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Investigation into the formation of a protein-protein
complex between ATP sulfurylase and APS
reductase in onion (*Allium cepa* L.).

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This thesis has attempted to obtain evidence, both *in vitro* and *in vivo*, of complex formation between chloroplastidic isoforms of APS-reductase (APSR; EC 1.8.4.9) and ATP-sulfurylase (ATPS; EC 2.7.7.4) of *Allium cepa* (onion).

Genes encoding APSR and ATPS from onion were expressed in *E. coli*, and the recombinant proteins were expressed and purified with glutathione Sepharose 4B chromatography and ion exchange chromatography. SDS-PAGE separation and Coomassie blue staining revealed an ATPS recombinant protein with a molecular mass of 50 kDa, and full length and a truncated form of APSR, with molecular masses of 55 kDa and 45 kDa, respectively.

Three different approaches were used to investigate complex formation *in vitro*. Using an ELISA-based technique, an association of recombinant ATPS with recombinant APSR was shown at pH 7.4, with a proposed 1:1 stoichiometry. However, when these ELISA experiments were conducted at pH 9.4, no evidence for complex formation was observed, suggesting that the complex is dependent on the pH of the buffered solution used.

The second method was the ligand binding assay where recombinant protein APSR was immobilised onto PVDF membrane and then incubated at 25 °C with a solution containing ATPS protein. The detection of bound ATPS was achieved using anti-ATPS IgG, and it was possible to detect a putative ATPS-APSR complex at pH 7.4, although the complex was unable to be detected at pH 7.8 or pH 9.4.

The third method used was the immunoprecipitation assay, where anti-ATPS IgG that was conjugated onto Sepharose resin was used to precipitate recombinant ATPS (and any proteins bound to ATPS). The proteins precipitated were identified firstly by their molecular mass and subsequent western analysis with either biotinylated ATPS IgG or APSR IgG antibodies. Using this technique, recombinant APSR was able to be precipitated from solution using recombinant ATPS, at pH 7.4.

To investigate the role of glutathione as a possible co-factor in the mediation of the protein complex, the tripeptide was added to the buffer used (final concentration between 0-10 mM) in the ELISA experiments. These experiments showed that glutathione had no effect on the formation of the complex. However investigation of the role of glutathione using the immunoprecipitation assay (added at 5.0 mM) demonstrated that glutathione did result in an increase in the amount of the ATPS-APSR complex.

The immunoprecipitation technique was also used in attempt to isolate the complex *in vivo* from onion chloroplast extracts. However the ATPS-APSR complex was unable to be detected using this technique. In this thesis, the term *in vivo* is used to refer to experiments with chloroplast extracts and so will include ATPS, APSR and other proteins that may also contribute to any ATPS-APSR complex. The term *in vitro* refers to the direct interaction between recombinant ATPS and APSR only.

To determine if the ATPS-APSR complex had any effect on the control of ATPS activity, the specific activity of ATPS was measured *in vitro* using recombinant ATPS and APSR. Results from these experiments demonstrated that the complex formation did not alter the activity of ATPS.

A second technique was used to detect the ATPS-APSR complex *in vivo*. An ATPS affinity column, made with anti-ATPS IgG antibodies conjugated to Sepharose, was firstly incubated with recombinant ATPS, and the chloroplast extract from onion was then passed through the column and any bound proteins were eluted with solutions with high or low pH. Using this technique, APSR was unable to be identified as a protein that associated with ATPS, but a number of other proteins, with molecular masses of *ca.* 48 kDa, 45 kDa, 40 kDa and 28 kDa, were identified as being putative protein partners to ATPS.

Another approach to signal whether ATPS complex formation with other protein partners (including APSR) has occurred is to examine changes in the kinetic properties of the enzyme. To do this, the K_m values of ATPS for inorganic phosphate were determined in two cultivars of onions, grown hydroponically with varying sulfur supply. Measurements that were taken prior to and during bulbing showed that for at least one cultivar (Texas Grano) with sufficient sulfur, an increase in the K_m value from 3.8 nM (prior to bulbing), to 0.15 μ M (during bulbing) was observed. It is possible that this change in K_m value is due to the formation of a protein-protein complex.

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Table of Contents

Abstract.....	ii
Acknowledgments.....	iv
Table of contents.....	vi
List of figures.....	xii
List of tables.....	xv
List of abbreviations.....	xvi
1.0 Introduction.....	1
1.1 Overview.....	1
1.2 Cysteine biosynthesis.....	4
1.3 ATP-sulfurylase.....	7
1.4 APS-reductase.....	9
1.4.1 Overview of APS-reductase.....	9
1.4.2 Localisation of APSR.....	10
1.4.3 The reductase domain.....	10
1.4.4 The thioredoxin-like domain.....	11
1.4.5 The reaction mechanism of APSR.....	11
1.4.6 Kinetic properties of APSR.....	12
1.5 Regulation of the sulfur assimilation pathway.....	15
1.5.1 Developmental regulation of ATPS and APSR.....	15
1.5.2 Regulation by sulfur status and products of the sulfur assimilation pathway.....	15
1.5.3 Regulation by nitrogen.....	19
1.5.4 Regulation by carbon.....	20
1.5.5 Regulation by oxidative stress.....	21
1.6 Regulation of sulfur assimilation in onion.....	22
1.6.1 Overview of onion production in New Zealand.....	22
1.6.2 Impact of sulfur nutrition on the flavour of onions.....	22
1.6.3 Regulation of sulfur assimilation at a molecular level in onion.....	23
1.7 Complex formation and the regulation of sulfur assimilation in onion.....	24

1.7.1	Current knowledge that supports the formation of a protein complex between ATPS and APSR.....	25
1.8	Thesis aims.....	26
2.0	Methods.....	27
2.1	Plant growth conditions.....	27
2.1.1	Plant material.....	27
2.1.2	Growth of plant material for general experiments.....	27
2.1.3	Growth of plant material for sulfur deficiency experiments.....	28
2.1.4	Extraction of plant extracts for APSR activity assay.....	28
2.2	Preparation of whole chloroplast protein extraction.....	29
2.2.1	Whole chloroplast isolation.....	29
2.2.2	Preparation of percoll gradients.....	30
2.2.3	Preparation of chloroplast extracts.....	32
2.3	Growth of bacterial cultures.....	34
2.3.1	Making glycerol stocks, LB-Amp plates and LB media.....	34
2.3.1.1	Preparation of LB media and LB plates	34
2.3.1.2	Growth of <i>E. coli</i> cells.....	34
2.3.1.3	Glycerol stocks.....	35
2.4	Isolation of ATPS and APSR GST-fusion protein.....	35
2.4.1	Large scale preparation.....	35
2.4.1.1	Lysing <i>E. coli</i> cells using the French press.....	36
2.4.1.2	Lysing <i>E. coli</i> cells using the cell disruptor.....	37
2.4.1.3	Lysing <i>E. coli</i> cells using the sonicator.....	37
2.4.2	Isolation of the GST-fused protein using the GS4B Sepharose.....	37
2.5	Sephadex G-25 filtration chromatography.....	38
2.6	Anion exchange chromatography.....	39
2.7	Quantification of protein concentration.....	40
2.7.1	Bradford method.....	40
2.7.2	UV method for the quantification of IgG in solution.....	40
2.8	Electrophoresis of proteins.....	42
2.8.1	Mini Gel- SDS-PAGE.....	42
2.9	Coomassie [®] Brilliant blue staining.....	44
2.10	Western blot analysis.....	44

2.10.1	Wet blotting.....	44
2.10.2	Immunodetection of proteins immobilised on the PVDF membrane.....	45
2.11	Isolation of Immuno-globin G (IgG).....	48
2.12	Conjugation of IgG to CN-Br activated Sepharose 4B.....	49
2.13	Biotinylation of IgG using succinimide ester.....	50
2.14	Immunoprecipitation with recombinant proteins.....	50
2.15	Enzyme-linked immunosorbent assay (ELISA).....	52
2.16	Ligand binding assay.....	53
2.17	ATPS affinity column.....	54
2.18	Enzyme assays.....	56
2.18.1	ATPS assay.....	56
2.18.1.1	Determination of K_m for APS.....	58
2.18.2	APSR assay.....	60
2.18.2.1	Preparation of $AP^{35}S$ for APSR activity assay	60
2.18.2.2	APSR activity assay.....	60
2.19	Statistical analysis.....	62
3.0	Results.....	62
3.1	Production of recombinant proteins.....	62
3.1.1	Storage of recombinant ATPS.....	63
3.1.1.1	Activity determination of stored recombinant ATPS.....	63
3.1.1.2	Anion exchange chromatography purification of ATPS and the effect on protein storage.....	67
3.1.2	Activity of recombinant APSR.....	70
3.2	Determination of protein complex formation using ELISA-based method.....	73
3.2.1	Development of methodology.....	73
3.2.1.1	Determination of the pH for effective coating of APSR to microtitre plates.....	73
3.2.1.2	Determination of the optimal amount of protein for coating.....	73
3.2.1.3	Determination of the most appropriate antibody.....	75

3.2.1.4	Determining the binding affinity of the antibody to APSR.....	75
3.2.2	Analysis of the complex formation.....	76
3.2.2.1	Detection of a protein complex between APSR and ATPS at pH 9.4.....	76
3.2.2.2	The effect of glutathione on the formation of a complex between ATPS and APSR at pH 9.4.....	78
3.2.2.3	Detection of a complex between APSR and ATPS at pH 7.4.....	78
3.2.2.4	The effect of glutathione on the formation of a complex between ATPS and APSR at pH 7.4.....	80
3.3	Ligand binding assay to determine putative ATPS-APSR complexes.....	82
3.3.1	Development of methodology.....	82
3.3.1.1	Testing the specificity of the anti-ATPS and anti-APSR antibodies.....	82
3.3.1.2	Do the purified IgG recognise separated <i>E. coli</i> proteins.....	83
3.3.2	Ligand binding assay.....	86
3.3.2.1	Ligand binding assay with anti-APSR.....	86
3.3.2.2	Optimisation of protein binding by increasing the pH.....	86
3.3.2.3	Optimisation of protein binding by the inclusion of magnesium ions.....	89
3.4	Immunoprecipitation of putative ATPS-APSR complexes.....	92
3.4.1	Immunoprecipitation with protein G Sepharose.....	92
3.4.1.1	Immunoprecipitation of ATPS and APSR using protein G Sepharose.....	92
3.4.1.2	Investigation into the <i>ca.</i> 52 kDa protein identified in the western blots of immunoprecipitation experiments.....	94
3.4.1.3	Use of biotinylated antibodies in western blot analysis of immunoprecipitation experiments.....	94
3.4.2	Immunoprecipitation with CN-Br conjugated Sepharose.....	95

3.4.2.1	Immunoprecipitation with anti-ATPS conjugated Sepharose.....	95
3.4.2.2	The effect of glutathione on the ATPS-APSR protein complexes.....	100
3.4.2.3	Determination of the specificity of the interaction with ATPS.....	100
3.4.3	Immunoprecipitation with chloroplast extracts.....	103
3.5	Physiological significance of the ATPS-APSR complex.....	103
3.5.1	Significance on ATPS activity.....	103
3.5.1.1	The effect of APSR on ATPS activity.....	103
3.5.1.2	The effect of glutathione on ATPS activity.....	104
3.5.1.3	The effect of glutathione on ATPS activity when in a complex with APSR.....	104
3.5.2	The effect of glutathione on APSR activity	104
3.6	Detection of protein-protein complex in a chloroplast extract using an ATPS affinity column.....	108
3.6.1	Western analysis with biotinylated anti-ATPS IgG of eluates.....	108
3.6.2	Western analysis with biotinylated anti-APSR IgG of eluates.....	110
3.7	Modulation of ATPS activity by sulfur supply.....	115
4.0	Discussion.....	119
4.1	Part 1: Characterisation of the activity of recombinant of ATPS and APSR.....	119
4.1.1	Purification of recombinant proteins.....	119
4.1.1.1	Storage of recombinant ATPS.....	120
4.1.1.2	Activity of recombinant APSR.....	121
4.1.2	The effect of glutathione on the activity of recombinant proteins.....	122
4.1.3	The effect of glutathione on ATPS activity.....	122
4.1.3.1	The effect of glutathione on APSR activity.....	124
4.2	Part 2: Detection of a protein complex between ATPS and APSR from onion (<i>Allium cepa</i>).....	126

4.2.1	ELISA-based method for protein-protein complex detection.....	126
4.2.1.1	The pH dependence of protein complex between ATPS and APSR.....	128
4.2.2	Detection of a complex using immunoprecipitation.....	130
4.2.2.1	Development of method.....	130
4.3	Part 3: The effect of glutathione on mediating a protein complex between ATPS and APSR.....	132
4.4	Part 4: Detection of a protein complex between ATPS and APSR <i>in vivo</i> using chloroplast extracts.....	135
4.4.1	Immunoprecipitation of chloroplast extracts.....	135
4.4.2	Detection of a protein complex between ATPS and APSR using an ATPS affinity column.....	136
4.5	Part 5: Regulation of ATPS by sulfur supply in <i>A. cepa</i>	140
4.5.1	Comparisons of the K_m of ATPS for PP_i in Texas Grano.....	140
4.5.2	Comparisons of the K_m of ATPS for PP_i in W202A.....	141
4.6	Conclusions.....	144
	References.....	145

List of Figures

Figure 1.1	Sulfur assimilation in higher plants.....	3
Figure 1.2	Structural domains of APSR.....	13
Figure 1.3	Proposed mechanism for the catalytic cycle of APSR.....	14
Figure 2.1	An onion (<i>Allium cepa</i> L.) seeding at ca. 6 weeks.....	31
Figure 2.2	Typical appearance of Percoll gradient with macerated onion leaf after first centrifugation.....	33
Figure 2.3	Example of a BSA protein standard curve.....	41
Figure 2.4	Illustration of the Trans-blot [®] cassette used in the wet-blot transfer of proteins onto a PVDF membrane.....	47
Figure 2.5	The coupled reaction for the measurement of the reverse reaction of ATPS.....	56
Figure 2.6	Typical results of the ATPS activity assay on onion chloroplast extracts.....	59
Figure 3.1	Coomassie stain of the purification of recombinant ATPS separated by SDS-PAGE.....	64
Figure 3.2	Coomassie stain of the purification of recombinant APSR separated by SDS-PAGE.....	65
Figure 3.3	Accumulation of recombinant proteins incubated at various temperatures after induction with IPTG.....	66
Figure 3.4	Protein concentration (mg/ml) of fractions of the ATPS protein extract separated with a Mono-Q column.....	68
Figure 3.5	Recombinant ATPS activity after various storage conditions over 10 days.....	69
Figure 3.6	Change in protein concentration of recombinant APSR over a period of 3 hours.....	71
Figure 3.7	Change in specific activity of recombinant APSR over a period of 3 hours.....	72
Figure 3.8	Optimisation of APSR coating.....	74
Figure 3.9	ELISA to determine the binding affinity of antibodies to the coated recombinant APSR.....	77
Figure 3.10	Detection of a complex between ATPS-APSR using ELISA.....	79

Figure 3.11	Influence of reduced glutathione on the ATPS–APSR complex at pH 7.4.....	81
Figure 3.12	Western analysis with anti-ATPS (A-C) and anti-APSR (D-F) antibodies.....	84
Figure 3.13	Western analysis with anti-ATPS (A) and anti-APSR (B) antibodies of <i>E. coli</i> (strain DH-5 α) protein extracts.....	85
Figure 3.14	Ligand binding assay at pH 7.4.....	87
Figure 3.15	Ligand binding assay at pH 7.8.....	88
Figure 3.16	Ligand binding assay in the presence of 5.0 mM MgCl ₂ at pH 7.4.....	90
Figure 3.17	Ligand binding assay in the presence of 5.0 mM MgCl ₂ at pH 7.8.....	91
Figure 3.18	Western analysis after immunoprecipitation with protein G Sepharose.....	93
Figure 3.19	Western analysis with biotinylated antibodies after immunoprecipitation with protein G Sepharose.....	96
Figure 3.20	Western analysis after immunoprecipitation of ATPS and APSR with anti-ATPS IgG conjugated Sepharose.....	98
Figure 3.21	Western analysis after immunoprecipitation of APSR with CN-Br Sepharose.....	99
Figure 3.22	Immunoprecipitation of ATPS and MD-ACO2 with anti-ATPS IgG conjugated Sepharose.....	101
Figure 3.23	Immunoprecipitation of ATPS and SBTI with anti-ATPS IgG conjugated sepharose.....	102
Figure 3.24	Assay of recombinant ATPS activity in the presence of recombinant APSR and various glutathione treatments.....	106
Figure 3.25	Activity of recombinant APSR in the presence of reduced glutathione and oxidised glutathione.....	107
Figure 3.26	Western analysis of eluates from a CN-Br Sepharose coupled with anti-ATPS column.....	111
Figure 3.27	Western analysis of eluates from a CN-Br Sepharose coupled with anti-ATPS IgG column.....	112
Figure 3.28	Eluates from a CN-Br Sepharose coupled with anti-ATPS IgG column revealed by Coomassie staining.....	113

Figure 3.29	Western analysis with anti-APSR IgG of recombinant ATPS, APSR and the eluate of the CN-Br Sepharose column after the DEA, pH 11.0, wash.....	114
Figure 3.30	K_m values for ATPS of two cultivars of onion, under two sulfur treatments before and after bulbing.....	117
Figure 3.31	Western analysis of chloroplast extracts from onion.....	118

List of Tables

2.1	Composition of percoll gradient.....	30
2.2	Composition of 12 % acrylamide separating and stacking gels for Mini- PROTEAN [®] SDS-PAGE apparatus.....	43
2.3	Composition of the standard reaction mixture (2 x) for ATPS assay.....	57
2.4	Composition of ATPS assay.....	57
2.5	Composition of APSR activity mixture.....	61
3.1	ATPS activity assay of recombinant ATPS and APSR.....	103

List of Abbreviations

Ab _{280/ 340/405/ 590/595 nm}	Absorbance at 280, 340, 405, 590 or 595 nm
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACSO	sulfur-alk (en) yl cysteine sulfoxide
Alliin	3-(2-propenylsulfinyl)-L-alanine
Aliinase	allin alkyl-sulfenate-lyase
AMP	5' adenosine monophosphate
Amp ¹⁰⁰	ampicillin (100mg/ml)
AP	alkaline phosphatase
APS	5' adenosine phosphosulfate
AP ³⁵ S	5' adenosine phospho ³⁵ sulfate
APSK	5' adenosine phosphosulfate kinase
APSR	5' adenosine phosphosulfate reductase
ATP	5' adenosine triphosphate
ATPS	5' adenosine triphosphate sulfurylase
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
°C	degrees Celsius
ca.	circa.
cDNA	complementary DNA
cm	centimetre
cpm	counts per minute
Cys	cysteine
DEA	diethanolamine
DEAE	diethylammonioethyl
DNA	deoxyribonucleic acid
DTE	dithioerythritol
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECISO	sulfur-ethyl cysteine sulfoxide
EDTA	ethylenediamine tetra-acetic acid

ELISA	enzyme linked immunosorbent assay
ELSA	enzyme linked sorbent assay
Fd	ferredoxin
FW	fresh weight
FPLC	fast protein liquid chromatography
g	gram
g	acceleration due to gravity
Grx	glutaredoxin
GS4B	glutathione Sepharose 4B
GSH	glutathione (reduced)
GSSH	glutathione (oxidised)
GST	glutathione S-transferase
HPLC	high pressure liquid chromatography
hr	hour
Hsp	heat shock protein
IgG	immunoglobulin G
IPTG	isopropyl- β -D-thiogalactopyranoside
kDa	kilodalton
K_{eq}	equilibrium constant
K_m	substrate concentration at half maximum reaction rate
kPa	kilopascal
L	litre
LB	Luria-Bertani
M	Molar, moles per litre
mAmp	milliampere
MCSO	sulfur-methyl-L-cysteine sulfoxide
MD-ACO2	ACO-2 from apple (<i>Malus sp.</i>)
Meq	milliequivalent
Milli-Q	water that has been purified by Milli-Q ion exchange chromatography
min	minute
ml	millilitre
μ g	microgram
μ l	microtitre

μm	micrometer
μmol	micromole
mol	moles
M_r	relative molecular mass (g mol^{-1})
mRNA	messenger RNA
NAD^+/NADH	nicotinamide adenine dinucleotide reduced/ oxidised
NADPH	dihydrotriphosphopyridine nucleotide
NBT	<i>p</i> -nitro blue tetrazolium chloride
ng	nanogram
nmol	nanomole
NR	nitrate reductase
OAS	<i>O</i> -acetylserine
OAS-TL	<i>O</i> -acetylserine thiol lyase
<i>ox</i>	oxidised
1-PECSO	<i>trans</i> - sulfur- (1-propenyl)- L-cysteine sulfoxide
2-PECSO	<i>trans</i> - sulfur- (2-propenyl)- L-cysteine sulfoxide
PA	1, 10-phenanthroline
PAGE	polyacrylimide gel electrophoresis
PAPS	adenosine 3'-phosphate 5'-phosphosulfate
PBS	Phosphate buffered saline
PCSO	sulfur-propyl- L-cysteine sulfoxide
<i>pers. comm.</i>	personal communication
pH	$-\log [\text{H}^+]$
PP_i	inorganic phosphate
ppm	part per million
psi	pounds per square inch
PVDF	polyvinylidene difluoride
<i>red</i>	reduced
RO	reverse osmosis
rpm	revolutions per second
RUBISCO	ribulose bisphosphate carboxylase/oxygenase
s	second
SAT	serine acetyltransferase
SAT-p	plastid-localised serine acetyltransferase

SAT-m	mitochondrial-localised serine acetyltransferase
SBTI	soy bean trypsin inhibitor
SDS	sodium dodecyl sulfate
SiR	sulfite reductase
TBS	tris buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine
Thr	threonine
Tris	tris (hydroxymethyl)-aminomethane
Trx _{red/ox}	thioredoxin reduced/oxidised
Tween-20	poly (oxyethylene) sorbitane-monolaurate
UV	ultraviolet light
V _{max}	maximum rate of reaction
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

1.0 Introduction

1.1 Overview

Members of the genus *Allium*, which include Chinese chive (*A. tuberosum*), garlic (*A. sativum*) and onion (*A. cepa*), have unique intense flavours, often described as pungent. These flavours are popular in medicine, and in cooking, where they are used for their distinctive flavour and the ability to enhance the flavour of other foods. The distinct flavours of *Alliums* are due largely to a class of sulfur-containing compounds, termed sulfur-alk(en)yl cysteine sulfoxides (ACSO) that occur in high concentrations. ACSO compounds themselves are not volatile, but require the hydrolysis by an allin alkyl-sulfenate-lyase, commonly referred to as alliinase (EC 4.4.1.4), which is activated in response to wounding. The activity of alliinase on ACSO as a substrate produces the distinctive flavours (Randle and Lancaster, 2001).

Onion (*Allium cepa*), a common vegetable crop grown in New Zealand, contains five major ACSO compounds: (+)-S-methyl-L-cysteine sulfoxide (MCSO), (+)-S-ethyl cysteine sulfoxide (ECSO), (+)-S-propyl-L-cysteine sulfoxide (PCSO), *trans*- (+)-S- (1-propenyl)-L-cysteine sulfoxide (1-PECSO) and *trans*- (+)-S- (2-propenyl)-L-cysteine sulfoxide (2-PECSO), and it is the 1-PECSO that contributes the most to the typical onion flavour (Bernard, 1970).

Pungency is a heritable trait, and this allows cultivars to be selected on the basis of flavour, as intensity varies between cultivars. For example, 'Canterbury Longkeeper', a more pungent cultivar, would generally contain more of the non-volatile precursor ACSO compounds. In contrast, a less pungent cultivar, e.g. 'Houston Grano', has less detectable ACSO compounds, but more free sulfate (McCallum et al., 2002).

Differences in pungency between cultivars has recently been shown to be due to differences in the regulation of the sulfur assimilation pathway (Randle et al., 1995; McCallum et al., 2002), as the biosynthesis of ACSO compounds is dependent on reduced sulfate in the form of cysteine. In *Allium* species, cysteine is converted to various ACSO compounds (Lancaster and Shaw, 1989). However, very little is known about the

regulation of the sulfur-assimilation pathway in onion, or if this regulation differs between onion cultivars with different levels of ACSO compounds.

As part of this overall investigation of the regulation of the sulfur assimilation pathway in onion, this project proposes to investigate the significance of protein-protein interactions, principally between ATPS and APSR as a candidate for a major control point in the pathway. Further, cultivar differences may arise from differences in the efficiency of sulfur assimilation that is regulated by the formation of these complexes. However, a distinct comparison of these cultivars is not the immediate aim of this project. As a starting point, it is therefore necessary to introduce the reductive sulfur assimilation pathway in higher plants.

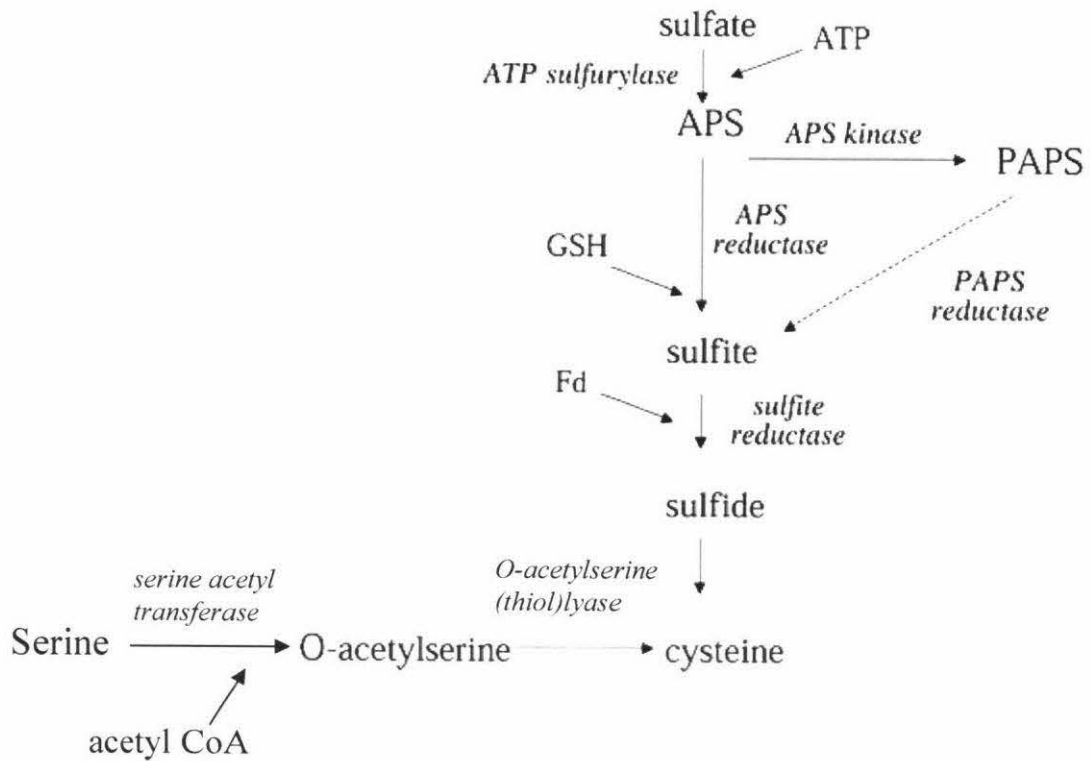
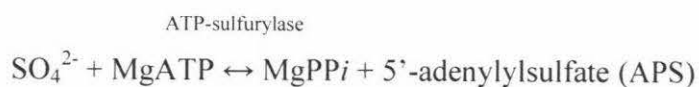


Figure 1.1: Sulfur assimilation pathway in higher plants. Derived from Kopriva and Koprivova (2004). Abbreviations; *ATP*, adenosine triphosphate, *APS*, adenosine-5'-phosphosulfate, *PAPS*, adenosine 3'-phosphate 5'-phosphosulfate, *GSH*, glutathione (reduced), *Fd*, ferredoxin (reduced), *CoA*, coenzyme *A*.

1.2 Cysteine biosynthesis.

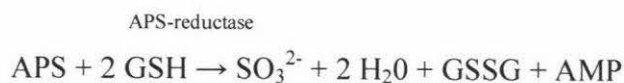
Sulfate is the major source of uptaken sulfur for higher plants and is usually obtained from the rhizosphere by the roots. Cysteine is the major product of the sulfur reduction pathway, which is regulated by a number of factors (Leustek et al., 2000). Transport of sulfate into cells is largely due to plasma membrane-localised H^+/SO_4^{2-} co-transporters that are dependent on the electrochemical gradient produced by a proton pump. In *Arabidopsis*, 14 genes have been identified that code for sulfur transport proteins (Takahashi et al., 1997; Takahashi et al., 1999; Takahashi et al., 1999; Takahashi et al., 2000; Vidmar et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). These are involved with the loading of sulfate into the root, long distance transport of sulfate through the plant, and for the uptake of sulfate into the root plastids or leaf chloroplasts. The site of sulfur assimilation occurs largely within the chloroplasts of leaves, although evidence suggests that limited sulfur assimilation also occurs in the cytoplasm, and in the plastids of roots (Lunn et al., 1990; Rotte and Leustek, 2000).

Assimilation of uptaken sulfate is initiated by the adenylation of sulfate by the magnesium-dependent enzyme, ATP-sulfurylase (ATPS; EC 2.7.7.4) which generates adenosine-5'-phosphosulfate (APS) and pyrophosphate (Renosto et al., 1993) (Reaction 1.1).



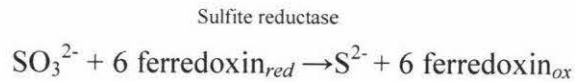
Reaction 1.1

Once this first activation step occurs, the reduction of activated sulfate (APS) takes place as two steps. The first reaction indirectly transfers two electrons from glutathione (GSH) to APS producing sulfite (Bick et al., 1998). This reaction is catalysed by APS-reductase (APSR; EC 1.8.4.9) (Reaction 1.2).



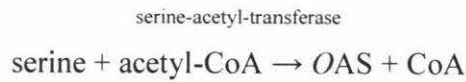
Reaction 1.2

Sulfite is then reduced further by sulfite reductase (SiR; EC 1.8.7.1), which catalyses the reduction of sulfite to sulfide through a 6-electron transfer from ferredoxin (Fd) (Reaction 1.3) (Nakayama et al., 2000).

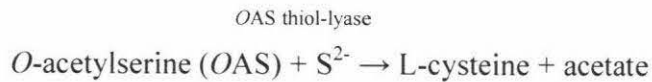


Reaction 1.3

Once sulfate is reduced to sulfide, it is then assimilated into the amino acid cysteine. Sulfide reacts with *O*-acetylserine (*OAS*) to form cysteine in a reaction that is catalysed by *OAS* thiol-lyase (*OAS*-TL; EC 4.2.99.8) (reaction 1.5). The formation of *OAS* occurs by the addition of acetyl CoA to serine and is catalysed by the enzyme serine-acetyltransferase (SAT; EC 2.3.1.30) (Reaction 1.4).



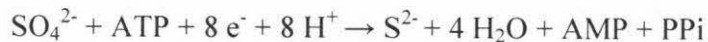
Reaction 1.4



Reaction 1.5

The reduction of sulfate involves an 8-electron transfer, which reduces sulfate to sulfide and also requires one ATP (for the ATP-sulfurylase reaction). The sulfide is largely incorporated into cysteine. From there, cysteine is incorporated into proteins and other sulfur-containing compounds including the ACSO compounds produced by members of the *Allium* genus (Lancaster and Shaw, 1989; Jones et al., 2004).

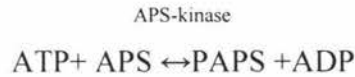
Reaction 1.6 shows the summary of the reduction of sulfate.



Reaction 1.6

Prior to 1996, before the activity of APS reductase was identified (Gutierrez-Marcos et al., 1996; Setya et al., 1996) it was thought that the sulfur assimilation pathway in higher plants was homologous to the pathway that operates in fungi and bacteria. In this pathway the activation of the sulfate is achieved in two steps rather than one. First, the sulfate is bound to an adenosine group in a reaction that is similar to that which occurs in higher plants (Reaction 1.2). Once APS is formed, an APS kinase (EC 2.7.1.25) adds an

additional phosphate group yielding adenosine 3'-phosphate 5'-phosphosulfate (PAPS) (Leyh, 1993) (Reaction 1.7).



Reaction 1.7

PAPS is then reduced by a thioredoxin-dependent reaction catalysed by PAPS reductase (EC 1.8.4.8) to form sulfite (Reaction 1.8). This pathway is referred to as sulfation.



Reaction 1.8

Two independent groups then determined that higher plants contain a APS-reductase (Gutierrez-Marcos et al., 1996; Setya et al., 1996), suggesting a pathway that by-passes the requirement for PAPS reduction. It is now generally agreed that this pathway (APS reduction) is the major route for reductive sulfate assimilation in higher plants (Bick and Leustek, 1998; Leustek et al., 2000). However, reduction of PAPS should not be excluded in plants since some evidence suggests that this pathway may be present. In the moss, *Physcomitrella patens*, mutants that had the APSR gene knocked out could grow on sulfate as the sole sulfur source. However, the authors did suggest that *P. patens* contained both pathways, and that higher plants have lost the sulfation pathway (Koprivova et al., 2002).

In *Arabidopsis*, at least three genes have been found to encode for an APS-kinase (Reaction 1.7), and at least one of these is located in the chloroplast (Lee and Leustek, 1998; Schiffmann and Schween, 1998), confirming that PAPS is formed in higher plants. However, it is unconfirmed as to whether higher plants contain PAPS reductase, as only one report exists in which PAPS reductase has been isolated from spinach leaves (Schween, 1989). A survey of the *A. thaliana* and rice (*Oryza sativa*) genomes do not contain any genes homologous to the *E. coli* PAPS reductase, other than domains within APSR. However it is possible that plant PAPS reductases have a different sequence and structure to *E. coli* APSR (Kopriva and Koprivova, 2004). In higher plants, PAPS can act as a sulfuryl donator, where a sulfate is added to oxygen by sulfotransferases thus

forming a sulfur ester bond. An example of this reaction is seen in the biosynthesis of the plant hormone group, the brassinosteroids (Rouleau et al., 1997).

In terms of the important enzymes in the pathway in onion, McCallum *et al.* (2002) identified ATP sulfurylase as a possible candidate for the observed differential regulation of the sulfur assimilation pathway between onions of high and low ACSO content. Also it is well known that the subsequent enzyme, APS reductase, has been identified as a key regulatory enzyme of the pathway. A number of factors have been described that effect gene expression or activity, including the levels of sulfur, carbon and nitrogen (Brunold et al., 1987; Kopriva et al., 2002), light (Kopriva et al., 1999), and oxidative stress (Bick et al., 2001). Thus APSR is another likely candidate responsible for cultivar-specific regulation of sulfur assimilation in onion. Therefore, these two enzymes will be introduced in more detail.

1.3 ATP-sulfurylase.

The first step of sulfur assimilation is the activation of the sulfate as a prelude to subsequent reduction. ATP sulfurylase (ATPS) catalyses the reaction which breaks the bond between the β - and the γ -phosphate of ATP and the sulfate is then subsequently bound to the γ -phosphate (Hiltz and Lipmann, 1955), resulting in the formation of APS and PP_i (Reaction 1.1). The energy stored between the β - and the γ -phosphate bond is essential for the successful reduction of the inert sulfate.

The genome of *Arabidopsis* contains four genes encoding ATP sulfurylase, designated *AtAPS1*, *AtAPS2*, *AtAPS3* and *AtAPS4* (Leustek et al., 1994; Klonus et al., 1995; Murillo and Leustek, 1995). Each of these genes encodes proteins that contain a chloroplast transit peptide, suggesting that each enzyme is localised in the chloroplasts. In spinach (*Spinacia oleracea*), an ATPS isoform has been shown to be localised in the cytosol (Lunn et al., 1990), but thus far in *Arabidopsis*, despite detectable ATPS activity in the cytosol (Rotte and Leustek, 2000), a gene coding for a putative cytosolic isoform is yet to be identified. Hatzfeld *et al.* (2000), however, suggest that *AtAPS-2* may play a cytosolic

role, since an alternative translation start point is proposed to result in an active *AtATPS-2* without the transit peptide (Hatzfeld et al., 2000).

Previous reports of ATPS kinetics in plants have reported K_m values using purified ATPS from the chloroplast of 0.046 mM for MgATPS and of 0.25 mM for SO_4^{2-} and for the reverse reaction K_m values of 64×10^{-85} nM for APS and of 10 μM for PP_i have been reported (Renosto et al., 1993).

In thermodynamic terms, the ATPS enzyme in all species carries out an unfavorable reaction, with a $K_{eq} \sim 10^{-8}$ M (Farooqui, 1980; Murillo and Leustek, 1995). To move the reaction in the forward direction, the products APS and PP_i , must be efficiently removed (Seubert et al., 1985). Since APS or PP_i cannot accumulate for sulfate assimilation to continue, an inorganic pyrophosphatase, ATP reductase and/or APS kinase must be readily available to accommodate this removal of substrate. To overcome this problem, other species have alternative methods for efficient APS synthesis. For example an ATPS isolated from *E.coli* was shown to contain two subunits, one that catalyses the APS synthesis reaction, while the other subunit is responsible for the hydrolysis of GTP. The hydrolysis of GTP is linked to APS synthesis and the transfer of energy is required for the reaction to occur (Leyh and Suo, 1992). So far, the GTPase subunit has not been identified in eukaryote ATPS proteins.

In several animal species, ATPS is conjugated with the subsequent enzyme, APS-kinase (Li et al., 1995; Jullien et al., 1997; Venkatachalam et al., 1998) but in plants and fungi these enzymes exist separately (Jain and Leustek, 1994; Murillo and Leustek, 1995). An alternative mechanism to encourage APS synthesis exists, in which APS channeling occurs between the ATPS and APS-kinase (Lyle et al., 1994).

In plants, the C-terminal domain of ATPS has high sequence homology and is structurally very similar with APS-kinase in yeast and *Penicillium chrysogenum* suggesting that ATPS may have evolved from APS-kinase. Despite the similarities ATPS does not act as a kinase, although in *P. chrysogenum* it does bind to PAPS, the product of APS-kinase, thus allowing allosteric control over ATPS (Martin et al., 1989; Renosto et al., 1990; MacRae et al., 2001). From an evolutionary perspective, plant ATPS enzymes, seem to be more closely related to animal and fungal ATPS enzymes than bacterial ATPS enzymes (Murillo and Leustek, 1995; Leustek, 1996).

