The Purification, Characterisation and Stabilisation of a Soluble Pyroglutamyl Aminopeptidase from Bovine Brain

Thesis Submitted for the Degree of

Master of Science

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science is entirely my own work and has not been taken from the work of others save and to the extent that such work has been sited and acknowledged within the text of my work.

Signed: Ultan McKeon **Date:** 5/1/99

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Abbreviations

The following abbreviations are used throughout this text.

AA-NHS	Acetic acid N-hydroxysuccinimide ester
Acryl	Acrylamide
ATS	Activated Thiol Sepharose
BCA	Bicinchoninic acid
Bisacryl	Bisacrylamide
βNA	β-Naphthylamide
BSA	Bovine Serum Albumin
CNS	Central Nervous System
Da	Daltons
DEAE	Diethylaminoethyl
DEPC	Diehtylpyrocarbonate
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EG-NHS	Ethylene Glycol-bis(succinic acid N-hydroxysuccinimide ester)
Ex/Em	Excitation/Emission
HCl	Hydrochloric acid
H.R.	High Resolution
IAA	Iodoacetic acid
K_i	Inhibitor Dissociation Constant
λ	Wavelength
LHRH	Luteinizing Hormone Releasing Hormone
MCA	7-Amino-4-Methyl-Coumarin
NaCl	Sodium Chloride
N.D.	Not Determined
NEM	N-Ethylmaleimide
NHS	N-Hydroxysuccinimide
PAGE	Polyacrylamide Gel Electrophoresis
PAP-I	Pyroglutamyl Aminopeptidase type-I

PAP-II	Pyroglutamyl Aminopeptidase type-II
PDMK	pGlu-Diazomethyl ketone
PEG	Polyethylene Glycol
pGCK	pGlu-chloromethyl ketone
pGlu	Pyroglutamic acid
PMSF	Phenylmethylsulphonylflouride
pNA	p-Nitroanilide
SD	Standard Deviation
rpm	Revolutions per minute
RSH	Thiol Reducing Agent
S_2	Supernatant
SDS	Sodium Dodecyl Sulphate
SH	Sulphydryl
T3	Triiodothyronine
T4	Tetraiodothyronine
TEMED	N, N, N, N'-Tetramethyl ethylenediamine
TNBS	2,4,6 Trinitrobenzene Sulphonic Acid
TNM	Tetranitromethane
TRH	Thyrotropin Releasing Hormone
Tris	Tris(hydroxymethyl)amino methane
v/v	Volume per volume
w/v	Weight per volume
Z-	N-Benzyloxycarbonyl-
Z-pGCK	Z-pGlu Chloromethyl ketone
Z-pGDK	Z-pGlu Diazomethyl ketone

Amino Acid Abbreviations

Ala/A	Ala	Leu/L	Leucine
Arg/R	Arginine	Lys/K	Lysine
Asn/N	Asparagine	Met/M	Methionine
Asp/D	Aspartic acid	Phe/F	Phenylalanine
Cys/C	Cysteine	Pro/P	Proline
Gln/Q	Glutamine	Ser/S	Serine
Glu/E	Glutamic acid	Thr/T	Threonine
Gly/G	Glycine	Trp/T	Tryptophan
His/H	Histidine	Tyr/Y	Tyrosine
Ile/I	Isoleucine	Val/V	Valine

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Abstract

Cytosolic bovine brain pyroglutamyl aminopeptidase was purified from whole brain by chromatography with DEAE sepharose, G100 gel filtration and ATS 4B affinity chromatography. An overall recovery of 14.4% and a purifiaction factor of 247 was achieved.

The relative molecular mass of PAP-I, determined by SDS PAGE, was found to be 24,680 Da. The partially purified and purified enzyme was found to be relatively unstable under assay conditions. Of all the additives tested as stabilisers only BSA was found to stabilise the enzyme, during assay and during storage. A minimum concentration of approximately 0.1% w/v BSA was found to achieve optimum stabilisation.

PAP-I was confirmed to be a cysteine protease based on its sensitivity to IAA, NEM and iodoacetamide. The enzyme was also inhibited by DEPC and EDC, suggesting that His and Glu and/or Asp are also involved in events at the active site. These results were confirmed by the protective effect of the competitive inhibitor TRH on inactivation of PAP-I by these modifying reagents. Cys and His may constitute the nucleophilic and imidazole residues of the active site, while Asp or Glu might constitute the third part of the active site. Tyr, Lys, Arg and Ser are not involved in the expression of activity.

