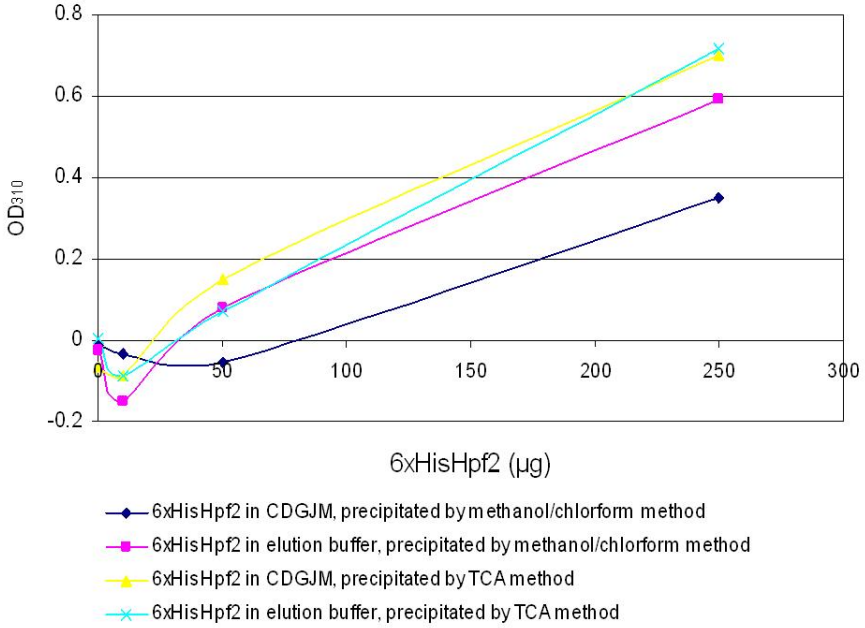


**Figure 4-2 Micro-biuret trial with 6xHisHpf2 in CDGJM or Ni-NTA elution buffer, precipitated using methanol/chloroform or TCA.**

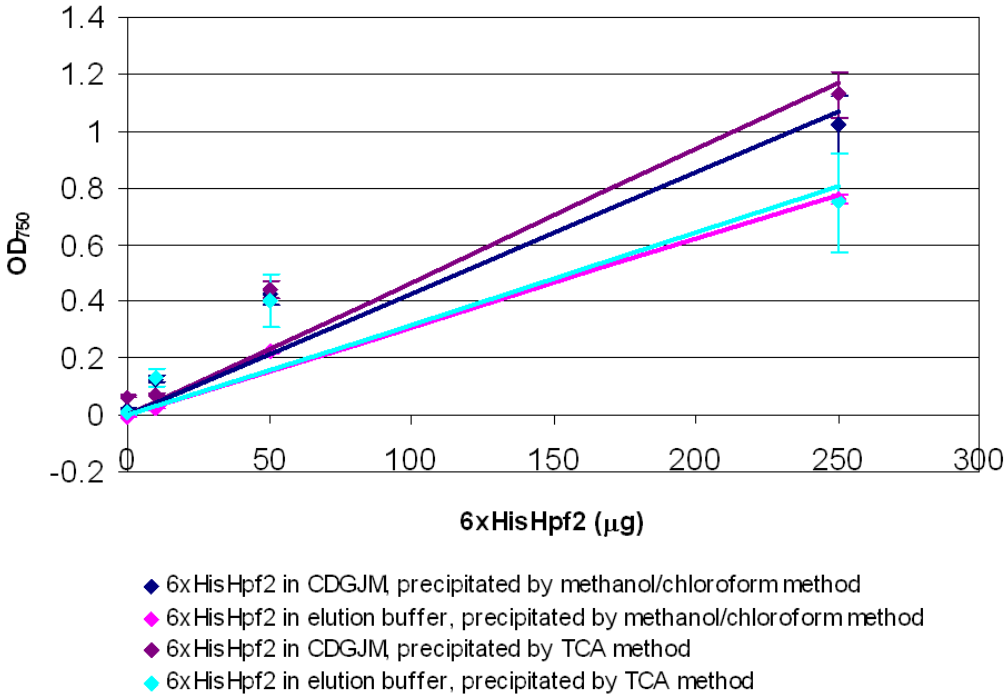


**Figure 4-3 Lowry trial with 6xHisHpf2 in CDGJM or Ni-NTA elution buffer, precipitated using methanol/chloroform or TCA.**

**Table 4-1 Relationships between the absorbance and 6xHisHpf2 concentration, and the corresponding regressions, for the Lowry method**

| Buffer | Precipitation method | Slope | Regression |
|---|---|---|---|
| CDGJM | Methanol/chloroform | $4.7 \times 10^{-3}$ | 0.9329 |
| Elution | Methanol/chloroform | $4.3 \times 10^{-3}$ | 0.9075 |
| CDGJM | TCA | $3.1 \times 10^{-3}$ | 0.9871 |
| Elution | TCA | $3.2 \times 10^{-3}$ | 0.777 |

#### *4.3.1.2 Extinction coefficient*

Absorbance readings at 205 nm and 280 nm were taken for a 6xHisHpf2 solution of known concentration, based on the dry weight of purified material. The extinction coefficient was calculated to be 0.917 based on a known concentration of 6xHisHpf2 used for the calibration. This was used for quantifying the concentration of 6xHisHpf2 in purified samples.

#### *4.3.1.3 HPLC*

Initial trials were conducted with samples of 6xHisHpf2 deglycosylated according to the PNGase F protocol (Sigma), which included SDS to denature the protein and TRITON X-100 to enable PNGase F activity in the presence of SDS, an inhibitor of PNGase F. Figure 4-4 shows a large peak at approximately 22 min, however this peak was also present in the negative control, the TRITON X-100 only sample, shown in Figure 4-5.

When the detergents were removed from the deglycosylation process, no significant peak was observed, as seen in Figure 4-6. Fractions were collected, including wash fractions at 95% acetyl nitrile, and checked by SDS-PAGE silver stain, but only small protein fragments were observed (data not shown), suggesting degradation or proteolysis on the column, possible by an impurity in the PNGase F preparation.

A second column was trialled, with a C4 stationary phase. 6xHisHpf2, deglycosylated with no detergents present, was loaded and fractions collected for Western blot analysis. Figure 4-7 shows that no significant peaks were seen and Figure 4-8 shows that the