The regulation of ATPS has been noted in a number of plant studies, but as yet no mechanisms have been described. In canola (*Brassica napus*), maize (*Zea mays*) and *Arabidopsis*, ATPS mRNA levels decline and an excess of reduced sulfur compounds (e.g. glutathione, cysteine) will also induce the down-regulation of ATPS mRNA after prolonged exposure to sulfur-deprivation (Lappartient and Touraine, 1996; Bolchi et al., 1999; Vauclare et al., 2002). However, the exposure of *Brassica juncea* to cadmium, which chelates glutathione and decreases the cellular concentration of glutathione, induces expression of ATPS mRNA (Heiss et al., 1999). Low nitrogen levels will also reduce the mRNA levels of not only ATPS, but also of APSR (Koprivova et al., 2000), in a coordinated manner.

In a detailed study, Rotte and Leustek (2000) reported that plastid-associated enzyme activity of ATPS in *Arabidopsis* is down regulated as the plant matures, but the activity of the cytosolic localised isoform of ATPS increases, suggesting a different function for the cytosolic form of ATPS.

1.4 APS-reductase

1.4.1 Overview of APS-reductase

APS reductase (APSR) catalyses the first reduction of activated sulfate (APS), where two electrons are transferred from a glutathione to the sulfate, producing AMP and sulfite (reaction 2). This step became universally accepted as a possible sulfate reduction step after the isolation of three isoforms of APSR in 1996 (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Before this, resolution of the APSR catalysing step, it was thought that an APS sulfotransferase activity existed, whereby the reduced sulfate is transferred to a thiol acceptor producing a thiosulphate, such as glutathione (Schmidt, 1975; Varin et al., 1997). This hypothesis was refuted when it was shown that the cloned APS sulfotransferase from *Lemna minor* (Suter et al., 2000) displayed very high identity to an APSR cloned from *A. thaliana* (Gutierrez-Marcos et al., 1996; Setya et al., 1996). Later, Suter et al. (2000) showed that APSR exclusively used APS to produce sulfite. Despite the identification of the APS reduction pathway, the presence of a PAPS pathway should not be excluded in plants, because of the identification of both APS kinase and PAPS

reductase enzyme activities (Schween, 1989; Lee and Leustek, 1998; Koprivova et al., 2002).

Three genes encoding APSR have been identified in the genome of *Arabidopsis*, which are designated *AtAPR1*, *AtAPR2* and *AtAPR3*. All contain a chloroplast transit peptide, a reductase domain and a thioredoxin-like domain (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Suter et al., 2000).

1.4.2 Localisation of APSR

Observation of the full length cDNA of all plant APSR enzymes reveals a chloroplast localisation sequence, suggesting that APSR is found in the chloroplast (Suter et al., 2000). By western blot analysis, APSR protein, before the entry into the chloroplast, is determined to be approximately 58 kDa, but APSR detected in the chloroplast is processed to give a 45 kDa product (Prior et al., 1999). Using immunogold electron microscopy, APSR was detected in the chloroplasts of three *Flaveria* species, with 70 % of detectable APSR localised near or associating with the thylakoid membrane (Koprivova et al., 2001).

1.4.3 The reductase domain

The reductase domain has high similarity with that of bacterial PAPS reductases, although one important difference between APSR and bacterial PAPS reductase exists. This is the presence of two additional cysteine pairs in the N-terminal domain of the plant APSR enzyme, which are essential for the binding of the cofactor [4Fe-4S], which confers specificity towards APS binding over PAPS (Kopriva et al., 2001; Kopriva et al., 2002). It is thought that this domain is responsible for the reduction of APS, although the reductase domain is unable to be catalytically active without the C-terminal domain or the addition of reduced thiols (Weber et al., 2000).

1.4.4 The thioredoxin-like domain

The C-terminal domain of APSR has sequence identity with thioredoxin (EC 1.8.4.10), a class of thiol:disulphide oxidoreductases (Gutierrez-Marcos et al., 1996; Setya et al., 1996). In *E.coli*, APSR activity with the C-terminal removed was low, but this was restored after the addition of thioredoxin (Bick and Leustek, 1998). In plants, despite the C-domain containing high sequence identity with thioredoxin, it is now accepted that the C-terminus domain acts as a glutaredoxin (which uses reduced glutathione) and not as a thioredoxin reductase (EC 1.8.1.9) (as the thioredoxin domain does). This conclusion was reached after a series of studies that showed firstly that glutathione was more effective as a hydrogen donor (Bick et al., 1998; Prior et al., 1999), that the C-terminal domain of the enzyme can catalyse glutathione-dependent reactions (Bick et al., 1998; Prior et al., 1999) and that it can also complement *E.coli* mutants lacking glutaredoxin (Bick et al., 1998). Taken together, these studies confirm the notion that the C-terminus acts as a glutaredoxin and not a thioredoxin.

1.4.5 The reaction mechanism of APSR

The catalytic mechanism of APSR can be divided into three steps (figure 1.3). For the first step, the sulfur from APS covalently binds to a conserved Cys₂₄₈ in *Arabidopsis* within the reductase region. Mutagenesis of the Cys₂₄₈ to Ser₂₄₈ resulted in the loss of activity due to the formation of a stable APSR-SO₃⁻ intermediate which does not require the interaction of the C-terminal (Weber et al., 2000). The second step releases the sulfite from the enzyme. This requires the reduced C-terminal domain which oxidises conserved cysteine residues forming a disulfide bond within the C-terminus and release of free sulfite. Sulfite is unable to be released if the C-terminal is absent, or if reduced thiols are not present, resulting in a stable intermediate (Weber et al., 2000). In order for active enzyme to be regenerated, two molecules of reduced glutathione are required, which reduce the di-sulfide bond between the cysteines of the C-terminal and N-terminal domains, restoring the APSR enzyme to an active state.

Consequently, APSR activity is increased by oxidative stress, *via* oxidation of the pair of cysteines required for function. This is caused by an abundant supply of oxidised

glutathione, a consequence of reactive oxygen species, and thus demonstrates a post translational mechanism for control of APSR (Bick et al., 2001).

1.4.6 Kinetic properties of APSR

The measured activity for APSR varies substantially between species with rates ranging from 0.4 to 0.8 nmol min⁻¹ mg⁻¹ protein in poplar (*Populus tremula* × *P. alba*) (Hartmann et al., 2004), 0.77 ± 0.09 nmol min⁻¹ mg⁻¹ protein in *Arabidopsis* (Tsakraklides et al., 2002) to 30-40 μmol min⁻¹ mg⁻¹ protein in *Lemna minor* (Kopriva et al., 2001). Kinetic properties have been determined for APSR in *L. minor*, where at an APS concentration of 40 μM, a K_m value of 16.8 ± 5.3 mM has been calculated for GSH and at 40 mM glutathione K_m value of 12.2 ± 4.6 μM has been calculated for APS (Suter et al., 2000).



Figure 1.2: Structural domains of APSR. The N-terminus includes the chloroplast transit peptide (TP), which is removed from the peptide as part of the mechanism of chloroplast entry. The reductase domain, homologous to the PAPS reductase domain in *E. coli*, contains a conserved cysteine that allows SO_4^{2-} to bind (Weber et al., 2000). The C-terminus contains a domain homologous to thioredoxin, yet acts like a glutaredoxin, oxidising glutathione (Bick et al., 1998). Reproduced from Bick and Leustek (1998)

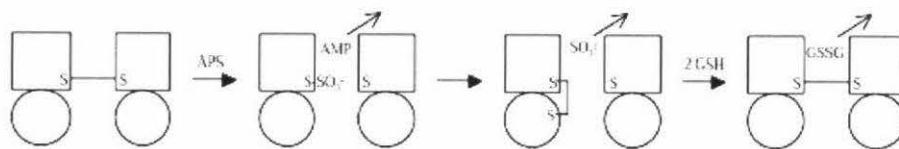


Figure 1.3: Proposed reaction mechanism for the catalytic cycle of APSR (see text for details). The circles and square represents the N-terminal and C-terminal domain of APSR, respectively. S represents the thiol group of catalytically significant cysteine residues. Adapted from Kopriva and Koprivova, (2004)

1.5 Regulation of the sulfur-assimilation pathway

Regulation of the sulfur assimilation pathway is important. The ability to sense environmental signals results in the regulation of metabolic processes in plants accordingly and thus in the efficient use of the nutrients. The regulation of sulfur assimilation is part of an intricate web of control which is dependent on the supply of sulfur, nitrogen, carbohydrates and the occurrence of oxidative stress (Prosser et al., 1997; Koprivova et al., 2000; Kopriva et al., 2002; Hesse et al., 2003; Nikiforova et al., 2003).

1.5.1 Developmental regulation of ATPS and APSR

At the gene level, the gene expression of ATPS and APSR are both regulated during plant development. In general, the highest expression of both genes occurs in the actively growing regions of the plant. The expression of ATPS and APSR mRNA declined in parallel with one another as the plant aged, suggesting similar regulatory cues.

For ATPS, Rotte and Leustek (2000) determined that the ATPS protein can be localised in either the cytosol or the chloroplast. The cytosolic and chloroplastic ATPS have different temporal patterns of abundance, where the chloroplastic, the most abundant isoform, is most active in the young growing leaves, and decreases as the plant develops (3-fold decrease). In contrast, the cytosolic isoform shows highest activity in the mature leaves (a 5-fold increase). Rotte and Leustek (2000) suggested that the cytosolic isoform functions to provide APS for sulfate biosynthesis in the cytosol.

1.5.2 Regulation by sulfur status and products of the sulfur assimilation pathway.

The external sulfur supply plays a major role in controlling the rate of sulfur assimilated. Under limited external sulfur supply, sulfate uptake, ATPS and APSR enzyme activities, an increase the transcript level of sulfate transporters, ATPS, APSR, and SAT are observed (Reuveny et al., 1980; Smith, 1980; Lappartient and Touraine, 1996; Takahashi et al., 1997; Yamaguchi et al., 1999). Under sulfur stress, the extracted protein levels,

activity and transcript levels of ATPS from roots of *A. thaliana* increase significantly after 3 days. Further, supplying sulfate to sulfur starved plants reduced the ATPS activity back to a basal level (Lappartient and Touraine, 1996; Lappartient et al., 1997). In another study, *Arabidopsis thaliana*, grown in sulfur deprived media for 2 days before northern blot analysis, showed an increase in transcription of a high affinity sulfate transporter and APSR in both the shoots and the roots (Takahashi et al., 1997). The mRNA levels of APSR in poplar (*Populus tremula* x *P. alba*) increased after 3 days of sulfur deprivation, but unlike herbaceous species, only occurred in the roots (and not in shoot tissues). Also, unlike *A. thaliana*, an increase of ATPS mRNA was observed only after prolonged sulfur deficiency (four weeks). It is suggested that poplar may have a larger store of sulfur which is a possible reason as to why an immediate down regulation of ATPS, observed in herbaceous species, is not observed (Kopriva et al., 2004). These observations suggest that APSR is more susceptible to control from sulfur stress, when compared with ATPS.

More recently, the role of reduced sulfur compounds such as glutathione, cysteine, *O*-acetyl-L-serine and H₂S have becoming increasingly apparent as important in sensing the sulfur levels, either as signals of sulfur stress or by directly regulating the sulfur assimilation pathway. (Neuenschwander et al., 1991; Lappartient and Touraine, 1997; Bolchi et al., 1999; Clarkson et al., 1999; Westerman et al., 2001; Vauclare et al., 2002) Glutathione (a product of the sulfur reduction pathway), which is commonly associated with scavenging of radicals produced by oxidative stress, is also linked with the storage and transport of reduced sulfur and as a modulator of enzyme activity (May et al., 1998; Noctor and Foyer, 1998). Glutathione is produced by the conjugation of cysteine to glutamate and glycine by γ -glutamyl-cysteine synthase (EC 6.3.2.2) and glutathione synthase, respectively (EC 6.3.2.3) (Hell and Bergmann, 1988, 1990). Glutathione is a likely candidate as a signaling molecule for sulfur stress. Here nutritional status can be determined in the roots and glutathione acts as a signal to the shoots, as it is easily translocated within the phloem (67–70% of reduced sulfur in the phloem is glutathione) (Rennenberg et al., 1979). Glutathione content also reflects the sulfur status of the plant, as the tripeptide requires reduced sulfur for synthesis (Meyer and Fricker, 2002). This ‘demand-driven’ model was developed by Lappartient and Touraine (1996) who demonstrated that glutathione transported in the phloem is the signal that controls sulfur

assimilation. Supplied glutathione and cysteine, either externally to the roots or internally through the phloem of canola (*Brassica napus* L. cv Drakkar), resulted in reduction of both ATPS transcript levels and ATPS activity and a reduction in sulfate uptake. This repression was inhibited if buthionine sulfoximine, a specific inhibitor of γ -glutamyl-cysteine synthase, which results in a decreased glutathione pool. This inhibitor study suggests that glutathione and not cysteine, is responsible for repression of the expression of ATPS mRNA. (Herschbach and Rennenberg, 1994; Lappartient and Touraine, 1996; Lappartient et al., 1999). However, conflicting results in *Arabidopsis thaliana* have been reported, whereby externally applied oxidised glutathione resulted in no reduction in either ATPS transcript or protein activity levels in root cultures. In contrast to ATPS, APSR mRNA, protein and activity reduced significantly in the presence of 0.2 mM oxidised glutathione, suggesting that APSR is more sensitive to glutathione when compared to ATPS (Vauclare et al., 2002). The regulation of APSR by glutathione has been reinforced in poplars, where exogenously applied glutathione resulted in a strong reduction in APSR mRNA and protein activity, yet no change in mRNA accumulation or protein activity of ATPS, APSR, SiR or OAS-TL was observed. Although this 'demand-driven' control of sulfur metabolism by glutathione is not applicable to all species, other reduced sulfur compounds have been suggested to play a regulatory role.

Bolchi *et al.* (1999) observed that transcript levels of both a sulfate transporter and ATPS increased under sulfur stress in maize (*Zea mays*), then reduced when sulfur supply was restored. Under sulfur stress, a reduction in glutathione pools was also observed. To test if the decrease in glutathione pools induced the increase in transcript levels, buthionine sulfoximine was added to inhibit glutathione synthesis. However, unlike previous reports (Lappartient and Touraine, 1996; Lappartient et al., 1999) reducing the glutathione pool was not sufficient for the up regulation of ATPS or the sulfate transporter. It was determined that L-cysteine, a major product of the sulfur assimilation pathway, successfully down-regulated the sulfate transporter and ATPS mRNA in maize (*Zea mays*) (Bolchi et al., 1999). This provides evidence of a mechanism of control in the absence of glutathione. It maybe that glutathione seems to have a predominate role in regulation in dicot plant species such as canola (*Brassica napus*), poplar (*Populus tremula*), *A. thaliana* but cysteine may be more important in monocots, such as maize.

In addition to ATPS and the sulfate transporter *s*, the activity of other enzymes in the sulfur assimilation pathway can also be influenced by L-cysteine. Serine acetyltransferase (SAT; EC 2.3.1.30) catalyses the formation of *O*-acetylserine from serine and acetyl-CoA, which will subsequently form cysteine after a reaction catalyzed by *O*-acetylserine (thiol) lyase (*OAS*-TL; EC 4.2.99.8). A number isoforms of SAT have been isolated and characterised from *A. thaliana*. These include SAT-c, a cytosolic localised SAT (Ruffet et al., 1995), SAT-p, localised in the plastids and SAT-m, of mitochondrial origin (Roberts and Wray, 1996; Hesse et al., 1999). Interestingly, it is only the SAT-c isoform and not SAT-p or SAT-m, which is sensitive to L-cysteine, allosterically inhibiting the protein activity (Noji et al., 1998). Regulation of cysteine sensitive SAT is due to a region of residues, from Pro276 to Phe285, that are different to the insensitive isoforms, providing evidence for a feedback regulatory mechanism that is specific to the isoform (Inoue et al., 1999).

In addition to L-cysteine, other reduced sulfur compounds, such as H₂S have been observed to have an effect on the sulfur reduction pathway. For example in *Brassica oleracea* L, a reduction in the activity of APSR in the shoots was observed only in the presence of H₂S. The activity of ATPS, SAT and *OAS*-TL were not effected in this treatment (Westerman et al., 2001).

O-acetylserine (*OAS*), a product of the reaction catalysed by SAT and is essential for the formation of cysteine, is also proving to play a major role in the regulation of the sulfur assimilation pathway. Unlike glutathione and cysteine, *OAS* seems to act as a positive regulator, increasing the capacity of the pathway under sulfur deprivation. Under conditions of low sulfur, levels of *OAS* increase in the plant cell (Kim et al., 1999) and are limiting in high sulfate conditions. *OAS* acts as a positive regulator of transcription, protein levels and activity of members of the sulfur reduction pathway. In maize (*Z. mays*), the external supply of *OAS*, with sufficient sulfur supply, leads to an increase in the mRNA of a high affinity sulfate transporter (Smith et al., 1997). Using cell suspensions of *Z. mays* in adequate sulfate nutrition, an increase in sulfate uptake and an increase in ATPS activity was observed after the addition of *OAS* (Clarkson et al., 1999). The activity of APSR has also been shown to be affected by *OAS*. The presence of *OAS* in the media of *Lemna minor* (L.) resulted in an increase in APSR activity but not of ATPS or *OAS*-TL (Neuenschwander et al., 1991). An increase of internal *OAS*

concentration obtained by over-expressing *O*-acetyl-serine mRNA, increased levels of cysteine and glutathione, probably due to increasing the substrate levels, as cysteine is known to be the limiting factor in the formation of glutathione. However, it is unknown if the increased levels of cysteine and glutathione observed, are due to an up-regulation of the sulfur assimilation pathway by *OAS*, as has been shown in previous studies (Blaszczyk et al., 1999; Harms et al., 2000; Youssefian et al., 2001).

Recent studies in potato (*Solanum tuberosum* L.) propose that *OAS* is not the signal for the increased gene expression of sulfur-reduction related genes in response to sulfur starvation. It is possible that other proposed signal molecules such as glutathione (Lappartient et al., 1999), cysteine (Bolchi et al., 1999) or sulfide (Westerman et al., 2001), are responsible, as *OAS* levels are thought to be too low to derepress expression at this stage. However, after prolonged sulfur starvation, the *OAS* pool does increase sufficiently, and this may lead to increased expression of sulfur reduction genes (Hopkins et al., 2005).

1.5.3 Regulation by nitrogen

In plants, it is becoming apparent that nitrogen and sulfur levels are tightly linked, whereby nitrogen status can influence the sulfur reduction pathway and vice-versa (Brunold and Suter, 1984; Yamaguchi et al., 1999; Koprivova et al., 2000; Prosser et al., 2001). In *Lemna minor*, the activities of ATPS, APSR and *OAS*-TL decrease under low nitrogen conditions, but are fully restored after the addition of ammonia or nitrate (Reuveny et al., 1980; Brunold and Suter, 1984). Addition of ammonia or nitrate to plants in nitrogen sufficient media resulted in an increase in APSR activity, but no increase in activity was observed for either ATPS or *OAS*-TL.

As described above, under sulfur stress, components of the sulfur assimilation pathway increase, to maximise sulfur uptake and reduction (Prosser et al., 1997; Takahashi et al., 1997). However, under nitrogen deprivation, this response is reduced, suggesting that the sulfur deprivation signal is dependent on the nitrogen supply (Yamaguchi et al., 1999). The regulation of the sulfate assimilation pathway by nitrogen levels is perceived to be possible due to *OAS* as a putative signal molecule. As discussed previously (see section 1.2.2), *OAS* has been seen to positively regulate components of the sulfate reduction

pathway. In *A. thaliana*, a reduction in APSR mRNA, protein levels and protein activity was observed under low nitrogen levels, but the application of OAS to these nitrogen deficient plants strongly increased the levels of mRNA of all isoforms of APSR, SiR and also chloroplastic OAS-TL. This supports evidence that OAS plays an important role as a possible connection between nitrogen and sulfur (Koprivova et al., 2000). Further, in DNA- microarray analysis of 9000 genes of *A. thaliana* isolated after treatment with 1.0 mM OAS, more than 100 genes were induced and 548 genes were repressed. Those induced included APSR, O-acetyl transferase and a sulfate transporter, although the correlation between the response to OAS and sulfur deprivation was not enough to suggest that OAS was the signal of sulfur status (Hirai et al., 2003).

1.5.4 Regulation by carbon

Components of the sulfur pathway have been shown to be under regulatory control by light, since the activity of ATPS and APSR and OAS-TL was shown to increase in the presence of light (Neuenschwander et al., 1991; Astolfi et al., 2001). A high density oligonucleotide microarray identified genes that exhibited a mRNA cycling which was dependent on a circadian pattern, and included two sulfate transporters, APSR and SAT (Harmer et al., 2000). In maize, APSR is known to be under a diurnal rhythm where maximal enzyme activity and mRNA levels occur during the light period. Interestingly, activity in the dark can be restored by the introduction of sucrose (Kopriva et al., 1999). However the control by light may or may not be attributed to the carbohydrates formed by photosynthesis. Omitting the CO₂ supply in the air resulted in the reduction of APSR mRNA levels and enzyme activity, suggesting transcriptional regulation of APSR. Further, the reduction is relieved if the plants are supplemented with sucrose (Kopriva et al., 2002). In other feeding experiments, 0.5 % (w/v) glucose but not sorbitol or mannitol, resulted in a significant increase in APSR mRNA, enzyme activity and protein accumulation, suggesting that the effect is not dependent on a change in osmotic potential (Hesse et al., 2003).

It is becoming apparent too that OAS, discussed earlier as a important molecule in the regulation of the sulfur assimilation pathway is a candidate that links the sulfur, nitrogen

and carbon nutritional status of the plant (Koprivova et al., 2000; Kopriva et al., 2002; Hesse et al., 2003).

1.5.5 Regulation by oxidative stress.

Oxidative stress is caused by active oxygen species which are commonly toxic to the plant cell. Oxidative stress can result from a number of environmental conditions, including light, chilling, drought, attack from pathogens and nutrient deficiency. One mechanism to reduce the concentration of active oxygen species is by using non-enzymatic antioxidants such as glutathione and ascorbate (May et al., 1998).

Not much is known about the effect of oxidative stress on the sulfur assimilation pathway.

In one study, canola (*Brassica napus*) treatment with H₂O₂ for 8 hrs increased ATPS activity, this effect was found to be independent of the control exerted by reduced glutathione and that an independent regulatory pathway is present (Lappartient and Touraine, 1997).

In contrast the APSR protein has been shown to be regulated by the redox state of glutathione, *Brassica juncea* treated with H₂O₂ resulted in a substantial increase both in APSR protein activity which was due to an increase in the oxidised glutathione: reduced glutathione ratio. However, the addition of reduced glutathione decreases the protein activity demonstrating an oxidative stress regulation dependent on glutathione (Bick et al., 2001). Bick *et al.* (2001) suggested that the regulation of APSR was due to a post-translational modification that may act through a mechanism involving disulfide bonds between conserved cysteine residues.

Alteration of the abundance of sulfur assimilation transcripts by overexpressing SAT and OAS-TL both resulted in an increased tolerance to oxidative stress. In both cases, glutathione and cysteine pools increased, indicating that the sulfur assimilation pathway is important to reactive oxygen resistance, by providing precursors for the antioxidant glutathione (Blaszczyk et al., 1999; Youssefian et al., 2001).

Chilling stress is also known to result in an increase in reactive oxygen and has also been shown to regulate components of the sulfur reduction pathway. Chilling *Z. mays* seedlings, by growing at 12 °C, resulted in an increase in mRNA level and protein

activity of APSR, also, under such conditions mRNA is detectable in the mesophyll cells, whereas seedlings grown at 25 °C, mRNA is localised exclusively in the bundle sheath cells, demonstrating a change in the distribution of APSR mRNA (Brunner et al., 1995; Kopriva et al., 2001). Interestingly, this treatment will also eliminate diurnal changes in the activities of APSR (Kocsy et al., 1997).

1.6 Regulation of the sulfur assimilation pathway in onion

1.6.1 Overview of onion production in New Zealand

The onion industry in New Zealand has traditionally been based on volcanic soils (sulfur-rich) in the South Auckland and Waikato region, but is increasingly moving to drier east coast areas on lighter soils under irrigation. As sulfate is highly mobile, the availability of sulfur is easily reduced on such soil, especially as growers have moved to the use of less super phosphate (high in sulfate) and more to the use of urea as fertiliser. It is likely too, that high nitrogen use on such soils can lead to S deficiency, compromising yield and storage as well as creating high nitrate levels in the crop, which is particularly undesirable. Therefore, a better understanding of the physiology of onion and the genetic variation in terms of N/S homeostasis will help develop better crop management systems to provide and optimise fertiliser regimes that can provide desired flavour quality while maximising crop productivity and nitrogen use efficiency.

1.6.2 Impact of sulfur nutrition on the flavour of onions

Agronomic and physiological studies have revealed a significant relationship between genotype and environment interactions, particularly in response to sulfur nutrition which effect the pungency of onions (Randle and Bussard, 1993).

Flavour intensity in onions are commonly measured indirectly by measuring the production of pyruvate produced in a reaction catalysed by alliinase (EC 4.4.1.4) (Randle and Bussard, 1993; Vavrina and Smittle, 1993), or directly, by use of high pressure liquid chromatography (HPLC) (Randle et al., 1995; Yoo and Pike, 1998).

It is known that flavour intensity varies between cultivars and is determined by the level of ACSO compounds.

It has also been suggested that the variability in flavour observed between onion lines is due to differences in sulfur uptake and its metabolism (Randle and Lancaster, 2001)

In general, sulfur nutrition plays an important role in the levels of sulfur compounds synthesised. While it is possible to reduce pungency and produce mild onions by reducing the sulfate levels to no greater than 50 ppm (Randle and Lancaster, 2001), this treatment, because of the requirement for sulfate during plant growth and development, will also reduce the bulb yield (Freeman and Mossadeghi, 1970; Hamilton et al., 1997). Onion varieties respond differently to sulfur nutrition although this commonly shows little correlation between total sulfur content and pungency (Randle, 1992; Randle and Bussard, 1993). It is thought that mild varieties accumulate more free sulfate and store it in the vacuole of cells, which excludes it from the incorporation into the flavour pathway (Randle et al., 1999). This is significant as it suggests that a major contributing factor in less pungent, milder lines (with less ACSO compounds) is that these lines can uptake sulfur, but are less efficient at the incorporation of the sulfur into thiols. Therefore, it is critical to define how the pathway of reduced sulfate into thiols and flavour precursors is regulated in mild and pungent varieties, and so test the hypothesis that the genetic variation in flavour is determined at this level.

1.6.3 Regulation of sulfur assimilation at a molecular level in onion

In terms of enzymes in the pathway, ATPS gene expression has been shown to increase with an abundance of sulfur, with notably higher expression in the more pungent cultivars. It is thought therefore that ATPS is an important candidate for the observed divergent sulfur metabolism between phenotypes (McCallum et al., 2002). ATPS could be a possible contender for pathway control because it catalyses the first step in the assimilation of sulfur, but the mechanism of how the differential expression of the ATPS gene regulation is unknown.

1.7 Complex formation and the regulation of sulfur assimilation in onion

Protein complexes do exist as part of the sulfur assimilation pathway in plants. A well characterised example is termed the cysteine synthase complex. This comprises SAT in complex with *OAS-TL* which catalyses the reaction that yields *OAS* from serine and acetyl CoA (reaction 1.4). Dissociated *OAS-TL* then catalyses the formation of L-cysteine from *OAS* and sulfide (reaction 1.5). Evidence of this complex has come from co-purification experiments with a number of plant species including rape, Chinese chives (*Allium tuberosum*) (Nakamura and Tamura, 1990) watermelon (*Citrullus vulgaris*) (Saito et al., 1995) and spinach (*Spinacia oleracea*) (Droux et al., 1992) and using yeast two-hybrid system in *A. thaliana* (Bogdanova and Hell, 1997).

It was assumed that the formation of the complex would result in a channeling effect, where the *OAS* formed by the activity of SAT would be converted to cysteine by the association by *OAS-TL* (Droux et al., 1992; Saito et al., 1995). However, investigation into the kinetic properties of bound and un-bound SAT and *OAS-TL* revealed that when these components are bound as a complex, the SAT activity increased, while *OAS-TL* activity reduced. Further, the proposed channeling was challenged when *OAS* was discovered to be released into the bulk solution. It is known that the formation of the complex is controlled by the level of substrates, whereby sulfide encourages the complex formation and *OAS* destabilises the association (Droux et al., 1998). These reports suggest a regulatory role for *OAS-TL*, controlling the catalytic activity of SAT, in addition to the catalytic function.

It is becoming increasingly apparent that more proteins are involved in protein complexes, either as a way of regulating the activity of the protein, or to change the flux of substrates to a desired product (Gontero et al., 2002). Thus in the sulfur assimilation pathway of onion, there may be other complexes that form. A candidate that is investigated in this thesis is the formation of a complex between APSR and ATPS, a major branch point between the reductive (towards cysteine) and oxidative (catalysed by APS kinase and sulfotransferase) sulfur assimilation pathways.

1.7.1 Current knowledge that supports the formation of a protein complex between ATPS and APSR.

Substrate channeling has been shown between ATPS and APS kinase catalytic sites if these exist as the bi-functional enzyme adenosine-5'phosphosulfate kinase (EC 2.7.1.25) in rat chondrosarcoma (Lyle et al., 1994). Using isotope dilution and enrichment with labeled APS, it was possible to show that during these reactions, the APS intermediate was not released into the bulk solution (Lyle et al., 1994). Further investigation in *Saccharomyces cerevisiae* revealed that the C-terminal domain of ATPS is responsible for channeling by forming a physical connection between the active sites of ATPS and APSK (Lalor et al., 2003).

In plants, ATPS activity in cabbage (*Brassica capitata* L.), was stimulated 60 % after the addition of APS kinase (APSK) from *Penicillium chrysogenum*, possibly as an interaction between the two enzymes that could facilitated substrate channeling (Osslund et al., 1982). However, the authors concluded that this is not the case because of the different origins of the proteins (Osslund et al., 1982).

In attempt to provide evidence for a protein complex between ATPS and APSK in plants, yeast two hybrid screens were performed with APSK from *Arabidopsis* as bait, but no complex was detected between ATPS and APSK (Lillig et al., 2001).

To date, there has been no evidence that APSR is involved substrate channeling, nor are there any reports of APSR involved in a protein complex. However, APSR has been shown to associate with the thylakoid membrane (Koprivova et al., 2001), the site of photosynthesis. APSR doesn't directly require photosynthetic products, but the formation of a protein complex between other proteins within the sulfur assimilation pathway, such as ATPS and SiR, which do require photosynthetic products (ATP and reduced ferredoxin respectively), would permit an effective supply of substrates. A similar model has been proposed for enzymes of the Calvin cycle that utilise photosynthetic products (Suss et al., 1993).

After sulfate is catalysed to APS by ATPS (reaction 1), APS can either be used as a substrate for APSR (reaction 2) or as substrate for APS kinase (reaction 7) (Burnell and Anderson, 1973; Lee and Leustek, 1998). Therefore both enzymes will compete for APS as a substrate. Interestingly, in *A. thaliana* APSK has a V_{max}/K_m ratio for APS of 2.8 x

$10^7 \text{ L mol}^{-1} \text{ s}^{-1}$, making it a more efficient enzyme than APSR by a factor of 10^3 - 10^4 for catalysing APS. This presumably this would limit the APS available for reduction (Lillig et al., 2001). However, this is not the case. It is unknown as to how the reduction pathway dominates over the sulfation pathway. It is possible that negative regulatory mechanisms control the APSK gene expression or protein activity allowing APS to be reduced by APSR. Regulation of APSK is yet to be described.

In onions, there is a high demand for reduced sulfate, which means that the reduction pathway is required to dominate over the sulfation pathway.

Therefore, a protein complex between ATPS and APSR would allow APS produced by ATPS to be passed to the active site of APSR without being released into the bulk solution, reducing the substrate available for APSK and directing the flux of APS towards a desired product.

One effect of forming a protein complex is to create a local environment where the concentration of an intermediate, shared by two enzymes, is at a higher concentration than that in the surrounding solution. The effect is that enzymes are able to produce more product and this is particularly useful if the protein has a high K_m value (Brooks et al., 1988). It known that ATPS has a very low equilibrium value ($K_{eq} \sim 10^{-8}$) (Farooqui, 1980), which requires the product (APS) to be removed quickly otherwise the reverse reaction will dominate over the forward reaction. A complex between ATPS and APSR would allow swift removal of APS by APSR *via* its reduction to sulfite which would reduce the negative effect of APS on the ATPS forward reaction (Leyh, 1993). The product of the APSR reaction (sulfite) is reactive in cells (as is the sulfide produced by sulfite reductase) and so the reduction of these compounds is also highly desirable to minimise oxidative damage to the cell and invites the possibility that SiR may form a complex with APSR.

1.8 Thesis aims

In summary, there is some conceptual evidence for the desirability of an ATPS-APSR complex. This thesis therefore sought experimental evidence for the complex using both *in vitro* and *in vivo* approaches.

To do this, the following objectives were designed:

- Express and isolate sufficient ATPS and APSR enzymes as recombinant proteins.
- Examine the formation of protein complexes *in vitro* using ELISA, the ligand binding assay and the immunoprecipitation assay.
- Provide evidence for protein complex *in vivo* using onion chloroplast extracts, affinity chromatography and kinetic data.

In this thesis, when defining experimental approaches, the term '*in vitro*' refers to the direct use of recombinant proteins in experimental assays. In contrast, the term '*in vivo*' is used to refer to techniques that use whole leaf onion extracts or onion chloroplast extracts and so will include all proteins extracted, including ATPS and APSR. It is understood, however, that the term *in vivo* should strictly refer to the study of interactions *in planta* using non-invasive techniques (e.g. protein complementation assay (PCA), fluorescence-resonance-energy transfer (FRET) or by bimolecular fluorescence complementation (BiFC)).

2.0 Materials and Methods

2.1 Plant growth conditions

2.1.1 Plant material

- Onion cultivar W202A (a pungent, high sulfur compound storage line sourced from Crop & Food Research, Lincoln)
- Onion cultivar Texas Grano (a mild, low sulfur compound storage line sourced from Crop & Food Research, Lincoln)
- Horticultural grade bark/ peat/ pumice (50: 30: 20) (Dalton Nursery Mix, Tauranga).

2.1.2 Growth of plant material for general experiments

Seedlings were germinated from seed, provided by John McCallum (Crop & Food Research, Lincoln) in horticultural grade bark/ peat/ pumice (50: 30: 20) (Dalton Nursery Mix, Tauranga). Seedlings were maintained in a temperature-controlled glasshouse, at the Plant Growth Unit (Massey University, Palmerston North), with a minimum temperature at 15 °C and venting at 25 °C. Plant growth and maintenance was undertaken by Ms. Susanna Leung (IMBS, Massey University).

2.1.3 Growth of plant material for sulfur deficiency experiments.

Germinated seedlings (*ca.* 10 to 20 days after germination, depending on variety) of W202A and Texas Grano were transferred to 'Oasis' horticultural foam blocks and once the second leaf had emerged, the plants were translocated to hydroponics tubs (18 L). The hydroponic solution used was Hoagland media (Hoagland and Arnon, 1938). Plants were either grown in deficient (0.1 Meq) or sufficient (4 Meq) sulfur environments. This was achieved by varying the concentration of MgSO₄, Mg (NO₃)₂ and Ca (NO₃)₂ in the Hoagland media. Plants were maintained in the glasshouse, as described in McCallum *et al.* (2002), at Crop and Food Research, Lincoln.

2.1.4 Extraction of plant extracts for APSR activity assay

Materials

- Extraction buffer containing 50 mM Na/KPO₄, pH 8.0; 30 mM Na₂SO₄, (made fresh), 500 μM AMP (stored at -20 °C) and 10 mM
- Dithioerythritol (DTE) (stored at -20 °C)
- Plant material
- Mortar and pestle
- Nappy liners (prepared by boiling in Milli-Q water for 5 min, rinsing several times with milli-Q water and then stored at 4°C)

For whole leaf extracts, unless specified, leaf material was sourced from onion (*Allium cepa*) genotype W202A. Approximately 70-90 mg plant material from the 2nd or 3rd leaf

of onion (see figure 2.1) was added to 1 ml of (cold) extraction buffer and the tissue macerated using a mortar and pestle. The slurry was filtered through one layer of the wet nappy liner, the filtrate collected and used immediately for the APSR activity assay (see section 2.18.2).

2.2 Preparation of whole chloroplast protein extracts

2.2.1 Whole chloroplast isolation

Materials

- Isolation medium containing; 2.0 mM Na₂ EDTA, pH 8.0; 1.0 mM MgCl₂; 50.0 mM Tricine-KOH, pH 7.9; Sorbitol 330 mM (stored at 4 °C)
- Ultraturrex blender (Janke and Kunkel GmbH and Co, IKA-Labortechnik, Britain)
- Nappy liners (prepared as described in 2.1.3)
- Percoll gradient (see section 2.2.2)

Five grams of leaf tissue from the 2nd and 3rd leaves of onion (see figure 2.1) was macerated in 25.0 ml of cold (4 °C) isolation medium using the Ultraturrex blender set at 10,000 rpm, for 5 seconds. The macerated tissue was filtered through two layers of nappy liners, and the filtrate centrifuged at 1,400 xg for 3 min at 4 °C. The pellet was resuspended in 5 ml of cold isolation medium, and layered onto the top of a 40 %-80% percoll gradient. The gradient was centrifuged at 3,300 xg for 10 min at 4 °C, where chloroplasts settle at the interface between the 40 % and 80 % percoll solutions. The top layer of solution (40 % percoll) was then discarded leaving the intact chloroplasts and the percoll gradient was then diluted by mixing with additional isolation medium. The suspension was then centrifuged at 4,100 xg for 10 min at 4 °C, the layer of liquid above the chloroplasts was again removed and fresh isolation medium added. After centrifugation at 4,100 xg for 10 min at 4 °C, the chloroplasts pelleted at the bottom of the corex tube. The chloroplasts were suspended in 0.5 ml of isolation medium and the suspension stored at -20 °C until further use.

2.2.2 Preparation of percoll gradients

Materials

- 0.5M Na₂ EDTA, pH 8.0 (BDH laboratory supplies, Poole, England)
- 1.0 M MgCl₂ (Sigma Chemical Co. Saint Louis, MO, USA)
- 5.0 M Tricine-KOH, pH 7.9
- Sorbitol (Sigma Chemical Co.)
- Percoll (Sigma Chemical Co.)
- 30 ml Corex tubes

Table 2.1 Composition of percoll gradient. .

Gradient components	Stock concentrations	40%	80%
Percoll		4.0 ml	8 ml
Na ₂ EDTA, pH 8.0	0.5 M	40 µl	40 µl
MgCl ₂	1.0 M	10 µl	10 µl
Tricine, pH 7.9	0.5 M	1.0 ml	1.0 ml
Sorbitol		0.6 g	0.6 g
Milli-Q water		<u>4.95 ml</u>	<u>950 µl</u>
Total Volume		100 ml	100 ml

The 40 % and 80 % percoll solutions are made separately. To pour the gradient, in each Corex tube 10 ml of 40 % percoll solution was added with a plastic pasteur pipette into the corex tube, and then 10 ml of the 80 % percoll solution is carefully placed underneath the 40 % percoll solution. The gradients were stored at 4 °C until further use, but usually overnight, to allow the interface to resolve.

