

WAITE INSTITUTE  
11.8.86  
LIBRARY

THE ENZYMES OF NITRATE ASSIMILATION IN  
*SCLEROTINIA SCLEROTIORUM*

by

Maskuntjir Abdul Rachim, B.Sc., Ir.

A thesis submitted in fulfilment of the requirements  
for the degree of

Master of Agricultural Science

Department of Agricultural Biochemistry  
Waite Agricultural Research Institute  
The University of Adelaide.

February, 1986

*Awarded 29-4-86*

PREFACE

Part of the work described in this thesis has been presented at the Australian Biochemical Society Conference (Canberra, 1985) and published in the following journals:

1. Some properties of glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*.  
M.A. Rachim and D.J.D. Nicholas (1985)  
*Proc. Aust. Biochem. Soc.* 17, 21
2. Glutamine synthetase and glutamate synthase from *Sclerotinia sclerotiorum*.  
M.A. Rachim and D.J.D. Nicholas (1985)  
*Phytochemistry* 24, 2541-2548
3. Some properties of nitrate reductase from *Sclerotinia sclerotiorum*.  
M.A. Rachim and D.J.D. Nicholas (1986)  
*Phytochemistry* 25 (in press)

ACKNOWLEDGEMENTS

I wish to express my deepest and sincerest thanks to my supervisor, Prof. D.J.D. Nicholas, Chairman of the Department of Agricultural Bio-chemistry, Waite Agricultural Research Institute, The University of Adelaide for his constant encouragement, guidance and constructive criticism throughout the progress of the present investigation and in the preparation of manuscript.

I would also like to thank Mr. D. Hein for his assistance with the  $^{15}\text{N}$  experiments, Mr. B.A. Palk for preparing the photographic prints, Mrs. M. Brock for the skilful typing of the thesis and to all others who helped me from time to time.

I am grateful to my wife Erna for her patience and unfailing encouragement during the course of this investigation.

The postgraduate scholarship from International Development Programme of Australian Universities and Colleges is gratefully acknowledged.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief no material described herein has been previously published or written by another person except when due reference is made in the text.

If accepted for the award of a M.Ag.Sc. degree, this thesis will be available for loan and photocopying.

---

M.A. RACHIM

ABBREVIATIONS

The abbreviations for chemicals and symbols in general follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem. J.* (1966) 101: 1-7) or the Instruction to Authors for the Phytochemistry (*Phytochemistry* (1983) 22: 1-7).

CHEMICALS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
BV	Benzyl viologen
BVH	Benzyl viologen (reduced)
CDP	Cytidine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
CMP	Cytidine 5'-triphosphate
Cyt <i>b</i> <sub>557</sub>	Cytochrome <i>b</i> <sub>557</sub>
DCPIP	2,6-Dichlorophenolindophenol
DEAE-cellulose	Diethylaminoethyl cellulose
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavin adenine dinucleotide
FADH <sub>2</sub>	Flavin adenine dinucleotide (reduced)
FMN	Flavin mononucleotide
FMNH <sub>2</sub>	Flavin mononucleotide (reduced)
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
IDP	Inosine 5'-diphosphate
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
MSX	L-methionine-DL-Sulphoximine
MV	Methyl viologen
MVH	Methyl viologen (reduced)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide
p-CMB	p-chloromercuribenzoate
SDS	Sodium dodecyl sulphate
-SH	Sulphydryl group
SVD	Snake venom phosphodiesterase
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
UDP	Uridine 5'-diphosphate
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate

Symbols and Units

A	absorbance
°C	degree Celcius (centigrade)
cm	centimeter
g	gram
g	unit of gravitational field
hr	hour(s)
kDa	kilo dalton(s)
$K_i$	inhibitor constant
$K_m$	Michaelis-Menten constant
L	litre
M	molar
mA	milliampere
mg	milligram
min	minute(s)
ml	millilitre
mm	millimeter
mM	millimolar
mmole	millimole(s)
MW	molecular weight
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
µmole	micromole(s)
N	normal
nm	nanometer
nmole	nanomole(s)
%	percent
$P_i$	inorganic phosphate
p.s.i.	pound per square inch
U.V.	ultra violet
v	volume
w	weight