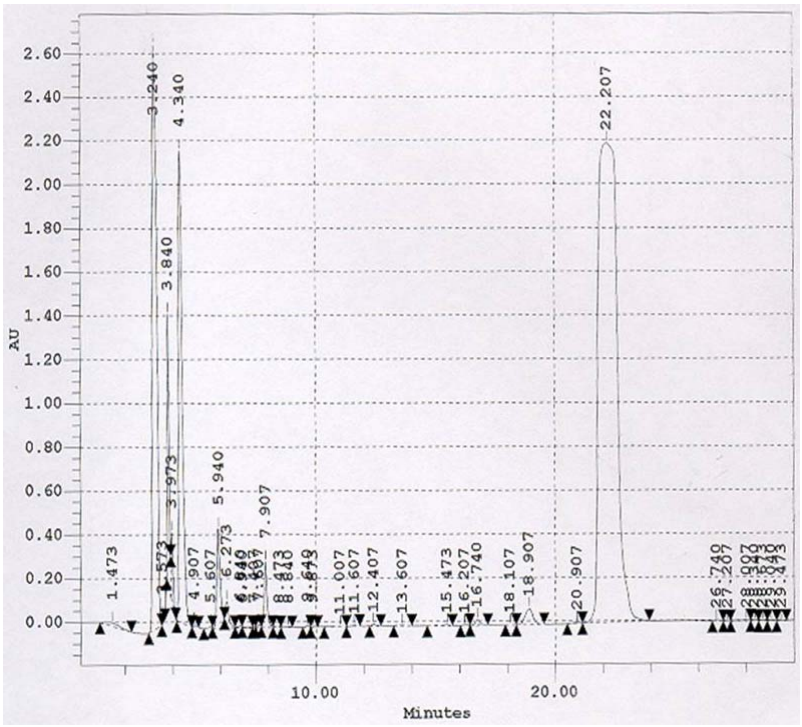


**Figure 4-4 Deglycosylated 6xHisHpf2 sample run on C8 HPLC column, prepared with TRITON X-100.**

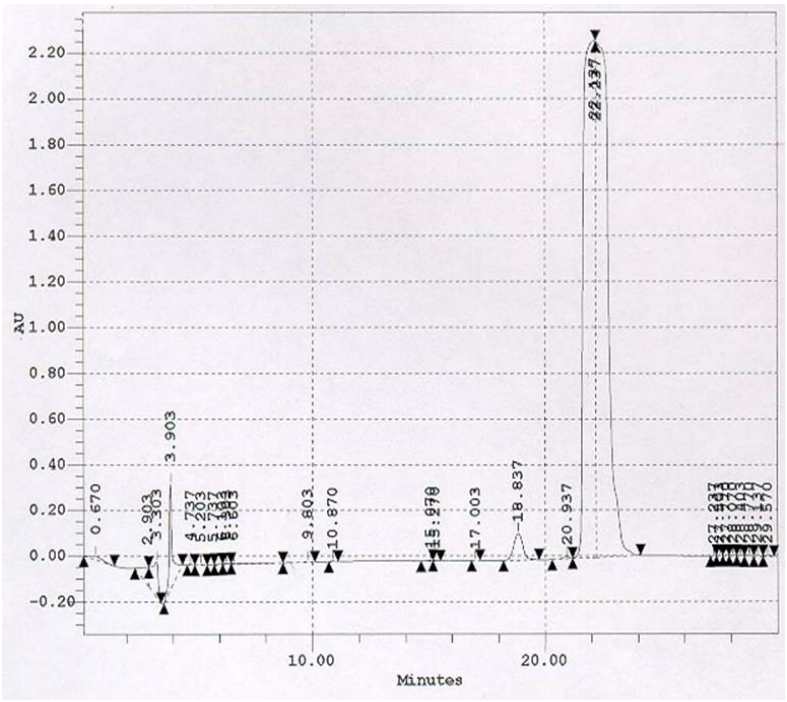


**Figure 4-5 TRITON X-100 sample run on C8 HPLC column.**

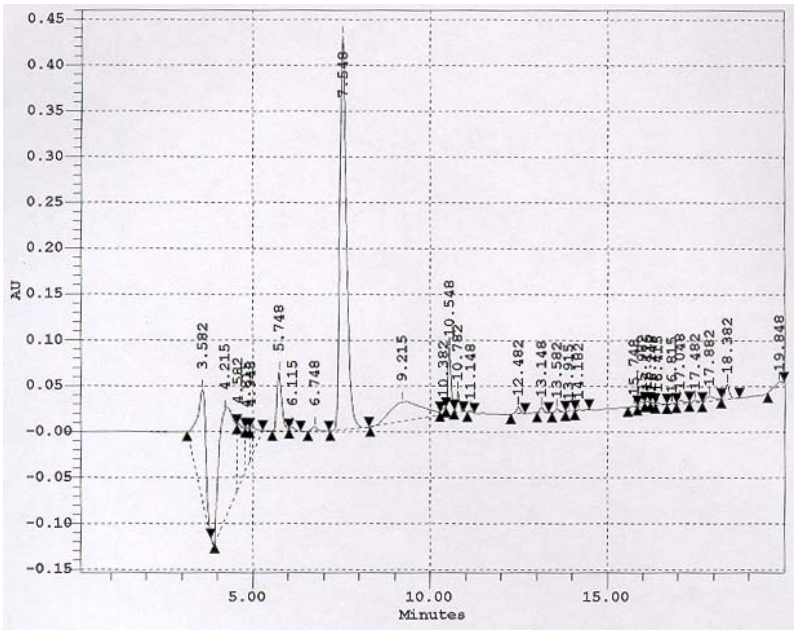


**Figure 4-6 Deglycosylated 6xHisHpf2 sample run on C8 HPLC column, prepared with no detergent present.**

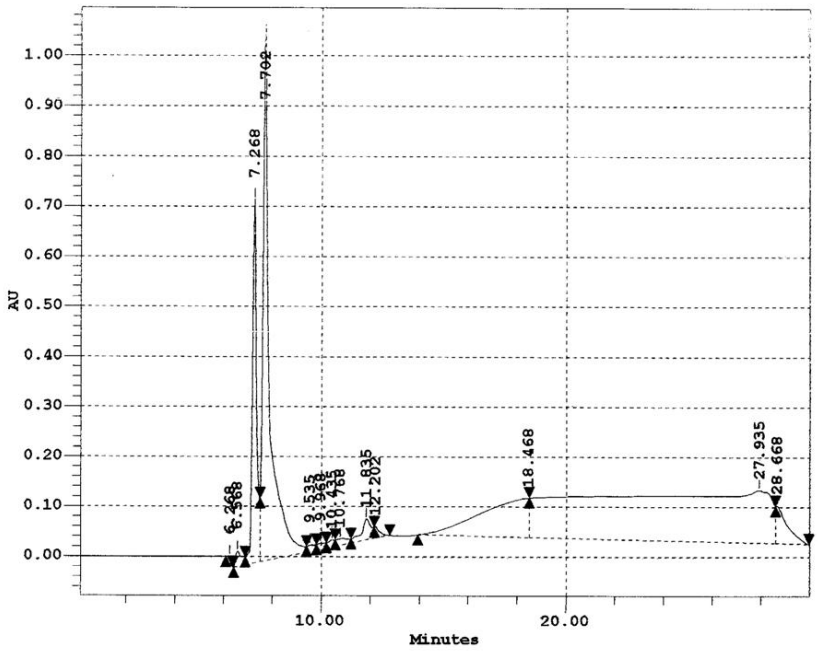


**Figure 4-7 Deglycosylated 6xHisHpf2 sample run on C4 HPLC column, prepared with no detergent present.**

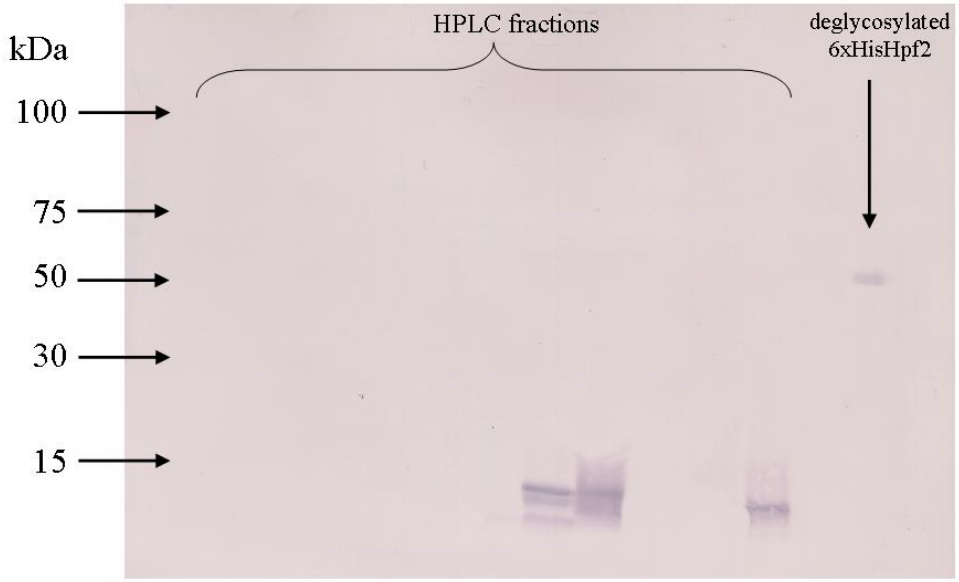


**Figure 4-8 Western blot of fractions from deglycosylated 6xHisHpf2 sample run on C8 HPLC column, prepared with no detergent present.**

fractions that were collected from the column contained 6xHis-tagged proteins that were much smaller than 6xHisHpf2 (MW of approximately 45 kDa), again indicating degradation or proteolysis.

Due to the problems with detergent interference and likely degradation of the protein, HPLC analysis for quantification was not further pursued.

#### *4.3.1.4 ELISA*

The ELISA method was trialled by running samples from different stages in the purification process and measuring the absorbance at 590 nm after probing with the antibodies and detection by the NBT/BCIP substrate. A standard curve was prepared for concentrations of 6xHisHpf2 up to 1 µg per well, based on dry weight, shown in Figure 4-9.

Samples from one purification batch were analysed, and the amount of 6xHisHpf2 was determined, as shown in Figure 4-10. The calculated amount of 6xHisHpf2 in 4 L of supernatant was found to be 34±23 mg while the eluate fractions combined were found to contain 226±20 mg. This variability is likely caused by binding differences due to different buffers being present. A second attempt was made, resulting in standard deviations of more than 300% from quadruplicate samples. This variability between replicates and binding differences due to buffer conditions is unacceptable in a quantification method, so improvements were attempted.

High protein binding plates were used in conjunction with pNPP as the phosphate substrate, with the absorbance was read at 405 nm. In this case, no relationship could be found between the absorbance readings and the protein concentration for the standards, shown in Figure 4-11.

Overall, ELISA gave inconsistent results both between replicates and for samples through the purification process, and attempts at this method for quantification were discontinued.

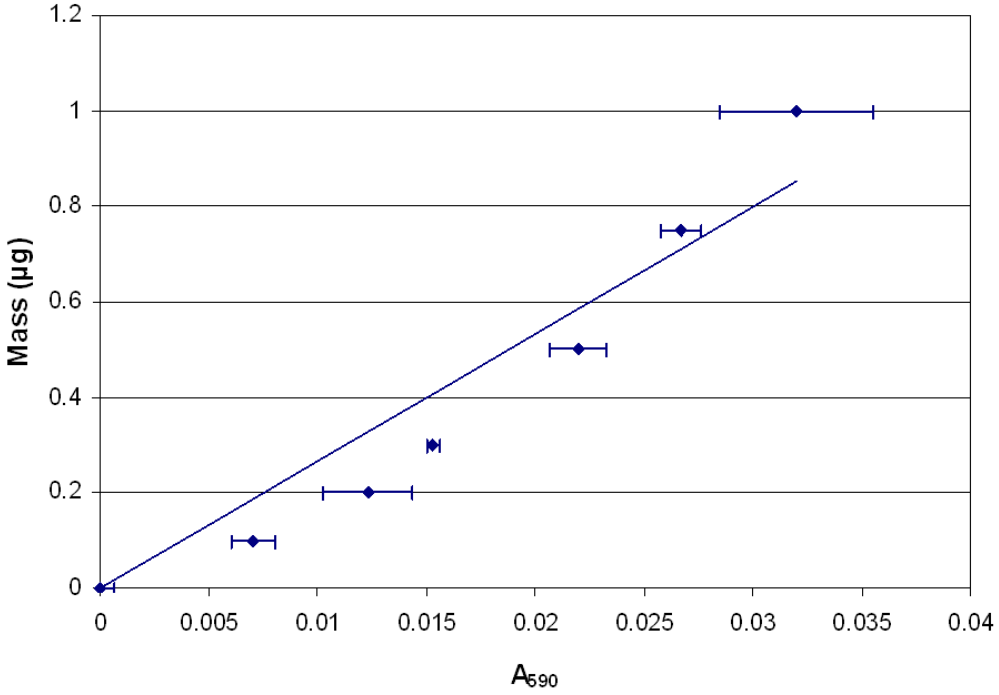


**Figure 4-9 ELISA standard curve for 6xHisHpf2 from 0 to 1 µg per well.**

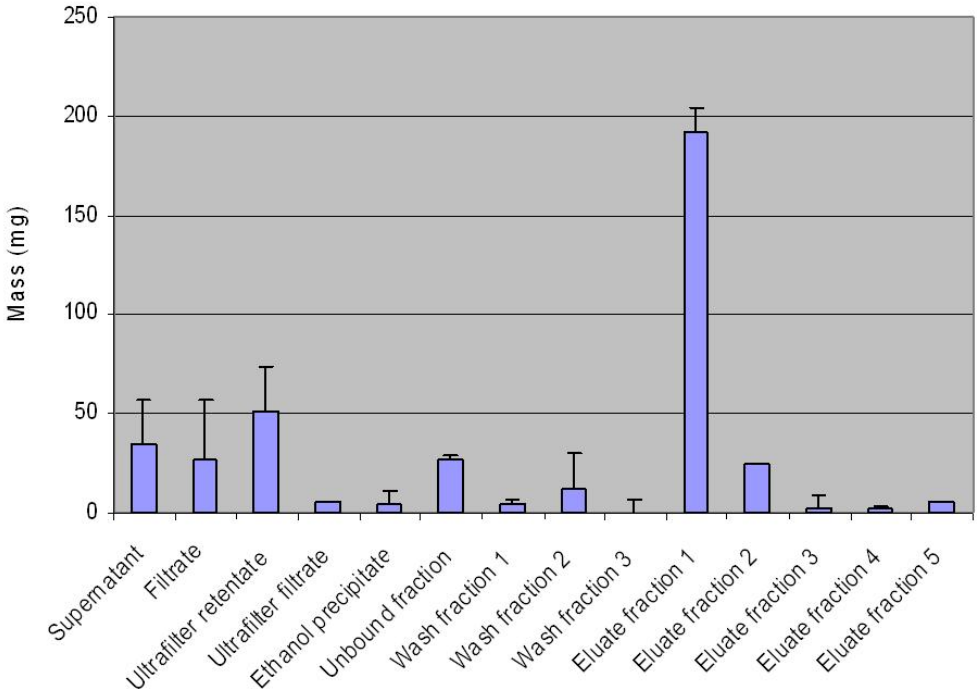


**Figure 4-10 6xHisHpf2 levels in purification samples as determined by ELISA.**

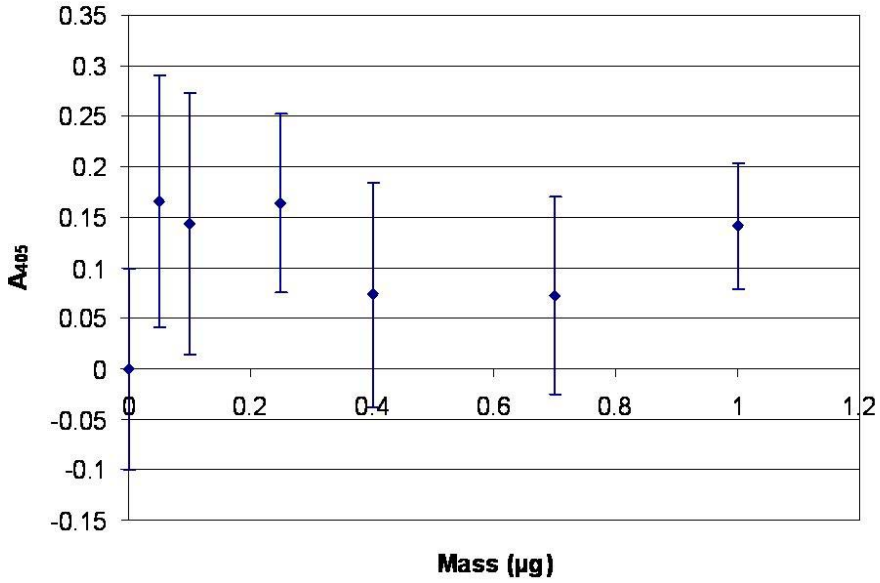


**Figure 4-11 Plot of absorbance against 6xHisHpf2 concentration with high protein binding plates and pNPP.**

#### *4.3.1.5 Slot blot*

The slot blot method was first attempted with the 6xHis ladder (Qiagen), purified 6xHisHpf2 and deglycosylated purified 6xHisHpf2. It was found that the untreated 6xHisHpf2 was not detected well, either due to inefficient binding or low detection by the primary antibody, shown in Figure 4-12, while the deglycosylated 6xHisHpf2 gave a good signal, so this was further investigated.

Some variation was observed in subsequent slot blots and further investigation revealed that the SDS and TRITON X-100 in the denaturing buffer were interfering with the binding of the protein. This is shown in Figure 4-13, where samples of equal 6xHisHpf2 concentration are prepared with varying levels of detergent.

A standard curve was prepared to enable the 6xHis ladder to be used as a standard in subsequent blots. This is shown in Figure 4-14, where the 6xHis ladder concentration ranges from 0 to 250 ng per well and the 6xHisHpf2 concentration ranges from 0 to 2000 ng per well.

Once the relationship between the 6xHis ladder and 6xHisHpf2 was established, samples from the purification batches were quantified. Each blot had the 6xHis ladder standard curve and six purification samples, all in quadruplicate. An example is shown in Figure 4-15, and all purification samples were tested this way to establish efficiencies in purification. Samples that fell outside the standard curve were re-analysed at higher or lower concentrations.

### 4.3.2 Purification method

Purification batches were grown in four 1 L cultures, and these 4 L batches were purified separately. Variations were introduced during this process in an attempt to improve the expression levels from the culture and the purification efficiency. Four typical batches are summarised in Table 4-2.