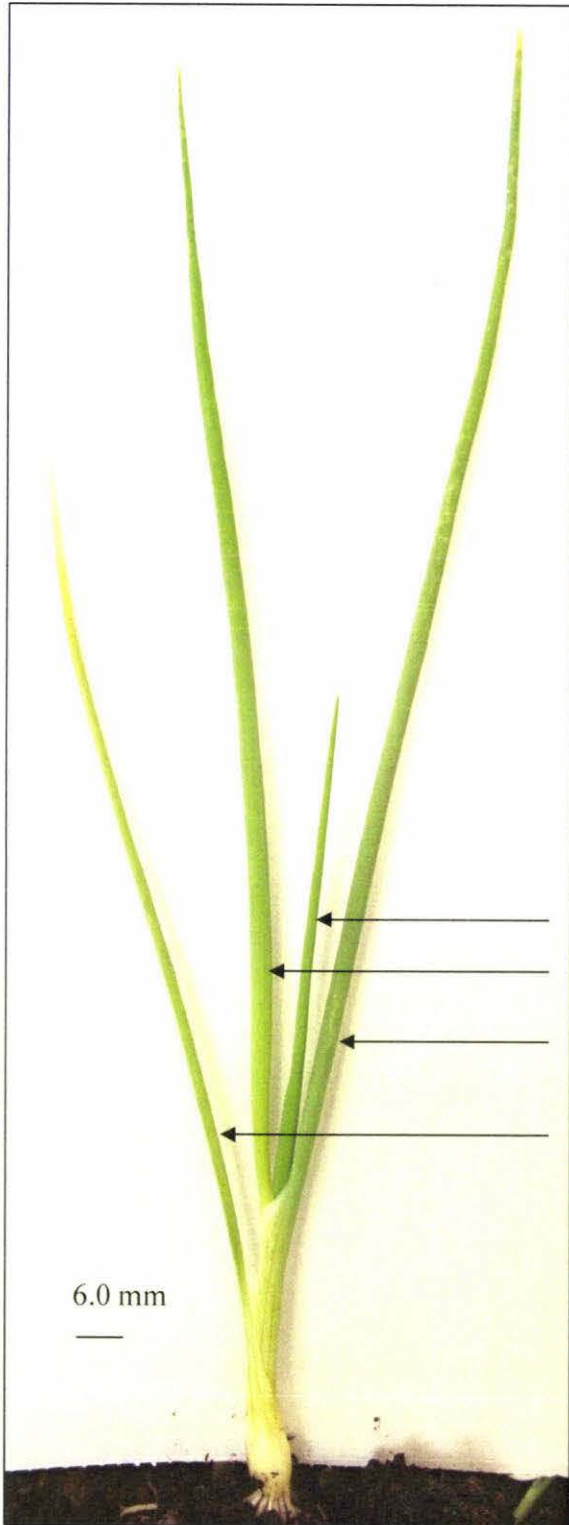


Figure 2.1: An onion (*Allium cepa* L.) seedling at *ca.* 6 weeks. Onion seeds were germinated and propagated as described in 2.1.1.

Leaves are numbered from youngest (1) to the oldest leaves (4).

1

2

3

4

Leaf
number

2.2.3 Preparation of chloroplast extract.

Materials

- Whole chloroplasts suspended in isolation medium (see section 2.2.1)
- 50 mM Tris-HCL, pH 7.8 (BDH chemicals, NZ)
- Acid-washed sand
- Mortar and Pestle

Whole chloroplast suspensions were thawed in 50 mM Tris-HCl (pH 7.8) at a 3:1, buffer: chloroplast suspension ratio. The diluted chloroplasts were ground with a mortar and pestle and a little acid-washed sand, before centrifugation of the chloroplast extract at 20,800 xg for 5 min at 4 °C. The supernatant was then collected and stored at -20 °C until further use.

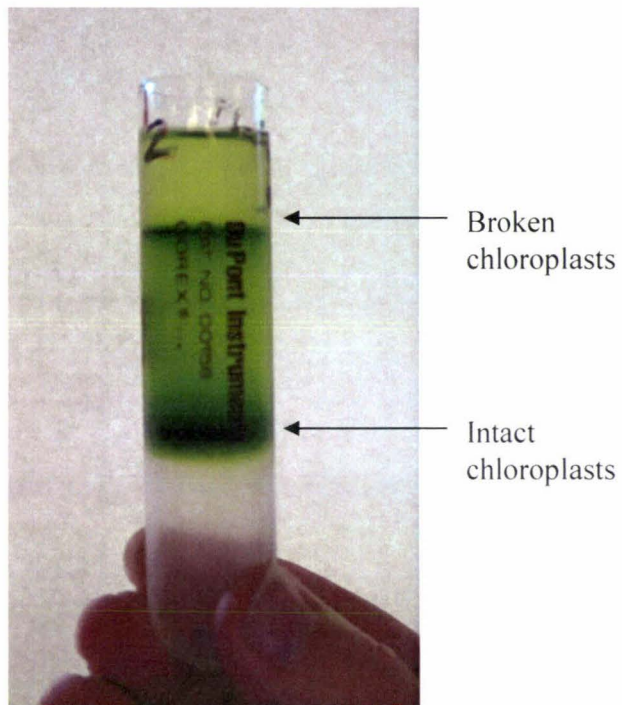


Figure 2.2: Typical appearance of Percoll gradient with macerated onion leaf after first centrifugation.

2.3 Preparation, maintenance and growth of bacterial cultures

2.3.1 Making glycerol stocks, LB-Amp plates and LB media

Materials

- Luria-Bertani (LB) broth [0.5 % (w/v) yeast extract (Merck, Darnstadt, Germany), 1 % (w/v) Tryptone (Merck), 1 % (w/v) NaCl, pH 7.5 (Univar, NSW, Australia)]
- 100 mg ml⁻¹ Ampicillin stock (Amp¹⁰⁰) (Sigma)
- 100 % (v/v) Glycerol (Merck, NZ Ltd.)
- Agar (Oxoid ltd., Basingstoke, Hampshire, England)

2.3.1.1 Preparation of LB media and LB plates

LB media was made fresh and before use and was routinely sterilised by autoclaving at 103 kPa for time periods dependent on volume (500ml or less, 20 min; 1 L, 30 min; 2 L, 45 min). When the LB had cooled to *ca.* 40 °C, 100 µl of Amp¹⁰⁰ was added to every 100 ml of LB, to give a final concentration of 100 µg ml⁻¹.

For LB-Amp¹⁰⁰ plates, 1.5 % (w/v) agar was added to the LB media before sterilisation. Once LB has cooled, Amp¹⁰⁰ is added to give a final concentration of 100 µg/ µl. The molten media (*ca.* 40 °C), was then poured into 9 cm diameter microbiology plates under a sterile lamina flow hood, and left to set. After this, the plates are wrapped in parafilm and stored at 4 °C until further use.

2.3.1.2 Growth of *E.coli* cells

An inoculation loop was held over a Bunsen flame until 'red hot' and after cooling was used to sample cells from the appropriate glycerol stocks. The sampled cells on the loop were either streaked on to a LB-Amp¹⁰⁰ agar plate or suspended the cells into a LB-Amp¹⁰⁰ broth (10 ml)

Streaked plates were grown, inverted at 37 °C overnight (*ca.* 16 hrs) and these cultures were stored on the agar plates for up to two weeks at 4 °C, before being replaced.

With liquid broth, the flasks were stoppered with cotton wool and shaken vigorously (180 rpm) over night (*ca.* 16 hrs) at the appropriate temperature. These cell cultures were used immediately.

2.3.1.3 Glycerol stocks

To make glycerol stocks, liquid broth of *E.coli* cells were grown overnight at 37 °C with vigorous shaking (180 rpm) in LB-Amp¹⁰⁰ broth. After the incubation, 850 µl of the inoculated LB broth was mixed with 150 µl of glycerol in cryotubes (Nunc) and frozen in liquid nitrogen. Glycerol stocks were stored at -80 °C until use.

2.4 Isolation of ATPS and APSR GST-fusion protein

2.4.1 Large scale preparation

Materials

- *E. coli* strains, which have been previously transformed with p-Gex vector containing onion (*A. cepa*) ATPS or APSR cDNA.
- Luria-Bertani (LB) broth 100 mg ml⁻¹ containing Ampicillin stock (Amp¹⁰⁰) (prepared as described in 2.21)
- 100 mM Isopropyl β-D- thiogalactopyranoside (IPTG) (filter sterilised)
- 10 mM (NH₄)₂ Fe (SO₄)₂ (sterilised by autoclave)
- Cleavage buffer, which consists of 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 1 mM Na-EDTA, 1 mM Dithiothreitol (DTT), pH 7.0 (cool to 4 °C before use, store up to 4 days at 4 °C).
- Glutathione Sepharose 4B (GS4B) (Amersham Pharmacia) (store at 4 °C)
- PreScission Protease™ (Amersham Pharmacia) (store at -20 °C)
- Bench top cell disruptor 2 plus, 1.1 kW (NZ Medical and Scientific Limited, Auckland, NZ)

- Soniprep 150 Ultrasonic Disintegrator sonicator (MSE Scientific Instruments, Manor Royal, Sussex, England)
- French press (AMINCO. Inst, Co. Int. Silver Spring, Maryland, USA) (components stored at 4 °C)
- Novaspec II spectrophotometer (Pharmacia)
- 500 ml Oakridge centrifuge tubes
- 50 ml Oakridge centrifuge tubes
- *ca.* 0.1 % (v/v) Virkon
- 70 % (v/v) Ethanol (Merck, NZ Ltd.)

LB-Amp¹⁰⁰ were inoculated with an *E. coli* colony from a previously streaked LB-Amp¹⁰⁰ plate (see section 2.3), incubated overnight (16 hrs) at 37 °C, with vigorous shaking at 180 rpm. Twenty ml of this culture was used to inoculate 4 x 500 ml LB-Amp¹⁰⁰ broths which were then cultured at 37 °C at 180 rpm for *ca.* 5 hrs until the absorbance reading at Ab_{590 nm} reached 0.6. Filter sterilised IPTG was then added to the culture to give a total concentration of 0.6 mM. If APSR GST-fusion protein was being prepared, (NH₄)₂ Fe (SO₄)₂ solution was also added to the culture, to a final concentration of 0.01 mM. This is proposed to increase the specific activity of the APSR protein (Kopriva, *S. pers. comm.*) Incubated overnight (16 hrs), cultures were then grown at the appropriate temperature, before transfer to 500 ml Oakridge centrifuge pots and the centrifuged using a Sorvall GS3 rotor, at 4,200 xg at 4 °C for 10 mins. The supernatant was removed and the pellet suspended in cold cleavage buffer (4 °C, 30 ml) before lysis. It was possible to store the pelleted cells at -20 °C prior to suspension in cleavage buffer.

2.4.1.1 *Lysing E. coli cells using the French press*

The components of the French press were constructed and the cell suspension (see section 2.4.1) was poured into the chamber. The T-valve was opened so that the pressure lies between 6000 and 8000 psi and the lysed bacteria collected. It became routine to repeat the pressing of the cell suspension at least two more times to ensure complete cell

lysis. The components were washed by rinsing with 70 % (v/v) ethanol and then with RO water.

2.4.1.2 *Lysing E.coli cells using the cell disruptor*

Firstly the cell disruptor was washed with at least 1 L of RO water, by operating the cell disruptor at 10,000 psi. The cell suspension (see section 2.4.1) was then placed in the sample holder and the cell disruptor run at 15,000 psi. The lysed cell suspension was commonly passed through again at 15,000 psi. The cell disruptor was cleaned afterwards by putting 2 L of virkon solution, followed by 1 L of RO water, then 50 ml of 70 % (v/v) ethanol, though at 10,000 psi.

2.4.1.3 *Lysing E.coli cells using the sonicator*

The sonicator was tuned before use and the cell suspension tube (see section 2.4.1) was placed on ice to maintain a low temperature during the process. Using the small probe (diameter =3 mm) at a power setting of 2.5, the cell suspension was sonicated for 4 x 30 seconds, letting the sample stand for 30 seconds in ice between each sonication. After use, the probe was cleaned with 70 % (v/v) ethanol.

2.4.2 Isolation of GST-fused protein using GS4B Sepharose

The lysed cells (see sections 2.4.1.1, 2.4.1.2 and 2.4.1.3) were centrifuged in 50 ml Oakridge tubes at 12,000 xg for 20 min at 4 °C. During this time, 0.75 ml bed volume (1.3 ml of 75 % (v/v) GS4B slurry) per 30 ml of cell lysate was washed with 5 cycles of centrifugation at 500 xg for 5 min at 4 °C, removal of supernatant, addition of cold cleavage buffer (*ca.* 50 ml) and mixed by inversion.

Once the cell lysate had completed the final centrifugation step (after 5 washes), the supernatant was transferred to two 50 ml Falcon tubes, the washed GS4B resin was separated into two equal portions, and added to the supernatant of the lysed cells. The mixture was then incubated at 25 °C for 1-2 hrs with end-over-end mixing. The resin was washed, with 3 cycles, each consisting of centrifugation at 500 xg for 5 min at 4 °C,

removal of supernatant and addition of cold cleavage buffer (*ca.* 30 ml) and gentle mixing. After the final wash, the resin was centrifuged at 500 xg for 5 min at 4 °C, the supernatant poured off until *ca.* 1 ml remains, the remaining resin slurry transferred into a 1.5 ml Eppendorf tube which was spun at 500 xg for 5 min at 4 °C and the cleavage buffer (*ca.* 1 ml) replaced. Ten µl of PreScission Protease™ was then added to each Eppendorf tube and incubated at 4 °C overnight (16 hrs), with end-over-end mixing. The mixture was centrifuged at 500 xg for 5 min at 4 °C and the supernatant collected (designated as elution 1). Fresh cleavage buffer then added and the resin slurry incubated for a further 4 hrs at 4 °C with end-over-end mixing. The supernatant was collected (designated as elution 2) after centrifugation at 500 xg for 5min at 4 °C. The protein concentrations of the elution samples were determined using the Bradford method (see section 2.7.1) and the elutions stored at either 4 °C, or at -20 °C.

2.5 Sephadex G-25 filtration chromatography

Materials

- Syringe barrel (Becton-Dickinson, Lincoln Park, NJ, USA)
- Sephadex G-25 resin (Pharmacia Biotech), stored at 4°C
- GF/A micro fibre filter (Whatman International, Maidstone, England)
- Column buffer, which consists of 50 mM Tris-HCl pH 7.5 with HCl and 5 mM MgCl₂ (buffer chilled to 4 °C before use)
- 0.2 M NaOH
- pH paper (Whatman International, Maidstone, England)

The Sephadex resin was prepared by firstly washing the resin with 2-3 volumes of 0.2 M NaOH and then re-equilibrating with column buffer until the pH of the filtrate reached pH 7.4. Columns were prepared by placing two layers of GF/ A micro fibre filters at the base of a 10 ml syringe barrel. The Sephadex resin slurry was poured quickly to ensure minimal bubbles. The recombinant protein extract (from 2.3) was pipetted carefully onto the top of the resin, so as not to disturb the resin, and once the recombinant protein extract had run into the column, 300 µl aliquots of column buffer was added. Fractions

(0.5 ml) were collected, and protein concentrations for each fraction was determined using the Bradford method (see section 2.7.1). Fractions with high protein concentrations were pooled and immediately used for FPLC with Mono-Q column (see section 2.6). The resin was stored in 20 % (v/v) ethanol at 4°C until regeneration with 0.2 M NaOH.

2.6 Anion exchange chromatography with the FPLC

Materials

- Fast Protein Liquid Chromatography (FPLC) apparatus, (Pharmacia Biotech. Controller LCC-501 plus)
- Mono-Q pre-packed HR 5/50 column (Amersham Biosciences, Tricon, Sweden)
- Buffer A, which consists of 50 mM Tris-HCl, pH 7.5, 10 µM 1,10-phenanthroline (PA) (dissolved in 100 µl methanol), 2 mM Dithiothreitol (DTT) and 5 mM MgCl, filtered through a 0.20 µm filter (Sartorius, Goettingen, Germany) (and then cooled to 4 °C before use)
- Buffer B, which consists of 50 mM Tris-HCl, pH 7.5, 10 µM 1,10-phenanthroline (PA) (dissolved in 100 µl methanol), 2 mM Dithiothreitol (DTT) and 5 mM MgCl, filtered through a 0.20 µm filter (Sartorius), before the addition of, 1 M NaCl (and then cooled to 4°C before use)

The Mono-Q HR 5/50 column was equilibrated by passing buffer A through for 20 min at a rate of 0.25 ml/min, during which 1-2 ml of protein extract was loaded into the sample loop of the FPLC. Fractions (0.5 ml) were collected for *ca.* 3 min before the sample was loaded onto the column and then after loading. As a standard procedure, a gradient of buffer B was used from 0 % to 100 % over 1 hr at a flow rate of 0.25 ml/min. Fractions (0.5 ml) were collected and the Bradford method was used to determine the protein concentration. It was expected that recombinant ATPS will elute within the first few fractions (figure 3.4), but only if MgCl was present in the buffer. Those fractions that contained high protein concentrations were pooled and stored at 4 °C. The Mono-Q column was washed after use by passing 4 ml of 1 M NaCl, then 1 M NaOH, then 1 M

HCl, then 1 M NaCl and finally with 2ml of filter sterilised milli-Q backwards through the column, at 0.5 ml/min. The column was stored in 20 % (v/v) ethanol between use.

2.7 Quantification of protein

2.7.1 Bradford method

Materials

- Micro-titre plate (Nunc, A/S, Roskilde, Denmark)
- Protein assay reagent (BIO-RAD)
- BSA (0.1mg/ml stock, stored at -20 °C)
- Anthos HT II plate reader (Anthos labtech Instruments, Salzburg, Austria)

Samples for measurement were diluted appropriately with water up to a volume of 160 μ l in a micro-titre plate. Forty μ l of protein assay reagent was added, and mixed well with the diluted protein sample. This was left to stand in the absence of light for 5 min, before the absorbance was read at 595 nm ($Ab_{595\text{ nm}}$) with the Anthos HT II plate reader.

A standard curve was produced using known amounts of BSA, which allowed a relationship between $Ab_{595\text{ nm}}$ and protein concentration to be determined. The standard curve was used to determine protein concentrations of protein extract with unknown protein concentration (figure 2.3).

2.7.2 UV method for the concentration of IgG in solution

Materials

- UV-visible spectrophotometer (UV-160A, Shimadzu Corporation, Tokyo, Japan)

Samples were diluted as appropriate with water and the spectrophotometer was calibrated to zero using water as a blank at $Ab_{280\text{ nm}}$. The diluted samples were measured at $Ab_{280\text{ nm}}$ and the protein concentration was calculated using the following formula.

$$(Ab_{280\text{ nm}} \times 1.4) \times \text{Dilution factor} = \text{mg/ml}$$

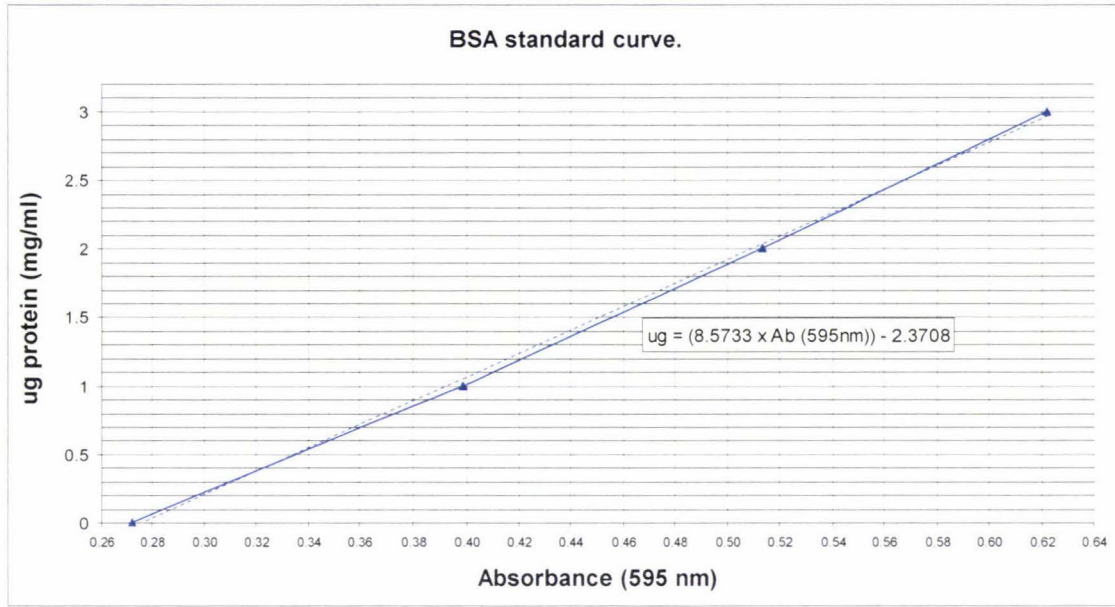


Figure 2.3: Example of a BSA protein standard curve. (see section 2.7.1 for details). Protein concentration of a BSA stock, 10 mg/ml. was measured using the Bradford method (1970). A range of dilutions were made in a total of 160 µl of water. 40 µl of protein determination dye was added, mixed and allowed to sit for 5 min. The absorbance was measured at $Ab_{595\text{ nm}}$ using an Anthos HT II plate reader. Results are mean values of 2 replicates, and the data shown is one example of 3 repeated experiments.

2.8 Electrophoresis of protein

2.8.1 Mini Gel- SDS-PAGE

Using electrophoresis, it is possible to separate proteins on the basis of differences in relative molecular mass using the method described by (Laemmli, 1970)

Materials

- Mini-PROTEAN[®] II cell (BIO-RAD)
- Power pack (BIO-RAD or Pharmacia Biotech)
- Low range pre-stained SDS-PAGE standards (BIO-RAD) (stored at -20 °C)
- 40 % (w/v) acrylamide/bis stock solution (BIO-RAD), (stored at 4 °C)
- 10 % (w/v) ammonium per sulfate (APS) (Univar, Auburn, NSW, Australia), (made fresh)
- N, N, N', N'-tetramethylethylenediamine (TEMED) (Riedel de Haen ag seelze, Hanover, Germany) (stored at 4 °C)
- SDS reducing buffer (2 x), which consists of 100mM Tris -HCl, pH 8.8, 20 % (v/v) glycerol, 5 % SDS, 2 % (w/v) Bromophenol blue (BDH chemicals NZ), pH 6.8 (store at -20 °C) Before use, add 5 % (v/v) 2-mercaptoethanol (BDH AnalaR) (stored at -20 °C)
- Separating buffer (4 x), which consists of 1.5 M Tris-HCl, pH 8.8, 0.4 % (w/v) SDS (stored at room temperature)
- Stacking buffer (2 x), which consists of 0.25 M Tris-HCl, pH 6.8, 0.2 % (w/v) SDS (stored at room temperature)
- SDS-PAGE running buffer (10 x), which consists of 250 mM Tris-HCl, 1.9 M Glycine, 1 % (w/v) SDS, pH *ca.* 8.2 (stored at room temperature)
- Water bath, 100 °C

Components	Separating gel (ml)	Stacking gel (ml)
40 % Acrylamide/bis	1.5 ml	250 μ l
Milli Q	2.25 ml	1.625 ml
4 x Separating buffer	1.25 ml	0 ml
4 x Stacking buffer	0 ml	625 μ l
TEMED	5 μ l	3.2 μ l
10 % APS	50 μ l	25 μ l

Table 2.2 Composition of 12 % acrylamide separating and 4 % stacking gels for Mini-PROTEAN[®] SDS-PAGE apparatus.

Glass plates of the Mini-PROTEAN[®] apparatus were washed with ethanol before setup as instructed by BIO-RAD. Components of the 12 % acrylamide separating gel were combined as described in table 2.2, ensuring that the 10 % APS is added last. The components were well mixed before pipetting the solution between the glass plates to a height of *ca.* 1 cm below the base of the sample wells. Immediately after, a *ca.* 1 cm layer of Milli-Q water was carefully pipetted onto the top of the separating gel mixture. Polymerisation of the separating gel required *ca.* 1 hr, after which, the Milli-Q water layer was removed. The stacking gel was prepared as described in table 2.2 and then poured onto the top of the polymerised separating gel and the well combs inserted to produce wells of a depth of *ca.* 1 cm. After *ca.* 30 min, the stacking gel polymerised and the gel was inserted in to the Mini-PROTEAN[®] cell. 1 x SDS-PAGE running buffer was added, sufficient to cover the entire gel and the comb was then carefully removed. Samples were prepared by the addition of 2 x SDS reducing buffer to equal amount of sample, incubating in a boiling water bath for 5 min and then centrifuging for 5 min at 20,800 xg at room temperature.

At least one lane was reserved to load 5-10 μ l of low range pre-stained SDS-PAGE standards. Electrophoresis was performed at 150 V for 1 hr and 10 min.

2.9 Coomassie[®] Brilliant blue staining

To visualise protein bands that have been separated by SDS-PAGE gel, Coomassie[®] Brilliant blue stain was used.

Materials

- Coomassie stain, which consists of 0.2 % (w/v) Coomassie[®] Brilliant blue R-250 (Sigma), 40 % (v/v) methanol, 10 % (v/v) glacial acetic acid
- Coomassie de-stain solution, which consists of 30 % (v/v) ethanol

After electrophoresis, the Mini-PROTEAN[®] II cell was dismantled and the gel was removed. The gel was placed in Coomassie stain, enough to cover the entire gel, before gently shaken at room temperature for 30 min. After this time, the stain was removed and replaced with Coomassie de-stain solution. The de-stain and gel were shaken gently overnight (*ca.* 16 hrs) before the stained image was recorded.

2.10 Western blot analysis

2.10.1 Wet-Blotting

Materials

- Mini Trans-blot[®] cell (BIO-RAD)
- Transfer buffer (1 x), which consists of 25 mM Tris, pH *ca.* 8.5, 190 mM Glycine, 10 % (v/v) Methanol (added prior to use).
- Methanol
- Polyvinylidene difluoride (PVDF) membrane, pore size 0.2 μ m (Immobilin-P, Millipore Corporation, Bedford, MA, USA)
- Chromatography paper (Whatman)

The components of the Trans-blot[®] (Chromatography paper, Scotch pad), were initially soaked in 1 x transfer buffer before the construction of the cassette. The PVDF

membrane was soaked briefly in methanol before soaking in 1 x transfer buffer. The Trans-blot[®] cassette was constructed as illustrated (figure 2.4). It was important that no air bubbles form between the PVDF membrane and the SDS-PAGE gel. The complete cassette was placed in the Mini Trans-blot[®] cell and surrounded with 1 x transfer buffer. Blotting was conducted at 250 mA, for 45 min with the transfer buffer mixed with a magnetic flea during transfer period.

2.10.2 *Immuno-detection of proteins immobilised on the PVDF membrane*

Materials

- Phosphate buffered salt (PBS, 1 x), which consists of 50 mM Na₂HPO₄·2H₂O, 250 mM NaCl. Adjust pH to 7.4 with, 50 mM NaH₂PO₄·2H₂O, 250 mM NaCl
- Blocking solution, which consists of 12 % (w/v) skim milk powder (Pams) in 1x PBS (solution made fresh)
- Primary antibody, IgG isolated from anti-ATPS or anti-APSR rabbit serum (stored at – 20 °C)
- Primary antibody, IgG isolated from anti-ATPS or anti-APSR rabbit serum, conjugated with biotin ester (see section 2.13) (stored – 20 °C)
- Secondary antibody, anti-rabbit (whole molecule) alkaline phosphatase conjugate (Sigma)(stored at 4 °C)
- Secondary antibody, streptavidin alkaline phosphatase conjugate (Sigma) (stored at 4 °C)
- PBS-Tween, which consists of 1 x PBS, ca. 0.1 % (v/v) Tween-20
- Substrate buffer, which consists of 100 mM Tris-HCl, 100 mM NaCl, pH 9.6
- 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) (Sigma) stock, which consists of 10 mg/ml BCIP dissolved in Dimethyl Formamide (store at -20 °C)
- Nitro blue Tetrazolium (NBT) (Sigma) stock, which consists of 20 mg/ ml NBT suspended in substrate buffer (as above) (stored at -20 °C)

- 1 M MgCl₂
- Developing buffer, which consists of 50 ml Substrate buffer (as above), pH 9.6, 500 µl 1 M MgCl₂ (10 mM), 500 µl BCIP stock (100 µg/ml), 500 µl NBT stock (200 µg/ml)(made fresh)

After the transfer of proteins onto the PVDF membrane, the membrane was submerged in blocking solution and incubated overnight (16 hrs) at 4 °C, or gently shaken for 1.5 hrs at 37 °C. The blocking solution was removed, and the membrane with PBS-Tween with gentle shaken for 5-15 min (the PBS-Tween was replaced a total of three times). After washing, the membrane was incubated with the primary antibody, diluted in PBS at a ratio 1:200-1:10,000 for 1 hr at 37 °C with gentle shaking. The membrane was then washed as described before and then incubated with secondary antibody diluted in PBS, either at 1: 5000 (for the streptavidin alkaline phosphatase conjugate) or 1: 10,000 (for the anti-rabbit (whole molecule) alkaline phosphatase conjugate). The membrane was incubated at either 25 °C (for streptavidin alkaline phosphatase conjugate) or at 37 °C (anti-rabbit (whole molecule) alkaline phosphatase conjugate) for 1 hr with gentle shaking.

The membrane was washed 3 times with PBS-Tween as described above, and then rinsed at least twice with substrate buffer before the membrane was incubated in developing buffer, at room temperature with gentle shaking, for *ca.* 1 minute or until visually desirable. The reaction was stopped by rinsing the membrane under RO water for *ca.* 30 seconds.

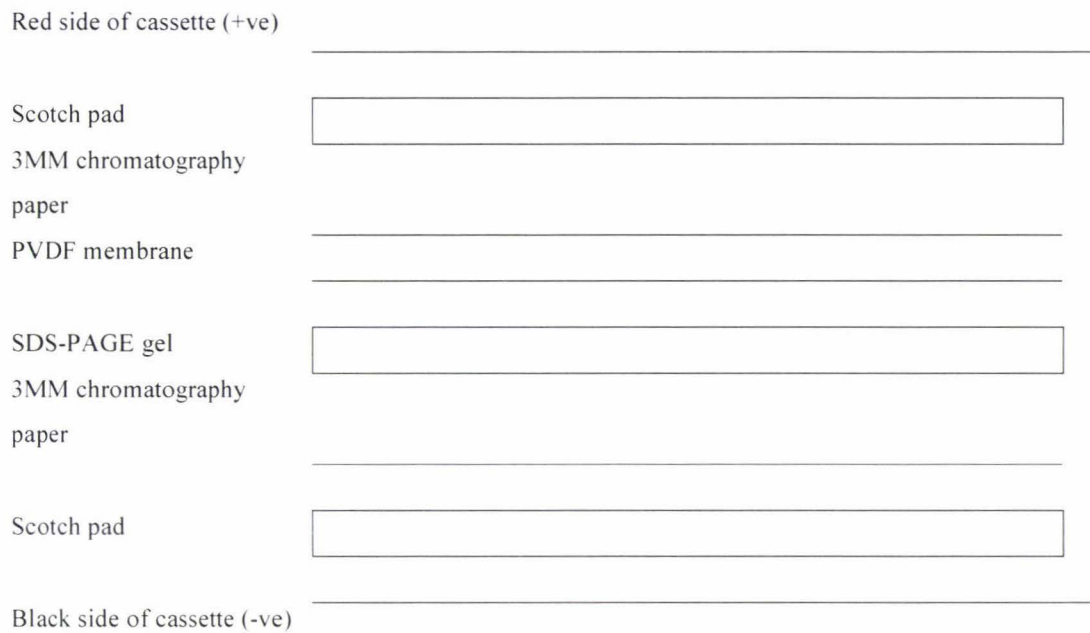


Figure 2.4: Illustration of the Trans-blot[®] cassette used in wet-blot transfer of proteins onto a PVDF membrane.

2.11 Isolation of Immuno-globin G (IgG)

Materials

- Rabbit serum (either anti-ATPS or anti-APSR)
- PBS
- Saturated ammonium sulfate solution: 40 g of ammonium sulfate dissolved in 50 ml of Milli-Q water with heating. The dissolved solution was cooled on ice and crystallised for 18 hrs at 4 °C
- 70 mM sodium phosphate buffer, pH 6.3 containing 250 mM sodium chloride
- Dialysis tubing (18/20, GIBCO BRL)
- Diethylaminoethyl (DEAE) Sephacel resin CL-6B (Pharmacia Biotech)
- GF/A micro fibre filter
- Anthos HT II plate reader (Anthos labtech Instruments)

For each 1 ml of serum, 1 ml of 1 x PBS, pH 7.4 was added, and 2 ml of saturated ammonium sulfate was added slowly over 30 min, with stirring at room temperature. The mixture was then centrifuged at 10,000 xg for 15 min at 4 °C, the supernatant removed and the pellet re-suspended in 1 ml of 70 mM sodium phosphate buffer, pH 6.3 and then transferred to dialysis tubing. The dialysis tubing was placed in 2 L of 70 mM sodium phosphate buffer, pH 6.3 and dialysis undertaken for 24 hrs at 4 °C with stirring. The buffer was replaced 4 times during this time period.

To prepare an ion-exchange column, two volumes of DEAE Sephacel resin CL-6B was pipetted into a 10 ml syringe barrel, with two layers of GF/A micro fiber filters lining the bottom. The DEAE Sephacel resin CL-6B was then washed with 3 volumes of 70 mM sodium phosphate buffer, pH 6.3 at room temperature. The dialysis mixture is loaded on to the column and eluted with 70 mM sodium phosphate buffer, pH 6.3. Eluted fractions (0.5 ml) were collected and the protein concentration determined using the UV-method (see section 2.7.2).

2.12 Conjugation of IgG to CN-Br activated Sepharose 4B

Materials

- 0.1 M NaHCO₃, pH 8.3 containing 0.5 M NaCl (adjust pH with 50 mM Na₂CO₃)
- Dialysis tubing (18/20) (GIBCO BRL)
- UV-visible spectrophotometer (UV-160A, Shimadzu Corporation, Tokyo, Japan)
- 1 mM HCl
- 0.1 M Sodium acetate, pH 4.0 containing 0.5 M NaCl
- 0.1 M Tris-HCl, pH 8.0 with HCl
- 0.1 M Tris-HCl, pH 8.0 with HCl containing 0.5 M NaCl
- 1 M ethanolamine
- CN-Br activated Sepharose 4B (Amersham Biosciences) (stored at 4°C)
- Sintered glass funnel

IgG (see section 2.11), which was stored in PBS, pH 7.4, was prepared for conjugation firstly by dialysis against 0.1 M NaHCO₃, pH 8.3 containing 0.5 M NaCl, over night (16 hrs) at 4°C. During this time, the CN-Br activated Sepharose medium was prepared by swelling sufficient freeze dried CN-Br activated Sepharose powder in 1 M HCl to provide 1 ml of swollen Sepharose per 1 mg of IgG (*ca.* 1 g freeze dried CN-Br activated Sepharose powder gives about 3.5 ml of swollen medium). The swollen Sepharose was then washed for at least 15 min using a sintered glass funnel with at least 60 ml of 1 M of HCl for each volume of swollen medium.

To conjugate, the IgG was diluted to 5 mg/ml with 0.1 M NaHCO₃, pH 8.3 containing 0.5 M NaCl, and the suspension added to the washed Sepharose. The mixture was then incubated by end-over-end mixing for 1 hr at room temperature or overnight (16 hrs) at 4°C. After this time, excess IgG was washed away, by transferring the medium to the sintered glass funnel, and the swollen Sepharose with IgG bound was washed with 5 volumes of 0.1 M NaHCO₃, pH 8.3. To block any remaining active groups, the swollen Sepharose was incubated with 0.1 M Tris-HCl, pH 8.0, for 2 hrs at room temperature (20

°C). After the incubation, the swollen media was transferred to a sintered glass funnel and washed with three cycles of buffer with alternating pH. Each cycle consisted of 0.1 M sodium acetate, pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris-HCl, pH 8.0 containing 0.5 M NaCl. For each cycle, the swollen Sepharose was washed with at least 5 volumes of each buffer and then the IgG-conjugated CN-Br activated Sepharose 4B was stored finally in 0.1 M NaHCO₃, pH 8.3 containing 0.5 M NaCl at 4 °C until further use.

2.13 Biotinylation of IgG using succinimide ester

Materials

- N-hydroxysuccinimide biotin (Sigma), 10 mg/ml (store at 4 °C, in dimethyl sulfoxide)(made fresh)
- Sodium Borate buffer 0.1 M Na₂B₄O₇, pH 8.8 (BDH chemicals, NZ), (pH with 0.1 M Boric acid)
- 1 M NH₄Cl
- PBS, pH 7.5
- Dialysis tubing (18/20) (GIBCO BRL)

An IgG solution with a concentration of 1-3 mg/ml was dialysed in sodium borate buffer overnight (16 hrs) at 4 °C. N-hydroxysuccinimide biotin in dimethyl sulfoxide was then added until 200 µg of ester for each milligram of IgG was reached and the solution was mixed over a magnetic stirrer for four hrs at room temperature (20 °C).

Sixteen µl of 1 M NH₄Cl was then added for every 200 µg of ester added and mixed for 10 min at room temperature (20 °C). The biotinylated IgG was dialysed against PBS (pH 7.5) to remove any uncoupled biotin over twenty four hrs. The buffer was replaced at least six times during this period, and at the conclusion the biotinylated IgG was stored at -20 °C until further use.