TABLE OF CONTENTS

	<u>Page No:</u>
PREFACE	i
ACKNOWLEDGEMENTS	ii
DECLARATION	iii
ABBREVIATIONS	iv
TABLE OF CONTENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	xi
SUMMARY	xiv
1. INTRODUCTION	1
1.1 The biology of <i>Sclerotinia sclerotiorum</i>	1
1.2 Nitrate assimilation in microorganisms	3
1.2.1 Nitrate reductase	4
1.2.2 Nitrite reductase	8
1.2.3 Metabolism of ammonia into amino acids	11
1.2.3.1 Glutamine synthetase	12
1.2.3.2 Glutamate synthase	16
1.3 Aims of the study	18
2. MATERIALS AND METHODS	19
2.1 Culturing <i>Sclerotinia sclerotiorum</i>	19
2.1.1 Growth conditions	19
2.1.2 Harvesting	19
2.2 Enzyme methods	20
2.2.1 Nitrate reductase assay	20
2.2.1.1 Colorimetric	20
2.2.1.2 Spectrophotometric	21
2.2.2 Nitrite reductase and hydroxylamine reductase assays	21
2.2.2.1 Colorimetric	21
2.2.2.2 Spectrophotometric	23

	<u>Page No:</u>
2.2.3 Glutamine synthetase assay	23
2.2.3.1 Transferase reaction	23
2.2.3.2 Biosynthetic reaction	24
2.2.4 Glutamate synthase assay	25
2.2.5 Determination of Michaelis-Menten constant ( $K_m$ )	25
2.2.6 Determination of inhibitor constant ( $K_i$ )	25
2.2.7 Determination of molecular weight	25
2.2.7.1 Gel filtration	25
2.2.7.2 Electrophoresis	26
2.2.8 Separation and identification of flavin from nitrate reductase	27
2.2.9 Adenylylation and deadenylylation of glutamine synthetase	27
2.3 General techniques	29
2.3.1 Incorporation of $^{15}\text{N}$ into washed felts	29
2.3.2 Preparations of columns	29
2.3.2.1 Matrex Gel Blue A	30
2.3.2.2 Blue Sepharose CL-6B	30
2.3.2.3 Sepharose 6B	30
2.3.2.4 DEAE-cellulose	31
2.4 Other determinations	31
2.4.1 Nitrite	31
2.4.2 Ammonia	32
2.4.3 Protein	32
2.5 Biochemicals, chemicals and other materials	33
2.5.1 Biochemicals and chemicals	33
2.5.2 Other materials	34
3. RESULTS	35
3.1 Nitrate reductase	35
3.1.1 Purification	35
3.1.2 Properties	36
3.1.2.1 Molecular weight	36
3.1.2.2 Effect of pH	36
3.1.2.3 Electron donors	36
3.1.2.4 $K_m$ values for substrate, reductant and cofactor	41
3.1.2.5 Characterization of flavin isolated from the purified enzyme	41
3.1.3 Inhibitor studies	47



	<u>Page No:</u>
3.2 Nitrite reductase	55
3.2.1 Purification	55
3.2.2 Properties	56
3.2.2.1 Effect of pH and time of incubation	56
3.2.2.2 Electron donors	60
3.2.2.3 Requirement for flavin	60
3.2.2.4 $K_m$ values for substrate, reductant and cofactor	60
3.2.2.5 Stoichiometries for NADPH, nitrite and ammonia	66
3.2.3 Inhibitor studies	66
3.2.4 Inactivation of nitrite and hydroxylamine reductases by NADPH in the presence of FAD	70
3.3 Incorporation of [ $^{15}\text{N}$ ]-labelled $(\text{NH}_4)_2\text{SO}_4$ into cell-nitrogen	70
3.4 Glutamine synthetase	75
3.4.1 Purification	75
3.4.2 Properties	77
3.4.2.1 Molecular weight	77
3.4.2.2 Effect of pH and incubation time	77
3.4.2.3 Divalent cation requirement	77
3.4.2.4 Nucleotide specificity	82
3.4.2.5 $K_m$ values for substrate	82
3.4.3 Inhibitor studies	84
3.4.4 Adenylation/deadenylation	89
3.5 Glutamate synthase	95
3.5.1 Purification	95
3.5.2 Properties	99
3.5.2.1 Molecular weight	99
3.5.2.2 Effect of pH	99
3.5.2.3 Substrate requirement	99
3.5.2.4 $K_m$ values for substrates and NADPH	99
3.5.3 Inhibitor studies	103
4. DISCUSSION	112
4.1 Nitrate reductase	112
4.2 Nitrite reductase	117
4.3 Pathway of ammonia assimilation	122
4.3.1 Glutamine synthetase	123
4.3.2 Glutamate synthase	127
5. BIBLIOGRAPHY	131