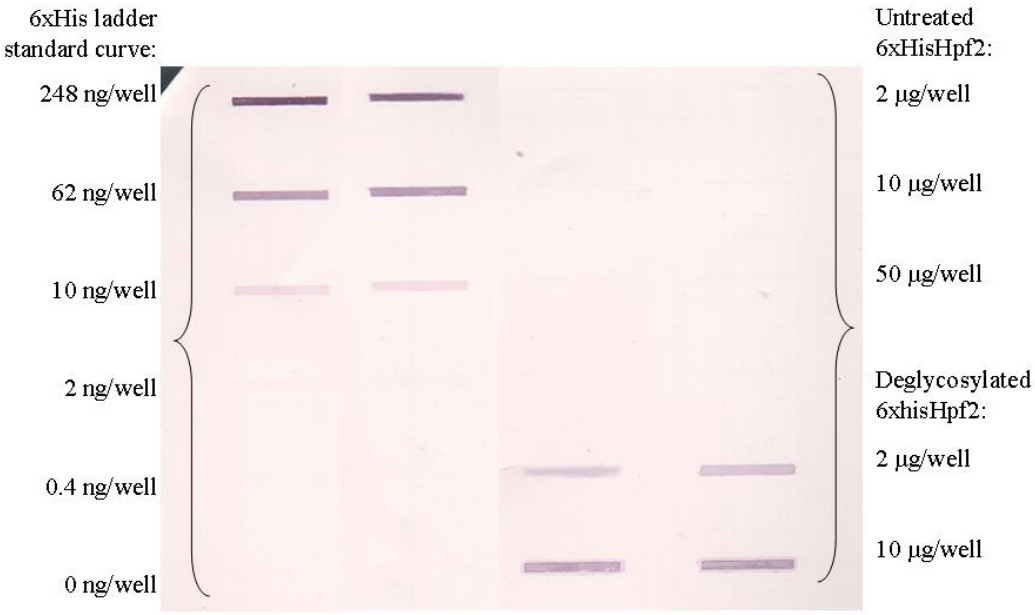


**Figure 4-12 Slot blot of 6xHis ladder and 6xHisHpf2 (untreated and deglycosylated). All samples in duplicate.**

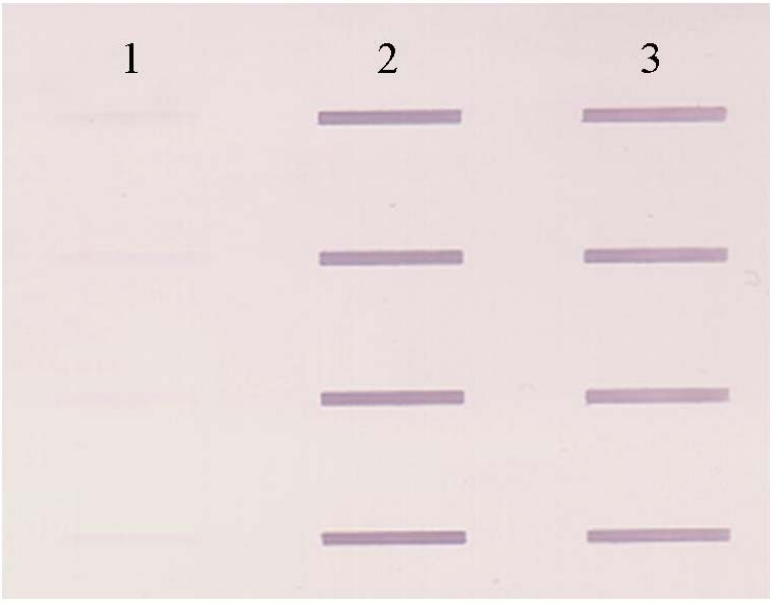


**Figure 4-13 Interference of detergents in binding. All wells loaded with 1 mg 6xHisHpf2 and all samples in quadruplicate. 1: 6xHisHpf2 with 1 µL TRITON X-100; 2: no TRITON X-100 present; 3: 6xHisHpf2 with 1 nL TRITON X-100.**

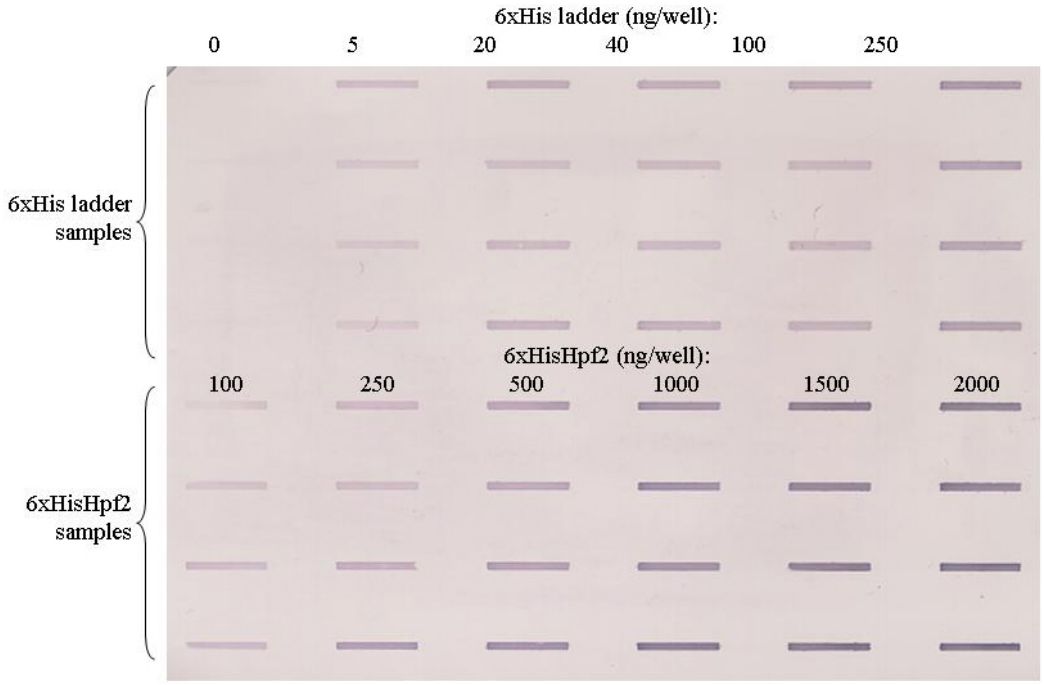


**Figure 4-14 Standard slot blot relating the 6xHis ladder to the deglycosylated 6xHisHpf2 standard.**

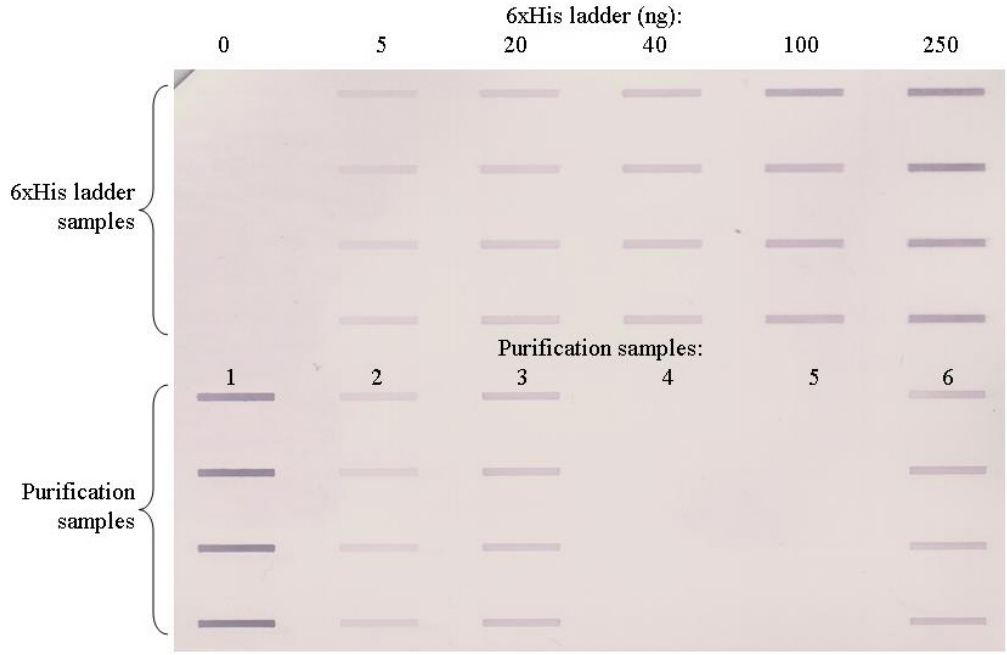


**Figure 4-15 Example slot blot showing fractions from purification batch D. 1: filtered supernatant; 2: Ni-NTA unbound fraction; 3: eluate fraction 2; 4: eluate fraction 3; 5: eluate fraction 4; 6: purified 6xHisHpf2.**

**Table 4-2 Summary of example purification batches**

| | Batch A | Batch B | Batch C | Batch D |
|---|---|---|---|---|
| **Expression (h)** | 32 | 48 | 40 | 32 |
| **Supernatant recovery** | Centrifuge and filtration | Centrifuge and filtration | Centrifuge and filtration | Centrifuge and filtration |
| **Volume reduction** | Ultrafiltration | Ultrafiltration and ethanol precipitation | Ultrafiltration and ethanol precipitation | Ultrafiltration and ethanol precipitation |
| **Metal affinity chromatography** | Ni-NTA | Ni-NTA | Ni-NTA | Ni-NTA |
| **Desalting** | Econo-pac column and dialysis | Econo-pac column and dialysis | Dialysis | Dialysis |
| **Finishing** | Lyophilisation | Lyophilisation | Lyophilisation | Lyophilisation |

### 4.3.3 Efficiency of purification

Twenty purification batches were expressed, for a total of 80 L of culture. The total dry weight of purified 6xHisHpf2 was 1.23 g, for an overall recovery rate of 15.4 mg $L^{-1}$.

The original concentration of 6xHisHpf2 in the JM59 culture was not able to be determined directly, so the initial concentration is taken to be the recovered supernatant, which has been centrifuged and filtered through a 0.2 μm filter. The concentration in the recovered supernatant was found to range between 50 and 100 mg $L^{-1}$, giving an overall production efficiency of approximately 15 to 30%.

Table 4-3 shows the recovery of 6xHisHpf2 at each stage of the purification process. These values were determined by slot blot analysis after production of 6xHisHpf2 was complete.

**Table 4-3 Recovery efficiencies of each stage in the purification process. Recovery is expressed as percent of initial concentration, with the step recovery in brackets.**

| Recovery (%) | Batch A | Batch B | Batch C | Batch D |
|---|---|---|---|---|
| Volume reduction | 91.0 | 98.0 | 90.7 | 99.2 |
| Metal affinity chromatography | 84.6 (93.0) | 52.3 (53.4) | 23.3 (25.7) | 32.9 (33.1) |
| Desalting | 37.5 (44.3) | 14.8 (28.3) | 11.3 (48.4) | 37.1 (113.0) |
| Finishing | 43.3 (115.7) | 9.0 (60.9) | 5.2 (45.8) | 30.0 (80.9) |

### 4.3.4 Purity of 6xHisHpf2 product

The protein content, determined from amino acid analysis, was 2.68 µg per 10 µL sample, which corresponds to 0.268 mg $mL^{-1}$. The mannose concentration was found to be 0.843 mg $mL^{-1}$, giving the total sample concentration as 1.102 mg $mL^{-1}$, lower than the expected 2 mg $mL^{-1}$ based on dry weight. The proportion of amino acids in the sample was found to be 24.3% by weight, very close to the theoretical value of 24.5%.

The purified sample was analysed by SDS-PAGE silver stain at two loadings, shown in Figure 4-16. It can be observed that the final product did not contain any other detectable proteins.

## 4.4 Discussion

Several commonly used quantification methods were assessed for their suitability in detecting 6xHisHfp2 in various buffers. A SDS-PAGE silver stain of supernatant samples, shown in Figure 4-1, revealed that the over-expressed secreted 6xHisHpf2 is the most abundant protein in the culture, so two non-specific protein quantification methods were considered. The first of these was the micro-biuret method described by Itzhaki and Gill (1964). This technique did not allow a useful relationship to be developed between the absorbance readings and the protein concentration, as low protein levels resulted in absorbance readings lower than the blank reading, shown in Figure 4-2. The micro-biuret method has been used to estimate protein concentrations

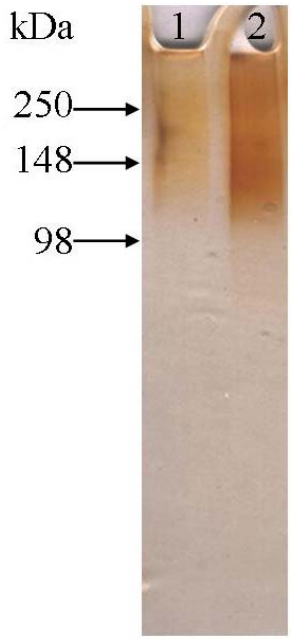


**Figure 4-16 SDS-PAGE Silver stain of purified sample of 6xHisHpf2. 1: 2 µg sample; 2: 10 µg sample.**

successfully over many years (Van Gelder and Krechting 1973; Semino et al. 1985; Zamer et al. 1989). Semino et al. 1985 also investigated glycoproteins and showed that this method was successful after treating the samples with exoglycosidases to reduce the glycosylation. In addition, previous studies have suggested that the sensitivity and accuracy of this method can be improved by the addition of detergents in the final colorimetric step (Johnson 1978), however these possible improvements to the method were not further pursued.

The Lowry assay was more promising. Previous investigations had found this method to be robust and adaptable to a automated microtiter plate format (Peterson 1977; Harrington 1990), and it has also been shown to be more accurate and reliable than the Bradford assay (Kirazov et al. 1993). In the current study, a linear relationship was found between the absorbance values and the protein concentrations (Figure 4-3), however this method was not robust, with at least a 50% error in concentration estimation resulting from the methanol/chloroform precipitation. Four combinations of precipitation technique and buffer were trialled, and different curves were found for each. Higher absorbances were observed for the samples prepared in CDGJM than for those in the elution buffer, indicating that this method would not be suitable for samples in different buffers.