Initial chemical modification studies demonstrated that EG-NHS modified PAP-I showed almost a two fold increase in thermostability over the native enzyme. AA-NHS modified enzyme, did not show any increase in thermostability.

1. Introduction

1.1 Pyroglutamic acid

Pyroglutamic acid (pGlu), also known as pyrrolidone carboxylic acid (PCA, pyr) and 5-oxo-L-proline is a cyclical amino acid with unique properties. Although it can be formed non-enzymatically (Sanger *et al.*, 1955; Winstead and Wold, 1962), the enzymatic formation of pGlu from glutamic acid has been well established (Orlowski and Meister, 1971). This reaction is catalysed by D-glutamic acid cyclotransferase. It has been suggested that the role of this enzyme may be to detoxify D-glutamic acid, which can be formed by the intestinal flora, or introduced through the diet (Orlowski and Meister, 1971).

L-glutamine cyclotransferase, discovered in Papaya latex by Messer (1963), catalyses the conversion of glutamine and glutaminyl peptides to pyroglutamic acid and pyroglutamyl peptides respectively. This enzyme has not yet been found in animal tissue, however, the conversion of glutamine to pyroglutamic acid by a two step enzyme catalysed reaction has been observed. Glutamine can be converted to γ-glutamyl-glutamine by the enzyme γ-glutamyl transpeptidase and subsequently converted to pyroglutamic acid by γ-L-glutamyl-cyclotransferase (Orlowski *et al.*, 1969). This enzyme is widely distributed in animal tissues with the highest levels of activity being found in the brain. Orlowski and Meister (1971) suggest that this wide distribution implies significance for this enzyme. A mammalian glutaminyl cyclase, capable of converting glutaminyl peptides into peptides was identified by Fischer and Spiess (1987).

The enzymatic synthesis of pGlu suggests that this residue may have important biological and physiological functions. Consistent with this is the observation that the N-terminus of many proteins and bioactive peptides ends in pGlu (see table 1.1) and that the activity of some bioactive peptides is usually associated with the presence of an N-terminal pGlu (Abraham and Podell, 1981). Moreover, as a free acid, pGlu has been found in the tissues of patients with Hawkinsinuria disease (Borden *et al.*, 1992). An increased level of free pGlu has been shown in the plasma of patients with Huntington's disease (Uhlhass and Lange, 1988). This amino acid has been found to prevent scopolamine-induced amnesia and electroconvulsive shock in rats (Spignoli *et al.*, 1987) and to improve learning and age associated memory loss (Grioli *et al.*, 1990). In addition to the aforementioned physiologically relevant properties of pyroglutamic acid, its presence at the N-terminus of polypeptides may minimise their degradation, or provide them with a particular function.

Peptide/Protein	Sequence
TRH	pGlu-His-Pro-NH_2
Neurotensin	pGlu-Leu-Try-Glu-Asn-
Bombesin	pGlu-Gln-Arg-Leu-Gly-
Eledoisin	pGlu-Pro-Ser-Lys-
Anorexogenic	pGlu-His-Gly-OH
Fibrinopeptidase:- Human	pGlu-Gly-Val-Asp(NH_2)-
- Reindeer	pGlu-Leu-Ala-Asp-
- Bovine	pGlu-Phe-Pro-Thr-Asp-
Gastrin:- Human	pGlu-Gly-Pro-Trp-Leu-
- Porcine	pGlu-Gly-Pro-Trp-Met-
Vasoactive Polypeptide	pGlu-Val-Pro-Trp-
Heavy Chain from Human Pathological IgG	pGlu-Val-Thr-
Heavy Chain of Human γG Immunoglobulin	pGlu-Val-Gln-Leu-
Heavy Chains of Rabbit Anti-Hapten Antibodies	pGlu-Ser-Leu-Glu-Glu-
	pGlu-Ser-Val-Glu-Glu-
Heavy Chains of Rabbit IgG	pGlu-Ser-Val-Glu-Glu-
	pGlu-Ser-Leu-Glu
	pGlu-Glu(NH_2)
Mouse λ Chains	pGlu-Ala-Val-Val-
Human apoLp-Gln-II	pGlu-Ala-Lys-Glu-Pro-
Thymic from Porcine Serum	pGlu-Ala-Lys-Ser-Gln-
Peptide Inhibiting Epidermal Mitosis	pGlu-Glu-Asp-Cys-Lys-OH
Colon Mitosis Inhibitory Peptide	pGlu-Glu-His-Gly-OH
Caerulein	pGlu-Gln-Asp-Try(SO_3H)-
Levitide	pGlu-Gly-Met-Ile-Gly-Try-
Human Monocyte Chemoattractant	pGlu-Pro-Asp-Ala-Ile-
Growth Hormone from Tilipia	pGlu-Gln-Ile-Thr-Asp-