LIST OF TABLES

<u>Table</u>		<u>Page No:</u>
1	Purification of nitrate reductase	37
2	Electron donors for the purified nitrate reductase activity	40
3	Effects of metal inhibitors on the MVH-dependent nitrate reductase activity	48
4	Effects of inhibitors on NADPH-dependent nitrate reductase activity	49
5	Effects of inhibitors of sulphhydryl-groups and flavin respectively on MVH-nitrate reductase activity	50
6	Purification of nitrite reductase	57
7	Effects of various electron donors on nitrite reductase activity	61
8	Effects of flavins on NAD(P)H-nitrite reductase and its associated diaphorase activities	62
9	Stoichiometries for NADPH, $\text{NO}_2^-$ and $\text{NH}_3$ for nitrite reductase	67
10	Effects of metal binding agents on NADPH-dependent nitrite reductase	68
11	Effects of inhibitors of sulphhydryl-groups and flavin on NADPH-nitrite reductase	69
12	Effects of preincubation of nitrite reductase with reductant, cofactor and/or substrate on its activity	73
13	Effects of L-methionine-DL-sulphoximine (MSX) and azaserine on the incorporation of $^{15}\text{N}$ -labelled $(\text{NH}_4)_2\text{SO}_4$ into washed cells	74
14	Purification of glutamine synthetase	76
15	Transferase and biosynthetic activities with various divalent cations	81
16	Effects of various nucleotides on transferase and biosynthetic activities	83
17	Effects of various concentration of L-methionine-DL-sulphoximine on transferase activity	88
18	Effects of various amino acids on glutamine synthetase activity	90

<u>Table</u>		<u>Page No:</u>
19	Effects of organic acids on glutamine synthetase activity	91
20	Effects of snake venom phosphodiesterase on glutamine synthetase activity	96
21	Purification of glutamate synthase	98
22	Substrates and NADPH requirements for glutamate synthase activity	102
23	Effects of amino acids on glutamate synthase activity	107
24	Effects of various metabolites on glutamate synthase activity	108
25	Effects of various concentrations of azaserine on glutamate synthase activity	109
26	Effects of various inhibitors on glutamate synthase activity	110

LIST OF FIGURES

<u>Figure</u>		<u>Page No:</u>
1	A. Molecular weight determination of nitrate reductase by gel filtration B. Estimation of subunit molecular weight of the purified enzyme by SDS-polyacrylamide gel electrophoresis	38
2	Effect of pH on nitrate reductase activity	39
3	Double reciprocal plots of the effects of various nitrate concentrations on NADPH- and MVH-dependent nitrate reductase activities	42
4	Double reciprocal plots of the effects of various concentrations of nitrate on FMNH <sub>2</sub> - and FADH <sub>2</sub> -linked enzyme activities	43
5	Double reciprocal plots of the effects of varying NADPH and FAD concentrations respectively on NADPH-dependent nitrate reductase activity	44
6	A double reciprocal plot of the effects of various concentrations of reduced methylviologen on nitrate reductase activity	45
7	Separation of the flavin component of the purified nitrate reductase by paper chromatography	46
8	Inhibitory effects of chlorate and bromate on MVH-nitrate reductase activity	51
9	A Dixon plot of the inhibitory effects of various concentrations of chlorate on the MVH-nitrate reductase activity	52
10	A Dixon plot of the inhibitory effects of various concentrations of bromate on the MVH-nitrate reductase activity	53
11	Effects of various concentrations of nitrite on NADPH-nitrate reductase activity	54
12	Effects of pH on nitrite reductase and hydroxylamine reductase activities	58
13	Effects of incubation times on nitrite reduction	59
14	Effects of various concentrations of substrates on NADPH-nitrite reductase and NADPH-hydroxylamine reductase activities	63

<u>Figure</u>		<u>Page No:</u>
15	Double reciprocal plots of the effects of various concentrations of NADPH on NADPH-dependent nitrite reductase and NADPH-dependent hydroxylamine reductase activities	64
16	Double reciprocal plots of the effects of various concentrations of FAD on NADPH-dependent nitrite reductase and NADPH-hydroxylamine reductase activities	65
17	Effects of various concentrations of cyanide on NADPH-dependent nitrite reductase activity	71
18	Effects of various concentrations of sulphite on NADPH-dependent nitrite reduction	72
19	A. Molecular weight determination of glutamine synthetase by gel filtration B. Estimation of subunit molecular weight of the purified enzyme by SDS-polyacrylamide gel electrophoresis	78
20	Effects of pH on glutamine synthetase activity	79
21	Effects of incubation times on glutamine synthetase activity	80
22	Effects of various concentrations of substrates on glutamine synthetase-transferase activity	85
23	Effects of various concentrations of substrates on glutamine synthetase-biosynthetic activity	86
24	Effects of various concentrations of ADP and ATP on glutamine synthetase activity	87
25	Inhibitory effects of glutamate and $\text{NH}_4\text{Cl}$ on glutamine synthetase-transferase activity	92
26	A Dixon plot of the effects of various concentrations of glutamate on glutamine synthetase-transferase activity	93
27	A Dixon plot of the effects of various concentrations of ammonium chloride on glutamine synthetase-transferase activity	94
28	A. Molecular weight determination of glutamate synthase by gel filtration B. Estimation of subunit molecular weight of the purified enzyme by SDS-polyacrylamide gel electrophoresis	100