2.14 Immunoprecipitation with recombinant proteins

Materials

- Fast flow Protein G Sepharose (Amersham Biosciences), store in 20 % (v/v) ethanol at 4 °C
- Primary antibody: IgG isolated from anti-ATPS rabbit serum (see section 2.11)(stored at -20 °C)
- CN-Br activated Sepharose 4B conjugate (Amersham) with anti ATPS conjugated (see section 2.12) (stored in 0.1 M NaHCO₃ containing 0.5 M NaCl, pH 8.3 at 4 °C)
- Tris buffer saline (TBS), which consists of 50 mM Tris-HCl, pH 7.4 containing 100mM NaCl
- Phosphate buffered salt (PBS), which consists of 50 mM Na₂HPO₄.2H₂O, 250 mM NaCl. Adjusted pH to 7.5 with NaH₂PO₄.2H₂O, 250 mM NaCl
- SDS reducing buffer (2 x), 100mM Tris-HCl, pH 8.8 containing 20 % (v/v) glycerol, 5 % (w/v) SDS, 2 % (w/v) Bromophenol blue. pH 6.8 (store at -20 °C). Before use, add 5 % (v/v) 2 -mercaptoethanol

One µg of recombinant ATPS or APSR was incubated with 0, 1 µg and 2.5 µg of recombinant ATPS or APSR, the volume adjusted with PBS to a total volume of 100 µl and incubate at 25 °C for 1 hr with end-over-end mixing. In some cases, glutathione was added to the PBS buffer to a final concentration of 5 mM. If protein G Sepharose was used, it was necessary to incubate the mixture with IgG specific to ATPS, for 1 hr at 25 °C. After which time, 20 µl of protein G Sepharose slurry was added to each sample and incubated for 2 hrs at 25 °C with end-over-end mixing. If CN-Br activated Sepharose 4B conjugated with anti-ATPS IgG was used, the addition of IgG was not required, but alternatively 20 µl of the 50 % (v/v) resin slurry was added, and the mixture incubated for 2 hrs at 25 °C with end-over-end mixing. After the protein mixture was incubated sufficiently with the Sepharose (either protein G or CN-Br Sepharose), the Sepharose was washed by centrifuging at 500 xg for 5 min at 4 °C, removal of the supernatant and then resuspension with PBS. This was repeated at least 3 times. The sample tubes are then centrifuged finally at 1000 xg for 5 min at 4 °C to ensure that the resin pellets strongly, before removing all the buffer solution. Fifteen µl of SDS reducing buffer was added and

the suspension prepared for SDS-PAGE, before being loaded immediately onto a SDS-PAGE gel (see section 2.8) or stored at -20 °C until further use.

2.15 Enzyme-linked immunosorbent assay (ELISA)

Materials

- Coating buffer, which consists of 50 mM Na₂CO₃ / NaHCO₃ , pH 9.7
- Tris buffer saline (TBS), which consists of 50 mM Tris-HCl, pH 7.4 containing 100 mM NaCl
- Nunc- Immuno-modules (microtitre plates) (Nunc, A/S, Roskilde, Denmark)
- 0.5 % (w/v) skim milk powder in either coating buffer or TBS (solution made fresh)
- Tween-20 0.05 % (v/v) in either coating buffer or TBS
- Primary antibody (IgG isolated from anti-ATPS or anti-APSR rabbit serum)(stored at -20°C)
- Secondary antibody (anti-rabbit (whole molecule) alkaline phosphatase conjugate (Sigma))(stored at 4 °C)
- ELISA developing substrate, which consists of coating buffer containing 8.0 mM MgCl₂, 0.1 mM p-Nitrophenol-phosphate (Sigma)(store in dark at 4 °C for up to two weeks)
- 10 M NaOH
- Anthos HT II plate reader (Anthos labtech Instruments, Salzburg, Austria)

Firstly, protein extract (designated the primary protein) at the appropriate concentration (see section 3.2.1.2) was coated onto the base of the micro-titre plate in 100 µl of either coating buffer or TBS, over night (16 hrs) at 4 °C. After coating was completed, the excess protein was washed from the micro-titre plate by replacing the buffer with 0.05 % (v/v) Tween in either coating buffer or TBS, ensuring that the wash solution was not transferred from one well to another, and allowing the plate to sit for 5 min. This process was repeated at least three more times.

To ensure minimal background response, blocking was achieved by the addition of 120 μ l of 0.5 % (w/v) skim milk powder in coating buffer or TBS, to the micro-titre plate. This was left to stand for either 1 hr at 37°C, or overnight (16 hrs) at 4°C. The plate was washed as described before with 0.05 % (v/v) Tween in either coating buffer or TBS. The coated proteins were then incubated with another protein extract (designated the secondary protein), containing a possible protein partner of the coated primary protein in either coating buffer or TBS for 1.5 hrs at 37 °C. After incubation, the wells were washed as described before with 0.05 % (v/v) Tween in either coating buffer or TBS. IgG raised against the secondary protein (designated the primary antibody), was then diluted in either coating buffer or TBS (1:5000) and subsequently incubated with the coated protein for 1 hr at 37 °C before washing as described previously. Anti-rabbit antibody conjugated with alkaline phosphatase (designated the secondary antibody) was then diluted in coating buffer or TBS (1: 10,000) and incubated at 37 °C for 1 hr. Excess secondary antibody was then removed by washing the wells with a solution of 0.05 % (v/v) Tween as described previously. The ELISA was developed by the addition of 100 μ l of ELISA developing substrate to each well and the reaction placed in the dark at room temperature (20 °C). After *ca.* 10 min, the development was stopped by the addition of 20 μ l of 10 M NaOH and the microtitre plate was then immediately measured spectroscopically at $\text{Ab}_{405\text{nm}}$ using the Anthos HT II plate reader.

2.16 Ligand binding assay

Materials

- Phosphate buffered salt (PBS), which consists of 50 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 250 mM NaCl. Adjusted pH to 7.5 with $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 250 mM NaCl
- TBS, pH 7.8
- Blocking solution, which consists of 12 % (w/v) skim milk powder in PBS (made fresh)
- PBS-Tween 20, which consists of *ca.* 0.1 % (v/v) Tween 20 in PBS
- Materials required for wet blot method (see section 2.10)

Strips of PVDF membrane containing immobilised protein standards, ATPS and APSR were produced by SDS-PAGE (see section 2.8) and subsequent transfer of proteins (see section 2.10). The PVDF membranes were incubated with blocking solution either overnight (16 hrs) at 4 °C, or shaken gently for 1.5 hrs at 37 °C. To remove excess blocking solution, the membrane was washed by replacing the solution with PBS-Tween 20 and gently shaking for 5 min. This step was repeated at least 3 times.

After the final wash, membranes were incubated in 20 ml of either PBS or TBS, each containing 1-5 µg of recombinant APSR and gently shaken over 1 hr at room temperature. In some cases, MgCl₂ was added to the solution to give a final concentration of 5.0 mM. After the incubation period, the membrane was washed with PBS-Tween 20 as described previously and to detect the presence of a protein complex, proteins were detected using the immuno-detection method described in 2.9.1, using the appropriate antibodies.

2.17 ATPS affinity column

Materials

- Syringe barrel (5 ml capacity) (Terumo medical corporation, MD,USA)
- GF/A Glass microfibre filters (Whatman International Ltd, Maidstone, England)
- 2120 varioperpex[®] II pump (Watson Victor ltd., Sweden)
- 50 mM Tris-HCl, pH 7.4 containing 100 mM NaCl (TBS)
- 50 mM Diethanolamine (DEA), pH 11.0 (made fresh)
- 200 mM Glycine, pH 2.5
- Recombinant ATPS (see section 2.4.1)
- Chloroplast extract from W202A (see section 2.2)
- Micon Bioseparations, Centriprep[®] YM-3 centrifugal filter device (Millipore corporation, Bedford, MA, USA)
- Nanosep[®] 10 K Omega centrifugal device (Pall life science, MI, USA)

Three ml of a 50 % (v/v) Sepharose slurry conjugated with anti-ATPS IgG (see section 2.12) was poured into a 5 ml syringe barrel containing two layers of glass microfibre filter placed at the bottom of the syringe barrel.

The top of the barrel was blocked with a stopper and a syringe needle attached to the pump was inserted till the end of the needle was within the syringe barrel.

The Sepharose was washed at 0.5 ml/min with 10 volumes of TBS, pH 7.4, 2 volumes of DEA, pH 11.0, 10 volumes of TBS, pH 7.4, 2 volumes of Glycine, pH 2.5 and then 10 volumes of TBS, pH 7.4.

After washing, 0.5 mg of recombinant ATPS was passed through the column, at 0.5 ml/min, the eluate was collected and pumped back through the column continuously for 2 hrs at room temperature (20 °C). After this step, unbound ATPS protein was washed from the Sepharose with 10 volumes of TBS, pH 7.4, before 13 ml of chloroplast extract (*ca.* 4.5 mg of protein) was loaded on the column, at a flow rate of 0.5 ml/min. The eluate was collected and recycled through the column over a period of 2 hrs, at room temperature.

At the conclusion of this period, unbound proteins were removed with 10 volumes of TBS, pH 7.4, after which no proteins could be detected by western analysis with anti-ATPS and anti-APSR IgG. Proteins that may have associated with the ATPS bound Sepharose were eluted from the column with 1 volumes of DEA, pH 11.0, 1 volumes of TBS, pH 7.4, 2 volumes of Glycine, pH 2.5 and then 2 volumes of TBS, pH 7.4. The elutions were collected after each solution wash and concentrated with a Centriprep[®] YM-3 centrifugal filter devices by centrifuging at 3,000 xg at 4 °C for 20-40 min or until the volume was reduced to 1 ml. Samples were then concentrated further using Nanosep[®] 10 K Omega centrifugal devices, at 20,800 xg for 5 min at 4 °C, until the total volume reached *ca.* 80 µl. Samples were then prepared for SDS-PAGE electrophoresis and subsequent western blot analysis with biotinylated anti-ATPS and biotinylated anti-APSR antibodies (see sections 2.8 and 2.10).

2.18 Enzyme assays

2.18.1 ATPS assay

The measurement of ATPS activity *in vitro*, measures the reverse reaction of ATPS (Segel et al., 1987).

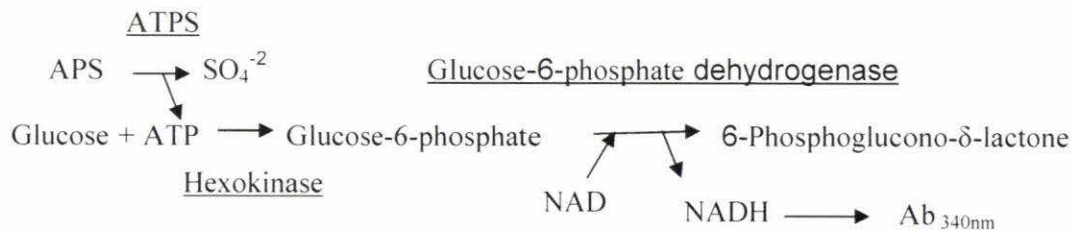


Figure 2.5: The coupled reaction for the measurement of the reverse reaction of ATPS.

The reverse reaction of ATPS provides ATP, if APS and Mg^{+2} are present and this is consumed in the reaction catalysed by Hexokinase that produces Glucose-6-phosphate. Glucose-6-phosphate dehydrogenase, an enzyme that produces NADH, if NAD^+ and glucose-6-phosphate are present then converts Glucose-6-phosphate to 6-Phosphoglucono- δ -lactone and the reaction is quantified by a change in the accumulation of NADH, which is measured as absorbance at 340 nm. The enzyme assay was started by adding $\text{Na}_4\text{P}_2\text{O}_7$.

Materials

- Micro-titre plate (Nunc, A/S, Roskilde, Denmark)
- Anthos HT II plate reader (Anthos labtech Instruments, Salzburg, Austria)
- 1 M Tris-HCl, pH 8.0
- 1 M MgCl_2
- 100 mM Glucose
- 6 mM NAD^+ (made fresh)
- 7.5 mM APS (stored at -20°C)

- Hexokinase (Sigma)(stored at 4 °C)
- Glucose-6-phosphate dehydrogenase (Sigma)(stored at 4 °C)
- 10 mM Na₄P₂O₇

Component	Stock concentration	Volume (µl)	Final concentration
Tris	1 M	100	50 mM
MgCl ₂	1 M	10	5 mM
Glucose	100 mM	100	5 mM
NAD ⁺	6 mM	100	0.3 mM
APS	7.5 mM	27	0.1 mM
H ₂ O	-	663	-

Table 2.3: Composition of the standard reaction mixture (2 x) for ATPS assay

Hexokinase and Glucose-6-phosphate dehydrogenase, which were stored in ammonium sulfate, were centrifuged at 20,800 xg for 10min at 4 °C. The pellet was suspended in equal volume of 1 M Tris-HCl.

The reaction was set up in microtitre plates.

Component	Volume
Reaction Mixture (2 x)	100 µl
Extract	*see below
Hexokinase	5 U
Glucose-6-phosphate	5 U
H ₂ O	make up to 180 µl
10 mM Na ₄ P ₂ O ₇	20

Table 2.4: Composition of ATPS assay.

* 20 µl of chloroplast extract, or 0.1 µg of recombinant ATPS.

To start the coupled reaction, $\text{Na}_4\text{P}_2\text{O}_7$ was added and absorbance readings at 340 nm were taken every 5 min for 30 min (for chloroplast extracts), or every 30 seconds for 5 min (for the recombinant protein). Over this period, the reaction was performed at room temperature (20 °C), in the absence of light.

The reaction rate was expressed as $\mu\text{mol}/\text{sec}/\text{mg}$, which was obtained by calculating the change in $\text{Ab}_{340\text{nm}}/\text{sec}/\text{mg}$ protein. This was then divided by the molar extinction coefficient for NADH, (6.2×10^3) to get $\text{mol}/\text{sec}/\text{mg}$ protein extract.

$$\frac{(\text{Slope}_{\text{Ab}_{340\text{nm}}/\text{sec}} / 6.2 \times 10^3)}{\text{Volume of extract in assay (ml)}} \times \text{protein concentration (mg/ml)} = \text{rate (mol/sec/mg)}$$

2.18.1.1 *Determination of K_m for APS*

The K_m was determined for pyrophosphate (PP_i), a substrate for the reverse reaction, by measuring the ATPS activity in presence of a range of $\text{Na}_4\text{P}_2\text{O}_7$ concentrations from 0.0 mM to 4 mM. The average rate of ATPS activity ($\mu\text{mol}/\text{sec}/\text{mg}$) was determined from duplicates and Lineweaver-Burk plots produced by plotting $1/(\mu\text{mol}/\text{sec}/\text{mg})$ vs. $1/\text{Na}_4\text{P}_2\text{O}_7$ concentration.

Using the Lineweaver-Burk plot, the K_m was determined by the following formula manually using a spreadsheet:

$$K_m = (-1/x \text{ intercept})$$

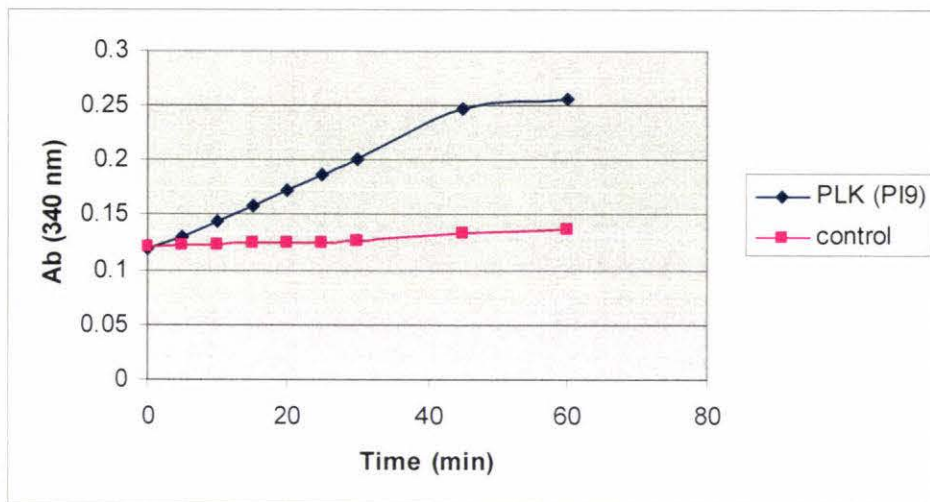


Figure 2.6: Typical results of ATPS activity assay using onion chloroplast extracts. The ATPS assay shown was conducted on onion (*Allium cepa* x *Pukekohe Longkeeper*) chloroplast extract. The assay designated as the control, was the ATPS assay without added chloroplast extract. Results are mean values of 2 replicates.

2.18.2 APSR activity assay

2.18.2.1 *Preparation of AP³⁵S for APSR activity assay.*

The APSR activity assay requires AP³⁵S as a substrate and preparation of this requires ATP, ³⁵SO⁻² and the ATPS enzyme to catalyse the reaction. This process was performed by Martin Shaw (Crop and Food research, Lincoln) using the method described by Setya et al.(1996).

2.18.2.2 *APSR activity assay*

The activity of APSR in a protein extract was determined based in a method described by Setya et al.(1996).

Materials

- 1 M Tris-HCl, pH 9.0
- 2 M MgSO₄
- 0.2 M DTE
- 3.75 mM AP³⁵S (specific activity 20,000-30,000 cpm /10 µl)
- 1 M NaSO₃
- 1 M triethanlamine
- 1 M H₂SO₄
- scintillation cocktail (Optiphase super mix)
- Plant extract
- Scintillation vials

Components	Stock concentration	Volume (μl)
Tris, pH 9.0	1 M	50
MgSO ₄	2 M	200
DTE	0.2 M	10
AP ³⁵ S	3.75 mM	10
NaSO ₃	1 M	100
Milli-Q water		110
Protein extract (see section 2.1.4)		20

Table 2.5: Composition of APSR activity mixture.

The APSR activity mixture (table 2.5) was made up in 1.5 ml Eppendorf tubes which were vortexed and incubated for 30 min at 37 °C. At the end of the assay, the Eppendorf lids were removed and the tubes transferred into scintillation vials that contained 0.8 ml 1M triethanlamine. Two hundred μl of 1 M H₂SO₄ was added to the reaction mix before the scintillation vials were quickly closed. The reactions were incubated at room temperature overnight (16 hrs), after which time the Eppendorf tubes were removed after washing the bottom of each tube with 200 μl Milli-Q into the scintillation vials. One ml of scintillation cocktail was then added, mixed well and the radioactivity was measured with a scintillator counter.

The activity of APSR in the protein extract was calculated from the following equation.

$$\frac{37.5 \times \text{cpm}}{\text{cpm}_{\text{APS}} \times c_{\text{prot}} \times V_{\text{E}} \times 30} = (\text{nmol}/\text{min}/\text{mg protein})$$

Where:

cpm = cpm recorded for reaction mixture

cpm_{APS} = specific activity of APS

c_{prot} = protein concentration (mg/ml)

V_E = Volume of extract in the assay (ml)

2.19 Statistical analysis

For this thesis, statistical analyses were conducted using the Microsoft[®] Excel spreadsheet programme (2002). Unless specified, presented data are sample means of two duplicates. Standard deviations were calculated using 3 replicates.

3.0 Results

3.1 Production of recombinant proteins.

Previous researchers had cloned onion ATPS and APSR sequences (Genbank No. AF403295 and AF212155, respectively) into the pGEX-6P-3 expression vectors and transformed these into the *E.coli* strain BL-21 (Lambert, A. and Scott, R. *pers. comm.*). In this thesis, *E.coli* were grown until the exponential stage at 37 °C (indicated by an absorbance of *ca.* 0.6 at 590 nm), before inoculation with 100 mM IPTG (to give a total concentration of 0.6 mM). The recombinant ATPS protein was isolated by GS4B chromatography (see section 2.4), and the recombinant protein detected by separation with SDS-PAGE. Coomassie blue staining revealed three proteins after treatment with PreScission Protease[™] treatment with molecular masses of *ca.* 50 kDa, 60kDa and 70 kDa (figure 3.1). Subsequent N-terminal sequencing has shown that the 70 kDa protein is *E.coli* Hsp 70, the 60 kDa protein is *E.coli* Hsp 60 and the 50 kDa protein is recombinant ATPS (Leung, S. *pers. comm.*). Isolation of recombinant APSR after GS4B chromatography (see section 2.4) revealed two proteins with molecular masses of *ca.* 55 kDa and 45 kDa (figure 3.2) Subsequent N-terminal sequencing has shown that the 55 kDa protein is the full length APSR protein, while the 45 kDa protein is a truncated APSR protein (McManus, M.T. *pers. comm.*) The identity of the larger molecular mass protein in lanes 6-7 of figure 3.2 (*ca.* 80 kDa) has not been determined. To maximise the yield of soluble protein produced, the cells were incubated at 17 °C, 20 °C or 25°C after the addition of IPTG. Coomassie staining showed that for the recombinant ATPS protein, the greatest yield was achieved when the inoculated *E.coli*

cells were incubated at 25 °C (figure 3.3 A), whereas for recombinant APSR protein, the greatest yield was achieved when the *E.coli* cells were incubated at 20 °C (figure 3.3 B).

3.1.1 Storage of recombinant ATPS

3.1.1.1 *Activity determination of stored recombinant ATPS*

During the initial purification of recombinant ATPS, it was noted that activity was lost during storage. To investigate the storage conditions further (for use in this thesis), recombinant ATPS after GS4B chromatography, was stored at 4 °C and -20 °C and activity was measured over 10 days (figure 3.5).

Comparisons of the rate of activity showed that the activity of ATPS at -20 °C was at least 50 % of the corresponding sample at 4 °C. Therefore recombinant ATPS, which was not purified further by FPLC, was stored at 4 °C.

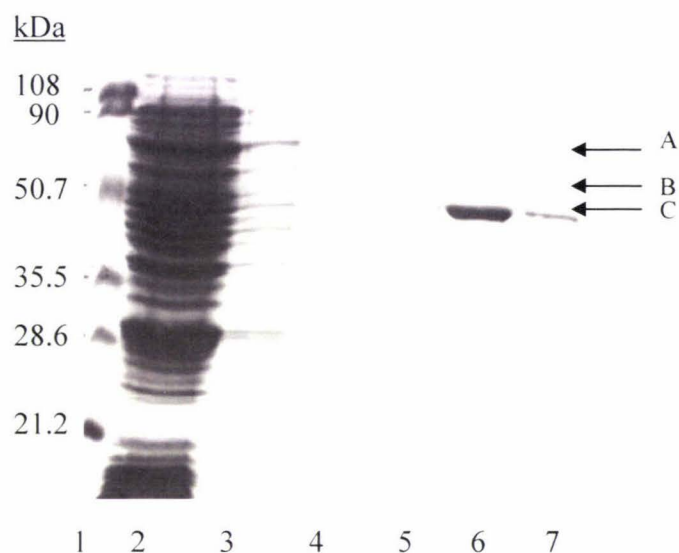


Figure 3.1: Coomassie stain of the purification of recombinant ATPS separated by SDS-PAGE. Samples were taken during the batch purification process with GS4B Sepharose of recombinant ATPS. Arrows indicate: A) *E.coli* Hsp 70, B) *E.coli* Hsp 60 and C) recombinant ATPS.

Lane 1: SDS protein standard

Lane 2: Supernatant of lysed *E.coli* cells

Lane 3: First wash with cleavage buffer, pH 7.0

Lane 4: Second wash with cleavage buffer, pH 7.0

Lane 5: Third wash with cleavage buffer, pH 7.0

Lane 6: First elution after PreScission Protease[™] treatment

Lane 7: Second elution after PreScission Protease[™] treatment

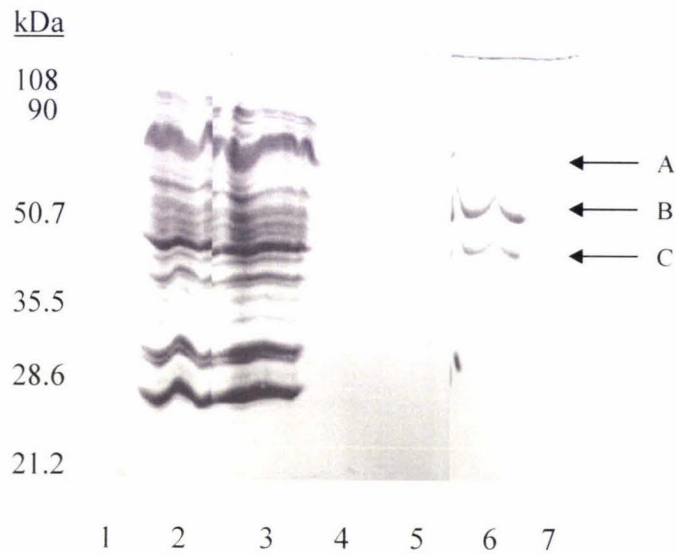


Figure 3.2: Coomassie stain of the purification of recombinant APSR separated by SDS-PAGE. Samples were taken during the batch purification process with GS4B Sepharose of recombinant APSR. Arrows indicate: A) Unidentified protein, B) Full length recombinant APSR and C) Truncated recombinant APSR

Lane 1: SDS protein standard

Lane 2: Total lysate after cell lysis

Lane 3: Supernatant of the lysed *E.coli* cells

Lane 4: First wash with cleavage buffer, pH 7.0

Lane 5: Second wash with cleavage buffer, pH 7.0

Lane 6: First elution after PreScission Protease™ treatment

Lane 7: Second elution after PreScission Protease™ treatment

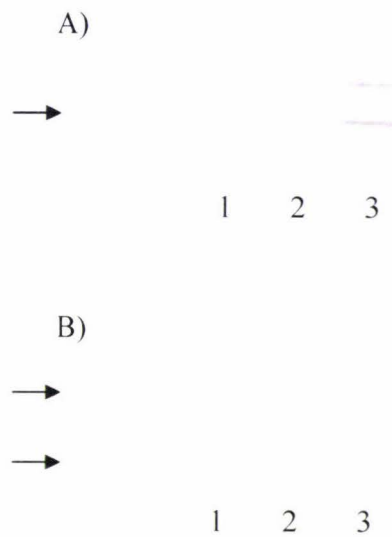


Figure 3.3: Accumulation of recombinant proteins incubated at various temperatures after induction with IPTG. Recombinant proteins were separated by SDS-PAGE and stained with coomassie blue. A) Expression of ATPS. The arrow indicates recombinant ATPS protein. B) Expression of APSR. The arrows indicate recombinant APSR protein and truncated recombinant APSR protein.

Lane 1: Cells incubated at 17 °C after IPTG induction.

Lane 2: Cells incubated at 20 °C after IPTG induction.

Lane 3: Cells incubated at 25 °C after IPTG induction.

3.1.1.2 *Anion exchange chromatography purification of ATPS and the effect of protein storage.*

A method was developed to remove the co-purifying chaperone proteins (Hsp 70, Hsp 60) and so further purify ATPS after GS4B resin chromatography. This involved firstly passing the recombinant ATPS through a Sephadex G25[®] column to exchange the recombinant proteins into buffer A and then chromatography through a MonoQ HR 5/50 ion-exchange column. If MgCl₂ is present in buffer A, ATPS is unable to bind to the Mono-Q column and it is eluted in the first few fractions (fraction number 6-10, figure 3.4).

To determine the effect of this additional purification step on ATPS activity after storage, the activity of FPLC-purified ATPS was measured over ten days and at two different storage temperatures (4 °C and -20 °C)(figure 3.5).

The results showed that over the storage period (10 days) at 4 °C, the activity of FPLC purified ATPS reduced from 0.5 nmol/min/mg to less than 0.2 nmol/min/mg (figure 3.5). In contrast, storage of FPLC-purified ATPS at -20 °C resulted in rapid reduction in activity, such that 75 % of activity was lost over only 3 days of storage. In contrast to these results, ATPS activity without FPLC purification shows that at 4 °C there is no significant change in activity over the storage period (figure 3.5). Also, the determined activity is at least 2 times higher than ATPS stored in other conditions. Storage of ATPS without FPLC purification at -20 °C over the 10 day storage period maintained 90 % of the original activity (figure 3.5).

In summary, a comparison of the activity of stored ATPS, with and without FPLC purification, shows that FPLC purification reduces the ability of ATPS to maintain activity during storage (figure 3.5). Therefore further ATPS activity assays using recombinant ATPS used freshly made protein that was not passed through a Sephadex G-25[®] column or a Mono-Q column.

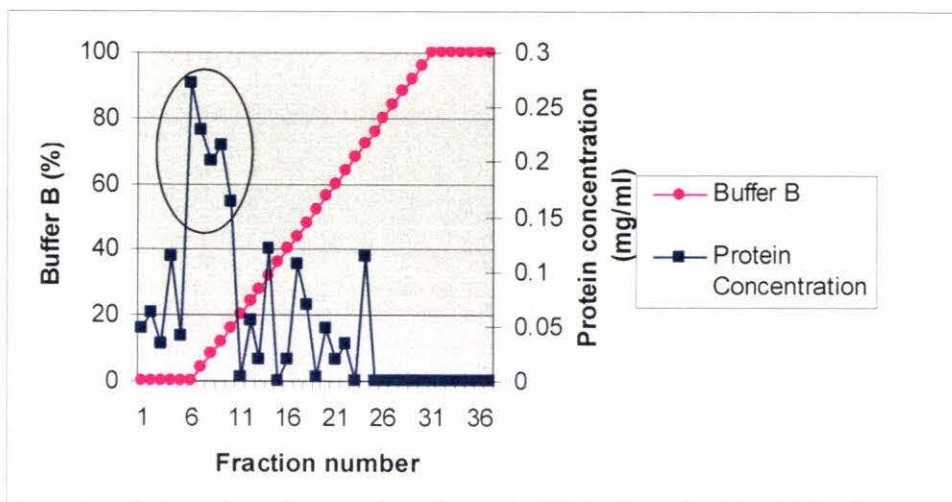


Figure 3.4: Protein concentration (mg/ml) of fractions of ATPS protein extract separated with a Mono-Q column. ATPS extract in buffer A, containing MgCl_2 was loaded onto Mono-Q column. Proteins were eluted from the column by increasing the NaCl concentration over 1 hr. Fractions (0.5 ml) were collected and the protein concentration was determined using the Bradford method. Those fractions which eluted early and showed high protein concentration (above 0.15 mg/ml) (circled) were collected and pooled. Results are mean values of 2 replicates.

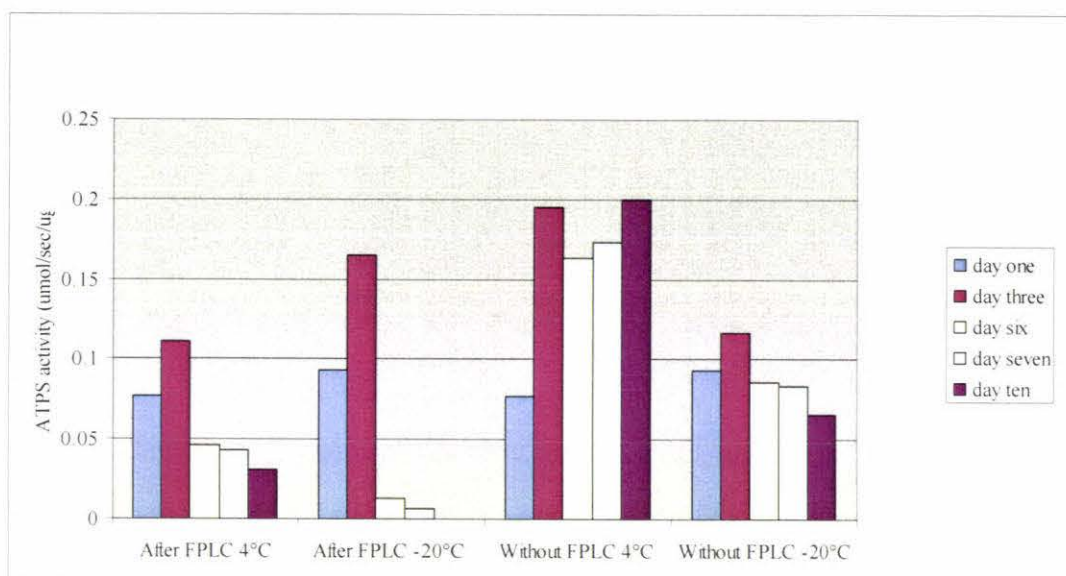


Figure 3.5: Recombinant ATPS activity after various storage conditions over 10 days. Recombinant ATPS with and without FPLC purification was stored at either 4 °C or -20 °C as indicated. The specific activity of ATPS was determined over ten days. Results are mean values of 2 replicates, and the data shown is an example of 2 repeat experiments.

3.1.2 Activity of recombinant APSR

Previous data on APSR activity (Kopriva, *S. pers. comm.*) has shown that APSR is an unstable protein and rapidly loses activity. To determine if recombinant APSR was active, the APSR assay was performed on freshly isolated APSR. Using scintillation meter to measure radioactivity, it is possible to determine the rate at which ^{35}APS is converted to ($^{35}\text{SO}_4^{2-}$) by APSR (see section 2.18.2).

Protein eluates after precision protease mediated elutions from the APSR-GST-GS4B Sepharose were tested at hourly intervals over a 3 hour time course.

The counts per minute (cpm) values, at each time point do not change significantly, but as the protein concentration, determined using Bio-Rad dye for protein determination, increased over time (figure 3.6), a decrease in the specific activity of APSR was observed (figure 3.7).

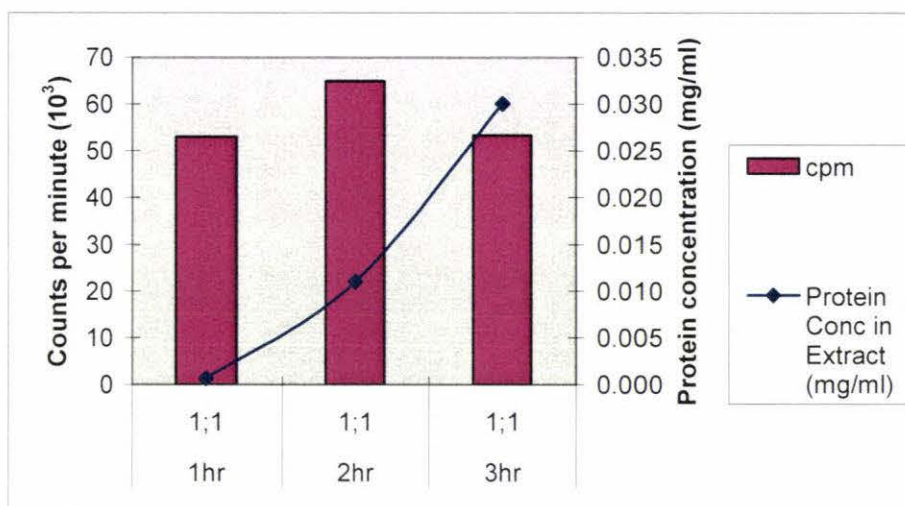


Figure 3.6: Change in protein concentration of recombinant APSR over a period of 3 hrs. Samples were collected at 1hr, 2hr, and 3hr intervals after precision protease treatment of APSR bound to GS4B. APSR activity was assayed at the dilutions indicated. Protein concentration was determined using the Bradford method (1976) at each time point. Results are mean values of 2 replicates.

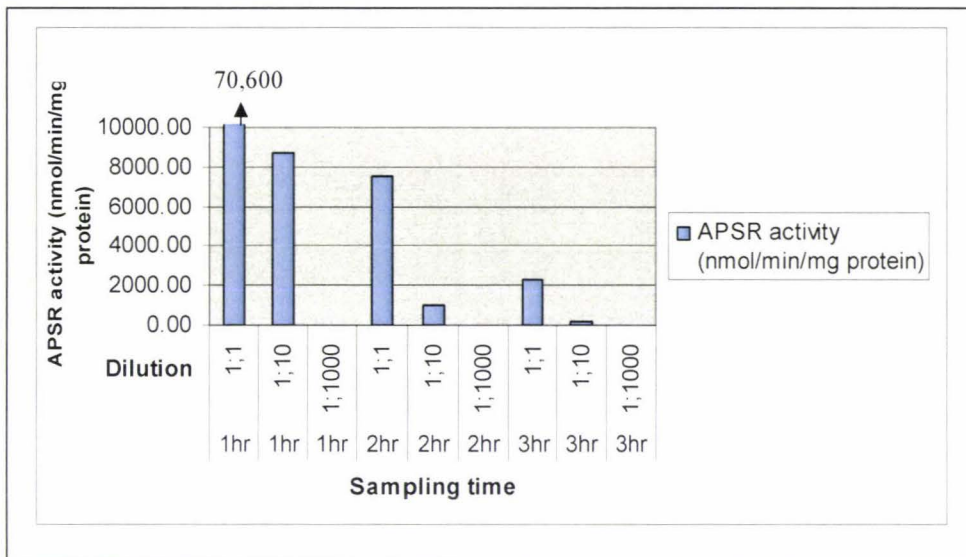


Figure 3.7: Change in specific activity of recombinant APSR over a period of 3 hrs. Samples were collected at 1hr, 2hr, and 3hr intervals after precision protease treatment of APSR bound to GS4B. APSR activity was assayed at the dilutions indicated. Results are mean values of 2 replicates.

3.2 Determination of protein complex formation using an ELISA- based method.

3.2.1 Development of methodology

3.2.1.1 Determination of the pH for effective coating of APSR to micro-titre plates

To determine the pH that would allow maximum protein coating, recombinant APSR protein was coated onto the microtitre plate with either sodium carbonate buffer at pH 9.4 or Tris-buffed saline (TBS) at pH 7.4. The standard ELISA method was followed (see section 2.15)

A comparison between the two buffers showed no significant difference. However, the samples coated with sodium carbonate buffer (pH 9.4) resulted in, on average, a slightly higher absorbance reading, at 405 nm, when compared to those coated with TBS (pH 7.4) (data not shown). Therefore, for further ELISA experiments, sodium carbonate buffer (pH 9.4) was used for coating unless specified.

3.2.1.2 Determination of the optimal amount of protein for coating.

A range of recombinant APSR protein concentrations (between 0.1 $\mu\text{g}/100 \mu\text{l}$ and 5.0 $\mu\text{g}/100 \mu\text{l}$) were coated onto the micro-titre plate. The standard ELISA method was then used (see section 2.15) using the anti-APSR antibody (K33 serum) at a 1:5000 dilution. The results produced a standard curve (figure 3.8) that indicated the maximum protein level (μg) which could be coated in the wells of the plate in 100 μl of solution. Observation of the standard curve indicates that above a concentration of *ca.* 1.0 $\mu\text{g}/100 \mu\text{l}$, the surface of the plate is saturated with protein (figure 3.8).

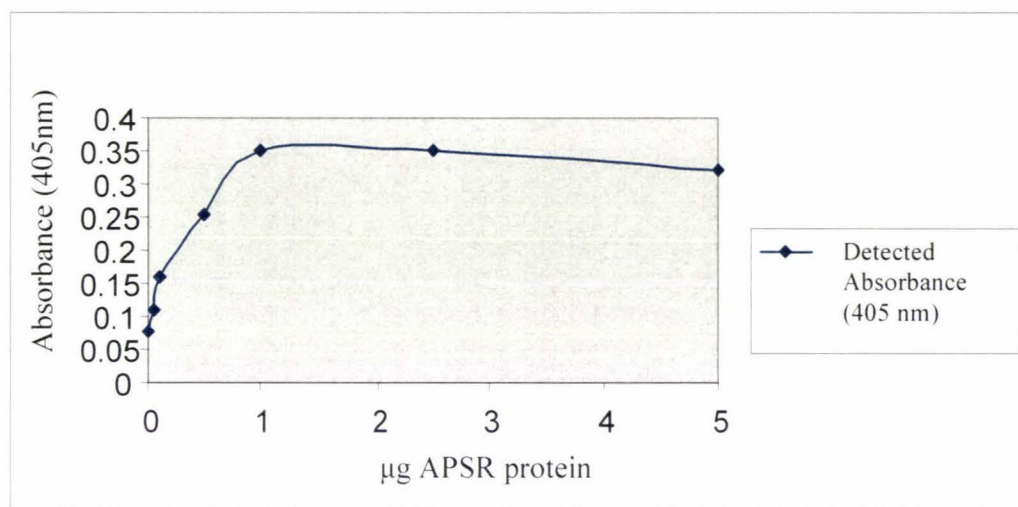


Figure 3.8: Optimisation of APSR coating. Recombinant APSR protein, at the amounts indicated, were coated and the ELISA method was conducted. Detection of coating protein was carried out using anti-APSR antibodies (K33 serum), anti-rabbit alkaline phosphatase secondary antibodies and measured the alkaline phosphatase reaction at $A_{405\text{ nm}}$. Results are mean values of 2 replicates, and the data is one example of 2 repeat experiments.