Two HPLC methods were then assessed. HPLC for protein estimation has been used for more than 20 years for estimating the protein content of wines (Tyson et al. 1981; Waters et al. 1995a; Nordestgaard et al. 2007), as well as for other sources of proteins (Sitaramamma et al. 1998; Pechenov et al. 2004). Initially a C8 HPLC column was trialled, using purified deglycosylated 6xHisHpf2. Figure 4-4 shows a large peak observed at 22 min of a 30 min gradient, yet this was also observed in the negative control sample containing just TRITON X-100 (Figure 4-5), indicating that the detergent was generating this peak, rather than the protein. The detergent was removed from the deglycosylation procedure and the final concentration of the eluting acetyl nitrile was increased to 95%, as it was predicted that the deglycosylated protein would be strongly hydrophobic. This result is shown in Figure 4-6, with a peak observed between 7 and 8 min, but no 6xHisHpf2 could be detected in this sample by SDS-PAGE silver stain.

As no 6xHisHpf2 could be detected being eluted from the column, it was thought that it might still be bound. The wash fractions at 95% acetyl nitrile were collected and analysed, but the protein was still not detected. A less hydrophobic column, the C4, was then investigated, again with a gradient of up to 95% acetyl nitrile to elute the 6xHisHpf2. The HPLC result is shown in Figure 4-7, with the fractions collected and analysed by Western blot (Figure 4-8) showing small 6xHis-tagged protein fragments. The faint band in the final lane of Figure 4-8 shows part of the 6xHisHpf2 fraction that was loaded onto the column, indicating the correct size of the protein. This suggests that the protein was degraded, possibly by acid hydrolysis due to the presence of trifluoroacetic acid in the elution buffer, or alternatively by an impurity with proteolytic activity in the PNGase F. When checked by SDS-PAGE silver stain, the PNGase F sample has been shown to contain many contaminating proteins (data not shown), which may be activated in the HPLC environment. These possibilities were not further explored, and a HPLC method was not further considered.

An ELISA was the first method trialled that was based on the presence of the 6xHis tag. This assay has been used for protein estimation in many different applications, such as medical (O'Callaghan 1991; Collé et al. 1992; Bakheit et al. 2004; Reddy et al. 2004; Jaganathan et al. 2005) and environmental (Wirsing et al. 1999). Kramer et al. 1995 adapted the binding step to include propanol in an effort to increase the binding efficiency of the hydrophobic protein being studied. In the current study, a standard curve was prepared using purified 6xHisHpf2 with a known dry weight, shown in Figure 4-9. The standard curve showed a strong relationship between the protein concentration and the absorbance, indicating that this assay might be suitable for 6xHisHpf2 quantification. However, when samples from the purification process were run, the levels of 6xHisHpf2 were inconsistent, with the total amount of protein calculated to be 34 mg in the supernatant and over 200 mg in the Ni-NTA elute fractions. This implied that the buffer the protein was in affected either the binding capacity or the sensitivity of the assay. Also, large variations of up to 300% were seen in the quadruplicate samples, indicating that the assay would not give consistent results.

Wirsing et al. 1999 used pNPP, a more sensitive phosphatase substrate, as the substrate for the ELISA. In an attempt to improve the ELISA technique, high protein binding plates and pNPP were tested, shown in Figure 4-11. This did not improve the detection,

rather it lead to a loss of any relationship for the assay, leading to the rejection of this assay as a means to quantify 6xHisHpf2.

The second assay to make use of the 6xHis tag for detection of 6xHisHpf2 was the slot blot assay. This assay has been used for many types of protein estimation, predominantly in the medical field (Brito et al. 2004; Sultana et al. 2004; Sultana et al. 2005). Both untreated and deglycosylated samples were run to assess the detection efficiencies. As shown in Figure 4-12, the deglycosylated protein is detected more effectively than the untreated protein. Further trials indicated that the detergents in the deglycosylation process interfered with the binding, as shown in Figure 4-13. Once this problem was resolved, a standard curve was established to relate the 6xHisHpf2 to the 6xHis ladder, so further analysis could be conducted using the standard product rather than preparing fresh 6xHisHpf2 for each blot (Figure 4-14), and the slot blot method was chosen as the quantification technique for 6xHisHpf2. Samples from the purification batches were then analysed to determine the purification efficiency and recovery rates of the process, with an example shown in Figure 4-15.

The purification method was varied initially, with the conditions given in Table 4-3, although at the time no quantitative method was established to determine whether this improved the yield. Due to time restrictions, the efficiencies of the four methods of 6xHisHpf2 purification were compared after sufficient protein was produced for sensory analysis, shown in Table 4-3. As a result, the purification process was not further optimised, although it could be seen which steps need further development to improve the recovery. Overall, it was observed that the changes to the purification process did not affect the recovery efficiency greatly.

The volume reduction step, either ultrafiltration on its own or in combination with an ethanol precipitation, was found to be very efficient, with more than 90% recovery in all batches. This was expected for the ultrafiltration step, as the MW cut-off was 10 kDa and 6xHisHpf2 is approximately 180 kDa. The ethanol precipitation was also highly efficient, indicating that the conditions used do not need further improvement.

The metal affinity chromatography step was found to have very variable recovery, as low as 25.7% for batch C, and this may be because the binding of the 6xHis tag is very

sensitive to the buffer pH or the elution may be very sensitive to the histidine concentration. Fresh buffers were prepared for each batch, and these may have varied. The unbound and wash fractions were checked for 6xHisHpf2 by SDS-PAGE silver stain and slot blot (data not shown), but this was never detected in significant concentrations. After the protein was eluted, the column was washed with two column volumes of 2 M NaOH. This was discarded and thus not tested but it may have contained tightly bound 6xHisHpf2. To increase the recovery from the column, it is suggested that higher histidine concentrations are considered for the elution buffer, or alternatively, the protein could be eluted by pH changes in the elution buffer (Qiagen 2003b).

Two types of desalting techniques were used for the purification. Initially size exclusion chromatography columns were used after the Ni-NTA elution step, however it was suspected that there were large losses from these columns (Pocock, K.F, personal communication, June 2006). As a result, the samples were treated by dialysing against Milli-Q $H_2O$ instead. Batches A and B went through both desalting steps, while batches C and D underwent dialysis only, and the recovery was higher in this case. Any further production of 6xHisHpf2 should only utilise the dialysis stage to desalt the samples after elution.

The final step in the production was drying of the product. This involved lyophilisation for all batches. The variation in recoveries may be due to the difficulty of transferring the samples from dialysis tubing to the bottles used for lyophilisation. Some sample was lost at this point as it remained in the tubing. This could be improved by better handling techniques, such as careful rinsing of the tubing after transferring the sample to the bottle.

The purity of the finished purified sample was assessed by amino acid analysis and a mannose assay. The weight determined by these methods found that the dry weight was over-estimating the protein weight, with a calculated concentration of 1.1 mg $mL^{-1}$ compared to the dry weight estimate of 2 mg $mL^{-1}$. This is most likely caused by incomplete drying in the lyophilisation process, as the mannose chains are very hydrophilic and bind water molecules tightly. The SDS-PAGE silver stain of the

sample (Figure 4-16) shows that the 6xHisHpf2 is relatively pure, with no significant contaminating proteins.

## 4.5 Conclusions

Many different quantification methods were assessed for use with 6xHisHpf2, although the only one that was successful was the slot blot assay. This still has some problems associated with it, as the samples must be deglycosylated and then diluted before analysis, with each step introducing more errors. Despite this, it was able to be used to assess the concentrations of various purification samples to allow the efficiencies and recovery rates to be determined for a range of purification batches.

If no improvements were made to the purification methods, the process used in batch D should be used if further production was required. This gave a 30% recovery of 6xHisHpf2. Although the recovery in batch A was slightly higher, the extra desalting step used was deemed to be unnecessary. However, by further improving the metal affinity chromatography and desalting steps in particular, large improvements in recovery rates may be achievable.

Although the purification efficiency was quite low, the final product was shown to be very pure by animo acid analysis, mannose assay and SDS-PAGE silver stain of the final purified protein, and enough material was produced to allow the sensory studies to be conducted.

# 5 SENSORY ANALYSIS OF INVERTASE AND 6XHISHPF2 IN WINE

## 5.1 Introduction

The aim of this project was to determine the effects of the addition of Hpf2 on the appearance, aroma and palate of wine, to establish whether the product could be a feasible alternative to bentonite fining. Invertase, a related yeast mannoprotein with haze protective activity, was also investigated. In order to perform the sensory analysis with both aroma and palate assessment, food grade invertase and 6xHisHpf2 were used. Invertase is commonly used in the manufacture of confectionary, so acquiring it from this source ensures that it is safe for consumption. 6xHisHpf2 was produced using dedicated new glassware and the production process was approved by the University of Adelaide Human Ethics Committee for sensory analysis. All wine samples were to be expectorated after tasting, so none of the invertase of 6xHisHpf2 should be consumed.

Previous studies have considered the sensory effects of up to 1.5 g $L^{-1}$ purified yeast mannoprotein in a Riesling wine (Will et al. 1991). No statistical difference was found in sensory quality, either with triangle or ranking tests. The study found no increase in viscosity of the treated wine (Will et al. 1991).

The effect of an invertase fragment with haze protective activity has also been studied (Rowe 2002). Sensory analysis of red and white wine showed no statistical significant difference in aroma or palate between the treated and untreated wines. As this fragment is assumed to behave in a similar nature to Hpf2, it is expected that Hpf2 will have little or no effect on the sensory properties of wine.

Prior to bentonite treatment, different wines have different potentials for developing haze and different protein concentrations (Kock and Sajak 1959; Bayly and Berg 1967; Ferreira et al. 2002). There does not appear to be a clear correlation between the haze potential of a wine and the protein concentration, MW and pI (Moretti and Berg 1965; Bayly and Berg 1967; Mesrob et al. 1983; Heatherbell et al. 1984; Lamikanra and Inyang 1988), so it is important to assess the haze potential in each wine by a heat test assay.

Sensory analysis can involve several different tests. Informal assessment allows the different wine attributes to be discussed and a suitable wine selected for further study. Duo-trio tests are useful for determining whether differences result from small changes to the product, normally due to ingredients or process techniques. The test can be used to determine if an overall difference exists, without specifying which attributes may be affected. Reasonably large panels are required, with more than 15 needed, but up to 30 is preferable (Meilgaard et al. 1991). The test is presented with a known control to the left, and then the control and the changed product are presented in random order. Panellists are trained in the methodology of the testing and the product should be familiar to them.

Statistical analysis was conducted on all the sensory results, with the data interrogated for differences and similarity. The difference testing determined whether there was a significant difference due to the addition of either 6xHisHpf2 or invertase, based on the P-value found using FIZZ software. The P-value is the probability of obtaining a result at least as extreme as a given data point, assuming that data point was the result of chance alone (Lawless and Heymann 1998). The similarity testing allowed an estimate of the proportion of discriminators amongst the general population, as well as an uncertainty in this value, or standard error.

## 5.2 Materials and methods

### 5.2.1 Preparation of wine

Three wines were used in this study, and all were sourced in 2006. One Sauvignon blanc wine was from the Adelaide Hills, while a Chardonnay and a second Sauvignon blanc were from the Riverland. The wine was allowed to cold-settle at 4°C until yeast was collected at the bottom of the tank. 40 L of each wine was racked into 2x 20 L containers. The wine was filtered (0.2 μm) into bottles after the addition of potassium metabisulfite to a concentration of between 25 and 30 mg $L^{-1}$ free sulfur dioxide. All wine was stored at 4°C in darkness until required.

### 5.2.2 Heat test assay

The heat test assay was based on the method of Pocock and Rankine (1973), with a modification of the micro-method described by Waters et al. (1991) and Stockdale (2000). 6xHisHpf2 was made up to 5 mg $mL^{-1}$ in each wine and the invertase was prepared as a 20% dilution in each wine (Enzyme Systems). The 5 mg $mL^{-1}$ solution of 6xHisHpf2 was diluted into the wines to a range from 50 to 500 mg $L^{-1}$, and the 20% invertase solution was diluted from 0.25 to 2%. Four replicates of each sample were prepared in PCR strip tubes (Eppendorf) and heated to 80°C for 6 h and then cooled to 4°C for at least 12 h in an MJ Research PTC-100 programmable thermal cycler. Samples were allowed to come to room temperature and were transferred to a 96-well flat bottom plate (Greiner). The optical density at 490 nm was measured using a SpectraMax plate reader. The minimum haze (0%) was set by the $OD_{600}$ of the unheated wine samples. The haze produced from the heated wine with no additions was classified as the maximum (100%).

### 5.2.3 Wine analysis

Ethanol, glucose and fructose, free and total sulfur dioxide, titratable acidity, and volatile acidity concentrations and pH specific gravity were measured by WineScan using the method described by (AWRI Analytical Service 2007b).

### 5.2.4 Protein content

The protein concentration was determined by HPLC as described by Pocock et al. (1998). Briefly, the samples were diluted in Milli-Q $H_2O$ appropriately and injected into a C8 column previously equilibrated in solvent A (Appendix 1). Components were eluted by a gradient of 0 to 80% of solvent B (Appendix 1) over 30 min. The thaumatin and chitinase concentrations were calculated separately as equivalents in mg $L^{-1}$ of a standard protein, cytochrome c.

### 5.2.5 Measurement of glycerol concentration

Glycerol in the invertase sample was measured by HPLC using a Bio-Rad HPX-87H column (Nissen et al. 1997).