<u>Figure</u>		<u>Page No:</u>
29	Effects of pH on glutamate synthase activity	101
30	Effects of various concentrations of glutamine on glutamate synthase activity	104
31	Effects of various concentrations of $\alpha$ -keto-glutarate on glutamate synthase activity	105
32	Effects of various concentrations of NADPH on glutamate synthase activity	106

SUMMARY

1. This thesis embodies results of an investigation on some biochemical aspects of enzymes involved in nitrate assimilation in a plant-pathogenic fungus, *Sclerotinia sclerotiorum*. The enzymes are nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthase.
2. Nitrate reductase, purified 118-fold, had a molecular weight of 210 kDa and was composed of 2 dissimilar subunits of 123 and 107 kDa. In addition to using NADPH as an electron donor, the enzyme also utilized reduced viologen dyes and reduced flavin nucleotides as reductants.
3. FAD was isolated from the purified enzyme; however, exogenous FAD was required for maximal activity of NADPH-dependent nitrate reductase *in vitro*. FMN did not substitute for FAD.
4. The enzyme activity was inhibited by metal chelating agents and by flavin and sulphhydryl group inhibitors. Azide markedly restricted both NADPH- and MVH-dependent reactions, but inhibition by p-CMB, NEM and amytal was more marked when NADPH was the reductant.
5. Nitrite inhibited nitrate competitively in NADPH-dependent nitrate reduction *in vitro*; however, it is unlikely to inhibit nitrate reductase activity under physiological conditions because the  $K_i$  for nitrite was three-fold greater than the  $K_m$  for nitrate.
6. Nitrite reductase, the second enzyme in the nitrate assimilation pathway utilized NAD(P)H, reduced viologen dyes and reduced flavin nucleotides as electron donors. NADPH was the most effective electron donor. Maximal activity of the NADPH-dependent nitrite reductase

was achieved by adding FAD to the assay mixture; FMN however, was less effective.

7. Associated with the nitrite reductase enzyme was a hydroxylamine reductase activity as well as diaphorase type activities utilizing either ferricyanide, DCPIP or cytochrome c as an electron acceptor.
8. The product of both nitrite and hydroxylamine reductases was ammonia but hydroxylamine was not an intermediate product of nitrite reductase. The stoichiometry of NADPH oxidized to nitrite utilized and ammonia produced in NADPH-dependent nitrite reduction was 3:1:1 and the ratio of NADPH oxidized to ammonia formed in the hydroxylamine reductase-mediated reaction was 1:1.
9. In the presence of FAD, nitrite reductase was inactivated by preincubation with NADPH, but NADP<sup>+</sup> was without effect. This inactivation was offset by the substrates (nitrite and hydroxylamine).
10. Nitrite reductase was sensitive to metal binding agents as well as flavin- and sulphhydryl group-inhibitors. The inhibition by p-CMB and mepacrine respectively, were reversed by adding cysteine and FAD.
11. Washed felts readily incorporated <sup>15</sup>NH<sub>4</sub><sup>+</sup> into cell-nitrogen but this effect was inhibited by both MSX and azaserine, inhibitors of glutamine synthetase and glutamate synthase respectively. Since glutamate dehydrogenase was not detected in cell-free preparations, this is further evidence that the glutamine synthetase/glutamate synthase pathway is the main route for the assimilation of ammonia into amino acids.
12. Glutamine synthetase, purified by ion exchange and affinity chromatography, had a molecular weight of 490 kDa. The enzyme was composed of 8 identical subunits of 60 kDa. The transferase activity of the



enzyme required  $Mn^{2+}$  and ADP for maximal activity whereas the biosynthetic activity required  $Mg^{2+}$  and ATP.

13. The enzyme was regulated by feedback inhibition involving amino acids and organic acids and by a adenylylation/deadenylylation mechanism. The transferase activity of the enzyme was also inhibited competitively by the substrates of the biosynthetic reaction, namely glutamate and  $NH_4Cl$ , with respect to glutamine.
14. Glutamate synthase had a molecular weight of 220 kDa and was composed of 4 subunits of 53.7 kDa. The enzyme had a specific requirement for NADPH,  $\alpha$ -ketoglutarate and glutamine as the electron donor, amino acceptor and amino donor, respectively.
15. Glutamate synthase was regulated by feedback inhibitors including amino acids, organic acids and nucleotides. The enzyme was markedly inhibited by p-CMB (this effect was reversed by cysteine), O-phenanthroline,  $\alpha, \alpha'$ -dipyridyl and azaserine.