For the purposes of this thesis, a protein concentration of less than 1.0 $\mu\text{g}/100 \mu\text{l}$ was chosen as this will theoretically allow space between coated proteins. The space will accommodate a protein complex by providing room for the possible protein partner and so a concentration of 0.7 $\mu\text{g}/100 \mu\text{l}$ or 0.8 $\mu\text{g}/100 \mu\text{l}$ of coated APSR was used routinely in further experiments.

3.2.1.3 *Determination of the most appropriate antibody*

Two anti-APSR antibodies (designated as K32, K33), which were produced from immunisation of two individual rabbits with recombinant APSR protein were available for use in this thesis. The serum from the rabbits containing the antibodies were collected and stored at -20°C before use. The standard ELISA method was carried out using each of the two sera at two different dilutions and the results showed that at the same antibody dilution, K32 produced a higher Ab_{405} when compared with K33 (data not shown). This may indicate that K32 has a higher affinity to the coated APSR protein extract. Although it should be noted that using this method gives no indication of the specificity of the antibody. However, despite the higher absorbance produced by K32, K33 was used in further ELISA experiments due to the higher availability of this sera. Unless specified, anti-APSR IgG isolated from K33 (see section 2.11) was used in further ELISA experiments.

3.2.1.4 *Determining the binding affinity of the anti-ATPS antibody to APSR*

In a similar manner to APSR, anti-ATPS antibodies were produced by immunisation of three individual rabbits with purified recombinant ATPS (see section 2.4), producing three batches of antibodies labeled K22, K34 and H21 that were available for use in this thesis. After each bleed, the serum was stored at -20°C .

To use the ELISA-based method to determine protein complexes between ATPS and APSR, it is essential that the ATPS antibody does not recognise the APSR protein if this is coated first. To determine the affinity of anti-ATPS antibodies to APSR, recombinant APSR was coated onto the micro-titre plate and anti-ATPS antibody was added and the

standard ELISA method carried out. A range of antibody dilutions were used and the results obtained using the anti-ATPS antibodies were compared to those using the anti-APSR antibody on coated APSR, to determine the dilution that produced the largest difference. This would thus determine the optimal dilution that would allow specific binding of anti-ATPS antibody without the antibody binding in an unspecific manner. Each of the anti-ATPS antibodies (designated K22, K34 and H21) were tested in this manner, and compared with the anti-APSR antibody, K33 serum, as a standard. Overall, the anti-APSR antibody produced a higher absorbance than the anti-ATPS antibodies, particularly at the lower dilutions used (1:1000 and 1:1500). At the lower antibody concentrations, anti-APSR gave results similar to that of anti-ATPS antibodies. Comparisons of K22 with K34 showed that there was a larger difference between K34 and the anti-APSR antibody than with K22 (figure 3.9).

Assuming that both ATPS antibodies have similar binding affinity to ATPS, the results suggest that the K22 binds to other non-target proteins. Further experiments with H21 IgG gave results similar to that of K34 serum (data not shown), indicating that H21 IgG was also an appropriate antibody for this application. Further ELISA experiments were conducted with anti-ATPS K34 serum or anti-ATPS H21 IgG.

3.2.2 Analysis of complex formation.

3.2.2.1 *Detection of a complex between APSR and ATPS at pH 9.4*

To give an indication if ATPS forms a complex with APSR, a range of ATPS protein was incubated with a single concentration of coated APSR protein at pH 9.4. Any bound ATPS protein (to APSR) will be detected with the use of the anti-ATPS antibody.

Observations of the results showed that there was no significant change in absorbance over the range of ATPS protein concentrations tested (figure 3.10), suggesting that ATPS does not bind to the coated APSR at pH 9.4.

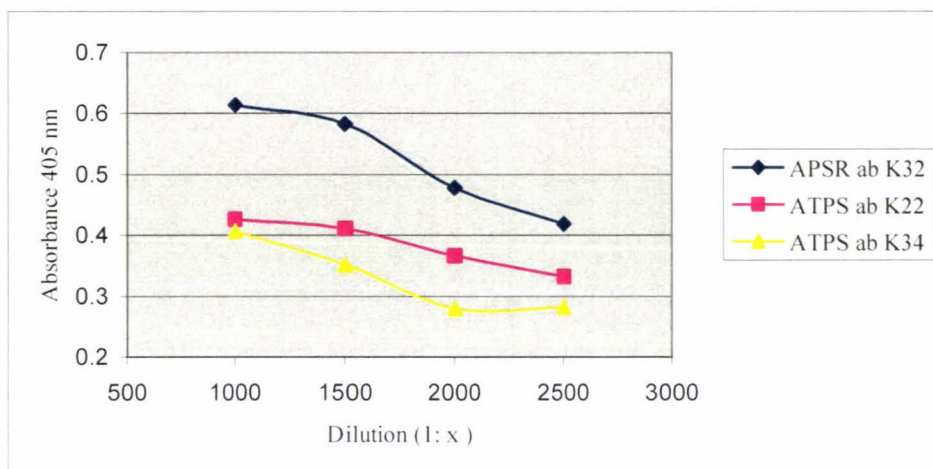


Figure 3.9: ELISA to determine the binding affinity of antibodies to coated recombinant APSR. The ELISA method was conducted with each antibody at the concentrations shown. Results are mean values of 2 replicates, and the data shown is one example of 2 repeat experiments.

3.2.2.2 *The effect of glutathione on the formation of a complex between ATPS and APSR at pH 9.4*

Glutathione is a down stream product of the sulfur assimilation pathway and so it may play an important role in mediating a complex between the two proteins ATPS and APSR (and so regulate its own biosynthesis).

However, using the ELISA-based method, when 5.0 mM glutathione was present in the buffers used, no evidence of promotion of a complex formation, at pH 9.4, was evident between ATPS and APSR proteins (data not shown).

3.2.2.3 *Detection of a complex between APSR and ATPS at pH 7.4*

To determine if the complex formation was dependent on the pH of the buffering medium, all incubation and coating steps were conducted using TBS as a buffer at pH 7.4.

As shown in figure 3.10, the results of the ELISA showed an increase in absorbance as more ATPS protein was present during incubation. This indicates that ATPS is associating with the coated APSR protein. Increasing the amount of ATPS above *ca.* 1 μ g results in a very small change in absorbance, indicating that no further ATPS is associating with the coated APSR.

If no APSR is coated to the plate, so that only proteins within the blocking solution are coated, no absorbance change is observed. This illustrates that ATPS is associating with APSR and not with any of the proteins present in the blocking solution.

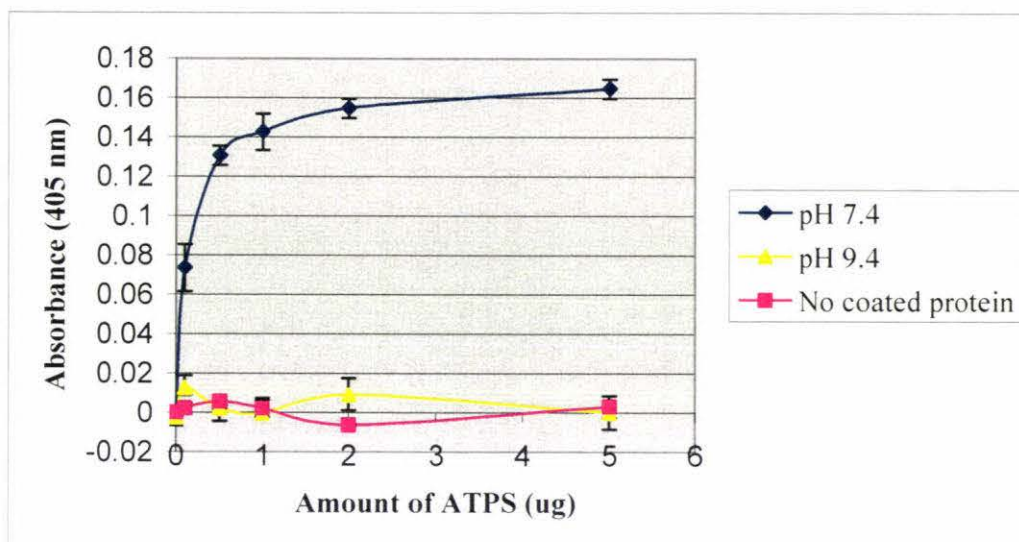


Figure 3.10: Detection of a complex between ATPS-APSR using ELISA. Increasing amounts of ATPS, as indicated, were incubated with a single amount of coated APSR (0.7 μg) at the pH indicated. Detection of any associated ATPS was carried out using anti-ATPS, anti-rabbit alkaline phosphatase and measuring the alkaline phosphatase reaction at $A_{405 \text{ nm}}$. To standardise the results, they were subtracted from the 0.0 μg ATPS value. Results are mean values of 2 replicates, and the data shown is one example of 3 repeat experiments. Error bars represent one standard deviation from the mean.

3.2.2.4 *The effect of glutathione on the complex between ATPS and APSR at pH 7.4.*

To determine if the complex formation can be further enhanced with the addition of glutathione, incubation with ATPS, containing reduced glutathione in a range of concentrations (between 0.1 mM and 10 mM) was tested.

Results from the ELISA experiment indicated that there is no significant difference in the ATPS-APSR binding when glutathione is added. (see figure 3.11). This suggests that glutathione doesn't improve the binding between ATPS and APSR.

To reveal if the redox state of glutathione is important in complex formation between ATPS and APSR, the ELISA experiment at pH 7.4 (see section 3.2.2.3) was repeated with oxidised glutathione. The results showed no difference between reduced glutathione and oxidised glutathione, implying that the redox state of glutathione is not important to the formation of a complex between ATPS and APSR (data not shown).

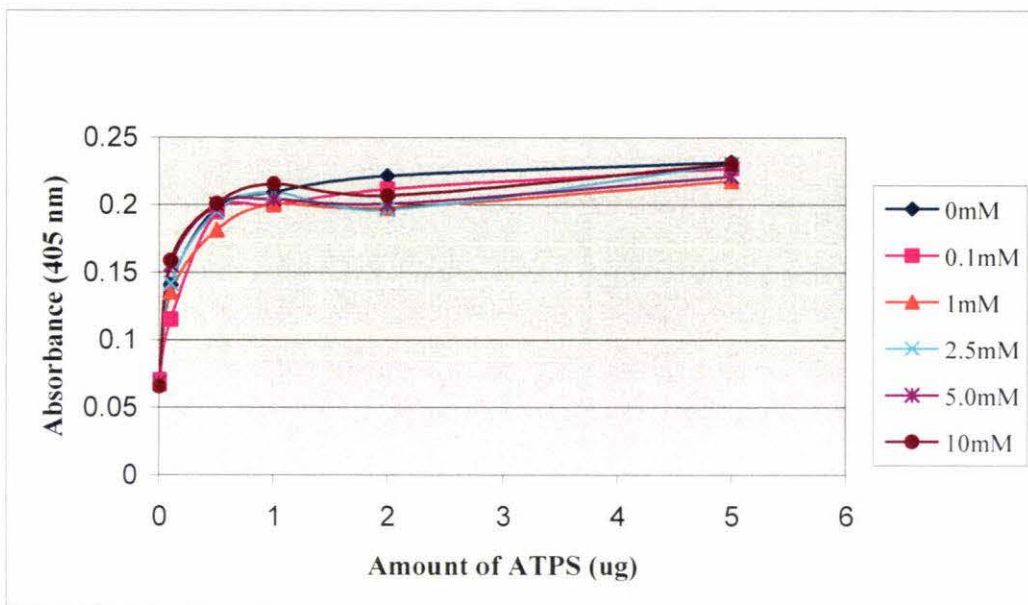


Figure 3.11: Influence of reduced glutathione on the ATPS–APSR complex at pH 7.4. Increasing amounts of ATPS (as indicated) and reduced glutathione at different concentrations were incubated with a single amount of coated APSR (0.7 μ g) at pH 7.4. Detection of associated ATPS was carried out using anti-ATPS antibody, anti-rabbit alkaline phosphatase and measuring the alkaline phosphatase reaction at $Ab_{405\text{ nm}}$. Results are mean values of 2 replicates, and the data shown is one example of 2 repeat experiments.

3.3 Ligand binding assay to determine putative ATPS-APSR complexes

3.3.1 Development of methodology

3.3.1.1 *Testing the specificity of the anti-ATPS and anti-APSR antibodies.*

To determine if the filter-based ligand binding assay can be used to detect putative ATPS-APSR protein complexes, it is important that the anti-ATPS antibody does not recognise APSR, or similarly, that the anti-APSR antibody does not recognise ATPS when either protein is immobilised on the PVDF membrane. Western blot analysis was used with each protein (recombinant ATPS or APSR) immobilised and then incubated with either anti-ATPS or anti-APSR antibodies as appropriate (data not shown). Western analysis showed that the anti-ATPS antibody (designated as K34) recognised three bands (*ca.* 90 kDa, 50 kDa, and 45 kDa) in the recombinant ATPS preparation, which was isolated using GS4B resin. The *ca.* 50 kDa band, which is similar to the predicted molecular mass of ATPS, produces the densest band (figure 3.12, A-C lane 2) and corresponds with the major staining band revealed by Coomassie blue staining (figure 3.1, lanes 6-7). The other two bands (*ca.* 90 kDa and *ca.* 45 kDa), are recognised very weakly, and in some cases do not appear (data not shown).

A comparison of blots challenged with different anti-ATPS concentrations, show that at the higher concentrations (1:500 and 1:3000); two bands are detectable (*ca.* 60 kDa, 55 kDa) in the APSR protein solution (figure 3.12, A-C, lane 3). However, at a dilution of 1:5000, no bands were detectable in the APSR lane and so this dilution was used for further western blots with anti-ATPS antibody.

In the anti-APSR western analysis, the anti-APSR antibody recognised three major bands (*ca.* 78 kDa, 55 kDa, 45 kDa) and a number of minor bands in the APSR preparation, ranging from *ca.* 20-90 kDa. The bands of *ca.* 55 kDa and 45 kDa correspond to the major staining bands revealed by Coomassie blue staining (figure 3.2, lanes 6-7), which have been determined previously by N-terminal sequencing to be APSR and a truncated APSR product, respectively (M.T. McManus, *pers.comm.*). As the dilution of anti-APSR increases, the density of minor bands reduces in the APSR lane (figure 3.12, D-F, lane 3). In the lane containing recombinant ATPS, an almost indistinguishable band of *ca.* 50 kDa

is detectable at all three dilutions which corresponds to the molecular mass of ATPS (figure 3.12, D-F, lane 2). Further experiments used the anti-APSR antibody at a dilution of 1:5000. At this dilution, recognition of the putative *ca.* 50 kDa ATPS band was minimal.

3.3.1.2 *Do the purified IgG recognise separated E.coli proteins?*

The method used for the production of recombinant ATPS/APSR proteins uses *E.coli* as a host vector. It is possible that proteins of *E.coli* origin (Hsp 70, Hsp 60) remain during the purification steps. So to determine if purified anti-ATPS IgG or anti-APSR IgG recognises any of the *E.coli* proteins that may not be removed over the purification process, whole *E.coli* extracts (strain DH- 5 α) were blotted and incubated with both antibodies. Examination of the western analysis using anti-ATPS and anti APSR both showed that the purified IgG recognised proteins in these *E. coli* extracts, only at a background level (figure 3.13, A-B, lane 3).

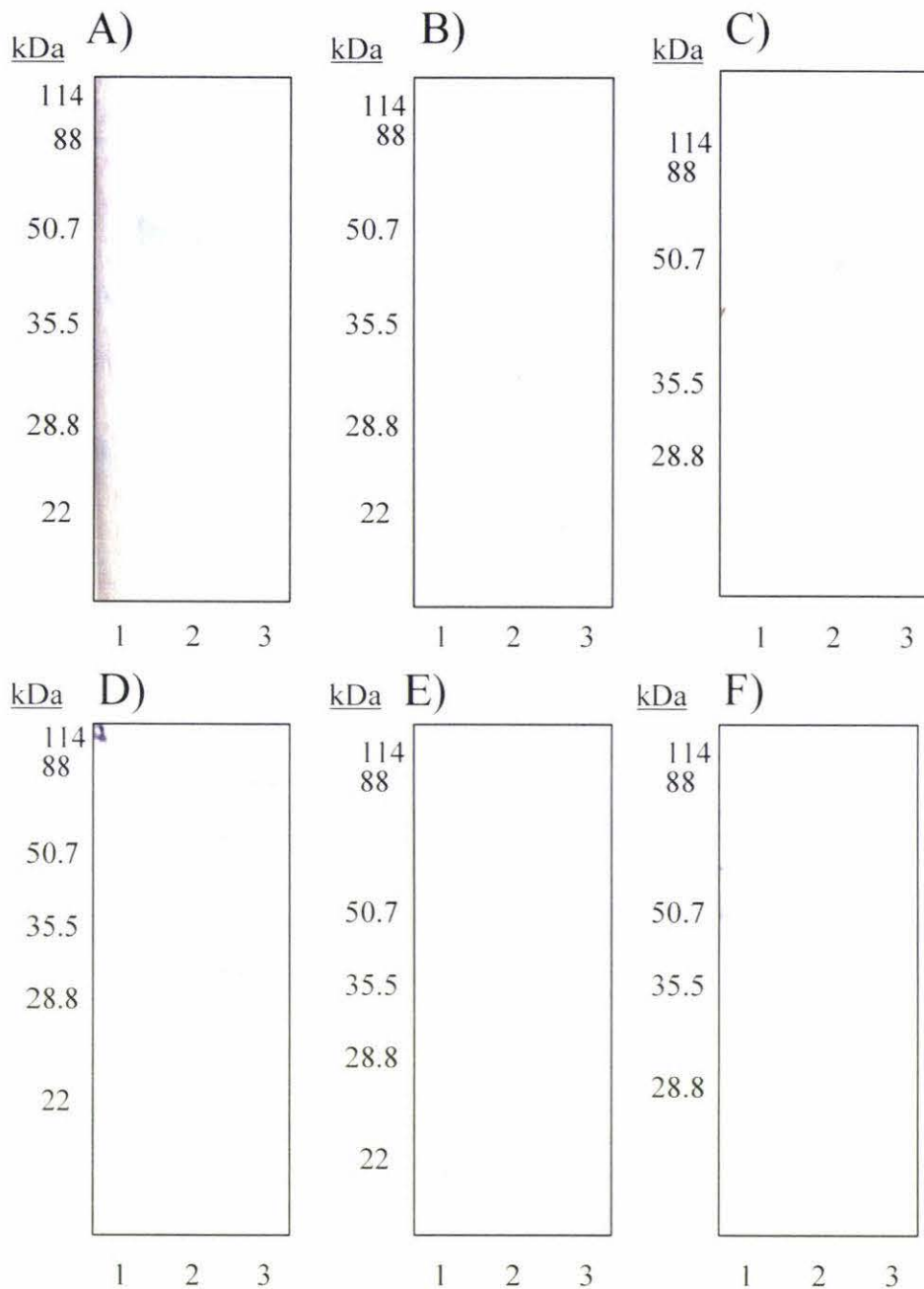


Figure 3.12: Western analysis with anti-ATPS (A-C) and anti-APSR (D-F) antibodies. Recombinant ATPS and APSR were blotted onto a membrane and challenged with a range of antibody dilutions.

In each blot; Lane 1: Marker; Lane two: ATPS; Lane three: APSR

- | | |
|-------------------------------------|-------------------------------------|
| A) Western with anti-ATPS at 1:500 | D) Western with anti-APSR at 1:1000 |
| B) Western with anti-ATPS at 1:3000 | E) Western with anti-APSR at 1:3000 |
| C) Western with anti-ATPS at 1:5000 | F) Western with anti-APSR at 1:5000 |

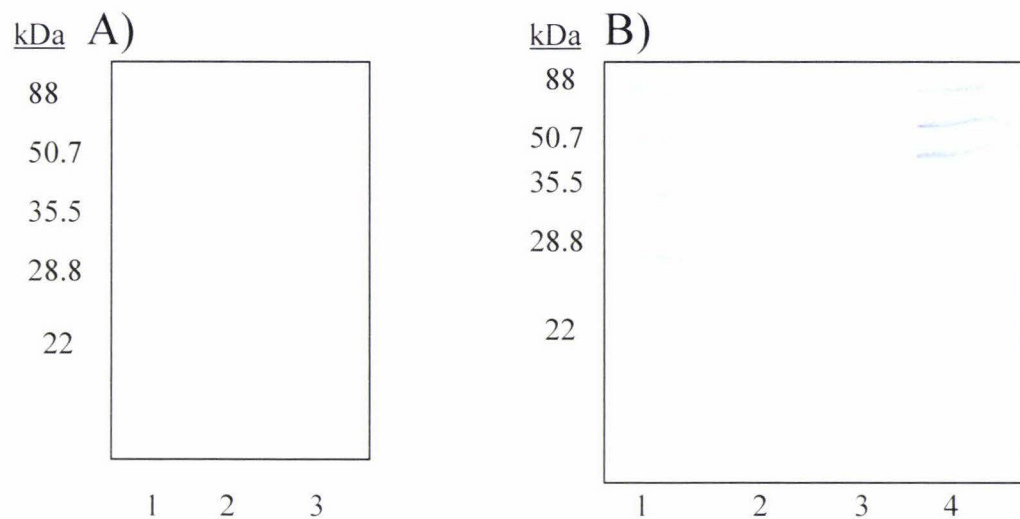


Figure 3.13: Western blot analysis with anti-ATPS (A) and anti-APSR (B) antibodies of *E.coli* (strain DH-5 α) protein extracts.

Lane 1: Protein standard

Lane 2: Recombinant ATPS

Lane 3: *E.coli* (DH-5 α) cell extract

Lane 4: Recombinant APSR (blot B only)

3.3.2 Ligand binding assay

3.3.2.1 *Ligand binding assay with anti-APSR*

To determine whether ATPS binds to APSR, separated ATPS is first blotted onto a membrane after SDS-PAGE, and then incubated with a solution containing free APSR, before detection of any APSR protein binding with anti-APSR antibody.

Identical blots were incubated with different concentrations of APSR protein (figure 3.14). Examination of these blots revealed that the anti-APSR antibodies recognised the APSR protein as predicted (figure 3.14, lane 1). In the ATPS lane of each blot (figure 3.14, lane 2), a band of molecular mass of *ca.* 50 kDa is recognised by the anti-APSR antibody that is similar in size to that of ATPS when compared with the protein standard (figure 3.14, lane 3). Closer inspection of this band over the three blots reveals a slight increase in density, as more APSR was present during the incubation (figure 3.14 A-C, lane 2, arrowed). If unspecific binding of anti-APSR IgG occurred, it would be unlikely to result in the increase in density observed, because the same amount of ATPS protein was blotted onto each of the blots. This may suggest that the free APSR is associating with the membrane-bound ATPS as a complex.

It should also be noted that in general the intensity of the APSR bands (*ca.* 78 kDa, 55 kDa, 45 kDa) increase in intensity when the immobilised APSR is incubated with increasing amounts of free APSR protein (figures 3.14, 3.15, 3.16, 3.17). This may indicate the formation of an APSR dimer or some other protein complex involving APSR.

3.3.2.2 *Optimisation of protein binding by increasing the pH of the incubation media*

To determine if the association between APSR and ATPS can be further improved by altering the pH, the experiment described in section 3.2.2.1, which was carried out at pH 7.4 was repeated at pH 7.8 (figure 3.15).

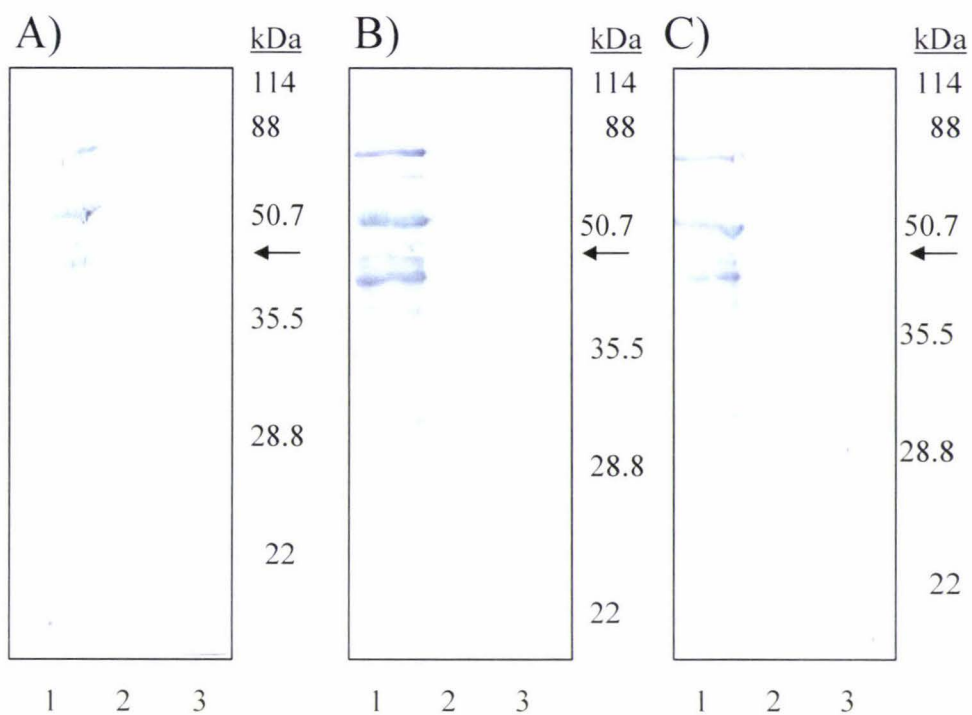


Figure 3.14: Ligand binding assay at pH 7.4. Recombinant APSR (lane 1 in each blot) and ATPS protein (lane 2 in each blot) were blotted onto PVDF membrane and incubated with 0 μg (A), 1 μg (B) and 5 μg (C) of APSR protein. Western analysis was performed with the anti-APSR antibody (A-C). Lane 3 contains a protein standard. The arrows indicate the expected molecular mass of ATPS.

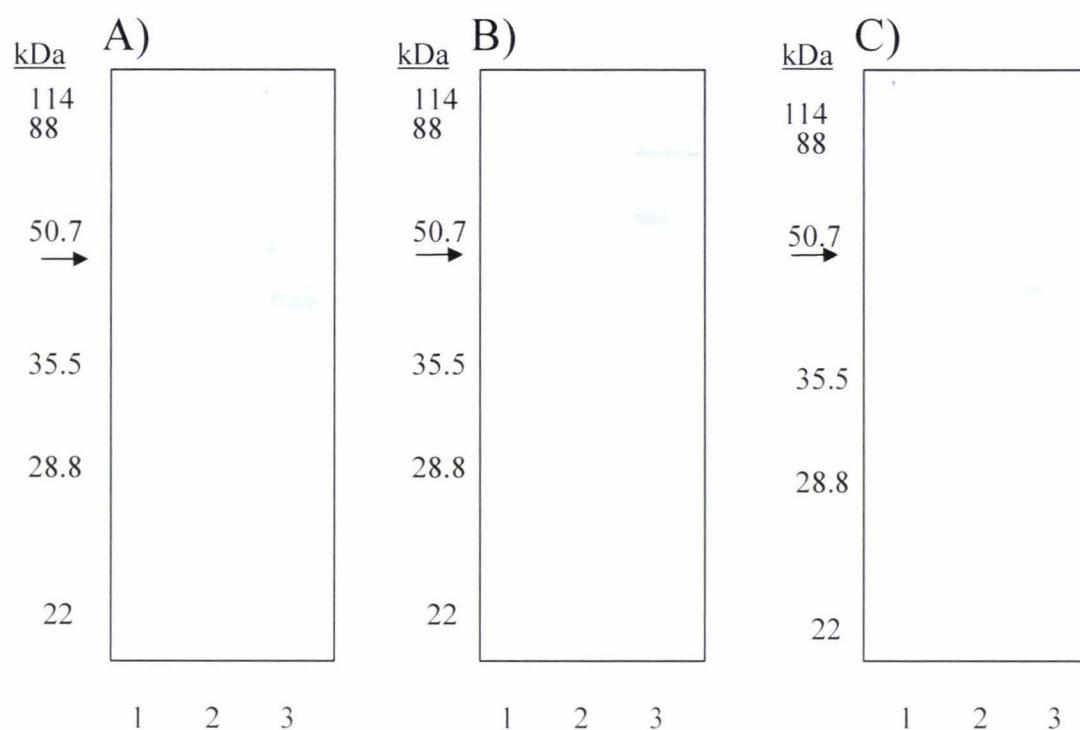


Figure 3.15: Ligand binding assay at pH 7.8. Recombinant APSR (lane 3 in each blot) and ATPS protein (lane 2 in each blot) were blotted onto PVDF membrane and incubated with 0 μg (A), 1 μg (B) and 5 μg (C) of APSR protein in PBS buffer at pH 7.8. Western analysis was performed with anti-APSR antibody (A-C). Lane 1 contains a protein standard. The arrows indicate the expected molecular mass of ATPS.

Examination of the western analysis with anti-APSR showed that in the ATPS lane (figure 3.15, A-C, lane 2), there was little or no change in intensity of the band of molecular mass of *ca.* 50 kDa, in response to different APSR incubation. This result, when compared to the ligand binding assay conducted at pH 7.4 (figure 3.14), suggests that an increase in pH, from 7.4 to 7.8, results in a reduction of protein complex formation between ATPS and APSR.

3.3.2.3 *Optimisation of protein binding by the inclusion of magnesium ions*

To investigate if magnesium plays a role in the mediation of a protein complex between ATPS and APSR, 5.0 mM MgCl₂ was included in the solution during incubation steps. Three identical blots were undertaken and incubated with different levels of APSR at pH 7.4 (figure 3.16) and pH 7.8 (figure 3.17). The results of the western blot analysis showed that in the ATPS lane of each APSR/MgCl₂ treatment, little difference in the intensity of APSR recognition was observed. This result was common to both pH incubations.

This result, if compared to the ligand-binding assay that contains no MgCl₂, suggests that the presence of MgCl₂ reduces or inhibits the ability of APSR to associate with the ATPS protein. However, to show this with more clarity, a range of magnesium concentrations need to be trialed.

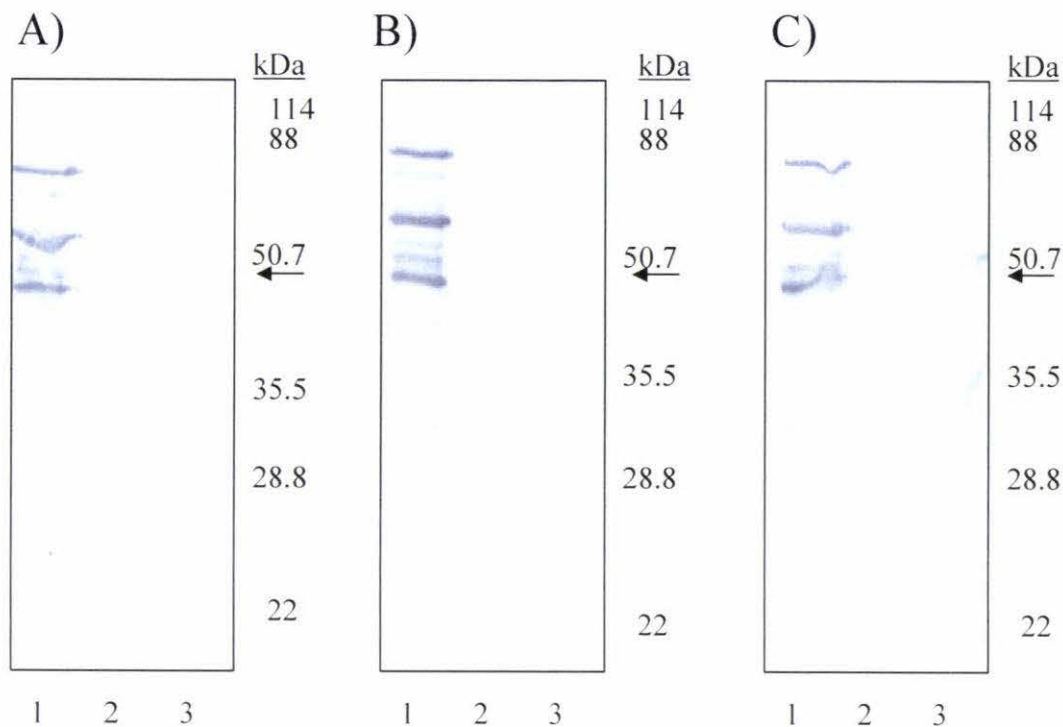


Figure 3.16: Ligand binding assay in the presence of 5.0 mM MgCl₂ at pH 7.4. Recombinant APSR (lane 1 in each blot) and ATPS protein (lane 2 in each blot) were blotted onto PVDF membrane and incubated with 0 μg (A), 1 μg (B) and 5 μg (C) of APSR protein in PBS buffer at pH 7.4 containing 5.0mM MgCl₂. Western analysis was performed with anti-APSR antibody (A-C). Lane 3 contains a protein standard. The arrows indicate the expected molecular mass of ATPS protein.

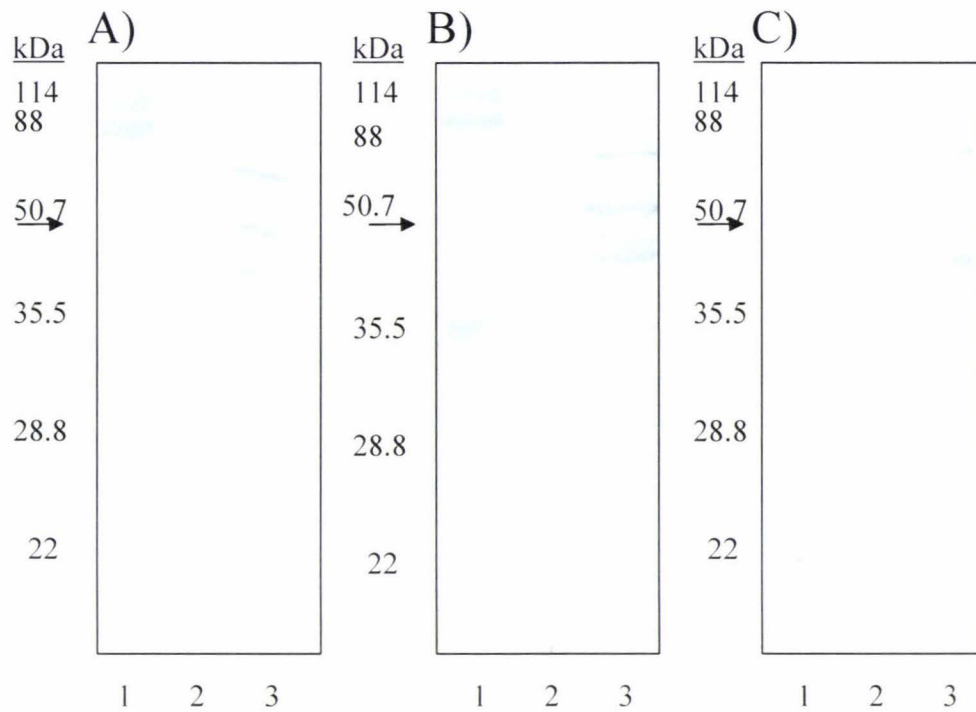


Figure 3.17: Ligand binding assay in the presence of 5.0 mM MgCl₂ at pH 7.8. Recombinant APSR (lane 3 in each blot) and ATPS proteins (lane 2 in each blot) were blotted onto PVDF membrane and incubated with 0 μg (A), 1 μg (B) and 5 μg (C) of APSR protein in a PBS buffer at pH 7.8 containing 5.0mM MgCl₂. Western analysis was performed with anti-APSR antibody (A-C). Lane 1 contains a protein standard. The arrows indicate the expected molecular mass of ATPS.

3.4 Immunoprecipitation of putative ATPS-APSR complexes

3.4.1 Immunoprecipitation with protein G Sepharose

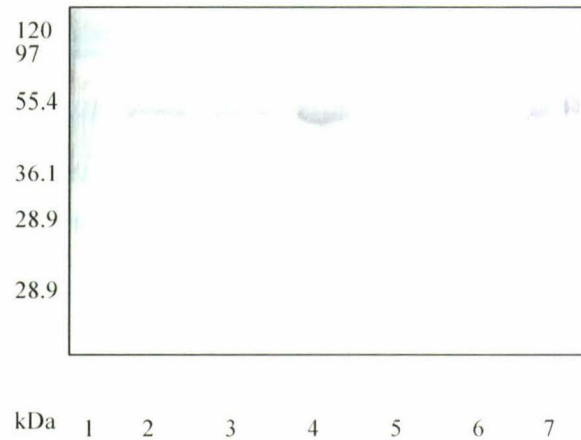
3.4.1.1 *Immunoprecipitation of ATPS and APSR using protein G Sepharose.*

Immunoprecipitation was performed by incubating 1 μg of ATPS with 0 μg , 1 μg or 5 μg of APSR. The protein mixture is then incubated with a specific antibody (anti-APSR IgG, K33) and any antibody-protein aggregates are isolated with the addition of protein G Sepharose, which binds to the IgG and is easily collected by a simple centrifugation step. Once isolated, western analysis are carried out on the samples using both anti-ATPS and anti-APSR antibodies (figure 3.18 A and B).

Results of the anti-ATPS western show a large thick band of molecular mass of *ca.* 52 kDa, which is present in all lanes (figure 3.18). This band may represent the ATPS protein, as it is the same estimated molecular mass, but is also visible in the lane where no ATPS is present (figure 18, A-B, lane 2).

Results from the western incubated with the anti-APSR antibody (figure 3.18 B), also showed the same dense band (*ca.* 52 kDa) seen in the anti-ATPS in all lanes confirming that this band is not ATPS. Above the dense band (at *ca.* 55 kDa), is a faint band that is present in all lanes, at approximately the same density. This is most likely the higher molecular mass APSR protein present (1 μg) in all of the immunoprecipitation samples, with the truncated form possibly occluded by the major recognised band (*ca.* 52 kDa).