### 5.2.6 Informal descriptive analysis

Approximately one year after bottling, informal descriptive analysis was undertaken. The three untreated wines were presented to four judges, experienced in assessing wines. The panel was asked to describe the aroma and palate of the wines.

### 5.2.7 Informal sensory analysis of 6xHisHpf2 and invertase treated wines

6xHisHpf2 and invertase treated wines were informally assessed in three different base wines (two Sauvignon blanc and one Chardonnay). The invertase and 6xHisHpf2 wines were prepared by the addition of food grade invertase to 2% or 250 $mgL^{-1}$ 6xHisHpf2 to the untreated wine. The amount was based on the heat test result to reduce haze to the minimum possible. A panel of eight assessors was used. An untreated sample (control) of each base wine was also assessed. Samples of 25 mL were presented in three sets of three, each set being one wine, with the untreated as the first wine, the invertase treated wine second and the 6xHisHpf2 treated wine as the third wine. Subjects were asked to describe appearance, aroma and palate of all the wine samples, and particularly to note difference in these areas between the wines in each set.

### 5.2.8 Formal sensory analysis – duo-trio test

Wines were prepared as in described in Section 5.2.7. As bubbles and foaming were noticed in the informal assessment, the wines were presented in black glasses to prevent this being used to distinguish the wines. Sodium lights were also used to try to mask the bubbles and foaming. Glycerol was added to the control wine to match the glycerol in the invertase solution. The testing took place in sensory booths under controlled conditions. The wines were presented as a set of three with a known control presented first (reference sample), then one control sample and one treated sample presented second or third in random order. Each set was presented in duplicate.

### 5.2.9 Statistical analysis

The sensory data from the formal duo-trio testing was subjected to difference and similarity analysis. Each panellist repeated the duo-trio test in each session, effectively

doubling the number of samples for difference testing. The statistical software package FIZZ was used to determine the P-values for the difference testing. A P-value of more than 0.1 suggests that the samples are not statistically different, while a value of less than 0.1 suggests differences between the samples.

The test for significant similarity uses the two sets for each taster, with a 1 in 4 chance of correctly guessing the different samples in both duo-trio sets. The number of discriminators, D, is calculated from the number of correct responses, X, and the total number of responses, N. Equation 5-1 shows the general formula for a 1 in 4 chance (Lawless and Heymann 1998).

$$X = D + \frac{1}{4}(N - D) \qquad \textbf{5-1}$$

Rearranging Equation 5-1, the number of discriminators can be found:

$$D = \frac{4}{3}X \; - \; \frac{1}{3} \qquad \textbf{5-2}$$

The standard error, SE, can be found:

$$SE = \sqrt{\frac{\left(X/N\right)\left(1 - X/N\right)}{N}} \qquad \textbf{5-3}$$

## 5.3 Results

### 5.3.1 Heat tests

#### *5.3.1.1 6xHisHpf2*

The haze protective activity of purified 6xHisHpf2 was assessed in heat tests in each of the three wines. Figure 5-1 shows the haze reduction in the three wines due to the addition of 6xHisHpf2. A plateau in haze occurred at approximately 250 mg $L^{-1}$ for each of the wines, and this level of addition was chosen for use in the sensory studies.

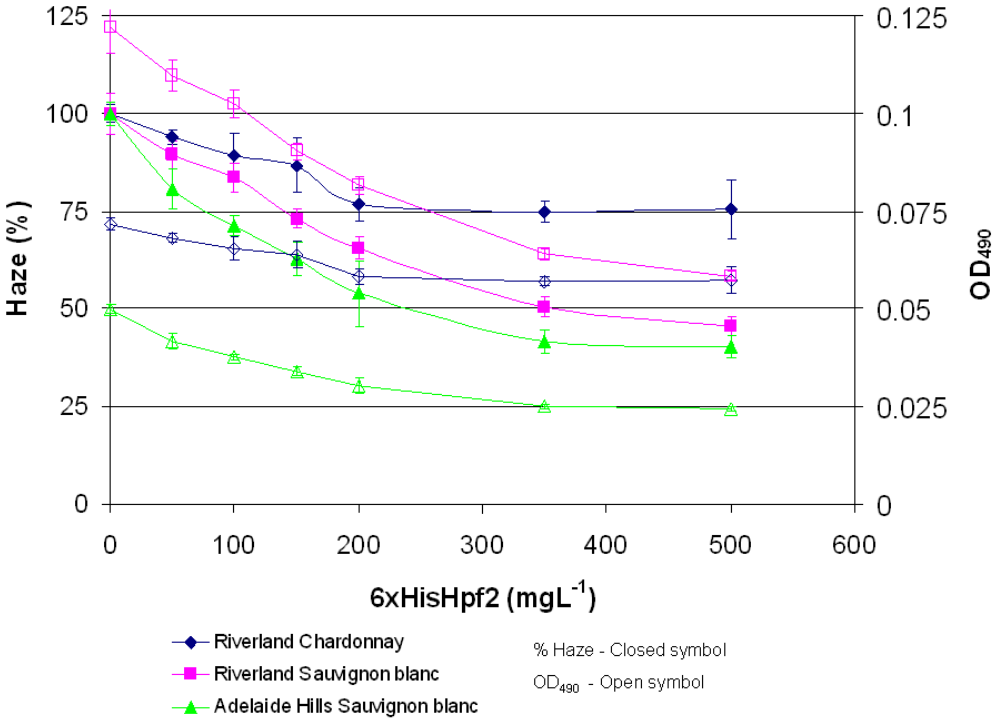


**Figure 5-1 Heat test assay of 6xHisHpf2 in three wines selected for sensory analysis.**

*5.3.1.2 Invertase*

The haze protective activity of invertase was determined in the three wines. The heat test assay is shown in Figure 5-2, and the haze reduction plateaus at 2%, and this value was used for the sensory studies.

### 5.3.2 Wine analysis and protein content

The composition of the wines and the protein concentrations are shown in Table 5-1.

**Table 5-1 WineScan and HPLC analysis of the three wines**

| Analysis | Riverland Chardonnay | Riverland Sauvignon blanc | Adelaide Hills Sauvignon blanc |
|---|---|---|---|
| Alcohol (% v/v) | 12.9 | 11.5 | 13.7 |
| Glucose and fructose ($g\ L^{-1}$) | 0.8 | 0.5 | 5.0 |
| pH | 3.45 | 3.16 | 3.17 |
| Specific gravity | 0.9916 | 0.9921 | 0.9916 |
| Free sulfur dioxide ($mg\ L^{-1}$) | 7 | 9 | 0 |
| Total sulfur dioxide ($mg\ L^{-1}$) | 100 | 71 | 62 |
| Titratable acid, pH 7.0 ($g\ L^{-1}$) | 5.3 | 5.9 | 6.6 |
| Titratable acid, pH 8.2 ($g\ L^{-1}$) | 5.9 | 6.2 | 6.6 |
| Volatile acidity ($g\ L^{-1}$) | 0.38 | 0.39 | 0.45 |
| Thaumatins ($mg\ L^{-1}$) | 70 | 132 | 91 |
| Chitinases ($mg\ L^{-1}$) | 5 | 88 | 40 |

### 5.3.3 Informal descriptive analysis of the untreated wines and difference testing of 6xHisHpf2 and invertase treated wines

The Riverland Chardonnay was described as very aromatic, with esters, bubblegum,

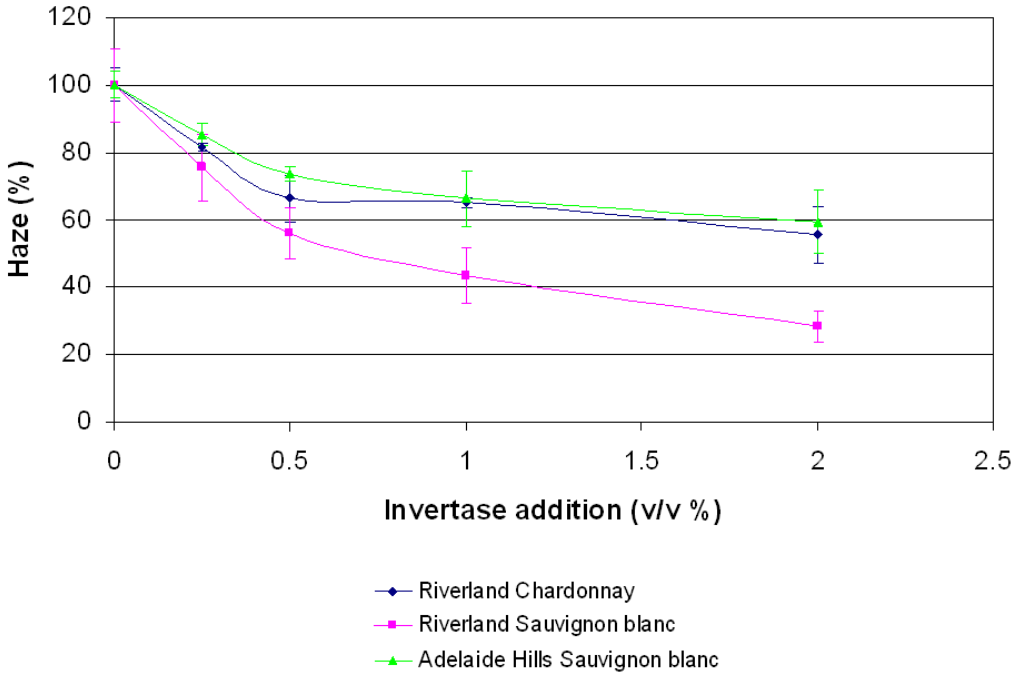


**Figure 5-2 Heat test assay of invertase in three wines selected for sensory analysis.**

fruit and oak being dominant and, to some extent, bitter on the palate. Descriptors used for the Riverland Sauvignon blanc included tropical fruit, plastic, and wet cardboard, and the wine was thought to be bland. The Adelaide Hills Sauvignon blanc was described as yeasty and green, with hydrogen sulfide or aldehyde characters. Phenolics were also noted and the wine was found to be sweet.

The wines were stored at 4°C in darkness, and approximately one year later, informal difference testing was undertaken. 6xHisHpf2 was added at 250 mg $L^{-1}$ and invertase was added at 2% to all three wines. A panel of eight assessors were presented with a set of three glasses per wine, with the set consisting of the untreated control, the invertase treated sample and the 6xHisHpf2 treated sample. Differences between the wines in each set were described.

The Chardonnay was described as oxidised, with a bruised apple character, and the 6xHisHpf2 made the wine 'harder', while the invertase addition lead to a creamier mouth-feel. In the Riverland Sauvignon blanc, the 6xHisHpf2 addition was the least preferred. The 6xHisHpf2 appeared to reduce or subdue the Sauvignon blanc character in the Adelaide Hills wine, and the control seemed more intense than either of the treated wines.

Bubbles and foaming was noticed in all the wines treated with either 6xHisHpf2 or invertase, and also both additions caused an increase in viscosity, noticed by eye and also in the mouth. To prevent the visual difference from influencing the formal panel, black glasses were used for the next stage of analysis.

Invertase addition was seen to increase the sweetness of the wines. HPLC analysis showed 16.3 g $L^{-1}$ glycerol in the invertase solution, so glycerol was added to the control wine to match the concentration in the invertase sample in the formal invertase testing.

Overall, 6xHisHpf2 did not appear to add any flavour or taint character, but masked or dulled the wine slightly. The differences seemed quite small, which warranted formal duo-trio testing. The Riverland Sauvignon blanc was chosen for the formal sensory

assessment as it was more bland, so any differences caused by the addition of either 6xHisHpf2 or invertase should be more apparent in this background.

### 5.3.4 Formal duo-trio

#### *5.3.4.1 Invertase*

Duo-trio tests were performed in duplicate for each subject, giving a 50% chance of guessing the correct answer for one set and a 25% chance of guessing the two sets correctly. A total of 30 subjects assessed the invertase samples, and the data could then be treated as 60 duo-trio tests for difference testing (1 in 2 chance of a correct guess) or 30 sets for similarity testing (1 in 4 chance of a correct guess). With the 2% invertase addition, 31 of 60 responses were correct, so no significant difference was found. Of the 30 sets, eight responses were correct, from which it was determined that 2.2% of tasters are discriminators and able to distinguish invertase in the wine sample. The standard error was 13.3%, indicating that actual number of discriminators is between 0 and 15.5%.

#### *5.3.4.2 6xHisHpf2*

The effect of 6xHisHpf2 in wine was tested twice, with the addition being made either on the day of the test or one month prior to the test. For the latter, the wine was then stored at 15°C until the day of the test. The difference testing showed no significant difference in either case, shown in Table 5-2, while the similarity testing, summarised in Table 5-3, found that approximately 10% of tasters can discriminate if 6xHisHpf2 is added on the day, but none can tell if it was added one month previously and stored in cellar-like conditions.