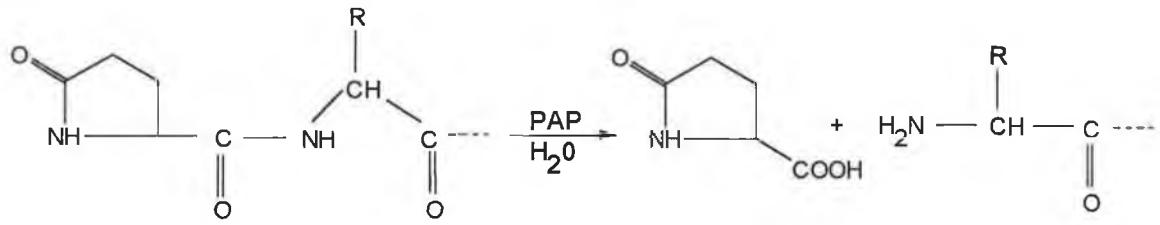
(Taken from Awade *et al.* (1994) and subsequently modified.)

Table 1.1 ***Some Peptides and Proteins with an N-terminal pGlu Residue***

1.2 Pyroglutamyl Aminopeptidases

Aminopeptidases (EC 3.4.11.1-14) are N-terminal exopeptidases involved in protein modification and in particular, protein and peptide metabolism. They exhibit specificity for the hydrolysis of peptide bonds which contain the aminoacyl residue present at the N-terminus of polypeptides. Omega peptidases (EC 3.4.19) are exopeptidases capable of removing terminal residues that (a) lack a free α-amino or α-carboxyl group (i.e. the pyroglutamyl and aminoacylamide groups), or (b) are linked through a sissile bond that involves a carboxyl or amino group which is not attached to an α-carbon (McDonald and Barrett, 1986).

Pyroglutamyl aminopeptidases (PAPs) represent one of a class of omega peptidases which specifically remove the L-pyroglutamyl residue from the amino terminus of polypeptides by hydrolysis (see fig 1.2). Pyroglutamyl aminopeptidase has also been referred to as pyrrolidonyl peptidase, pyrrolidonecarboxylyl peptidase, pyrrolidonecarboxylate peptidase, pyroglutamyl arylamidase, pyroglutamate aminopeptidase, pyroglutamyl peptidase, L-pyroglutamyl peptide hydrolase, PYRase and 5-oxoprolyl-peptidase (Awade *et al.*, 1994). To date, two classes of pyroglutamyl aminopeptidase have been characterised. The first includes bacterial pyroglutamyl aminopeptidase and animal type-I pyroglutamyl aminopeptidase (PAP-I), a soluble enzyme with biochemical characteristics similar to the bacterial PAPs. The second class includes mammalian type-II pyroglutamyl aminopeptidase (PAP-II), a membrane bound enzyme and serum PAP, also known as Thyroliberinase (Bauer *et al.*, 1981). Enzymes from these two classes present distinct differences in their cellular and subcellular localisation, molecular weights and enzymatic properties.



Pyroglutamyl-peptide Pyroglutamic acid n-1 peptide

Figure 1.2 ***Hydrolysis of pGlu from the N-terminus of L-pGlu-proteins or L-pGlu-peptides by PAP.***

1.2.1 Class-I: Animal PAP-I and Bacterial PAPs

PAP activity was first discovered in a strain of *Pseudomonas fluorescens* by Doolittle and Armentrout (1968) who found that a crude extract could hydrolyse the pyroglutamyl dipeptide L-pGlu-L-Ala to yield free pGlu and alanine. PAP has since been observed in the tissues of mammals, birds, fish, plants and bacteria.