A)



B)

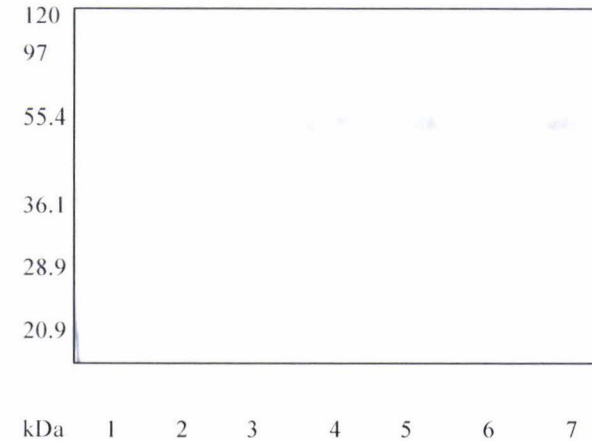


Figure 3.18: Western analysis after immunoprecipitation with protein G Sepharose. Immunoprecipitation was conducted with 1 μg of recombinant APSR and 0 μg , 1 μg or 5 μg recombinant ATPS as indicated, with and without 5 mM reduced glutathione, as indicated.

Western blot A was challenged with anti-ATPS IgG and western blot B, with anti-APSR IgG. Antibody binding was detected using anti-rabbit alkaline phosphatase.

Lane 1: SDS protein standard

Lane 2: Immunoprecipitation with 1 μg APSR and 0.0 μg ATPS

Lane 3: Immunoprecipitation with 1 μg APSR and 2.5 μg ATPS

Lane 4: Immunoprecipitation with 1 μg APSR and 5.0 μg ATPS

Lane 5: Immunoprecipitation with 1 μg APSR and 0 μg ATPS with 5.0 mM glutathione

Lane 6: Immunoprecipitation with 1 μg APSR and 2.5 μg ATPS with 5.0 mM glutathione

Lane 7: Immunoprecipitation with 1 μg APSR and 5.0 μg ATPS with 5.0 mM glutathione

3.4.1.2 *Investigation into the ca. 52 kDa protein identified in the western analysis of immunoprecipitation experiments.*

To determine the identification of the unknown dense band identified (figure 3.18), all species required in the immunoprecipitation were blotted individually against both anti-ATPS and anti-APSR.

Observation of the western blot analysis, reveal that the dense band resides in lanes that contain either K33, the anti-APSR IgG antibody or K34, the anti-ATPS IgG antibody (data not shown). This result is probably due to the secondary antibody which has a high affinity to rabbit antibody, recognising the primary anti-APSR and anti-ATPS IgGs that are used as part of the immunoprecipitation procedure.

3.4.1.3 *Use of biotinylated antibodies in western blot analysis of immunoprecipitation experiments.*

Because the secondary antibodies used previously recognise the antibodies used in the immunoprecipitation, an alternative secondary antibody was used. Strepavidin-alkaline phosphatase (AP) can be used as an alternative to anti-rabbit-AP, by recognising a biotin ester that can be conjugated to the primary antibody. Thus the biotin ester was attached to both the anti-ATPS and anti-APSR IgG proteins. To determine if the strepavidin-AP only recognised the biotinylated antibodies and not the non-biotinylated antibodies, western analysis was conducted on the immunoprecipitation samples and incubated with strepavidin-AP.

Observation of the western blot analysis showed that the strepavidin-AP distinguished the biotinylated anti-ATPS and anti-APSR (designated as H21-B and K33-B) from the non-biotinylated antibodies (H21 and K33) (data not shown).

To determine if ATPS and APSR forms a complex, immunoprecipitation was undertaken using protein G Sepharose. Western analysis was prepared using biotinylated antibodies and streptavidin-AP.

Results from the western blot analysis show that APSR is being immunoprecipitated with the protein G complex with ATPS. More APSR is detected as more protein is added to the mixture suggesting that APSR is associating with ATPS (figure 3.19 B, lanes 2-4). However, when immunoprecipitation of ATPS and APSR are undertaken without the use of IgG, distinct bands that correspond to both ATPS (figure 3.19 A, lane 7 and 9) and the full length APSR protein (figure 3.19 B, lane 8 and 9) are observed. This suggests that ATPS and APSR are able to bind in a non-specific manner to the Sepharose or to the protein G. Because of this, it is difficult to make any definite conclusions about the immunoprecipitation experiment as any interaction detected could possibly be due to the proteins binding to the Sepharose.

3.4.2 Immunoprecipitation with CN-Br conjugated Sepharose

3.4.2.1 *Immunoprecipitation with anti-ATPS conjugated Sepharose*

In attempt to reduce the amount of non-specific binding of proteins to the protein G Sepharose, the anti-ATPS (H21) IgG were conjugated to CN-Br activated Sepharose resin.

It was determined that between 0.5-1.0 μg of ATPS protein was able to saturate 5 μl of 50 % (v/v) anti-ATPS IgG conjugated Sepharose slurry (data not shown).

Immunoprecipitation experiments were carried out with 1 μg of ATPS and 0 μg , 1 μg or 5 μg of APSR added and any complex formation was detected by western blot analysis, again using biotinylated IgG and streptavidin alkaline phosphatase. The results show that in all immunoprecipitation assays, ATPS is identified in a consistent amount, as predicted, by the use of anti-ATPS IgG (figure 3.20 A). ATPS was unable to bind to uncoupled Sepharose demonstrating that conjugated anti-ATPS IgG is necessary for ATPS to bind to the resin (data not shown).

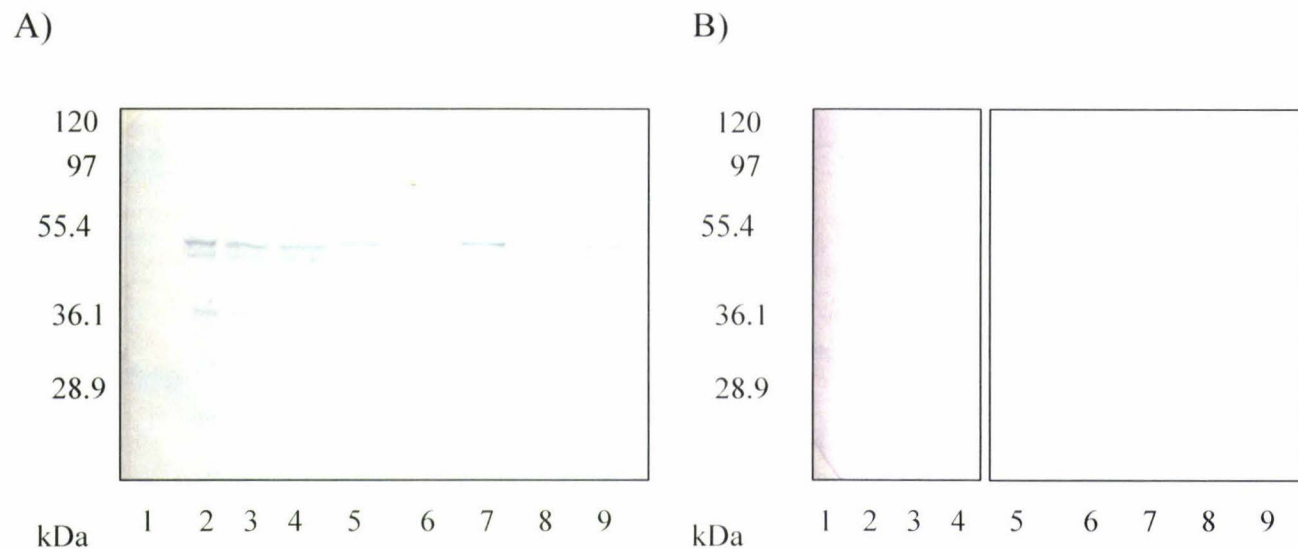


Figure 3.19: Western analysis with biotinylated antibodies after immunoprecipitation with protein G Sepharose.

Immunoprecipitation was conducted with 1 μ g of recombinant APSR and 0 μ g, 1 μ g or 5 μ g recombinant ATPS as indicated, with and without 5 mM reduced glutathione, as indicated. Western blot A, was challenged with biotinylated anti-ATPS IgG and western blot B was challenged with biotinylated anti-APSR IgG. Antibody binding was detected using alkaline phosphatase conjugated streptavidin.

Lane 1: SDS protein standard

Lane 2: Immunoprecipitation with 1 μ g ATPS and 0.0 μ g APSR

Lane 3: Immunoprecipitation with 1 μ g ATPS and 2.5 μ g APSR

Lane 4: Immunoprecipitation with 1 μ g ATPS and 5.0 μ g APSR

Lane 5: Recombinant ATPS before immunoprecipitation

Lane 6: Recombinant APSR before immunoprecipitation

Lane 7: Immunoprecipitation of 1 μ g ATPS without anti-ATPS IgG

Lane 8: Immunoprecipitation of 1 μ g APSR without anti-ATPS IgG

Lane 9: Immunoprecipitation of a 1 μ g ATPS and 1 μ g APSR mixture without anti-ATPS IgG

APSR was detected when present in the immunoprecipitation experiment (figure 3.20 B). As more APSR is added, more APSR is detected indicating that the APSR protein is being immunoprecipitated. Where no ATPS is present, APSR is detectable as two light bands, probably associating with the conjugated anti-ATPS IgG (figure 3.21). This result indicates that some of the APSR can bind in a non-specific manner to the resin. However, because the putative APSR-ATPS complex produces a darker band on the anti-APSR western analysis (figure 3.20 B), than without ATPS protein (figure 3.21), it is conceivable that some APSR is also associating with ATPS. To show this more convincingly, both complexes should be separated and challenged with the anti-APSR antibody on the same western blot.

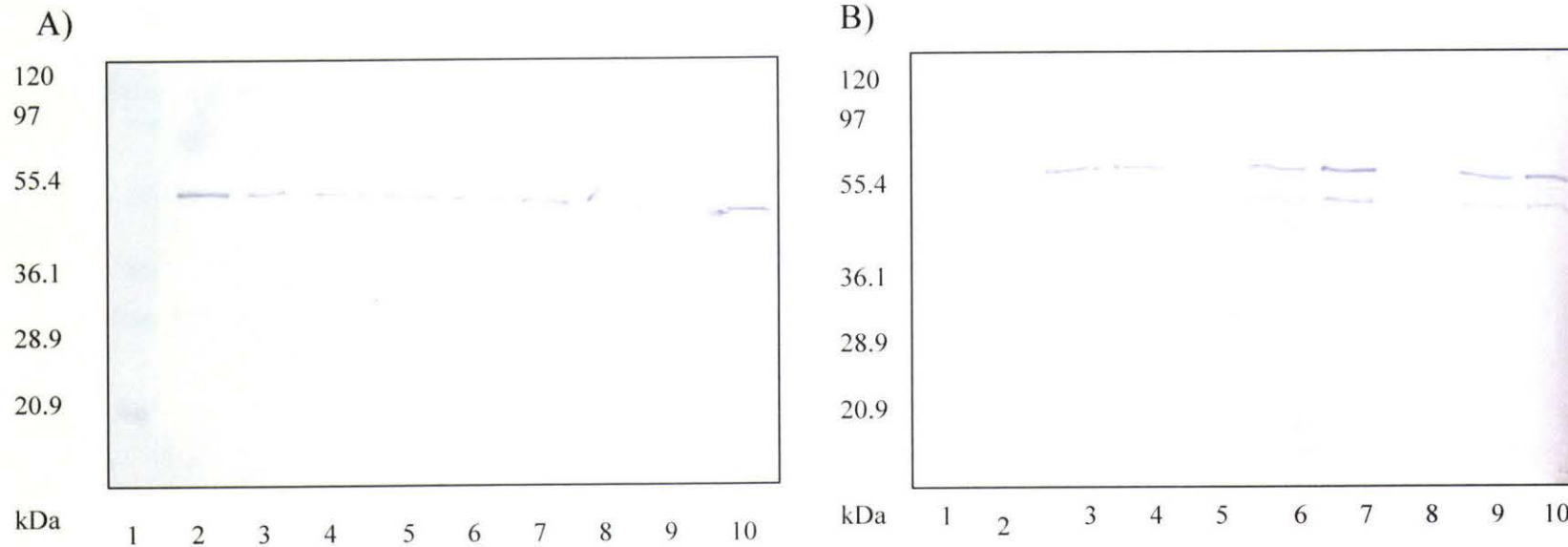


Figure 3.20: Western blot analysis after immunoprecipitation of ATPS and APSR with anti-ATPS IgG conjugated Sepharose. Immunoprecipitation was conducted with 1 μg of recombinant ATPS and 0 μg , 1 μg or 5 μg recombinant APSR as indicated, with and without 5 mM reduced or oxidised glutathione as indicated. Western blot A was challenged with biotinylated anti-ATPS IgG and western blot B with biotinylated anti-APSR IgG. Antibody was detected using alkaline phosphatase conjugated streptavidin.

Lane 1: SDS protein standard

Lane 2: Immunoprecipitation with 1 μg ATPS and 0.0 μg APSR

Lane 3: Immunoprecipitation with 1 μg ATPS and 2.5 μg APSR

Lane 4: Immunoprecipitation with 1 μg ATPS and 5.0 μg APSR

Lane 5: Immunoprecipitation with 1 μg ATPS and 0 μg APSR with 5.0 mM reduced glutathione

Lane 6: Immunoprecipitation with 1 μg ATPS and 2.5 μg APSR with 5.0 mM reduced glutathione

Lane 7: Immunoprecipitation with 1 μg ATPS and 5.0 μg APSR with 5.0 mM reduced glutathione

Lane 8: Immunoprecipitation with 1 μg ATPS and 0 μg APSR with 5.0 mM oxidised glutathione

Lane 9: Immunoprecipitation with 1 μg ATPS and 2.5 μg APSR with 5.0 mM oxidised glutathione

Lane 10: Immunoprecipitation with 1 μg ATPS and 5.0 μg APSR with 5.0 mM oxidised glutathione

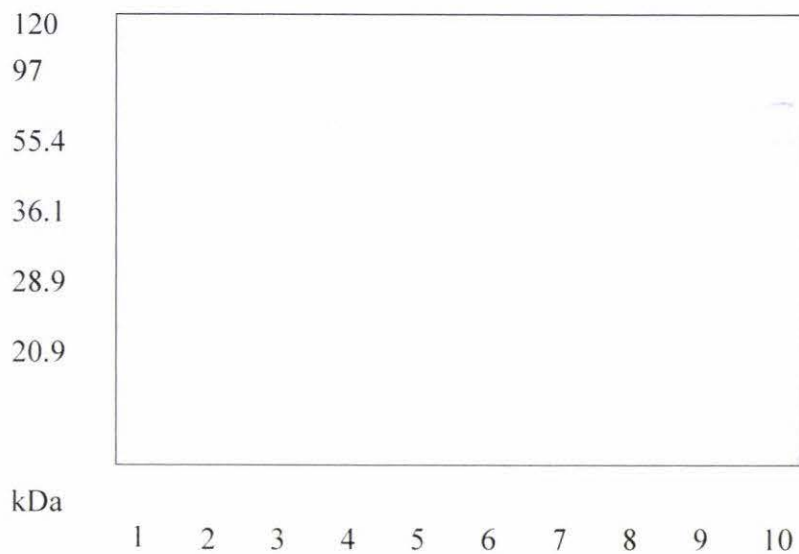


Figure 3.21: Western analysis after immunoprecipitation of APSR with CN-Br Sepharose. Immunoprecipitation was conducted without the 1 μ g of recombinant ATPS protein with 0 μ g, 1 μ g or 5 μ g recombinant APSR protein added. The experiment was also repeated with 5 mM oxidised and reduced glutathione, as indicated. Biotinylated anti-APSR antibody was used and antibody binding was detected using alkaline phosphatase conjugated streptavidin.

Lane 1: SDS protein standard

Lane 2: Immunoprecipitation with 0.0 μ g APSR

Lane 3: Immunoprecipitation with 2.5 μ g APSR

Lane 4: Immunoprecipitation with 5.0 μ g APSR

Lane 5: Immunoprecipitation with 0 μ g APSR with 5.0 mM reduced glutathione

Lane 6: Immunoprecipitation with 2.5 μ g APSR with 5.0 mM reduced glutathione

Lane 7: Immunoprecipitation with 5.0 μ g APSR with 5.0 mM reduced glutathione

Lane 8: Immunoprecipitation with 0 μ g APSR with 5.0 mM oxidised glutathione

Lane 9: Immunoprecipitation with 2.5 μ g APSR with 5.0 mM oxidised glutathione

Lane 10: Immunoprecipitation with 5.0 μ g APSR with 5.0 mM oxidised glutathione

3.4.2.2 *The effect of glutathione on the ATPS-APSR protein complexes.*

To determine if glutathione plays a role in mediating the complex formation between ATPS and APSR, either oxidised or reduced glutathione were included in the ATPS/APSR immunoprecipitation procedure.

Western blot analysis with anti-APSR shows that when reduced or oxidised glutathione is present during the immunoprecipitation experiment (figure 3.20 B, lanes 5-10), APSR bands increase in intensity when compared with the corresponding lanes without added glutathione (figure 3.20 B, lanes 1-3). This suggests that glutathione may play a role in mediating or stabilising a protein complex between ATPS and APSR.

3.4.2.3 *Determination of the specificity of the interaction with ATPS.*

It is possible that the interaction observed between ATPS and APSR may not be specific.

To test if the ATPS binds in a non-specific manner to other proteins,

immunoprecipitation was conducted with ATPS and two un-related plant proteins; the ACC oxidase, isoform 2, MD-ACO2 from Apple (*Malus sp.*), and the Kunitz trypsin inhibitor from Soya bean (SBTI)(*Glycine max*).

Observation of western analysis of immunoprecipitation of ATPS and an ACC oxidase isoform, show that ACC oxidase is not immunoprecipitated with ATPS (figure 3.22, B). A dark band is present at *ca.* 52 kDa, which is presumed to be the H21 antibody, as this western was incubated with anti-rabbit-AP. The molecular mass of ACC oxidase is *ca.* 35 kDa which when separated by SDS-PAGE, can be assessed clear of the IgG band (*ca.* 52 kDa).

When the soya bean trypsin inhibitor is immunoprecipitated with ATPS, SBTI was not detected by western analysis with the anti-SBTI monoclonal antibody (raised in mice), suggesting that SBTI does not associate with ATPS during immunoprecipitation (figure 3.23, B). The dense band visible (*ca.* 52 kDa) in the ACC oxidase blot (figure 3.22, B), is now not visible, since anti-mouse-AP was used, and this will not recognise the H21 antibody used in the immunoprecipitation.

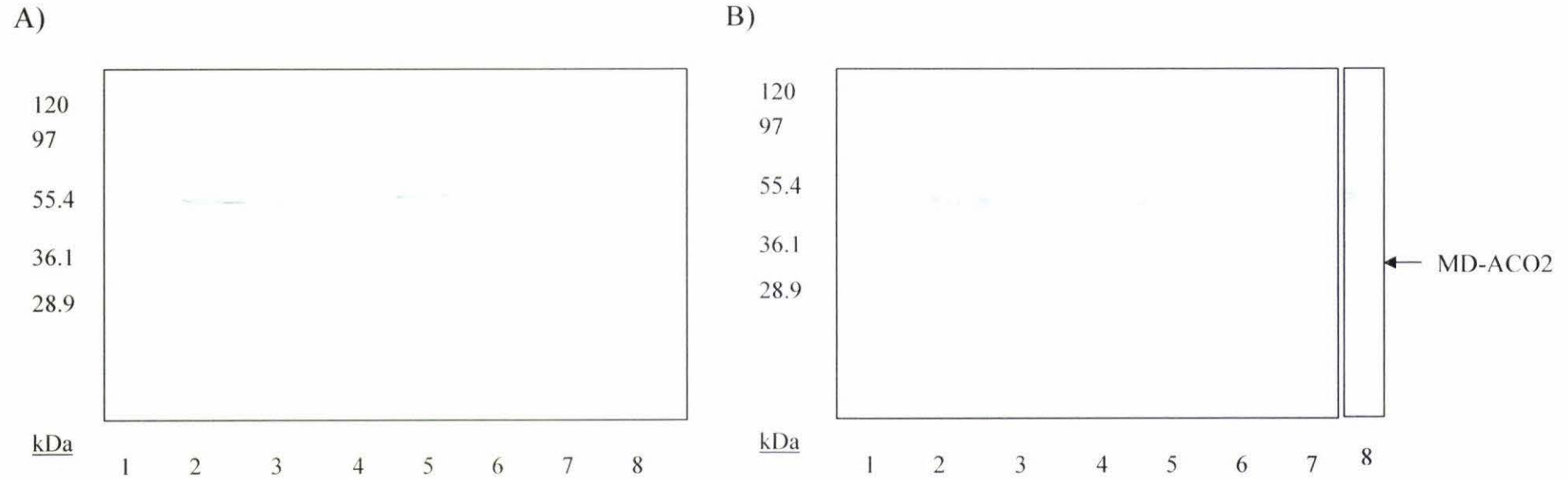


Figure 3.22: Immunoprecipitation of ATPS and MD-ACO2 with anti-ATPS IgG conjugated Sepharose. Immunoprecipitation was conducted with 1 μg of recombinant ATPS and 0 μg , 1 μg or 5 μg recombinant MD-ACO2, as indicated, with and without 5 mM oxidised and reduced glutathione, as indicated. Western blot A was challenged with biotinylated anti-ATPS and B challenged with anti-MD-ACO2 IgG antibody. Antibody binding was detected using alkaline phosphatase conjugated streptavidin (A) or anti-rabbit alkaline phosphatase (B). Arrow indicates the expected molecular mass of ACC oxidase-2 (*Malus sp.*)

Lane 1: SDS protein standard

Lane 2: Immunoprecipitation with 1 μg ATPS and 0.0 μg MD-ACO2

Lane 3: Immunoprecipitation with 1 μg ATPS and 2.5 μg MD-ACO2

Lane 4: Immunoprecipitation with 1 μg ATPS and 5.0 μg MD-ACO2

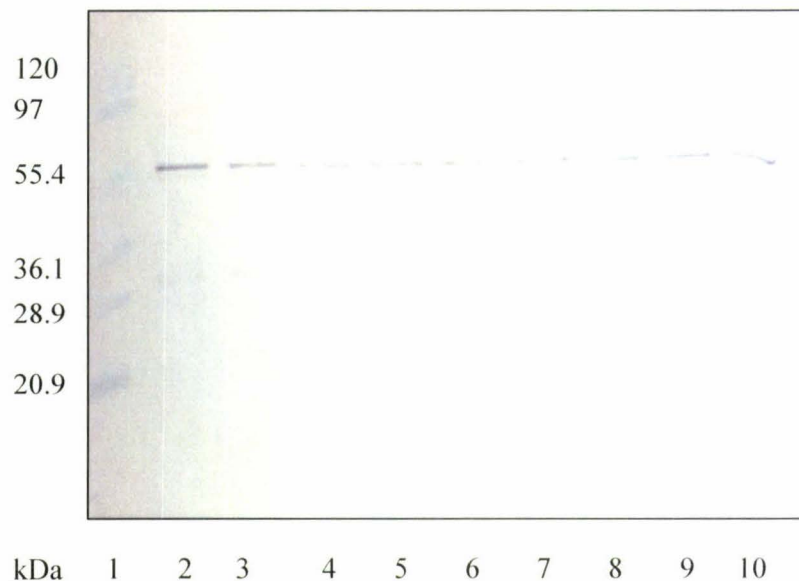
Lane 5: Immunoprecipitation with 1 μg ATPS and 0 μg MD-ACO2 with 5.0 mM reduced glutathione

Lane 6: Immunoprecipitation with 1 μg ATPS and 2.5 μg MD-ACO2 with 5.0 mM reduced glutathione

Lane 7: Immunoprecipitation with 1 μg ATPS and 5.0 μg MD-ACO2 with 5.0 mM reduced glutathione

Lane 8: 1 μg recombinant MD-ACO2

A)



B)

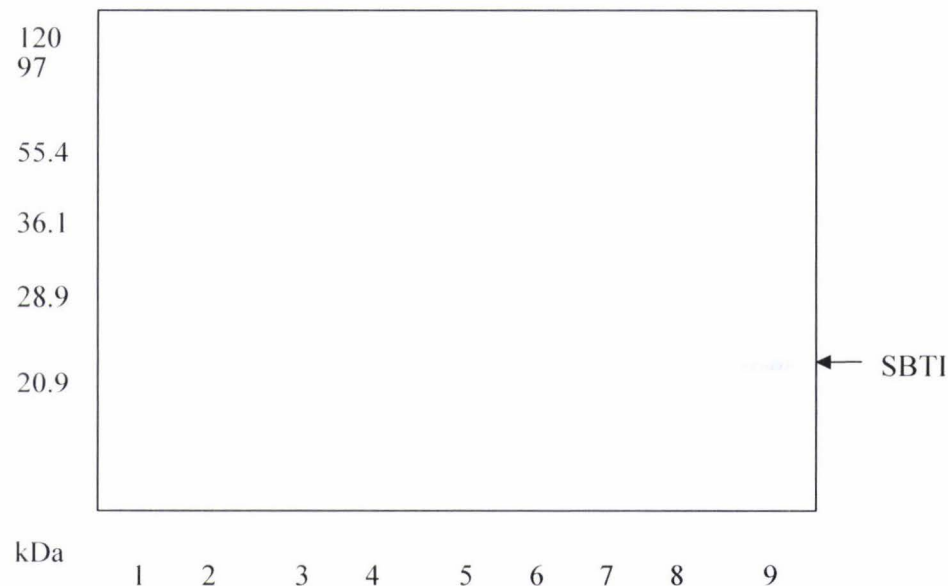


Figure 3.23: Immunoprecipitation of ATPS and SBTI with anti-ATPS IgG conjugated Sepharose. Immunoprecipitation was conducted with 1 μg of recombinant ATPS and 0 μg , 1 μg or 5 μg of commercially available SBTI, as indicated, with and without 5 mM oxidised and reduced glutathione, as indicated. Western blot A was challenged with biotinylated anti-ATPS IgG and B with anti-SBTI monoclonal antibody. Antibody binding was detected using alkaline phosphatase conjugated streptavidin (A) or anti-mouse alkaline phosphatase (B). Arrow indicates the expected molecular mass of soybean trypsin inhibitor.

Lane 1: SDS protein standard

Lane 2: Immunoprecipitation with 1 μg ATPS and 0.0 μg SBTI

Lane 3: Immunoprecipitation with 1 μg ATPS and 2.5 μg SBTI

Lane 4: Immunoprecipitation with 1 μg ATPS and 5.0 μg SBTI

Lane 5: Immunoprecipitation with 1 μg ATPS and 0 μg SBTI with 5.0 mM reduced glutathione

Lane 6: Immunoprecipitation with 1 μg ATPS and 2.5 μg SBTI with 5.0 mM reduced glutathione

Lane 7: Immunoprecipitation with 1 μg ATPS and 5.0 μg SBTI with 5.0 mM reduced glutathione

Lane 8: Immunoprecipitation with 1 μg ATPS and 0 μg SBTI with 5.0 mM oxidised glutathione (A): 0.75 μg SBTI (B)

Lane 9: Immunoprecipitation with 1 μg ATPS and 2.5 μg SBTI with 5.0 mM oxidised glutathione (A): 1.5 μg SBTI (B)

Lane 10: Immunoprecipitation with 1 μg ATPS and 5.0 μg SBTI with 5.0 mM oxidised glutathione

These results showing that neither SBTI nor ACC oxidase could associate with ATPS, providing evidence that the associations detected previously with APSR to ATPS, are not due to non-specific interactions, but may be due to a specific protein-protein interaction.

3.4.3 Immunoprecipitation with chloroplast extracts.

Immunoprecipitation was conducted to determine if the ATPS-APSR complex can be detected in chloroplast extracts. The H21-conjugated Sepharose was incubated with recombinant ATPS, before incubation with the chloroplast extract. Unfortunately, no APSR was detected in the western blots, suggesting that the APSR from the chloroplast did not associate with the recombinant ATPS under the conditions used (data not shown).

3.5 Physiological significance of the ATPS-APSR complex

3.5.1 Significance on ATPS activity

3.5.1.1 *The effect of APSR on ATPS activity*

To test if the complex between ATPS and APSR revealed in this thesis (see sections 3.2, 3.3, and 3.4) had any effect on ATPS activity, the two proteins were mixed in solution and then the activity of ATPS was measured. Observation of the results revealed that if the ATPS assay was conducted with APSR, the detected activity is higher than ATPS alone, by *ca.* 1.8×10^{-2} units. However, this difference is probably due to the production of NADH by APSR in the absence of ATPS, which was recorded at a rate of 9.2×10^{-2} $\mu\text{mol} / \text{sec} / \mu\text{g}$ (table 3.1).

	ATPS Activity (μmol - NADH formed/sec/ μg protein)
ATPS	9.2×10^{-2}
APSR	9.2×10^{-3}
ATPS and APSR	0.11

Table 3.1: Activity assay of recombinant ATPS and APSR

3.5.1.2 *The effect of glutathione on ATPS activity*

To determine if glutathione is able to mediate the activity of ATPS, the activity of recombinant ATPS was measured in the presence of a range of reduced and oxidised glutathione concentrations (from 0.1 mM to 5.0 mM). The results show that at a concentration of 1.0 mM oxidised or reduced glutathione, there was no change in ATPS activity (figure 3.24). However, when the concentration of glutathione was increased to 2.5 mM, a 90 % reduction in ATPS activity was observed. The activity of ATPS is reduced to zero if 5.0 mM glutathione is added. There is no difference between oxidised and reduced glutathione and neither oxidised nor reduced glutathione gave any detectable absorbance change when assayed in the absence of ATPS (data not shown).

These results suggest that glutathione may play a role in regulating the activity of ATPS, but that the redox state of glutathione is not important in this regulation.

3.5.1.3 *The effect of glutathione on ATPS activity when in a complex with APSR*

Immunoprecipitation experiments (see section 3.4) have indicated that glutathione may play a role in the formation of a complex between ATPS and APSR. To see if the response of ATPS activity to glutathione is altered if it is in a complex with APSR, ATPS was allowed to form a complex in buffer containing between 0.0-5.0 mM glutathione and the activity of ATPS was determined. The results show that the ATPS activity in response to glutathione is very similar to that of ATPS without APSR, where the activity reduces by 90 % at a glutathione concentration of 2.5 mM, and most if not all activity at a glutathione concentration of 5.0 mM (figure 3.24). The different redox states of glutathione did not have any difference in relation to ATPS activity, when in the ATPS-APSR complex.

3.5.2 *The effect of glutathione on APSR activity.*

To determine if the activity of the recombinant APSR is regulated by glutathione, the APSR assay was conducted in the presence of reduced and oxidised glutathione at different concentrations.

The results show that oxidised glutathione, had no significant effect on the activity of APSR. However, in the presence of reduced glutathione, the activity of APSR is more variable, but there is no significant trend is observed (figure 3.25). These results indicate that neither reduced glutathione or oxidised glutathione has any role in mediating APSR activity, at the concentrations tested.

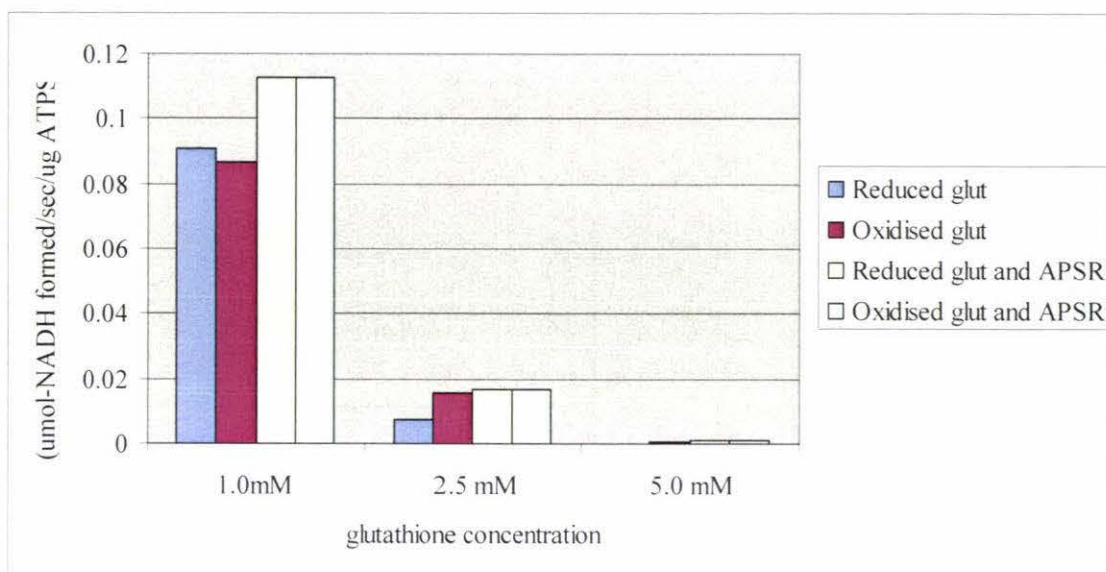


Figure 3.24: Assay of recombinant ATPS activity in the presence of recombinant APSR and various glutathione treatments. The ATPS activity assay was conducted with recombinant APSR, as indicated. The buffers used contained reduced or oxidised glutathione at the concentrations indicated. Results are mean values of 2 replicates, and the data shown is one example of 2 repeat experiments.

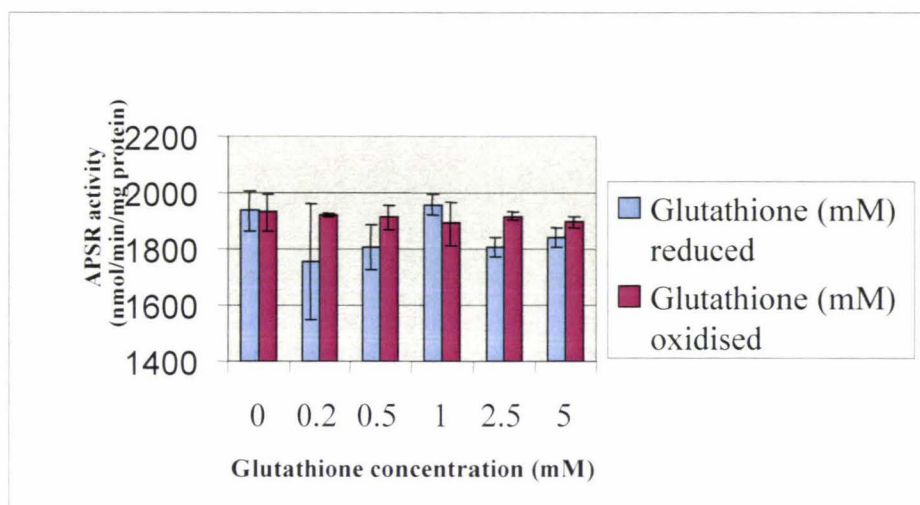


Figure 3.25: Activity of recombinant APSR in the presence of reduced glutathione and oxidised glutathione. APSR assay was conducted with recombinant APSR in the presence of various concentrations of either oxidised or reduced glutathione, as indicated. The error bars represent one standard deviation from the mean. Results are mean values of 3 replicates, and the data shown is one example of 2 repeat experiments.

3.6 Detection of protein-protein complex in a chloroplast extracts using an ATPS affinity column.

To detect the presence of any putative protein complex between ATPS and APSR in the chloroplast, an ATPS affinity column was assembled using Sepharose conjugated with anti-ATPS IgG (see section 2.12). Prior to the addition of the chloroplast extract from the *Allium cepa*, W202A cultivar (see section 2.2) to the affinity column, recombinant ATPS (see section 2.4) was added. To remove any bound ATPS or ATPS-APSR protein complexes, high pH and low pH elution buffers were used. Fractions were collected and then analysed by western analysis with biotinylated anti-ATPS IgG and biotinylated anti-APSR IgG.

3.6.1 Western analysis with biotinylated anti-ATPS IgG of eluates.

In the recombinant ATPS protein preparation used to pre-coat the column, two bands are visible by western analysis with anti-ATPS (figure 3.26 A, lane 2); a band with a molecular mass of *ca.* 50 kDa, the predicted size for ATPS and a unknown protein of *ca.* 30 kDa. In the chloroplast extract from *A. cepa*, ATPS was detectable as a light band of molecular mass of *ca.* 50 kDa (figure 3.26 A, lane 4). Unfortunately, this is difficult to observe in the figure shown. No ATPS was detected in the eluate after the chloroplast extract was incubated with the resin (figure 3.26 A, lane 5). It is probable therefore that ATPS in the chloroplast bound to the anti-ATPS IgG on the Sepharose, reducing the concentration in the eluate. The column was washed with TBS, pH 7.4, to remove unbound proteins. Western analysis of the initial eluate after the TBS wash, revealed a single protein with a molecular weight of *ca.* 52 kDa. At this molecular weight it is conceivable that this band represents IgG that has become uncoupled from the Sepharose and is recognised non-specifically by the biotinylated anti-ATPS IgG during western analysis. This is the same molecular weight observed for the immune-recognised IgG proteins observed previously in this thesis (see section 3.4.1.2).

An aliquot of Sepharose was also collected after the incubation with recombinant ATPS and chloroplast extract. SDS-reducing buffer was added to the Sepharose and boiled for 5 min before loading onto the SDS-PAGE gel followed by either Coomassie staining or western

analysis. Western analysis with biotinylated anti-ATPS IgG revealed three bands with molecular masses of *ca.* 52 kDa, 50 kDa and light band *ca.* 30 kDa. This is similar to the proteins detected in recombinant ATPS preparation before being bound to the Sepharose (figure 3.26 A, lane 2). The presence of a protein with the molecular mass of 52 kDa may be the anti-ATPS IgG, that was bound to the Sepharose (figure 3.26 A, lane 7) and is recognised in a non-specific manner by the biotinylated anti-ATPS IgG. Coomassie staining revealed 4 protein bands (figure 3.28, lane 2) of molecular masses of *ca.* 52 kDa (a major staining band) and three additional bands with molecular weights of *ca.* 33 kDa, 28 kDa and *ca.* 40 kDa (a light staining band). It is possible that the major protein detected at *ca.* 52 kDa was the IgG bound to the Sepharose, masking the protein band that represents ATPS (*ca.* 50 kDa). After removing the un-bound proteins with TBS, pH 7.4, any bound proteins were removed from the column by washing the column with DEA, pH 11.0 then glycine, pH 2.5 and then with TBS, pH 7.4. Fractions were collected after each buffer wash, concentrated and prepared for SDS-PAGE. The column was washed with TBS, pH 7.4, until no proteins were detected by western analysis with anti-ATPS IgG (figure 3.27 A, lane 2). However, after the wash with DEA, pH 11.0 (figure 3.27 A, lane 3), a number of protein bands are revealed. These include the two bands (*ca.* 50 kDa, 30 kDa) that correspond to the bands in the ATPS preparation (figure 3.26 A, lane 2), with additional bands with molecular masses of *ca.* 48 kDa, 45 kDa, 40 kDa and 28 kDa. Coomassie staining revealed only two proteins with molecular mass of *ca.* 50 kDa (figure 3.28, lane 3), likely to be ATPS, that had been washed from the column, and a *ca.* 40 kDa protein of unknown identity. No protein was detected in the eluate after the low pH wash (figure 3.27 A, lanes 4-5). After the elution process, an aliquot of the Sepharose column was subjected to western analysis with biotinylated anti-ATPS IgG and Coomassie staining. Western analysis with anti-ATPS IgG (figure 3.27 A, lane 6), showed light bands corresponding to proteins that appeared in the recombinant ATPS preparation (figure 3.26 A, lane 2). As well as a *ca.* 52 kDa protein was observed after the Coomassie stain (figure 3.28 A, lane 4), which probably represented the bound IgG. This suggested that a small amount of proteins within the ATPS extract remain bound even after the elution steps.