**Table 5-2 Difference testing of 6xHisHpf2 in Riverland Sauvignon blanc**

| Sample preparation | Number of assessors | Correct responses | P-value | Significance |
|---|---|---|---|---|
| On day of test | 62 | 33 | 0.3518 | Not significant |
| Stored for 1 month at 15°C | 60 | 23 | 0.9741 | Not significant |

**Table 5-3 Similarity testing of 6xHisHpf2 in Riverland Sauvignon blanc**

| Sample preparation | Number of assessors, N | Correct responses, X | Number of discriminators, D | Standard error range |
|---|---|---|---|---|
| On day of test | 31 | 10 | 9.5% | 0 – 23.5% |
| Stored for 1 month at 15°C | 30 | 4 | 0% | N/A |

## 5.4 Discussion

The initial analysis of the three wines showed some differences. The alcohol concentrations ranged from 11.5 to 13.7%, the latter being quite high for a white wine. Interestingly, this Adelaide Hills Sauvignon blanc wine with the high level of ethanol had 5g $L^{-1}$ residual glucose and fructose, leading to it being described as sweet by some of the panellists. The protein levels varied significantly, with the Riverland Sauvignon blanc having the highest levels. This may be why the haze in this wine was able to be reduced to a lower percentage than the other two wines, shown in Figure 5-1. This is supported by the raw absorbance readings, where the Riverland Sauvignon blanc produces the highest haze, with an $OD_{490}$ of almost 0.12 compared to just over 0.05 for the Adelaide Hills Sauvignon blanc.

The heat test assay for invertase (Figure 5-2) shows a similar pattern, with the plateau occurring at around 2% addition. This is specific for this invertase sample, which was supplied as a solution with no concentration given. It is assumed it is dissolved in water and it was shown to contain glycerol, so the final concentration of invertase required to reduce haze to a minimum is unknown. If this product was to be developed further, the invertase concentration should be quantified.

It should be noted that the heat test does not indicate that the haze was reduced to acceptable levels. To prevent visible haze, the $OD_{490}$ needs to be reduced to 0.018 (data not shown). This implies that even the best reduction in haze by either invertase or 6xHisHpf2 is not able to stabilise the wine completely, and so some bentonite fining would still be required, but the bentonite dosing would likely be greatly reduced, lowering the negative effects of the treatment, such as wine and aroma compound losses and financial losses to the winery.

During the informal difference testing, it was observed that the addition of either invertase or 6xHisHpf2 to wines leads to bubble formation. As a result, black glasses were used in the formal duo-trio testing so subjects were not able to judge the wines on appearance, as only the aroma and palate differences were to be assessed. However, if either product was to enter the market as a bentonite replacement, this problem would need to be resolved, as the consumer would be likely to reject a still wine which foams and bubbles in the glass.

The formal duo-trio test for the invertase showed no significant difference between the control wine and the invertase-treated wine. This is consistent with previous work on the sensory effects of mannoproteins in wines (Will et al. 1991). It was calculated that approximately 2% of the panel were discriminators, which is low enough to be negligible: 2% invertase could be added to wines and the general consumer would not notice the effect.

When 6xHisHpf2 was considered, no significant difference was observed, however the P-value was much lower, and approximately 10% of tasters were found to be discriminators. The test was repeated, but with the addition of 6xHisHpf2 one month prior to the tastings to more closely replicate the industrial situation and again no significant difference was found. The P-value increased from around 0.35 to 0.97. For this trial, no discriminators were detected, indicating any difference was indistinguishable.

## 5.5 Conclusions

Active concentrations of invertase and 6xHisHpf2 were determined in three different unfined wines with varying protein concentrations by heat test assays. To reach the maximum haze reduction, 2% addition of food grade invertase is required or 250 mg $L^{-1}$ 6xHisHpf2. The sensory effects of both invertase and 6xHisHpf2 were assessed informally in the three wines, and only minimal differences were noticed.

Formal sensory analysis showed no significant differences for either treatment, with 10% of the panel being discriminators for 6xHisHpf2 and only 2% for invertase. When

the 6xHisHpf2 trial was repeated, after storage of the treated wine for one month at 15°C, no discriminators were identified, implying that either of these products could be used in the wine industry in conjunction with a reduction in bentonite fining, without noticeable changes to the wine aroma or palate, although the foaming in the glass requires further investigation, as this is one aspect that could keep the product from the marketplace.

# 6 COMPARISON OF THE HAZE PROTECTIVE ACTIVITIES OF 6XHISHPF2 FROM *SACCHAROMYCES CEREVISIAE* AND *PICHIA PASTORIS*

## 6.1 Introduction

In a separate study, the role of the glycosylation of Hpf2 was examined. As part of this investigation, Hpf2 was expressed in a second yeast strain, *Pichia pastoris*. This methylotrophic yeast has been shown to produce proteins with different glycosylation patterns from *S. cerevisiae*. The expression was attempted with two different signal sequences for secretion, the alpha mating factor and the native Hpf2 signal, both from *S. cerevisiae*, and with the 6xHis tag at the N- and C-terminus of Hpf2. The most successful combination was the alpha mating factor with the C-terminus tag. This protein was over-expressed and purified from *P. pastoris* and characterised (Tan 2005).

The *P. pastoris* 6xHisHpf2 was found to be approximately 83 kDa by SDS-PAGE, with about 50% protein (Tan 2005). In contrast, the *S. cerevisiae* protein is approximately 25% protein, with a total weight of 180 kDa. This size difference suggests that the two proteins may have different haze protective activities. The extinction coefficient, $E_{280}$, was determined to be 0.49 (Nasution 2007), compared to 0.917 for the *S. cerevisiae* protein.

Two attempts were made to produce deglycosylated 6xHisHpf2. The first method involved expression of 6xHisHpf2 in the bacteria *E. coli*, as described in Chapter 2. Bacteria are unable to glycosylate proteins, so the protein expressed would have no glycosylation. However consistent expression and purification was not achievable from *E. coli*, so this method was dismissed. The second trial involved using HPLC to purify 6xHisHpf2 produced from *S. cerevisiae*, which had been treated with PNGase F to remove the N-glycosylation. This was trailed in conjunction with the HPLC quantification method, as described in Section 4.3.1.3. Again, this method was unsuccessful, so only the *P. pastoris* protein was compared to the original *S. cerevisiae* protein for this study.

The aim of this work is to study how the differences in glycosylation affect the haze protective activity of Hpf2 in wine, in an attempt to determine the mode of action of the protein in haze reduction.

## 6.2 Materials and methods

### 6.2.1 Wine preparation

The wine used for these studies was a 2004 Sauvignon blanc. It was cold-settled at 4°C for one month until yeast collected at the bottom of the tank. The wine was racked off, then filtered (0.2 μm) into bottles after the addition of potassium metabisulfite to a concentration of between 25 and 30 mg $L^{-1}$ free sulfur dioxide. All wine was stored at 4°C in darkness until required. HPLC analysis was performed to determine the protein levels of the wine.

### 6.2.2 Sample preparation

6xHisHpf2 from *S. cerevisiae* was prepared as described in Section 4.2.2. 6xHisHpf2 from *P. pastoris* was provided by collaborators (Nasution 2007). Briefly, *P. pastoris* over-expressing 6xHisHpf2 was grown in buffered methanol-complex medium for 3 days at 30°C. The supernatant was collected by centrifugation (14 000g, 40 min, 4°C), one volume of 5x Ni-NTA binding buffer was added for every four volumes of supernatant and the pH was adjusted to 8.0. The supernatant was loaded on a Ni-NTA column that had been pre-equilibrated with 1x Ni-NTA binding buffer. The column was washed with 1x Ni-NTA binding buffer supplemented with 20 mM imidazole. The 6xHisHpf2 was eluted with 1x Ni-NTA binding buffer supplemented with 250 mM imidazole. The eluate fraction was dialysed against Milli-Q $H_2O$ and then lyophilised.

For the 'total' fractions, the protein was made up in Milli-Q $H_2O$, wine, or a buffer of 20 mM citrate pH 4.0, 20 mM HEPES pH 7.0 or 20 mM Tris pH 8.0, at 2 or 5 mg $mL^{-1}$ and vortexed. For the 'soluble' fractions, the sample was mixed with the solute, vortexed well, then centrifuged (16 100 g, 10 min, 20°C). The absorbance at 280 nm ($A_{280}$) of the supernatant was measured to determine the protein concentration, as described in Section 4.2.1.4, and the supernatant was used immediately in a heat test.

### 6.2.3 Heat test assay

Heat tests were performed as described in Section 5.2.2. 6xHisHpf2 samples produced from both *S. cerevisiae* and *P. pastoris* were prepared in the same manner for each heat test assay.

## 6.3 Results

### 6.3.1 Protein concentration of the wine

The wine was analysed by HPLC and the concentrations of grape proteins was found to be 116 mg $L^{-1}$ thaumatins and 25 mg $L^{-1}$ chitinases.

### 6.3.2 Protein prepared in water

Lyophilised 6xHisHpf2 from both yeast hosts was suspended in Milli-Q $H_2O$ at a concentration of 2 mg $mL^{-1}$, and used in a heat test. It was observed that the *P. pastoris* sample did not dissolve well. Consequently, soluble samples of *S. cerevisiae* 6xHisHpf2 at 2 mg $mL^{-1}$ and *P. pastoris* 6xHisHpf2 at 5 mg $mL^{-1}$ were prepared. The absorbances at 280 nm were used to determine the solubility as a percentage of dry weight, shown in Table 6-1. The supernatants were used in a heat test, and the results are shown in Figure 6-1. It can be seen that the total *P. pastoris* 6xHisHpf2 fraction has some haze prevention activity, although the soluble fraction appears to contribute to the haze in the wine.

**Table 6-1 Percentage of Hpf2 that is soluble, as a percentage of dry weight, determined by $A_{280}$**

| Strain | In Milli-Q $H_2O$ | In citrate buffer |
|---|---|---|
| *S. cerevisiae* | 35 | 27 |
| *P. pastoris* | 64 | 75 |

### 6.3.3 Protein prepared in citrate buffer

Three buffers, citrate, HEPES and Tris, were trialled in an attempt to increase the solubility of the *P. pastoris* protein. The buffers were chosen as HEPES and Tris are

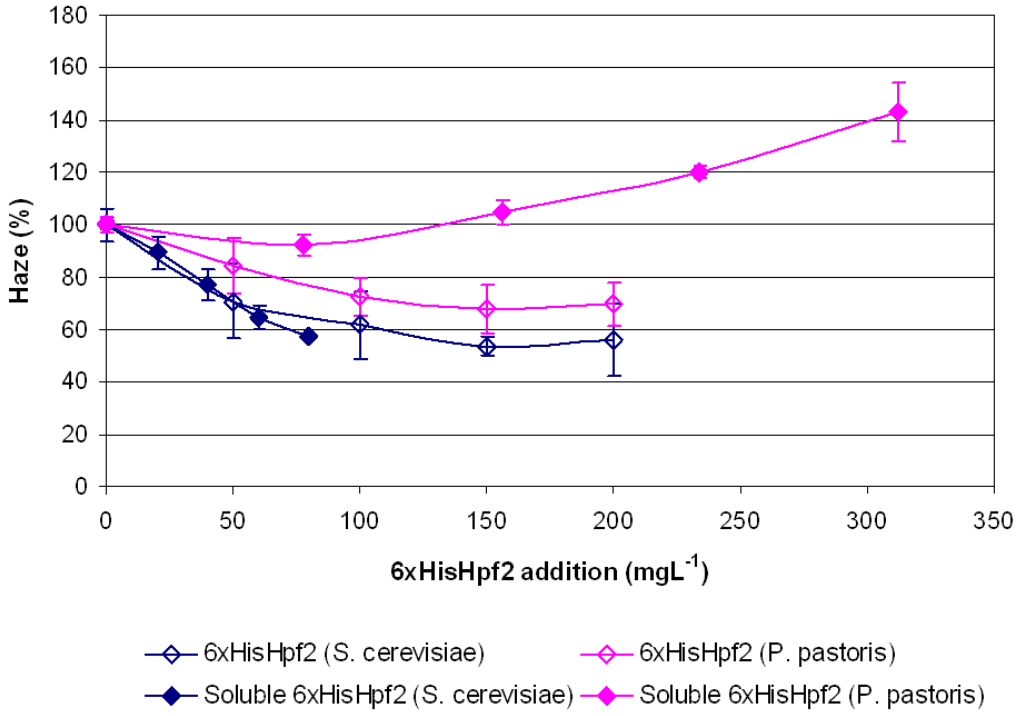


**Figure 6-1 The effect of sample preparation of the haze protective activity. 6xHisHpf2 (*S. cerevisiae* and *P. pastoris*) was prepared by suspension in Milli-Q $H_2O$ (open symbols) or by suspension in Milli-Q $H_2O$ followed by centrifugation to remove insoluble material (closed symbols).**

commonly used for protein studies, and citrate is an allowable additive to wine in Australia (AWRI Analytical Service 2007a). The *P. pastoris* 6xHisHpf2 did not dissolve well in the HEPES and Tris, as a precipitate was present after vortexing, (protein concentrations were not determined) although it appeared reasonably soluble in the citrate buffer, shown in Table 6-1. This total fraction in citrate buffer was run in the heat test assay, and the sample was centrifuged and the supernatant tested also. The protein concentration was estimated using the extinction coefficient. The results are shown in Figure 6-2. Some haze prevention activity is observed in the total fraction, but little or no haze protective activity is seen in the soluble fraction.