3.6.2 Western analysis with biotinylated anti-APSR IgG of eluates.

To detect if APSR bound to ATPS, western blot analysis with biotinylated anti-APSR was conducted. Observation of the western analysis revealed that the IgG did not recognise ATPS (figure 3.26 B lane 2), but did recognise two bands of molecular mass *ca.* 55 kDa (the expected mass of APSR) and 35 kDa in the chloroplast extract (figure 3.26 B, lane 4).

Fraction collected after the chloroplast extract had passed through the column (see section 2.17) showed a reduction in the concentration of these proteins.

No protein was detected by western analysis with anti-APSR when an aliquot of Sepharose was collected after the chloroplast extract was incubated with the column.

After elution with DEA, pH 11.0, the biotinylated anti-APSR IgG detected a number of proteins ranging from *ca.* 55 kDa to 30 kDa (figure 3.27 B, lane 3), including denser bands at *ca.* 52, 40, 35 and 30 kDa. No protein was detected in the eluate after the low pH wash (figure 3.27 B, lanes 4-5). Coomassie stain revealed two bands that had eluted after the DEA, pH 11.0, wash (figure 3.28, lane 3) with molecular masses of *ca.* 50 kDa and 40 kDa. One band of *ca.* 52 kDa was detected after the whole elution process.

Because the molecular weights are similar between ATPS and APSR, western analysis with biotinylated anti-APSR IgG was conducted on recombinant ATPS, recombinant APSR and the fraction after elution with DEA, pH 11.0 to compare the molecular weights of the protein bands (figure 3.29). These results showed that no bands correspond to the molecular weight of APSR, suggesting that APSR in the chloroplast extract was unable to associate with the recombinant ATPS protein when it was bound to the anti-ATPS IgG CN-Br Sepharose column.

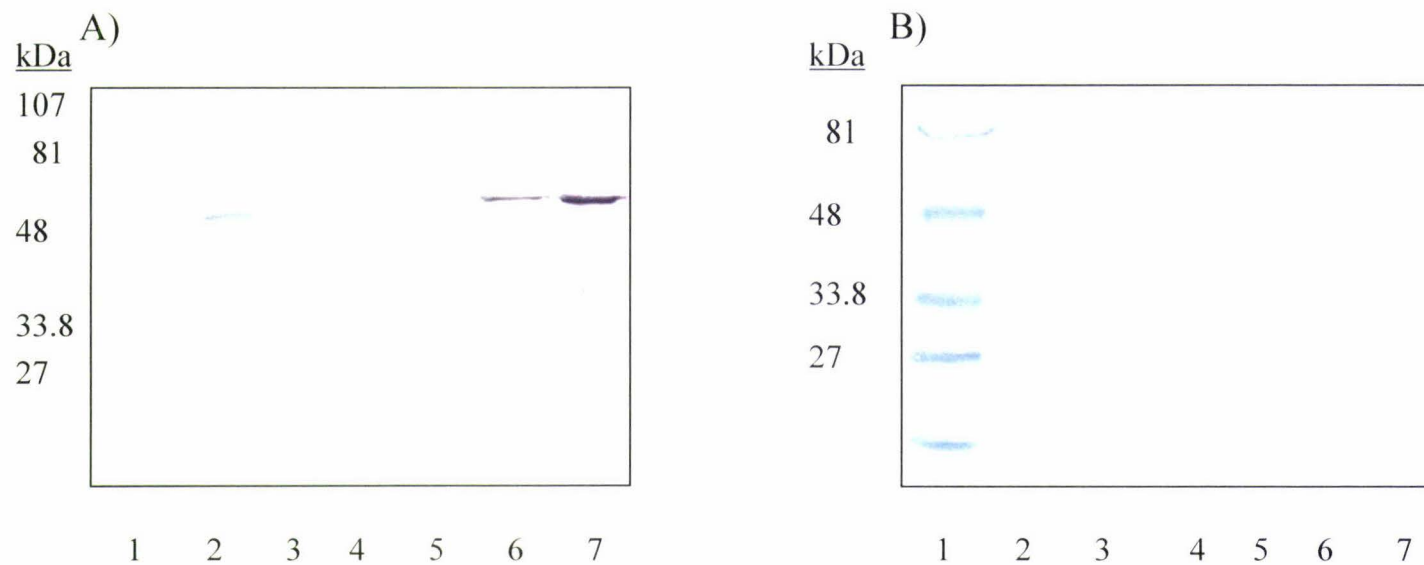


Figure 3.26: Western analysis of eluates from a CN-Br Sepharose coupled with anti-ATPS IgG column. Anti-ATPS IgG bound Sepharose was incubated firstly with recombinant ATPS, then a chloroplast extract, before the bound proteins were eluted from the column by a sequence of wash buffers. The eluate was collected and protein samples concentrated before proteins were separated using SDS-PAGE. A) Western analysis with the biotinylated anti-ATPS IgG. B) Western analysis with biotinylated anti-APSR IgG.

Lane 1: SDS protein standard

Lane 2: Recombinant ATPS prior to passage through column

Lane 3: ATPS after passage through the column for 2 hrs

Lane 4: Chloroplast extract prior to column

Lane 5: Chloroplast extract after passage through the column

Lane 6: Initial eluate after wash with TBS, pH 7.4

Lane 7: Sample of the Sepharose in SDS reducing buffer, after ATPS and the chloroplast extract have been passed through the column (but prior to treatment with DEA, pH 11.0 and glycine-HCl, pH 2.5).

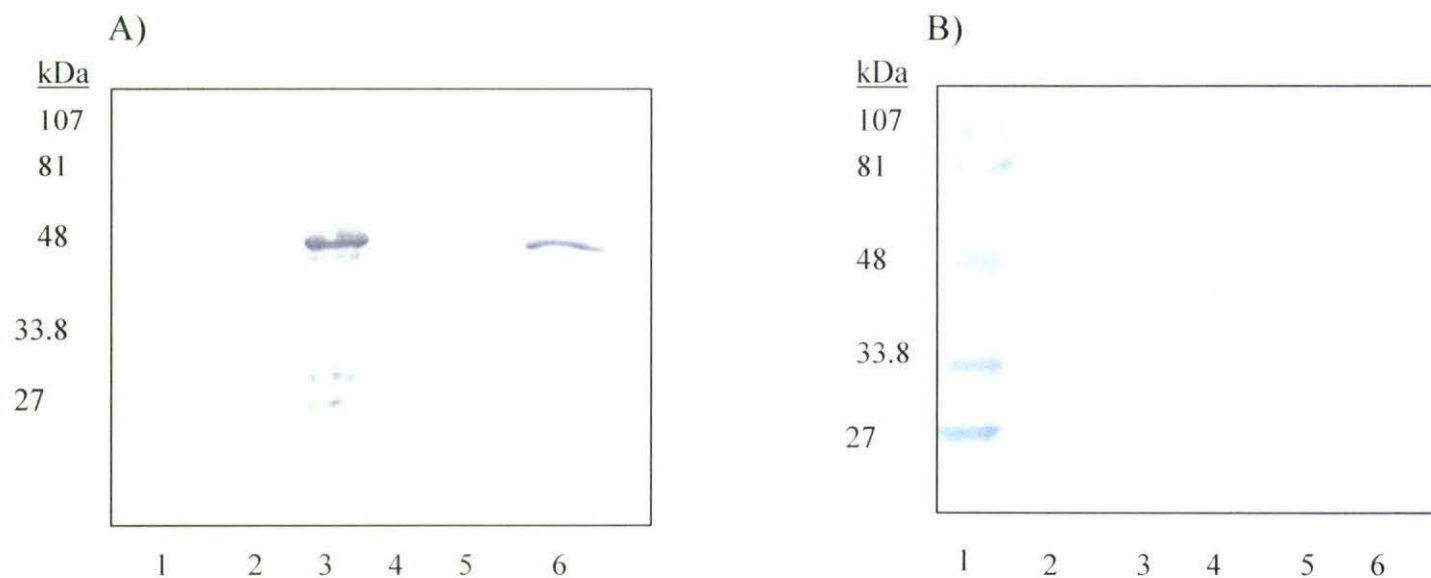


Figure 3.27: Western analysis of eluates from a CN-Br Sepharose coupled with anti-ATPS IgG column. Anti-ATPS IgG bound Sepharose was incubated firstly with recombinant ATPS, then a chloroplast extract before the bound proteins were eluted from the column by a sequence of wash buffers. The eluate was collected and protein samples concentrated before proteins were separated using SDS-PAGE. A) Western analysis with the biotinylated anti-ATPS IgG. B) Western analysis with biotinylated anti-APSR IgG.

Lane 1: SDS protein standard

Lane 2: Eluate after excessive wash with TBS, pH 7.4

Lane 3: Eluate after wash with DEA, pH 11.0

Lane 4: Eluate after wash with TBS, pH 7.4

Lane 5: Eluate after wash with glycine, pH 2.0

Lane 6: Sample of the Sepharose after elution steps in SDS reducing buffer

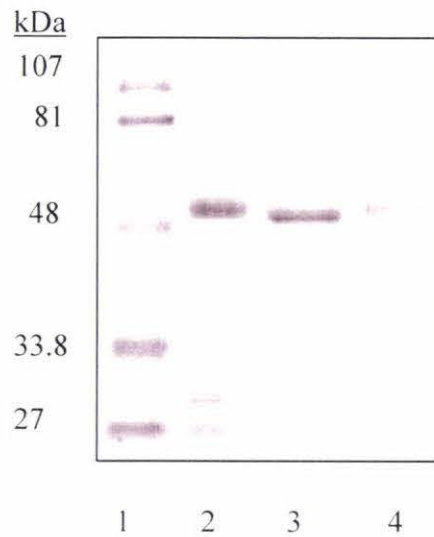


Figure 3.28: Eluates from a CN-Br Sepharose coupled with anti-ATPS IgG column revealed by Coomassie staining. Samples of the Sepharose were taken before and after the elution steps, as indicated.

Lane 1: SDS protein standard

Lane 2: Sample of the Sepharose after ATPS and the chloroplast extract have been passed through the column.

Lane 3: Eluate after wash with DEA, pH 11.0

Lane 4: Sample of the Sepharose after elution steps

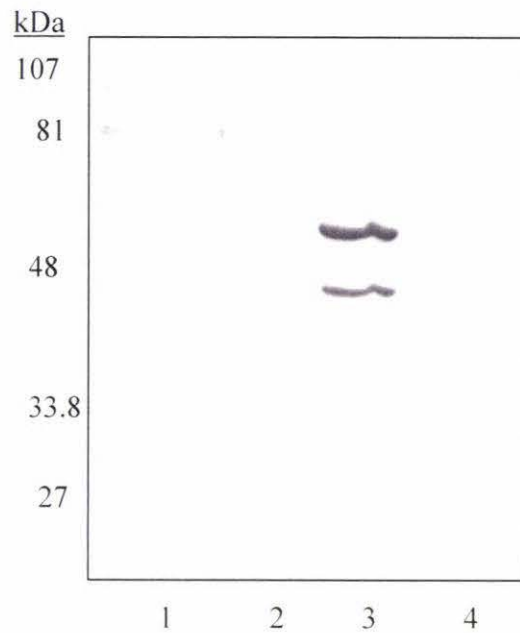


Figure 3.29: Western analysis with anti-APSR IgG of recombinant ATPS, APSR and the eluate of the CN-Br Sepharose column after the DEA, pH 11.0, wash. Western analysis was performed with biotinylated anti-APSR IgG.

Lane 1: SDS protein standard

Lane 2: Recombinant ATPS

Lane 3: Recombinant APSR

Lane 4: Eluate after wash with DEA, pH 11.0

3.7 Modulation of ATPS activity by sulfur supply

To determine if the ATPS enzyme undergoes any modulation of activity (either *via* complex formation or through post-translational modification), Lineweaver-Burk plots were used to determine the K_m values for PP_1 in chloroplast extracts from leaves of pre-bulbing (10-weeks after germination) and bulbing (18-weeks after germination) onions. These experiments were performed with chloroplast extracts from onion cultivars with a low pungency (Texas Grano) and a high pungency (W202A) grown in high and low sulfur conditions (see section 2.1.3). Observation of the pre-bulb data, show that Texas Grano with sufficient sulfur has a lower K_m value (0.038 mM) than Texas Grano grown in a low sulfur environment (0.92 mM) (figure 3.30 A). Comparison with the Texas Grano bulbing data, illustrates that under the low sulfur environment there is no change in K_m between the pre-bulbing and the bulbing chloroplast extracts (0.92 mM and 0.081 mM, respectively) (figure 30 A and C). However, for Texas Grano under high sulfur the K_m of ATPS increases, from 0.038 mM at pre-bulbing to 0.148 mM at bulbing, when there is a greater demand for sulfur during bulbing (figure 3.30 A and C). These results indicate in this cultivar, ATPS could be a point of regulation during sulfur starvation which is affected by sulfur demand and the formation of storage tissue (bulb).

The K_m values recorded for the onion line W202A, prior to bulbing, showed little difference between the two sulfur treatments (high sulfur 0.038 mM, low sulfur 0.073 mM) (figure 3.30 B). After bulbing, no significant difference was observed between the two sulfur treatments (high sulfur 0.44 mM; low sulfur 0.36 mM) (figure 3.30 D). Generally there was a slight increase in the K_m values in both sulfur treatments after the bulbs have formed, but it is unknown whether this difference is significant (figure 3.30 B and D). This data suggests that there is little regulation of ATPS, which will affect the K_m , during sulfur stress or transition to bulbing, in this onion cultivar.

However, a distinct difference is noted between the two cultivars. Texas Grano shows a noticeable affect by a high sulfur status whereas W202A is unaffected by the sulfur status. As well, the determined K_m values recorded are higher in Texas Grano than in W202A (figure 3.30 A, B, C, and D).

The chloroplast extracts used to determine the K_m values were used for western blot analysis with anti-ATPS IgG to estimate the relative quantity of ATPS protein in each sample (figure 3.31).

The blots showed two bands of *ca.* 50 kDa, the larger of the two, is likely to represent RUBISCO, the anti-ATPS IgG recognised this protein possibly due to the large quantities of the RUBISCO protein in the leaf extract. The recognition of RUBISCO allows a possible loading standard to which the abundance of ATPS can be compared by comparing the intensities of lower bands, it is possible to compare the levels of ATPS in the chloroplast extract. The amount of ATPS in the pre-bulb samples (figure 3.31, lanes 2-5) is lower than that in the bulbing samples (figure 3.31, lanes 6-9) in both cultivars. The ATPS levels in the pre-bulb samples are approximately the same between cultivars and sulfur levels (figure 3.31, lanes 2-5). However, in the post-bulb samples grown in deficient sulfur (figure 3.31, lanes 7 and 9) a higher level of ATPS protein is observed when compare to those grown in sufficient sulfur (figure 3.31, lanes 6 and 8).

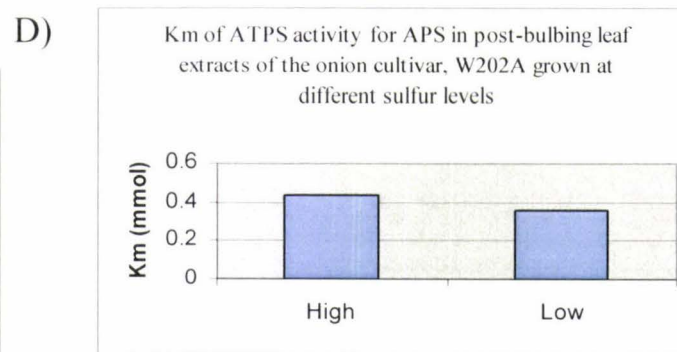
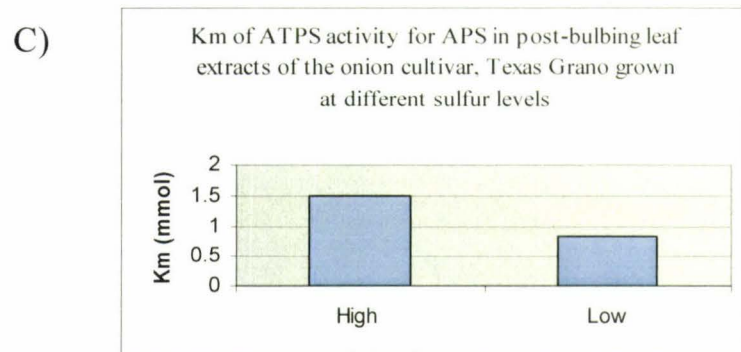
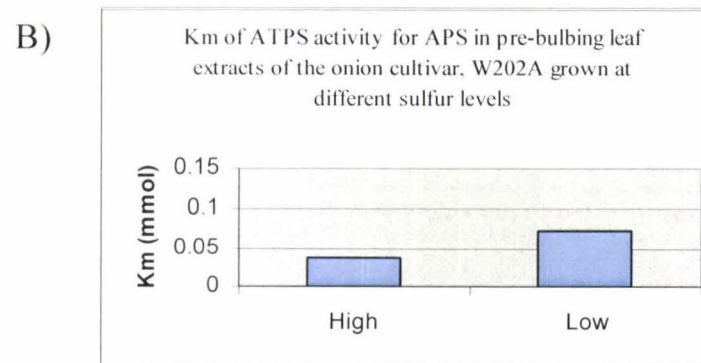
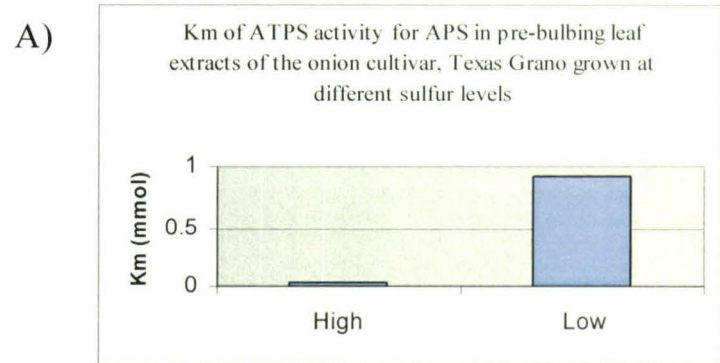


Figure 3.30: K_m values for ATPS of two cultivars of onion, under two sulfur treatments before and after bulbing.

Activity of the reverse reaction of ATPS was determined with various PP_i concentrations, and the K_m values for ATPS for PP_i were determined from Lineweaver-Burke plots. Results are mean values of 2 replicates.

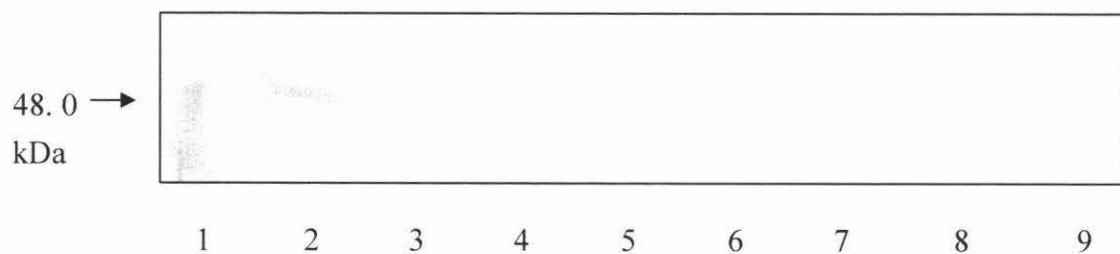


Figure 3.31: Western analysis of chloroplast extracts from onion.

Chloroplasts were isolated from either *A. cepa* varieties Texas Grano or W202A, grown hydroponically with sufficient or deficient sulfur. Plant samples were taken at two time points, before (10 weeks after germination) and at bulbing (18 weeks after germination).

Lane 1: SDS protein standard

Lane 2: Pre-bulb, Texas Grano, sulfur sufficient

Lane 3: Pre-bulb, Texas Grano, sulfur deficient

Lane 4: Pre-bulb, W202A, sulfur sufficient

Lane 5: Pre-bulb, W202A, sulfur deficient

Lane 6: Post-bulb, Texas Grano, sulfur sufficient

Lane 7: Post-bulb, Texas Grano, sulfur deficient

Lane 8: Post-bulb, W202A, sulfur sufficient

Lane 9: Post-bulb, W202A, sulfur deficient

4.0 Discussion

4.1 Part 1: Characterisation of the activity of recombinant of ATPS and APSR

For most experiments in this thesis, either recombinant ATPS or APSR was required. Therefore, it was important to maximise the yield of the protein obtained, from and to minimise the quantity of proteins that were incorporated into inclusion bodies. This occurs when the *E. coli* host is unable to facilitate the post-translational modifications required for correct folding of the protein (Baneyx and Mujacic, 2004). To avoid this, the *E. coli* cells were grown at a temperature sub-optimal for *E. coli* (less than 37 °C). However, if this temperature is too low, the growth of the cells will be restricted, resulting in a reduction in protein expression. Thus it is important to reduce the temperature during induction of recombinant protein expression, but only to a point where sufficient protein expression can still occur.

For maximum yield of proteins, the *E. coli* cells were incubated with IPTG at 25 °C for GST-fused ATPS protein and 20 °C for the GST-fused APSR protein (see section 3.1). This resulted in maximum yield of APSR or ATPS as soluble proteins (i.e. accumulation of the periplasm of the *E. coli* cell rather than being incorporated into the inclusion bodies).

4.1.1 Purification of recombinant proteins

Batch purification with GS4B-Sepharose resin resulted in sufficiently purified full length APSR protein (*ca.* 55 kDa). However, an additional protein with a molecular mass of *ca.* 45 kDa was routinely identified by Coomassie staining after SDS-PAGE. N-terminal sequencing of the proteins confirmed that the *ca.* 55 kDa protein was the full length APSR and that the *ca.* 45 kDa protein was a truncated product of the full length protein, in which the first 76 residues had been removed from the N-terminus (see section 3.1) (McManus, M. T. *pers. comm.*). It is unlikely that the PreScission™ Protease is responsible for this truncation since the *A. cepa* APSR nucleotide sequence does not contain the PreScission Protease™ cleavage recognition sequence (Leu-Glu-P₄-Leu-Phe-Gln/Gly-Pro where P₄ is Val, Ala, or Thr and ‘/’ represents the

cleavage site) (Cordingley et al., 1990). It is possible that the PreScission™ Protease cleaved the APSR protein at an alternative cleavage site on the APSR protein.

However, only a single protein of a molecular mass of *ca.* 30 kDa (which probably represents the GST) (data not shown) was observed after the addition of glutathione to the GS4B Sepharose. The addition of glutathione after the PreScission™ Protease step removes the GST tags from the GS4B Sepharose.

The purification of recombinant ATPS by batch purification with GS4B Sepharose resulted in the purification of proteins identified by N-terminal sequencing to be ATPS, and chaperone proteins, including heat shock protein 60, and heat shock protein 70 (McManus, M.T. *pers. comm.*). The occurrence of the *E. coli* heat shock proteins occurred most prevalently during the induction of ATPS. It is not observed during the induction of APSR (this thesis) or when other enzymes in the reductive sulfur assimilation pathway have been produced in the same strain of *E. coli* (e.g. *O*-acetyl serine (thiol) lyase, serine acetyltransferase; Leung, S. *pers. comm.*).

Homologues of the ATPS enzyme exist in *E. coli* (Leyh et al., 1992; Murillo and Leustek, 1995), and it may be that the host chaperone proteins recognise the plant ATPS as they are induced at 25 °C.

To isolate pure ATPS, ion exchange chromatography was employed. In the absence of MgCl₂ in the buffer, elutions containing ATPS were difficult to isolate from the Mono Q column (data not shown). However, if MgCl₂ was added to the buffer, ATPS was unable to bind to the column and was collected within the first fractions collected (figure 3.4). The effect of MgCl₂ on ATPS is unknown, but it is possible that Mg²⁺, which is known to be important as a co-factor of the ATPS reaction (Renosto et al., 1993) binds to ATPS and induces a conformational change, which inhibits column binding.

4.1.1.1 *Storage of recombinant ATPS*

During the development of the ATPS assay it was observed that the recombinant ATPS isolated by PreScission Protease™ treatment of the Sepharose, lost activity. For purpose of this thesis, it was important to determine the storage capabilities of recombinant ATPS. To do so ATPS enzyme activity was measured at two temperatures over 10 days. Observations revealed that the activity remained highest when stored at 4 °C. The loss of activity at -20 °C is probably due to repetitive

freezing and subsequent thawing, which is commonly attributed to loss of activity. ATPS activity was also tested after further purification by ion exchange chromatography. The results showed that the ATPS activity reduced to almost zero after the ten-day period at both 4 °C and at -20 °C (figure 3.5). It is possible that the chaperone proteins of *E. coli* origin, which are removed by the FPLC process, maintained the stability of the protein during storage. This may have been achieved by the chaperone proteins associating with the ATPS protein and facilitating correct folding and preserving the native state of the protein (Baneyx and Mujacic, 2004). Further, the presence of MgCl₂ (added during the FPLC purification) may result in the ATPS proteins observed decrease in activity during storage after FPLC (figure 3.5). Previous work has shown that high concentrations of MgCl₂, above 10 mM MgCl₂, inhibits the activity of ATPS in spinach leaves (*Spinacia oleracea*) (Shaw and Anderson, 1974), but this is unlikely to cause the inhibitory effect seen here, as 5 mM MgCl₂ was used in the assay. The addition or removal of salts including MgCl₂ and NaCl (which was removed prior to FPLC), has been shown to influence the stability of the protein (Kohn et al., 1997; Record et al., 1998). It is unknown whether ATPS stability is affected by NaCl or MgCl₂, but it may be a contributing factor to the loss of activity observed. Therefore, as a precaution, the protein was made fresh each time experiments were conducted.

4.1.1.2 Activity of recombinant APSR

In contrast to ATPS activity, recombinant APSR protein lost activity very rapidly after removal from the GS4B resin. The purification process for recombinant APSR that was employed featured binding the recombinant protein with a GST tag onto GS4B resin, which has a high affinity for GST tags. To remove the APSR protein, PreScission™ Protease was added and after a period of incubation, the resin was centrifuged and the supernatant, containing APSR protein, was collected.

To minimise any APSR activity lost during purification, samples were taken at hour intervals after protease was added (figure 3.6). During this period the APSR specific protein activity reduced significantly, over 90 % within the first hour. This decrease in activity loss was much more rapid than shown previously (Suter et al., 2000; Kopriva et al., 2001), and maybe due to reduction in the stability of the recombinant APSR from *A. cepa*.

It is possible that the folding of the onion APSR protein in the cytosol of *E. coli* was incorrect as APSR protein processing in plants occurs in the chloroplasts (Prior et al., 1999). Previous work on APSR have also reported a loss in protein activity during storage of APSR protein (Kopriva et al., 2001). Studies with recombinant APSR isolated from *Lemna minor*, showed significant reduction of activity after 5 days of storage at 4 °C. As well, APSR protein was observed to be unstable in extract of *L. minor* where it was observed that 50% of activity was lost within 6 hrs. It was noted that the specific activity of APSR could be maintained if 5.0 mM 5'-AMP was added to the protein extract (Suter et al., 2000), although this was not attempted as part of this thesis.

APSR activity has been observed to be related to the retention of iron bound as part of a [4Fe-4S] cluster (Kopriva et al., 2001). So, to increase the specific activity of recombinant APSR, it became routine to add $(\text{NH}_4)_2 \text{Fe} (\text{SO}_4)_2$ to the LB liquid media (total concentration 0.01 mM) during the IPTG induction of the recombinant protein (Kopriva, *S. pers. comm.*).

The observed results indicate that APSR activity assays with recombinant enzyme must be done at least within the first 3 hrs after the addition of PreScission™ Protease. To increase the stability further, it has also been suggested that the production of the protein should be done in anaerobic conditions (Kopriva, *S. pers. comm.*), but this was not tried as part of this thesis.

4.1.2 The effect of glutathione on the activity of recombinant proteins

4.1.2.1 *The effect of glutathione on ATPS activity*

Glutathione (γ -Glutamylcysteinylglycine) is a tripeptide that consists of the amino acids glutamine, cysteine and glycine. It is present in two forms, reduced and oxidised, with the reduced state maintained by a NADPH-dependent glutathione reductase (Noctor et al., 1998).

The synthesis of glutathione occurs in the chloroplast (Hell and Bergmann, 1990; Rueggsegger and Brunold, 1993) and is dependent on the availability of precursors, in particular reduced sulfur (Meyer and Fricker, 2002). Glutathione is known to be the signal for many stress responses, where one function is to regulate metabolic

pathways in response to environmental influences including high light, drought or heavy metal exposure (Danon and Mayfield, 1994; May et al., 1998). Because glutathione is an end product of the sulfur assimilation pathway, its localisation and its importance in the plant system, makes the compound a likely candidate as a feedback regulator.

Glutathione levels mimic those of SO_4^{2-} in the plant cell, where under sulfur deprivation glutathione levels will drop. Glutathione is a mobile compound in the plant, which can be found and transported in the phloem sap of plants (Rennenberg et al., 1979; Schneider et al., 1994). This mobility makes glutathione a suitable indicator and signal molecule for regulation of sulfur assimilation.

The effect of glutathione on ATPS mRNA expression, protein level and activity varies between species. It is reported that when Poplar (*Populus tremula* x *P. alba*) was transformed with γ -glutamylcysteine synthase, the cytosolic glutathione levels increased. However, this had no effect on ATPS mRNA or protein activity (Hartmann et al., 2004). Further, externally applied glutathione (0.5 mM) to *Arabidopsis thaliana* root cultures had no effect on ATPS protein accumulation or enzyme activity (Vauclare et al., 2002). However, supplying higher concentration of glutathione (1.0 mM) to root cultures of *A. thaliana* and canola (*Brassica napus* L. cv *Drakkar*) did result in a reduction of ATPS mRNA transcript, protein accumulation and enzyme activity (Lappartient and Touraine, 1996, 1997; Lappartient et al., 1999). The only report of ATPS regulation in monocots shows that cysteine, a precursor of glutathione, reduces the abundance of ATPS transcripts in maize (*Zea mays*), independent of glutathione (Bolchi et al., 1999).

In this thesis, to test whether ATPS activity is regulated by glutathione, activity was measured in the presence of both reduced glutathione and oxidised glutathione. A range of glutathione concentrations was used, between 1.0 mM and 5.0 mM which corresponded to the levels expected in the plant cell (Fricker et al., 2000).

ATPS activity was recorded to be between 0.09 to 0.12 $\mu\text{mol}/\text{sec}/\mu\text{g}$ (figures 3.5 and 3.24), but activities as high as 0.2 $\mu\text{mol}/\text{sec}/\mu\text{g}$ were observed in some cases (figure 3.5). At low concentrations of glutathione (1.0 mM) there was little to no effect on ATPS activity (figure 3.24) which is inconsistent with previous findings in *A. thaliana* and canola (*Brassica napus* L. cv *Drakkar*) (Lappartient and Touraine, 1996, 1997; Lappartient et al., 1999). These reports showed a significant reduction in activity with

1.0 mM glutathione. However, by increasing the glutathione concentration to 2.5 mM, the ATPS protein activity reduced to 90 % of the control (*ca.* 0.01 $\mu\text{mol}/\text{sec}/\mu\text{g}$). Further increases in the glutathione concentration resulted in a complete loss of activity. Since the glutathione levels are higher in *A. cepa* than in other species (Hartmann et al., 2004), a reduction in the sensitivity to glutathione would support the use of glutathione as a signal molecule in the regulation of ATPS.

Comparisons between the reduced and oxidised glutathione showed no difference in the regulatory control on ATPS (figure 3.24), which is consistent with other reports that show that the redox state of glutathione is not responsible for regulation of ATPS activity (Lappartient and Touraine, 1997). This also reinforces the demand driven theory proposed in previous studies (see section 1.5.2).

It is important to note here that the experiments conducted in this thesis with the recombinant ATPS represents only one ATPS isoform. It is known that this isoform is present in the chloroplast (McCallum, *J. pers. comm.*) but it is also possible that other isoforms of ATPS are present in *Allium cepa* and these may be regulated in a different manner by glutathione. However, only one isoform has been identified using an *Allium cepa* EST library (McCallum, *J. pers. comm.*).

4.1.2.2 *The effect of glutathione on APSR activity*

Glutathione, as discussed previously, is an indicator of sulfur status, and has been shown to decrease APSR activity according to some studies (Bick et al., 2001; Vauclare et al., 2002). However other studies have postulated that this is not always the case. For example, in the *Arabidopsis thaliana* mutant, *cad2*, which has reduced total glutathione content, no change in APSR activity is observed (Howden et al., 1995). In poplars (*Populus tremula* \times *P. alba*) transformed with γ -glutamylcysteine synthase which induces a 3 to 4-fold increase in glutathione levels, no difference in APSR activity was observed (Hartmann et al., 2004).

In this thesis, the activity of a recombinant APSR protein was measured in the presence of oxidised and reduced glutathione, at concentrations shown previously to inhibit APSR in other plant species (Lappartient and Touraine, 1996, 1997; Lappartient et al., 1999; Bick et al., 2001; Vauclare et al., 2002; Hartmann et al., 2004). Over the range of glutathione concentrations tested, there was no significant

difference in the activity of the APSR measured (figure 3.25). Reduced glutathione consistently decreased the APSR protein activity more than oxidised glutathione, but these differences were not significant (figure 3.25) indicating that the recombinant APSR is not regulated by the redox state of glutathione. Because the sequence is based on the APSR sequence identified in onion (*Allium cepa*), these results may give an indication on the regulation of APSR *in planta* in onion cells. In assessing the effect of glutathione on APSR activity, the cellular concentration of glutathione must be assessed. The concentrations of glutathione in the cell vary between species. In *A. thaliana*, the cellular glutathione concentration was estimated to range between 2-3 mM (Fricker et al., 2000; Meyer and Fricker, 2000; Meyer et al., 2001), where in Poplar, the glutathione levels are believed to range between 0.2-0.3 mM (Hartmann et al., 2003). It is possible that these differences in glutathione levels may result in different responses to glutathione, and may explain why no reduction on APSR activity was observed with the recombinant APSR from onion.

Before bulbing, the measured level of glutathione in onion is 300-350 nmol g⁻¹FW which is almost twice the amount measured in poplar (Hartmann et al., 2004), although after bulbing in onion, the level of glutathione decreases to between 70-180 nmol g⁻¹FW (Shaw, M. *pers. comm.*). It is possible, therefore, that APSR from *A. cepa*, is not regulated by glutathione, because of the high content within the cell at all times resulting in the compound being an unsuitable signal for sulfur status. It is also possible that because onion cells have a higher concentration of glutathione, the glutathione concentrations tested were too low.

The data obtained in this thesis contradicts that found in other species (Lappartient and Touraine, 1996, 1997; Lappartient et al., 1999; Bick et al., 2001; Vauclare et al., 2002; Hartmann et al., 2004). This observed difference may be due to differences in the regulation of sulfur assimilation in *A. cepa* and more particularly, this isoform of APSR. There have been accounts of APSR enzyme activity regulated by glutathione, particularly reduced glutathione. Exogenously applied reduced glutathione to poplars (*P. tremula*), resulted in a 50% reduction in activity (Hartmann et al., 2004). In *A. thaliana* root cultures, application of 0.2 mM reduced glutathione resulted in a decrease to an activity of 25% of the control (Vauclare 2002).

To investigate the importance of the redox state of glutathione, Bick *et al.* (2001) measured the activity of recombinant APSR in the presence of a range of thiols including oxidised glutathione and reduced glutathione. They found that reduced

glutathione decreased the APSR activity by 25%. Whereas APSR, with low activity due to a reductive environment, recovered after oxidised glutathione was added. They suggested that the oxidative environment post-translationally activated the APSR protein, possibly by the formation of a disulfide bond. Here it was suggested that disulfide bonds form between two pairs of cysteine residues at C202/C203 and at C385/C388, which are responsible for the regulatory control of APSR. In *A. cepa*, only the cysteine pair at C385/C388 is present in APSR. The absence of the cysteine pair at C202/C203 may result in APSR being unable to form the disulfide bond, which could account for the lack of control by oxidised glutathione.

In *A. thaliana*, APSR forms a homo-dimer estimated to have a molecular mass of 91 kDa, which comprises of two monomers each with a molecular mass of 43 kDa believed to be joined by a disulfide bond (Suter et al., 2000; Kopriva and Koprivova, 2004). It is possible that APSR activity is regulated by converting the APSR protein dimer to a less active monomer, by reducing the appropriate cysteine bonds, possibly regulated by the redox state of glutathione. If the APSR activity measured in this thesis were that of the monomer, then added glutathione would not have any effect. However, it might be expected that reduced glutathione should have increased the activity, perhaps by mediating formation of the homo-dimer. Or, it is possible that another component may be involved in the formation of the disulfide bond.

Further studies to identify other complex-mediating components as a mechanism for regulation should be explored. These may include other molecules which have been suggested in the regulation of sulfur assimilation proteins. These include; cysteine, shown to control activity of sulfate transporters and ATPS in maize (Bolchi et al., 1999); *O*-acetyl-serine (*OAS*), which induces activity of APSR in *L. minor* (Neuenschwander et al., 1991). These components may play a more important role in regulation of activity of APSR or ATPS of *A. cepa* through the mediation of a protein complex.

4.2 Part 2: Detection of a protein complex between ATPS and APSR from onion (*Allium cepa*).

4.2.1 ELISA-based method for protein-protein complex detection

To investigate the presence of a possible protein complex between ATPS and APSR, the ELISA method was used. This method has been used previously to illustrate the interaction between the eukaryotic initiation factor and eukaryotic binding proteins in human myoplasts (Kimball et al., 2004)

The method is depended upon the coated protein being able to bind to the micro-titre plate efficiently.

Proteins are able to bind to plate due to charges that exists on the surface of the protein. Thus pH is an important factor for binding, as pH is able to neutralise the charge and thus alter the binding affinity to the micro-titre plate. Because of this, APSR was bound to micro-titre plates at two different pH values, pH 9.4 and pH 7.4. Comparisons made between the binding affinities of APSR at each pH, showed little to no difference (see section 3.2.1.1). For the accommodation of a protein-protein complex, the amount of protein coated on the plate needs to be less than saturated, thus, theoretically, allowing room for any protein partners to associate. If the concentration of the coated protein is too high, then complexes may be prevented from forming through steric hindrance. Saturation of coated APSR was achieved at a concentration of 1 $\mu\text{g} / 100 \mu\text{l}$ (figure 3.8). Therefore, to accommodate the formation of any protein-protein complex, a concentration of 0.7-0.8 $\mu\text{g} / 100 \mu\text{l}$ APSR protein was used.

A second important factor in the success of this method is the specificity of the antibodies. The ATPS antibodies had a higher specificity than APSR to their corresponding proteins. Therefore APSR was chosen to coat the microtitre plate. To ensure that cross-reactivity of the antibodies is kept to a minimum, antibodies specific to ATPS were selected which recognised ATPS and not APSR. For this thesis, sera and isolated IgG from three rabbits, immunised with ATPS were available (designated K22, K34 and H21) and so the affinity of each to APSR was determined (see section 3.2.1.3). The comparison, using the ELISA method, revealed that IgG designated H21 and sera designated K34 (figure 3.9) were both appropriate, as they exhibited low affinity for the bound APSR.

It is difficult to determine if the antibody interaction observed is due to a specific interaction to ATPS using this method. It is possible that the anti-ATPS, or other antibodies within the sera, recognise other proteins in the protein extract particularly of *E.coli* origin. However, further testing of the antibodies by western analysis did indicate that the antibodies had a high affinity for ATPS (figure 3.12).

4.2.1.1 *The pH dependence of protein complex between ATPS and APSR*

To determine if the pH of the environment is important in the formation of a protein complex, the ELISA experiment was conducted using buffers of two different pH values (pH 7.4 and pH 9.4). Previous reports have used a similar method (the enzyme linked sorbent assay) to demonstrate the pH dependent interaction between gap junction protein channels, connexins and the SH3 domain of c-Src in mammals (Duffy et al., 2003).

Observation of the results from the ELISA experiment at pH 7.4 show an increase of absorbance as more ATPS was added until 1 μg of ATPS was added (see section 3.10). At this amount, an apparent point of saturation was reached. To ensure that the interaction is specific between ATPS and APSR, additional ELISA experiments were conducted to ensure the absorbance change observed (figure 3.10), was not due to the ATPS associating with the proteins within the blocking solution. These results revealed that ATPS was unable to associate with proteins within the blocking solution, verifying the complex detected was due to ATPS associating with APSR. The observed increase in absorbance up to the saturation point suggests that the ATPS protein is associating with APSR, possibly as a specific protein complex, and the apparent point of saturation at 1.0 μg suggests that the complex between ATPS and APSR has adopted a 1:1 stoichiometry.

It is a possibility that larger complexes were unable to form due to physical constraints, so it should not be discounted from these ELISA experiments that larger complexes form between ATPS and APSR. As a suggestion for further work, the ATPS protein could be coated and the APSR protein added. This experiment was not conducted in this study due to the low specificity of the anti-APSR antibody.

The ligand binding assay was also conducted as a method for detecting a protein complex. This method has been used previously to show that glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) and phosphoglycerate kinase (EC 2.7.2.3) enzymes in pea (*Pisum sativum*) can interact with each other (Wang et al., 1996). Using the ligand binding assay, evidence of a complex was detected when the buffer at pH 7.4 was used. This is consistent with the data obtained using the ELISA (see section 3.2) and in later immunoprecipitation techniques (see section 3.4).

It is possible that the bands observed (figure 3.14 lane 2) are due to non-specific interaction of the anti-APSR IgG recognising the ATPS, despite attempts to reduce this occurring (see section 3.3.1). However, the bands are only visible when APSR is incubated with the immobilised ATPS, suggesting that ATPS-APSR complex formation has occurred.

One restriction of this method is that immobilised ATPS was denatured during the SDS-PAGE. It is understood that proteins can partially refold with incubation with native buffer after immobilisation onto the PVDF membrane but it can't be assumed that this folding is correct. It is possible that APSR is unable to associate with the denatured state of ATPS with great efficiency or, alternatively, bind in a fashion that does not represent association *in vivo*. As an alternative the ligand binding assay could be performed using native gel electrophoresis and blotted onto the PVDF membrane in a native form. Using this method would reduce proteins associating to domains produced by incorrectly folded proteins.

Conducting the ELISA experiments with buffers at pH 9.4 (see section 3.2.2.1) resulted in no apparent complex formation, determined by an absorbance similar to the negative control (with no APSR present), indicating that ATPS is not associating with APSR at this pH (figure 3.10). The pH does not alter the amount of coated APSR, as APSR was found to bind to the microtitre plates with similar affinity at pH 9.4 as at pH 7.4 (see section 3.2.1.1). So, the observed results must be due to a reduction in the associated ATPS. This suggests that the association between ATPS and APSR is dependent on the pH of the environment. It is interesting to note that no association was detected when buffers at pH 7.8 were used in the ligand binding experiments, which indicates that the complex formation may have a sensitive response to pH (see section 3.3.2.2). This may indicate that the ATPS-APSR protein complex *in vivo* is transient, and under fine control. Protein complexes that form short-lived associations are often difficult to detect convincingly (Pettersson, 1991). However, such associations have been shown to redirect the flux of metabolites without the formation of a stable complex. For example, in the phenylpropanoid pathway in plants, phenylalanine ammonia lyase and cinnamate 4-hydroxylase were found not to form a stable complex but are able to change the metabolic flux away from phenylalanine towards p-coumarate *via* a transient association (Ro and Douglas, 2004).

The pH of the buffer used for the interaction will ultimately effect the electrostatic potential of the protein surface, and thus will contribute to the interactions between proteins and are significant factors in the formation of a protein complexes (Mauk et al., 1991; Muegge et al., 1998; Gong et al., 2000). It is important that the residues at the interface of association need to be in a specific ionisation state to mediate the formation of complex. For example if the charges on the interface of each protein are opposite, then it is possible that salt bridges can form and mediate protein-protein interactions (Drozdov-Tikhomirov et al., 2003). The pH dependent association between ATPS and APSR suggests that the complex may be formed due to electrostatic forces as a mechanism of interaction, and that the pH may play a role in the regulation of this interaction.

4.2.2 Detection of a complex using immunoprecipitation

4.2.2.1 *Development of method*

The immunoprecipitation method is based on the principle that specific antibodies are able to bind to a target protein or a protein complex containing the target protein. To isolate the complex (antibody, target protein and possible protein partner), the mixture is incubated with protein G Sepharose resin, which can specifically bind to the conserved domains of IgG, and then centrifugation is used to isolate the complex. A major factor in this method is the specificity of the antibodies. To test the specificity of the antibodies used in this thesis, western blot analysis was conducted. Initial screening of the serum showed that H21 (anti-ATPS), recognised three major bands *ca.* 95 kDa, 50.0 kDa and 45 kDa and a number of faint bands (figure 3.12 A-C, lane 2). To reduce the recognition of putatively non-specific bands, isolation of the H21 IgG was carried out, and this purified antibody recognised only one band of *ca.* 50 kDa, in a recombinant ATPS extract. Initial testing of the K33 (anti-APSR) serum, showed three major bands and a number of light bands (figure 3.12 D-F, lane 3) were recognised. After isolation of the IgG, two bands of *ca.* 55.0 kDa and 45 kDa were present. In some cases only the 55.0 kDa band is detectable. The size of this band is consistent with the estimated weight of the full-length APSR protein and the 45 kDa band is presumed to be the truncated form.

Initial experiments used the immunoprecipitation technique with protein G Sepharose to precipitate the antibody-protein complex and detection of the proteins by western analysis with anti-ATPS IgG and anti-APSR IgG (figure 3.18). Where APSR was included in the immunoprecipitation experiment (figure 3.18 B, lanes 3, 4, 6, 7), a faint band *ca* 55 kDa was detected. Also, in all immunoprecipitation experiments a dense band with a molecular mass of *ca*. 52 kDa was present. Further investigation found that when both K33 and H21 IgG which were used to immunoprecipitate the complex, were separated by SDS-PAGE they showed to have a molecular mass of *ca* 50 kDa. These IgG are able to be recognised by the anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase, used in the western analysis, since the IgG used are of rabbit origin. So due to this detection of the IgG that is removed from the protein G Sepharose as part of the SDS-PAGE, it was impossible to detect the presence or absence of ATPS in the western blot analysis with the anti-ATPS IgG. To help rectify this problem, an alternative secondary antibody system was employed. Strepavidin conjugated with alkaline phosphatase is able to recognise a biotin ester, which is conjugated to the IgG. With the strepavidin-AP, it was possible to distinguish between the biotinylated antibodies, which would be used in the western analysis and the IgG used in the immunoprecipitation method (which are unbiotinylated) (see section 3.4.1.3). Immunoprecipitation experiments were repeated and western analysis was conducted using the strepavidin-AP and the biotinylated IgG. Comparison of the western analysis showed that the strepavidin was unable to recognise the IgG used during the western analysis resulting in the detection of the ATPS protein (figure 3.19 A, lanes 2-4) present in the immunoprecipitation experiments. In immune-precipitation experiments with ATPS and APSR, the APSR protein was able to be immunoprecipitated from solution, and detected by western analysis (figure 3.19 B, lanes 3-4), suggesting that APSR was able to associate with ATPS. However, the negative control, where the immunoprecipitation experiment was conducted in the absence of IgG, showed that it was possible that both ATPS and APSR can bind to the protein G Sepharose without the specific IgG (figure 3.19 A, lane 7 and figure 3.19 B, lane 8). Because of this it is difficult to tell if the APSR that is detected, is present because it has formed a complex with ATPS, or it has bound to the protein G Sepharose.

To attempt to solve this problem, the immunoprecipitation method was altered to include an alternative resin to precipitate the complex; one that is designed to minimise the non-specific interactions that were observed with protein G Sepharose. The anti-ATPS IgG (designated H21), was chosen to be coupled covalently to CN-Bromide activated Sepharose 4B resin. This antibody-coupled resin was able to precipitate ATPS out of solution (figure 3.20 A, lanes 2-10), where the ATPS associates with the IgG and not the resin (as ATPS was shown to be unable to bind to the resin without the IgG coupled to the CN-Br Sepharose). The APSR protein could be immunoprecipitated with un-coupled Sepharose, suggesting that this protein was associating in a non-specific manner to the resin (figure 3.21). However, more APSR was able to be immunoprecipitated when anti-ATPS IgG-coupled Sepharose and ATPS protein were included in the incubation (figure 3.20 B, lanes 2-4). This suggests that at least a proportion of the recovered APSR was due to associations with ATPS. This comparison is based on the intensity of recognition by western blotting. To show this with more certainty, some qualitative measure of the amount of APSR precipitated would need to be developed.

The immunoprecipitation experiments were conducted using buffers at pH 7.4, as this was shown previously with other techniques to be an appropriate pH for protein complex between ATPS and APSR. However, incubation at other pH values were not tried, using this method.

Further immunoprecipitation experiments were conducted to ensure that the interaction observed is not due to non-specific protein-protein interactions. That is, that ATPS and APSR associate in a specific way. Using the immunoprecipitation technique, ATPS was shown to be unable to associate with the non-related plant proteins tested, ACC oxidase from apple (*Malus sp.*) (MD-ACO2) or Trypsin inhibitor from Soy bean (*Glycine max*) (SBTI) (figures 3.22 B and 3.23 B, respectively). This suggests that the association between ATPS and APSR is not due to non-specific binding of ATPS to APSR.

Western analysis with anti-MD-ACO after immunoprecipitation with MD-ACO2 and ATPS, revealed an intense band with a molecular weight of *ca.* 52 kDa in all lanes. This was due to the secondary antibody used, (anti-rabbit alkaline phosphatase) which was able to recognise the IgG that was bound to the Sepharose. However this was of no concern, as the MD-ACO2 protein has a molecular weight of *ca.* 35 kDa and can be assessed accurately.

4.3 Part 3: The effect of glutathione on mediating a protein complex between ATPS and APSR.

As of yet, there have been no record of glutathione participating in the mediation of complex formation. However thioredoxin, which like glutathione, is able to act as a reducing agent has been shown to be involved in the formation of protein complexes. For example, the complex between a heat-shock protein 70 chaperone and the co-chaperone heat shock protein 70-interacting like-protein in *A. thaliana*, has been shown to be sensitive to the redox state of each protein, which is regulated by thioredoxin (Vignols et al., 2003). Glutathione is necessary for the reduction of APS by APSR (reaction 1.2) and previous work has demonstrated that the association of reaction substrates can induce a complex formation. For example, the formation of the complex between cytochrome P450 and cytochrome P450 reductase is mediated by substrate binding (Paine et al., 2005). Taken together, such studies support the idea that glutathione may mediate complex formation in the reductive sulfur assimilation pathway, including between ATPS and APSR.

For the investigation of glutathione as a possible mediator of a protein complex between ATPS and APSR immunoprecipitation experiments were conducted in the presence of oxidised and reduced glutathione.

In these experiments, comparing the intensity of the bands on the anti-APSR western blots showed that the addition of oxidised glutathione increases the affinity of APSR to ATPS, suggesting that glutathione may play an important role in the formation in a complex between ATPS and APSR. The increase in the complex detected implies that oxidised glutathione plays a role in either attracting species within the complex or somehow stabilising the association between ATPS and APSR. However, when the immunoprecipitation experiment was conducted with reduced glutathione, it was shown to be more effective than oxidised glutathione for increasing the affinity complex formation. This suggests that reduced glutathione may play a bigger role in complex formation. In these experiments, it is difficult to say that the apparent increased complex formation is due to reduced glutathione specifically, or that the interaction was mediated due to the reductive environment, produced by the reduced glutathione. It is known that with complexes mediated by thioredoxin, the redox state was shown to be important in the control of complex formation between ribulose 1,5-

bisphosphate carboxylase-oxygenase and phosphoribulokinase in spinach (*Spinacia oleracea* L.) (Mulliert et al., 1993). However, in this thesis, because both forms of glutathione increased the formation of the complex, it is possible that the glutathione molecule is important for complex formation and that this is not dependent on the redox environment.

To further the investigation of the role of glutathione as a possible co-factor for protein complex between ATPS and APSR, the ELISA method was also used. Observation of the results showed that neither reduced and oxidised glutathione at concentrations between 0.1 mM and 10 mM, had any significant effect on the complex formation between ATPS and APSR (figure 3.11).

In the ligand binding assay conducted at pH 9.4 added glutathione promoted a very slight association. This may suggest that glutathione may play a role in mediating a complex in an unfavourable pH environment, but due to the detection method used it was difficult reach any conclusive results. Despite evidence of glutathione playing a role in increasing the detected ATPS-APSR complex using immunoprecipitation, the other methods do not support a role for glutathione in complex formation.

For further investigation into possible co-factors which mediate the formation of a protein complex, other compounds that have shown to regulate protein activities within the sulfur assimilation pathway, such as cysteine, (Bolchi et al., 1999) and *O*-acetyl-serine (*OAS*) (Neuenschwander et al., 1991) should be tested. It would also be interesting to investigate the role of 14-3-3 proteins. With a strong interaction between the sulfur and nitrogen pathways (Brunold et al., 2003; Hesse et al., 2004; Kopriva et al., 2004), it is suggested that the nitrogen level may be a candidate for mediating the formation of a protein complex between ATPS and APSR. Nitrate reductase (NR) (EC 1.7.1.1), a protein with a similar mechanism to APSR shows a similar regulatory pattern in response to carbon. Limiting sucrose also induces the formation of a protein complex between a 14-3-3 protein and NR, which then restricts the reduction properties of the enzyme and tags it for degradation (Comparot et al., 2003). The 14-3-3 protein has been shown to mediate protein activity of plasma membrane ATPases in *Nicotiana tabacum*, where binding of the 14-3-3 protein increases the ATPases activity by the formation of a hexamer (Kanczewska et al., 2005). The 14-3-3 protein can also act as a scaffold to recruit other proteins mediating a stable protein complex. Such phenomena is seen in animal systems between Raf-1 kinase and Bcr, where the complex will only form if a 14-3-3 protein

is present (Brasemann and McCormick, 1995). In wheat germ lysate, 14-3-3 protein associated to a phosphorylated motif on the transit peptide sequence of protein precursors. The complex also involves heat-shock protein 70 and improved the importation rate of protein precursors into the chloroplast and mitochondria (Maya and Solla, 2000). In plants there have been limited reports of 14-3-3 proteins as a scaffold, but up to 40 % of proteins contain the 14-3-3 binding consensus sequence RSXpSXP or RXXXpS/TXP (where R is arginine, X is any amino acid, S is serine, pS is phosphoserine and P is proline) (Moorhead et al., ; Yaffe et al., 1997; Sehnke et al., 2002). Of relevance to this study though is that within plants, proteins that include the 14-3-3 target sites include OAS-TL and ATP sulfurylase (Robert J. Ferl and Matt Reyes *pers. comm.*), leading to a possible role for 14-3-3 proteins in protein interactions between ATPS and APSR.

The APSR protein, due to the similarities with NR and the presence of 14-3-3 proteins in the chloroplast, may also be a candidate for associating with 14-3-3 proteins, either to mediate complex formation or as a regulatory mechanism. Although this will not be investigated in this thesis, it would be worth undertaking in further studies (Sehnke et al., 2000; Huber et al., 2002)

4.4 Part 4: Detection of a protein complex between ATPS and APSR in vivo using chloroplast extracts.

4.4.1 Immunoprecipitation of chloroplast extracts

After successful immunoprecipitation experiments with recombinant ATPS and APSR *in vitro*, it was important to attempt to detect such complexes *in vivo*. Earlier studies have noted that both ATPS and APSR protein are both localised in the chloroplast (Fankhauser and Brunold, 1978; Suter et al., 2000), so the immunoprecipitation assays were attempted with whole chloroplast extracts. In these experiments, recombinant ATPS was first incubated with the H21-conjugated CN-Br Sepharose, prior to the Sepharose being incubated with chloroplast extract. However, despite positive results with the recombinant APSR and immunoprecipitation, APSR in the chloroplast extract was not shown to associate with ATPS by western analysis (data not shown). Because only a small proportion of the proteins in the chloroplast are APSR, further improvements to the assay should be based around maximising the

concentration of any protein partners of ATPS that are recovered. These may include: (i) increasing the amount of ATPS-IgG coupled Sepharose, thus increasing the recoverable protein partners, (ii) increasing the protein concentration of the chloroplasts extract, which would improve the likelihood of the interaction occurring, (iii) using more sensitive detection mechanisms such as western analysis with horse radish peroxidase coupled secondary antibody and chemiluminescent detection system, or (iv) the use of 2D Blue native gel-electrophoresis, a new method that detects protein complexes that associate only weakly (Millar et al., 2004).

4.4.2 Detection of a protein complex between ATPS and APSR using an ATPS affinity column

As described in section 4.4.1, previous attempts to detect a putative complex between ATPS and APSR *in vivo* using chloroplast extracts of onion using the immunoprecipitation technique proved unsuccessful, so an alternative method was employed. Here, Sepharose conjugated to anti-ATPS IgG, was used to make an ATPS affinity column.

The principle of this method depends on successful binding of recombinant ATPS to the bound IgG. It has been determined previously using other techniques such as western analysis (figure 3.12) and ELISA (figure 3.9) that the anti-ATPS IgG is specific. Recombinant ATPS was first added to the column prior to the addition of the chloroplast extract, to ensure that the IgG associated with ATPS protein. The ATPS was shown to successfully bind to the column as a reduction in ATPS concentration in the extract after incubation was observed (figure 3.26 A, lanes 2-3) and ATPS was also detectable as being bound to the Sepharose after incubation (figure 3.26 A, lane 7).

A second important aspect of this method is that it is possible to elute bound proteins from the column. It is possible to elute bound ATPS from the column with at least 1 volume of DEA, pH 11.0, as this was shown by the detection of a number of proteins in the filtrate of the elution step including ATPS, which was identified by western analysis with anti-ATPS IgG (figure 3.27 A, lane 3). Thus a method was devised that allows sufficient bound ATPS, and efficient removal of bound ATPS proteins (and any putative proteins partners) from the column, resulting in an appropriate method for detection of a protein-protein complex that includes ATPS.

Once the ATPS was bound, the chloroplast extract was incubated with the column for 2 hrs. ATPS and APSR were both detected in the chloroplast extract by western analysis as protein bands of *ca.* 50 kDa and 55 kDa, respectively (figures 3.26 A, lane 4 and 3.27 B, lane 4). A reduction in ATPS concentration in the chloroplast extract was noted after the extract was passed through the column. A reduction was also observed in the APSR concentration as a result of incubation with the column, suggesting that at least a proportion of the APSR protein associated with the column either specifically to ATPS, or non-specifically, to the Sepharose.

Examination of a sample of the Sepharose after incubation of both ATPS and chloroplast protein extract indicated that not only had ATPS bound to the column (figure 3.26 A, lane 7) but other proteins of *ca.* 52 kDa and 33 kDa also had. The *ca.* 33 kDa protein was present in the ATPS preparation, eluted after the TBS, pH 7.4 wash, suggesting that this protein is weakly bound to the column (figure 3.26 A, lane 6). The *ca.* 52 kDa protein, detected by Coomassie blue staining (figure 3.28 lane 2 and 4) and by western analysis with biotinylated anti-ATPS IgG (figure 3.26 A, lanes 6 and 7, 3.27 A, lanes 3 and 6), is possibly IgG that was bound to the Sepharose, and is detected in the western analysis non-specifically by the biotinylated anti-ATPS IgG. The anti-ATPS IgG have been observed to have a molecular mass of *ca.* 50 kDa (see section 3.4.1.2) and so the presence of a protein with a molecular mass of 52 kDa in the eluate after a TBS, pH 7.4 wash (figure 3.26 A, lane 6), suggests that some of the bound IgG was removed. However, analysis of a sample of the Sepharose reveals that the majority remains bound (figure 3.17 A, lane 7).

Elution of the bound proteins with DEA, pH 11.0 resulted in the detection of a number of proteins by biotinylated anti-ATPS IgG and anti-APSR IgG. The proteins recognised by anti-ATPS IgG were of molecular weights of *ca.* 50 kDa, 48 kDa, 45 kDa, 40 kDa, 30 kDa and 28 kDa. Those proteins identified to have molecular mass of *ca.* 50 kDa and *ca.* 30 kDa, were also present in the recombinant ATPS preparation as ATPS and an unknown protein. However, proteins, other than the *ca.* 50 kDa and 30 kDa (found in the recombinant ATPS extract) that were also recognised by the biotinylated anti-ATPS IgG (*ca.* 48 kDa, 45 kDa, 40 kDa, 28 kDa) may represent possible protein partners with ATPS. Coomassie stain of this eluate revealed a protein of *ca.* 40 kDa, which was present in reasonable quantities and which is not observed in the recombinant ATPS preparation. These proteins that were revealed represent a group of unknown proteins that have either associated to the column in a

specific manner, associating to ATPS or, associating in a non-specific manner, either to unbound IgG, or to the Sepharose itself.

It is possible that these proteins represent a novel interaction with ATPS. Possible interactions include those with other proteins within the sulfur assimilation pathway. Previous attempts to identify a complex with ATPS included one with APS kinase, which was observed in *A. thaliana*. APSK has a molecular mass of *ca.* 23 kDa (Lillig et al., 2001), and in this thesis no protein at that mass was identified. However, both OAS-TL and SAT with a molecular masses of *ca.* 41.8 kDa (plastid isoform) (Hesse et al., 1999) and *ca.* 33 kDa (McManus et al., 2005), respectively (which are already known to form a protein complex with each other) (Bogdanova and Hell, 1997), could be possible candidates based on similar molecular masses to the proteins revealed in the DEA, pH 11.0 eluate (figure 3.27 A, lane 3). This could result in a multimeric protein complex incorporating a larger number of proteins within the same metabolic pathway such as complexes have been observed in the Calvin cycle, and the phenylpropanoid pathway in plants (Winkel, 2004). To identify these proteins more accurately it will be necessary to conduct western analysis with the appropriate antibodies or use sequencing methods such as N-terminus gas-phase sequencing or mass spectrometry.

The APSR protein was not detected by the biotinylated anti-APSR in the eluate of the DEA, pH 11.0, by western analysis (figure 3.27 B, lane 3), as no protein with a molecular mass of *ca.* 55 kDa was detected. This suggests that APSR was unable to bind to the column. The APSR was also unable to be detected in the sample after any of the elution steps, suggesting that if it did bind to the ATPS affinity column it would be expected to be eluted after the wash solutions (DEA, pH 11.0 and glycine, pH 2.5). A number of proteins were detected using western analysis with biotinylated anti-APSR IgG including the detection of the Sepharose bound IgG (figure 3.27 B, lanes 3 and 6). This was probably due to cross reactivity of the biotinylated anti-APSR IgG since all the protein bands are only very lightly stained after the addition of the western substrate. Direct comparison of the eluate after the DEA, pH 11.0 wash and recombinant ATPS and recombinant APSR by western analysis with the anti-APSR IgG, revealed that no bands in the eluate (figure 3.29, lane 4) corresponded to the molecular mass of the full length recombinant APSR (*ca.* 55 kDa) (figure 3.29, lane 3). However, the biotinylated anti-APSR was able to recognise recombinant ATPS at

a low background level (figure 3.29, lane 2). These results suggest that the APSR from the chloroplast was unable to bind to recombinant ATPS.

It is possible that the APSR was eluted using the low pH wash. Eluates of the glycine, pH 2.5 elution steps resulted in no detectable protein, but it is probable that during storage the proteins precipitated from solution, as a result of the particularly low pH. Lowering the pH is a common method employed to precipitate proteins. Such a phenomena may explain why proteins that were bound to the Sepharose, of molecular masses *ca.* 30 kDa and 27 kDa, were detected by Coomassie stain prior to the elution steps (figure 3.28 lane 2), but were not detected in either the DEA, pH 11.0 eluate (figure 3.28 lane 3) or in the sample of the Sepharose after both the elution steps (figure 3.28 lane 4). It is logical to think that these proteins eluted from the column after the glycine, pH 2.5 wash. Therefore, it could be conceivable that APSR protein eluted from the column at a low pH, and was subsequently precipitated from solution and thus are unable to be separated by SDS-PAGE, so therefore will not be detected by Coomassie staining or by western analysis. However, since ATPS eluted after the DEA, pH 11.0 wash, any APSR that eluted with the low pH wash, would have probably represented non-specific binding of APSR to the Sepharose.

Despite this, a number of unidentified proteins of *ca.* 48 kDa, 45 kDa, 40 kDa, 28 kDa, were detected that may represent possible protein partners to ATPS, leading to further work to identify these proteins.

Alternative methods to detect protein complexes *in vivo* could be employed for future work. These may include the use of bimolecular fluorescence complementation (BiFC), which is based on the association between two fragments of a fluorescent protein when they are brought together by an association between two proteins fused to the fragments. This method has been successful in *Nicotiana benthamiana* and *A. thaliana* (Bracha-Drori et al., 2004). Another method employs the use of TAP-tagged proteins. The TAP-tagged proteins, and any protein partners can be isolated by affinity chromatography (through the identity of the TAP-tag), and then the identity is determined by mass spectrometry. This method has been shown to be successful in detecting, *in vivo*, an interaction between Hsp70 and Hsp90 with the glucocorticoid receptor in *A. thaliana* (Rohila et al., 2004).

The sulfur status of the plant is known to have a marked effect on the control of sulfur assimilation, including the regulation of ATPS protein activity (Reuveny et al., 1980; Smith, 1980; Lappartient and Touraine, 1996; Takahashi et al., 1997; Yamaguchi et al., 1999). However, the mechanism has yet to be described. One possibility may be through the formation of a protein complex. In this thesis an attempt was made to identify complex formation between ATPS and APSR *in vitro*, and some evidence suggests that has been successful. However, attempts to show this complex formation *in vivo* were of limited success.

Evidence for protein complexes can also be gained when the kinetic properties of the putative protein partners are compared. Therefore, as another approach to look for evidence of ATP complex formation, the affinity (K_m) for inorganic phosphate of ATPS was measured.

In these experiments the K_m of ATPS for inorganic phosphate was measured for two cultivars of differing sulfur compound storage capacity, grown in either sufficient or deficient sulfur. These measurements were taken twice; firstly before bulbing had occurred, where the demand for sulfur is low, and again during bulbing, a period where the demand for sulfur is high due to the increased production of the sulfur containing flavour precursors, the ACSOs (Platenius and Knott, 1935).

The K_m values obtained were determined by measuring the reverse reaction of ATPS and would therefore reflect the K_m values of the back reaction. If this is the case, a high K_m value, would suggest that the back reaction is not favoured, therefore promoting the forward reaction.

These experiments assume that the levels of endogenous components that may affect the results of the ATPS assay, such as inorganic pyrophosphate, ATP, ATPases, NAD^+ reductases, were identical in all of the chloroplast extract samples.

4.5.1 Comparisons of the K_m of ATPS for PP_i in Texas Grano.

Texas Grano is known as a mild variety of onion, since it stores a proportionally lower amount of SO_4^{2-} as ACSO compounds when compared with other varieties (Pike et al., 1988).

It was observed that prior to bulbing the K_m value is considerably lower when the plant has adequate sulfur supply, than under low sulfur (figure 3.30 A). This suggests that the forward reaction of ATPS is more efficient in low sulfur conditions than in high sulfur. However, once the onion has bulbed, the K_m of ATPS when onions were grown in high sulfur increases from 0.038 mM (prior to bulbing), to 1.48 mM (during bulbing) (figure 3.30 A and C).

It could be thought that during bulbing, when there is a high demand for sulfur, and there is a high external supply of sulfur, that ATPS is up-regulated which increases the flux of sulfate to cysteine (the forward reaction) as is required for the formation of flavour compounds. The large difference in the K_m in this instance suggests a major change in the function of the protein, possibly a result of post-translational regulation. No significant change in K_m was observed between the pre-bulbing (0.92 mM) and post-bulbing (0.8 mM) when Texas Grano was grown in inadequate sulfur (figure 3.30 A and C). This suggests that the increase in K_m observed in adequate sulfur supply was dependent on not only an increase in sulfur demand during bulbing, but also because of the high sulfur supply.

Measurements of the specific protein activity show that prior to bulbing, in high sulfur, the activity is low, however after bulbing the activity increases (data not shown). The kinetic (K_m) data complements this, suggesting that the recorded changes in activity may be due to changes in the catalytic efficiency of ATPS.

Thus, the changes in catalytic efficiency may be due to the formation of a protein-complex with ATPS mediated by the sulfur demand.

4.5.2 Comparisons of the K_m of ATPS for PP_i in W202A.

The second cultivar examines, W202A, a line of onion bred for high pungency, due to the high level of sulfur containing flavour compounds stored in the vacuoles.

The K_m values determined for ATPS, prior to bulbing, showed no significant difference between the two sulfur treatments (high sulfur: 0.038 mM, low sulfur: 0.072 mM) (figure 3.30 B). Likewise, no difference was observed during bulbing in respect to the two sulfur treatments (high sulfur: 0.44 mM, low sulfur: 0.36 mM) (figure 3.30 D). These results show that in the W202A line of onion, the external sulfur conditions are unable to alter the K_m of ATPS for PP_i . Generally, the K_m values for pre-bulb are lower than calculated for post-bulb tissue, which may suggest that

control of K_m of ATPS, in W202A, is regulated more by the demand for reduced sulfate rather than the external supply (figure 3.30 B and D). However, more tests are needed to be conducted for the determination of the significance of these values.

The overall results suggest that W202A has lost the ability to regulate ATPS activity in response to either sulfur supply or sulfur demand. In onions, sulfur is stored in the vacuoles either as SO_4^{2-} or as accumulated ACSO compounds. The pungent varieties, such as W202A, partition more of the sulfur as ACSO compounds and so pungency is not due to an increase in sulfur uptake. Thus it is difficult to determine if the ATPS protein within W202A is primed (as determined by K_m) for high or low activity as there is poor correlation between pungency and the sulfur uptake (Randle, 1992; Randle and Bussard, 1993).

The K_m of ATPS measured in other studies in spinach (*Spinacia oleracea* L.) showed that the reaction is largely unfavourable, and the forward reaction is dependent on the swift removal of the products. Results gained in this study show the K_m of ATPS of onion ranges from 38 μM to 1.48 mM, which are higher to that shown in previous accounts which have shown that in spinach, the K_m for PP_i (the reverse reaction) to be between $10 \pm 2 \mu\text{M}$ – 16 μM using purified ATPS. I feel that these results are particularly high as I would have expected the K_m values to be lower because of the fact that onions are known as high sulfur accumulators. It should be noted that pure ATPS was not assayed in this thesis, and will have an impact on the accurate determination of the K_m .

In general, the K_m values for the back reaction were considered low compared to the K_m values determined for the forward reaction, due to a small calculated $E \cdot \text{APS}$ dissociation constant, which suggested that ATPS has a higher affinity for APS (Shaw and Anderson, 1974; Renosto et al., 1993).

Western analysis with anti-ATPS IgG can give an indication of the amount of ATPS in the chloroplast extract. Generally, the pre-bulb samples had less ATPS protein when compared to post-bulb onions (figure 3.31). This increase in ATPS protein levels could be a response to the high demand for sulfur during bulbing. However, no differences in ATPS transcript levels have been observed after bulbing in onions (McCallum, *J. pers. comm.*), which may suggest a higher protein half-life during the bulbing period. In terms of sulfur supply, the pre-bulb samples had a similar amount of ATPS protein despite the different sulfur conditions. However, the post bulb samples showed a higher ATPS protein levels in deficient sulfur. This report is

consistent with other reports in *A. thaliana* which demonstrated that ATPS protein levels increased during a period of sulfur deprivation (Lappartient et al., 1997). A likely signal molecule for demand-driven regulation is glutathione (Lappartient et al., 1999). It is known that during the development of the onion, the glutathione levels change. Prior to bulbing, the glutathione levels are measured to be between 300-350 nmol g⁻¹FW, but after bulbing has occurred, the glutathione levels drop to levels between 70-180 nmol g⁻¹FW (Shaw, M. pers. comm.). This decrease in glutathione levels after bulbing may relieve repression of ATPS activity. This repression by glutathione has been shown *in vitro* in this thesis (see section 3.5.1, figure 3.24), and this may act by a mechanism that alters the catalytic property of ATPS, and which may explain the differences between pre- and post- bulbing observed in the Texas Grano line. In this thesis it has been shown that glutathione probably does not mediate protein complex formation between ATPS and APSR *in vitro* (see section 3.2.2.4, figure 3.11) but it is possible that this is not the case *in vivo*. Complex formation has been shown in other systems to regulate K_m values. For example, in the Benson-Calvin cycle the K_m of the carbon fixing enzyme RUBISCO, when associated in a five-protein complex with other Calvin cycle enzymes, reduces from 0.140 mM ± 0.03 to 70 μM ± 6 (Gontero et al., 1994; Ricard et al., 1994). In contrast, the formation of a protein complex between two proteins within the sulfur assimilation pathway, OAS-TL and SAT form a protein-protein complex, where the formation of the complex reduces the catalytic efficiency of OAS-TL 7-fold, acting as a mechanism of control (Droux et al., 1998). In addition to protein-protein complexes, there are a number of other post-translational events such as phosphorylation and glycosylation, which could result in the change in K_m observed for ATPS, which need to be investigated further. To investigate if the formation of a protein-protein complex is a mechanism of control, ATPS activity assays were conducted *in vitro*, using recombinant proteins, which demonstrated that the formation of the ATPS-APSR complex resulted in a slight increase in ATPS activity. However, this difference could be explained by NADH produced by the APSR protein without ATPS present (figure 3.24 and table 3.1). This suggests that the ATPS-APSR complex does not alter the activity of ATPS *in vitro* and that it seems unlikely that the observed change in the K_m values in the Texas Grano line (figure 3.30), was not due to the formation of a complex between ATPS and APSR. It would be interesting to investigate the K_m of APSR using chloroplast extracts, and to

investigate the role, if any, of the ATPS-APSR complex on regulating the APSR activity.

It should also be noted that the ATPS activity measured may have been from several isoforms present in chloroplasts of onion. As of yet, only one isoform has been identified in an onion EST library (McCallum, J. *pers. comm.*). However, *A. thaliana* contains at least four isoforms of ATPS (three are localised in the chloroplasts) (Leustek et al., 1994; Klonus et al., 1995; Murillo and Leustek, 1995), suggesting that onion may have more than one. The response of ATPS isoforms to the external sulfur status, or to the demand for sulfur may differ between isoforms, as observed during the development of *A. thaliana* (Rotte and Leustek, 2000). To further this study, it would be required to investigate further isoforms and to determine each isoforms the response to sulfur status.

4.6 Conclusions

In this thesis, purification of recombinant ATPS and APSR was achieved, using a number of chromatography methods.

These purified proteins were used to identify a putative protein complex, using the *in vitro* techniques of ELISA, immunoprecipitation and ligand binding assay.

Characterisation of the complex determined that the ATPS-APSR complex was most stable at pH 7.4, and that glutathione may have a possible role in formation of the complex.

ATPS activity assays *in vitro* have shown that the formation of the complex had no impact on the activity of ATPS. However, glutathione, a suggested co-factor decreased the activity of ATPS in a dosage dependent manner.

In attempt to detect the ATPS-APSR complex *in vivo*, immunoprecipitation and an ATPS affinity column was employed and chloroplast extracts from onion were used. Unfortunately, despite success *in vitro*, the ATPS-APSR complex was unable to be detected. However a number of unknown proteins were observed using the ATPS affinity column and may represent other possible partners to ATPS.

In the light of other studies that have shown that the formation of protein complexes are able to modify the kinetic properties of proteins, the K_m values of ATPS were determined for PP_i of two onion cultivars (W202A and Texas Grano) with different ACSO storage capacity, grown in two sulfur treatments. Measurements were taken

prior to bulbing and during. Significant changes were observed in K_m values in one cultivar (Texas Grano) in response to the demand for reduced sulfur. It is possible that the change in kinetic properties of ATPS is due to formation of a complex with APSR. However, *in vitro* evidence collected as part of this thesis does not support this.

This thesis has provided evidence that ATPS and APSR do form a protein complex. However, this was only shown *in vitro* using recombinant ATPS and APSR. The ATPS-APSR complex was unable to be shown using chloroplast extracts. The formation of the protein-protein complex between ATPS and APSR gives a plausible explanation as to how the APS is directed towards the reductive pathway, despite the higher affinity towards the oxidative pathway. The ATPS-APSR complex could also channel APS to sulfite, thus reducing the negative effect of APS on the forward reaction of ATPS. However, these aspects will need to be investigated further. Despite the lack of evidence obtained *in vivo*, the results from the *in vitro* assays give grounds for further investigation into the formation of the ATPS-APSR complex *in vivo*.

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