### 6.3.4 Protein prepared in wine

In an attempt to overcome the solubility issues in water, 6xHisHpf2 samples were prepared directly in wine. The samples were prepared at 2 mg $mL^{-1}$, vortexed well, then diluted for the heat test. The results are shown in Figure 6-3. When prepared in wine, it appears that the *P. pastoris* 6xHisHpf2 does not have any haze prevention activity. It was observed that the *P. pastoris* sample did not dissolve well in the wine, however the solubility of the fraction was not determined as insufficient material was available for further study.

## 6.4 Discussion

The *P. pastoris* 6xHisHpf2 displayed some haze prevention activity when prepared in Milli-Q $H_2O$, although this activity was lost when the soluble only fraction was considered, shown in Figure 6-1. In fact, the soluble protein increased the haze when dissolved in water. Interestingly, the solubility problems had never been observed with the *S. cerevisiae* 6xHisHpf2 and its activity did not seem to be affected when only the soluble fraction was considered, suggesting that the solubility problem of the *P. pastoris* 6xHisHpf2 may be related to the haze prevention activity. Silver stains were conducted on the *P. pastoris* 6xHisHpf2 to determine if contaminating proteins with higher solubility may be responsible for this effect (Nasution 2007), however the analysis showed no contaminating proteins.

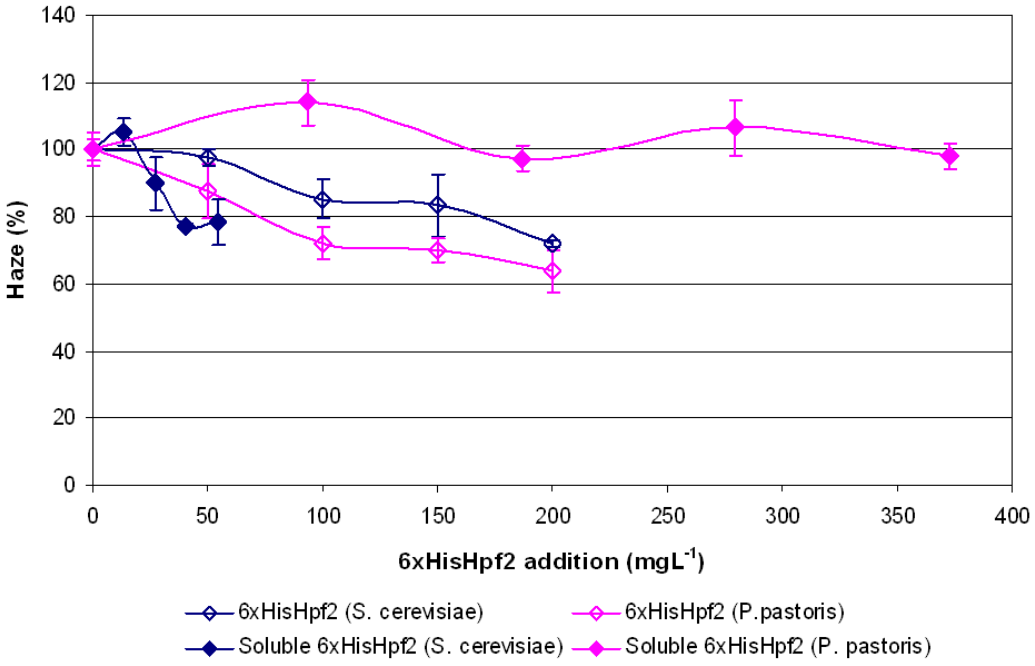


**Figure 6-2 The effect of sample preparation of the haze protective activity. 6xHisHpf2 (*S. cerevisiae* and *P. pastoris*) was prepared by suspension in citrate buffer (open symbols) or by suspension in citrate buffer followed by centrifugation to remove insoluble material (closed symbols).**

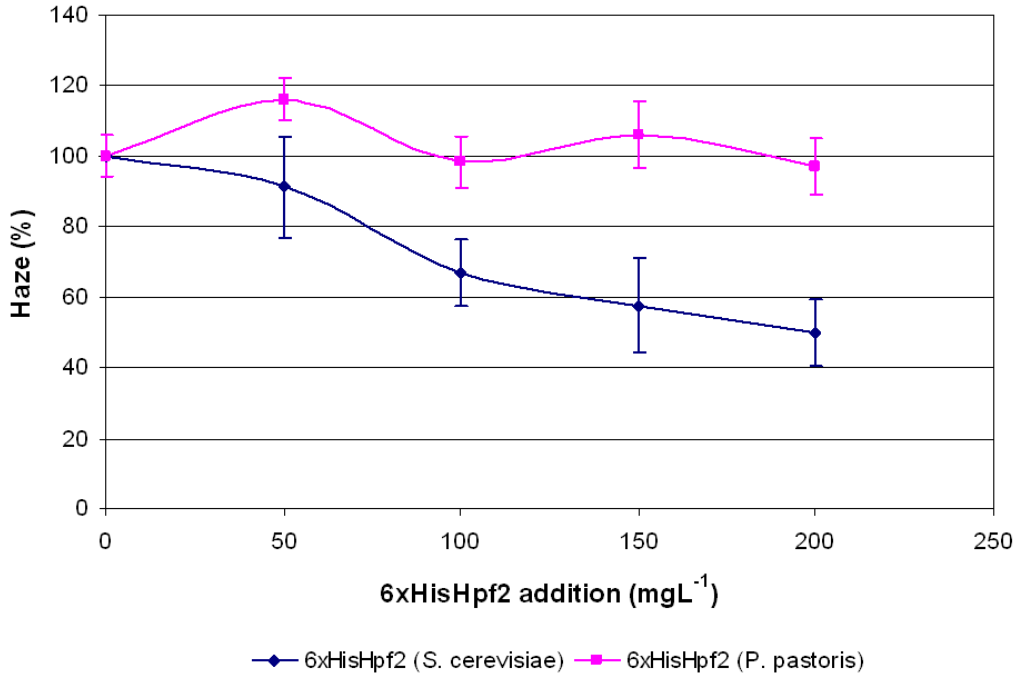


**Figure 6-3 The haze protective activity of 6xHisHpf2 from *S. cerevisiae* and *P. pastoris* in wine.**

As it was observed that the protein did not dissolve well in water, other buffers commonly used for protein studies were examined. Neither HEPES nor Tris at 20 mM were able to increase the solubility of the protein, but it dissolved well in 20 mM citrate at a lower pH. Figure 6-2 shows that when the *P. pastoris* 6xHisHpf2 was dissolved in the citrate buffer, it had some haze protection activity, but it again lost this ability when the soluble fraction was tested. This could possibly be due to the insoluble material being activated by the heat during the heat test, although no mechanism for its action has been determined.

A final test examined the activity of the two proteins prepared directly in wine, shown in Figure 6-3. The samples were mixed into the wine at a 10-fold higher concentration than required, and then diluted back to a maximum of 200 mg $L^{-1}$ for the heat test. Surprisingly, no activity was observed for the *P. pastoris* sample, although the *S. cerevisiae* protein gave a similar haze reduction to when it was prepared in water. This could be explained again by the insolubility of the protein in wine, as the *P. pastoris* 6xHisHpf2 did not dissolve well in the wine, and the calculated concentration was probably higher than the actual protein level in the heat test.

The three heat tests presented here provide some insight into the mode of action of 6xHisHpf2. The *S. cerevisiae* protein, which is approximately 75% mannose, and the *P. pastoris* protein, which is about 50% mannose, display similar haze protection when prepared in either Milli-Q $H_2O$ or 20 mM citrate buffer. However, when considering the soluble fraction, only the larger, more heavily glycosylated protein from *S. cerevisiae* has this activity. This suggests that the glycosylation is particularly important in solubilisation and possibly stability of the protein in solution, so the sugar chains both help the protein dissolve in the solvent and help prevent protein precipitation and denaturation. For the role that this protein would have in the wine industry, this is very important as the protein must remain in solution as well as stabilise the grape proteins in the wine and prevent them precipitating and denaturing during storage and transport. It would be interesting to consider the behaviour of the *P. pastoris* 6xHisHpf2 in storage trials in heat unstable wine, such as those with the *S. cerevisiae* 6xHisHpf2 (Brown et al. 2007), although more material would need to be prepared for any future studies.

## 6.5 Conclusions

The *P. pastoris* 6xHisHpf2 showed some haze prevention activity when prepared in either water or a mild citrate buffer, however when the soluble fraction was considered, this activity was lost in the citrate buffer and actually increased the haze in the water. It was not observed to be active at all when prepared directly in the wine. The solubility of the protein appears to be crucial. When dissolved completely, the activity is lower than a crude mix of the protein, although it is not clear why this behaviour occurs. Further studies are advised to confirm these results and to consider the long-term protein stability of wines treated with the *P. pastoris* 6xHisHpf2.

# 7 KEY FINDINGS AND PERSPECTIVE FOR FUTURE WORK

Protein haze in white wine is a constant problem in the wine industry. Bentonite fining is almost universally used to prevent the problem, but this method has some disadvantages, including losses of wine and aroma compounds. Alternatives are being investigated, such as proteolysis, heat treatment, ultrafiltration and proanthocyanidins, as well as the haze protective proteins from yeast.

This thesis has focused on one promising alternative treatment, the use of a yeast haze protective mannoprotein, Hpf2. The principle objective of the research was to evaluate the sensory impact of Hpf2 in wine at an active concentration. In order to achieve this, it was necessary to investigate the scale-up of methods for expression and production of Hpf2. In addition, the role of glycosylation on its activity in haze prevention was considered. The key findings from these studies are summarised below.

### *Escherichia coli* is not a suitable host for Hpf2 production

A 6xHis-tagged Hpf2 was cloned into the *E. coli* strain BL21(DE3) for expression of a degylcosylated version of the protein. Initial expression levels were very low and attempts were made to increase the yields and solubility of 6xHisHpf2, such as adjusting the time and temperature of expression, varying the biomass concentration at induction, and changing the expression media. The expression was inconsistent and the protein was not able to be purified despite several different methods being investigated.

In order to more deeply investigate this option, the solubility would need to be improved. Several options could be considered, including using different host strains which are designed for enhanced protein folding, such as the Origami strain (Novagen), or using other additives which have been shown to change the osmotic pressure on the cells and thus yield more soluble heterologous protein (Blackwell and Horgan 1991; Yu et al. 1995; Thomas and Baneyx 1997; Ghosh et al. 2004). Other possibilities could be to add ethanol to the media at inoculation to induce heat shock proteins and chaperones to help protein folding (Thomas and Baneyx 1997), co-expression of heat shock proteins or other co-factors (Thomas and Baneyx 1996; Weickert et al. 1996; Thomas and Baneyx 1997), or the addition of more soluble fusion partners (Kim and Lee 1996;

Nilsson et al. 1997; Davis 1999; Stevens 2000; Perrin et al. 2003; Fang and Ewald 2004; Turner et al. 2005).

**Expression levels could be increased significantly in *Saccharomyces cerevisiae***

An existing yeast plasmid for the over-expression of 6xHisHpf2 was transformed into a *S. cerevisiae* strain with no superfluous auxotrophies, which improved the yield over the existing strain. Further improvements were made by increasing the galactose concentration, the media pH, and the expression time. Any further optimisation should consider a multi-factorial approach, incorporating the slot blot quantification method established after the completion of this work.

**Slot blots provide a method for quantification**

Several quantification methods were considered for 6xHisHpf2, including traditional total protein assays such as the micro-biuret, Lowry and HPLC assays, and assays specific to the 6xHis tag, including the ELISA and the slot blot assay. Overall, the slot blot assay was found to be the most suitable. Two weaknesses remain with this technique: the samples must be deglycosylated and diluted prior to analysis. Despite this, it appears to be a useful method for quantification of the 6xHis-tagged protein.

**A suitable purification method was established**

An existing purification method was adapted to make it more suitable to repeated batches of 6xHisHpf2 production. The improved method consisted of ultrafiltration, ethanol precipitation, metal affinity chromatography, dialysis and lyophilisation. This resulted in a recovery of approximately 30%. Both the metal affinity chromatography and desalting resulted in significant losses of protein, so further improvement should focus on these steps and large improvements in recovery rates may be achievable. Overall, the final product was shown to be very pure by animo acid analysis, mannose assay and SDS-PAGE silver stain of the final purified protein, despite the low recovery rates.

**Active concentrations of invertase and 6xHisHpf2 do not affect wine sensory properties**

The concentrations of invertase and 6xHisHpf2 required to reduce protein haze were determined in three unfined wines by heat test assays. Initial informal sensory testing with invertase and 6xHisHpf2 at active concentrations in the three wines found only minimal differences. Further formal sensory analysis in one wine found no significant differences with either invertase or 6xHisHpf2 addition, with 2% of the panel being discriminators for invertase and 10% for 6xHisHpf2. In addition, control wine and 6xHisHpf2 treated wine was stored for one month at 15°C, and formal sensory analysis showed no significant difference between these samples, with no discriminators found on the panel. This implies that either invertase or 6xHisHpf2 could be used in the wine industry as an alternative to bentonite, without changing the sensory properties of the wines.

**6xHisHpf2 expressed from *P. pastoris* has less activity than from *S. cerevisiae***

In parallel work, 6xHisHpf2 was expressed and purified from the methylotrophic yeast *P. pastoris*. The protein was shown to be less than half the size of the *S. cerevisiae* protein, at about 83 kDa, and was approximately 50% mannosylated by weight (Tan 2005; Nasution 2007). The *P. pastoris* 6xHisHpf2 displayed some haze prevention activity when prepared in either water or a mild citrate buffer. Interestingly when the soluble fraction was considered, the sample prepared in water caused an increase in the haze, and the haze protection activity was lost in the citrate buffer. No activity was observed when the protein was prepared directly in wine. The solubility of the protein appears to be central: the activity of the *P. pastoris* 6xHisHpf2 is lower when dissolved completely than a crude mix of the protein is used. Further studies should be conducted to confirm these results and to understand the role of the glycosylation in haze protection in wine.

**Concluding remarks**

The work described in this thesis has brought the wine industry a step closer to employing a new method for protein haze prevention in white wines. The background

work in optimisation of Hpf2 production in a microorganism and the purification of the protein provide a starting point for large-scale production of not only Hpf2 but other large yeast mannoproteins that could be used in many other industries, such as biotechnology or pharmaceuticals. The finding that Hpf2 does not affect the sensory properties of wine is essential if Hpf2 is to be used commercially, as winemakers and wine consumers would most likely reject an additive that alters the wine aroma or palate. A comprehensive optimisation of Hpf2 expression and purification can now be undertaken with the aid of the slot blot quantification method, and a subsequent economic analysis should reveal whether Hpf2 addition can be a cost-effective alternative to bentonite fining.

Further work is required to fully elucidate the role of the glycosylation in the activity of the Hpf2 protein, but attempts to express the protein in *E. coli* and the heat test assay results of the *P. pastoris* protein suggest that the solubility is crucial to its function. This work may lead to a cost-effective method of protein haze prevention for the wine industry.

# APPENDIX 1

**Breaking buffer**

2% (v/v) TRITON X-100

1% (w/v) SDS

100 mM NaCl

10 mM Tris.Cl (pH 8.0)

1 mM EDTA (pH 8.0)

**Agarose gel**

For a small 1% gel:

0.5 g agarose

50 mL 1x TAE buffer

Microwave until boiling, stir, and allow to cool. Add 2.5 μL 10 g $L^{-1}$ ethidium bromide, then pour into casting well.

**50x TAE**

For 1 L:

242 g Tris base

57.1 mL Glacial acetic acid

100 mL 0.5 M EDTA (pH 8)

Add Milli-Q $H_2O$ to 1 L.

**Luria-Bertani (LB) medium**

For 1 L:

To 950 mL of Milli-Q $H_2O$, add:

10 g Bacto-tryptone

5 g Bacto-yeast extract

10 g NaCl

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 1 mL 1M NaOH. Adjust the volume of the solution to 1 L with Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

**M9 minimal medium**

Prepare 50 mL M9 media as follows:

5 mL 10x salts

5 mL 10x ammonium salts

0.75 mL Glucose stock

50 µL Calcium chloride stock

50 µL Magnesium sulfate stock

50 µL Thiamine stock

50 µL Biotin stock

Sterile Milli-Q $H_2O$ to 50 mL

- 10x salts

  67.8 g $Na_2HPO_4$ (anhydrous)

  30 g $KH_2PO_4$

  5 g NaCl

  Dissolve in 1 L Milli-Q $H_2O$, and adjust pH to 7.4. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

- 10x ammonium chloride

  10 g $NH_4Cl$

  Dissolve in 1 L Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

- Glucose stock

  20 g Glucose

  Dissolve in 100 mL Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

- Calcium chloride stock

  1.11 g $CaCl_2.2H_2O$

  Dissolve in 100 mL Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

- Magnesium sulfate stock

  24.6 g $MgSO_4.7H_2O$

  Dissolve in 100 mL Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

- Thiamine stock

  10mg Thiamine

  Dissolve in 1 mL Milli-Q $H_2O$. Sterilise by filtration.

- Biotin stock

  10 mg Biotin

  Dissolve in 1 mL sterile Milli-Q $H_2O$.

**2x YT medium**

For 1 L:

To 950 mL of Milli-Q $H_2O$, add:

16 g Tryptone

10 g Yeast extract

5 g NaCl

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 L with Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle.

**Lysis buffer (for denaturing Ni-NTA)**

50 mM $Na_2HPO_4$

0.3 M NaCl

6 M urea

10 mM β-mercaptoethanol

pH 8.0

**Equilibrium buffer (for denaturing Ni-NTA)**

50 mM $Na_2HPO_4$

0.5 M NaCl

6 M urea

pH 8.0

**Wash buffer (for denaturing Ni-NTA)**

50 mM $Na_2HPO_4$

0.5 M NaCl

6 M urea

20 mM imidazole

pH 8.0

**Eluate buffer (for denaturing Ni-NTA)**

20 mM Tris

0.1 M NaCl

6 M urea

300 mM imidazole

pH 8.0

**5x SDS-PAGE sample buffer**

0.5 M dithiothreitol

10% (w/v) SDS

40% (v/v) glycerol

300 mM Tris.HCl (pH 6.8)

A few specks of bromophenol blue

**SDS-Glycine running buffer**

For 5 L:

30.3 g Tris base

71.3 g Glycine

5 g SDS

Dissolve in 5 L Milli-Q $H_2O$.

**Coomassie blue stain**

40% ethanol

7% acetic acid

0.1% Coomassie R250

**Destain**

20% ethanol

7% acetic acid

**Transfer buffer**

25 mM Tris base

192 mM glycine

20% (v/v) methanol

pH should be 8.3 without adjustment. Store at 4°C.

**Blocking buffer**

5% skim milk powder in 1x TBS-T.

**10x TBS-T**

1.37 M NaCl

27 mM KCl

250 mM Tris base

Adjust to pH 8.0 with HCl, then add 1% Tween 20, and add Milli-Q $H_2O$ to required volume.

**10x TBS**

1.37 M NaCl

27 mM KCl

250 mM Tris base

Adjust to pH 8.0 with HCl, then add Milli-Q $H_2O$ to required volume.

**Alkaline phosphatase buffer**

100 mM Tris.HCl

150 mM NaCl

1 mM $MgCl_2$

**Synthetic complete medium (SC)**

For 1 L:

To 950 mL of Milli-Q $H_2O$, add:

6.7 g Difco yeast nitrogen base without amino acids

0.67 g Synthetic complete drop-out mix

20 g Glucose

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 L with Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle. Glucose can be replaced with galactose plus a non-repressing carbon source for expression from the *GAL1* promoter.

- Synthetic complete drop-out mix

  2 g Adenine hemisulfate

  2 g Arginine HCl

  2 g Histidine HCl

  2 g Isoleucine

  4 g Leucine

  2 g Lysine HCl

  2 g Methionine

  3 g Phenylalanine

  2 g Serine

  2 g Threonine

  3 g Tryptophan

  2 g Tyrosine

  1.2 g Uracil

  9 g Valine

  Omit appropriate components to prepare required dropout mix.

**Chemically defined grape juice medium (CDGJM)**

For 1 L:

20 g Glucose

2.5 g Potassium hydrogen tartrate

3 g L-Malic acid

1.23 g $MgSO_4.7H_2O$

1.14 g $K_2HPO_4$

0.2 g Citric acid

0.33 g Calcium chloride (anhydrous)

1.7 g Ammonium chloride

1 g Synthetic complete drop-out mix

0.1 g *Myo*-inositol

1 mL Trace element stock solution

1 mL Vitamin stock solution

Dissolve 2.5 g potassium hydrogen tartrate in 100 mL of water at 80°C with constant stirring. Add glucose at appropriate concentration in small amounts along with 900 mL of water. Add the remaining salts, minerals and vitamins. Adjust the pH to 3.2 with 5 M tartaric acid or to higher pH with sodium hydroxide and filter through a 0.2 µm membrane to sterilise. Glucose can be replaced with galactose plus a non-repressing carbon source for expression from the *GAL1* promoter.

- CDGJM trace element stock solution

  For 1 L:

  200 mg $MnCl_2$

  135 mg $ZnCl_2$

  30 mg $FeCl_2$

  15 mg $CuCl_2$

  5 mg $H_3BO_3$

  30 mg $Co(NO_3)_2.6H_2O$

  25 mg $NaMoO_4.2H_2O$

  10 mg $KIO_3$

- CDGJM vitamin stock solution

  For 1 L:

  2 g Pyridoxine hydrochloride

  2 g Nicotinic acid

  1 g D-Pantothenic acid (Hemi-calcium salt)

  500 mg Thiamin hydrochloride

  200 mg *p*-aminobenzoic acid

  200 mg Riboflavin

  125 mg Biotin

  200 mg Folic acid

**Lund defined medium (LDM)**

For 1 L:

20 g Glucose

5 g Ammonium sulfate

3 g Potassium phosphate

0.5 g $MgSO_4.7H_2O$

10 mLTrace element stock solution

1 mL Vitamin stock solution

Adjust the pH to 3.2 with 5 M tartaric acid or to higher pH with sodium hydroxide and filter through a 0.2 µm membrane to sterilise. Glucose can be replaced with galactose plus a non-repressing carbon source for expression from the *GAL1* promoter.

- LDM trace element stock solution

  For 1 L:

  450 mg $ZnSO_4.7H_2O$

  100 mg $MnCl_2.4H_2O$

  190 mg $CuSO_4$

  30 mg $CoCl_2.6H_2O$

  40 mg $Na_2MoO_4.6H_2O$

  450 mg $CaCl_2.2H_2O$

  21.46 mg $FeCl_2.6H_2O$

  100 mg $H_3BO_3$

  12.9 mg $KIO_3$

- LDM vitamin stock solution

  For 1 L:

  0.05 g Biotin

  1.0 g Calcium pantothenate

  1.0 g Nicotinic acid

  25.0 g *Myo*-inositol

  1 g Thiamine hydrochloride

  1 g Pyridoxol hydrochloride

  0.2 g *p*-aminobenzoic acid

**Delft defined medium (DDM)**

For 1 L:

20 g Glucose

10 g Potassium phosphate

2.5 g $MgSO_4$

3 g Urea

10 mL Trace element stock solution

15 mL Vitamin stock solution

Adjust the pH to 3.2 with 5 M tartaric acid or to higher pH with sodium hydroxide and filter through a 0.2 μm membrane to sterilise. Glucose can be replaced with galactose plus a non-repressing carbon source for expression from the *GAL1* promoter.

- DDM trace element stock solution

  For 1 L:

  5.75 g $ZnSO_4$

  0.32 g $MnCl_2$

  0.50 g $CuSO_4$

  0.47 g $CoCl_2$

  0.48 g $Na_2MoO_4$

  2.9 g $CaCl_2$

  2.8 g $FeSO_4$

- DDM vitamin stock solution

  For 1 L:

  0.05 g Biotin

  1.0 g Calcium pantothenate

  1.0 g Nicotinic acid

  25.0 g *Myo*-inositol

  1 g Thiamine hydrochloride

  1 g Pyridoxol hydrochloride

  0.2 g *p*-aminobenzoic acid

**Yeast peptone dextrose medium (YPD)**

For 1 L:

To 950 mL of Milli-Q $H_2O$, add:

10 g Bacto-yeast extract

20 g Bacto-peptone

20 g Dextrose

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 L with Milli-Q $H_2O$. Sterilise by autoclaving for 20 min at 15 psi on liquid cycle. Dextrose can be replaced with galactose plus a non-repressing carbon source for expression from the *GAL1* promoter.

**Development solution**

3 g $L^{-1}$ sodium carbonate

0.5 mL $L^{-1}$ 37% formaldehyde

4 μg $L^{-1}$ sodium thiosulfate

**Complex forming reagent for the Lowry assay**

Prepare the following solutions in Milli-Q $H_2O$:

Solution A – 20 g $L^{-1}$ $Na_2CO_3$

Solution B – 10 g $L^{-1}$ $CuSO_4.5H_2O$

Solution C – 20 g $L^{-1}$ potassium sodium tartrate

Prepare immediately before use by mixing solutions A, B and C in the proportion 100:1:1.

**Solvent A**

8% acetyl nitrile

92% Milli-Q $H_2O$

0.1% TFA

**Solvent B**

80% acetyl nitrile

20% Milli-Q $H_2O$

0.1% TFA

**Solvent C**

95% acetyl nitrile

5% Milli-Q $H_2O$

0.1% TFA

**PBS**

10 mM $Na_2HPO_4$

2 mM $KH_2PO_4$

0.15 M NaCl

3 mM KCl

Adjust pH to 7.4, then add Milli-Q $H_2O$ to required volume.

**5x Ni-NTA binding buffer**

For 1 L:

34.5 g $NaH_2PO_4,H_2O$

87.65 g NaCl

Adjust pH to 8.0 with NaOH pellets.

**1x Ni-NTA elution buffer**

For 1 L:

52.4 g histidine

200 mL 5x Ni-NTA binding buffe

Adjust the pH to 8.0 with NaOH pellets.

# APPENDIX 2

**Primers**

For cloning:

JM1: AATGCATCATCATCATCATCATGCTACTTCCTCTTCTTCCAGC

JM2 ATCAATTGCCTTTGGAACTCTTAGAGCT

For sequencing:

pETBlueUP: TCACGACGTTGTAAAACGAC

pETBlueDOWN: GTTAAATTGCTAACGCAGTCA

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