1.2.1.1 Biochemical and Biophysical Characteristics of Class-I PAPs

With the exception of *Klebsiella coacae* enzyme, which is associated with the particulate fraction (Kwiatkowska *et al.*, 1974), all of the bacterial PAPs studied to date have been shown to be soluble proteins located in the cell cytosol (Tsuru *et al.*, 1978; Awade *et al.*, 1992b). The hydrophobic character of four bacterial PAPs indicates that the charge of these enzymes is uniformly distributed along the polypeptide. This is consistent with the observation that the enzymes are soluble proteins (Kyte and Doolittle, 1982). Mammalian PAP-I (EC 3.4.19.3), like its bacterial counterpart, is also a soluble cytosolic enzyme (Mudge and Fellows, 1973; Browne and O'Cuinn, 1983; Lauffart *et al.*, 1988; Cummins and O'Connor, 1996). The enzyme has been found to be present in all mammalian tissues tested, with the exception of blood (Szewczk and Kwiatkowska, 1970). The regional distribution of the enzyme in the supernatant fractions of rat brain reveals less than two fold variation in regions from lowest to highest concentrations (Friedman and Wilk, 1986).

The mammalian PAP-I is a monomeric enzyme with a molecular weight of 22,000 to 25,000 Da (Mudge and Fellows, 1973; Browne and O'Cuinn, 1983, Mantle *et al.*, 1991; Cummins and O'Connor, 1996). The molecular weight of the bacterial enzyme, under non-denaturing conditions, has been shown to vary from 50,000 to 91,000 Da. Tsuru *et al.* (1978; 1984) have proposed that the *B. amyloliquefaciens* PAP, with a native molecular weight of 72,000 Da and a subunit molecular weight of 24,000 Da, probably functions as a trimer. More recently however, Yoshimoto *et al.* (1993) have cloned the gene for the *B. amyloliquefaciens* enzyme and, following its overexpression in *E. coli*, have shown that the recombinant enzyme appears to exist as a dimer. This suggests that the recombinant form of the enzyme differs from the natural form, possibly due to different post-translational processing patterns in the host cell. Other studies indicate that the recombinant PAPs from *B. subtilis* (Gonzales and Awade, 1992; Awade *et al.*, 1992b) and *S. pyrogens* (Awade *et al.*, 1992a) are probably tetramers, whilst the recombinant PAP from *P. fluorescens* (Gonzales and Robert-Baudouy, 1994) probably functions a dimer.

PAP-I and bacterial PAPs are members of the cysteine proteinase family. The cysteine residue at the active site of the enzyme is very labile and thus a thiol-reducing agent such as DTT or 2-

mercaptoethanol is required for enzyme activity. The enzymes are very sensitive to sulphydryl-blocking reagents such as N-ethylmaleimide, 2-iodoacetamide, iodoacetate and p-hydroxymercuribenzoate and also to trace amounts of heavy metals such as Hg^{2+}, Zn^{2+}, and Cu^{2+} (Doolittle and Armentrout, 1968; Tsuru *et al.*, 1978; Bauer and Kleinhauf, 1980; Browne and O'Cuinn, 1983; Cummins and O'Connor, 1996).

The overexpressed *Bacillus amyloliquefaciens* enzyme has been crystallised and therefore knowledge of PAP structure is imminent (Yoshimoto *et al.*, 1993). The bacterial PAP genes examined so far do not show any significant similarity to other known nucleotide sequences, nor do the deduced amino acid sequences compare favourably with protein sequences from other prokaryotic and eukaryotic sources. This lack of homology with other proteins, including other proteases, suggests that bacterial PAPs belong to a new and unique class of peptidases. All of the cloned bacterial PAPs contain a uniquely conserved cysteine residue, suggesting that this residue is directly involved in the catalytic site of these thiol dependent enzymes. This has been confirmed by alteration of the cysteine residue, which results in total loss of enzyme activity (Yoshimoto *et al.*, 1993). A histidine residue is also conserved, indicating that this residue may also be essential for enzyme activity or substrate binding (Gonzales and Robert-Baudouy, 1994).

Both the animal PAP-I and the bacterial PAPs are unstable and heat sensitive enzymes, being rapidly inactivated at temperatures above 50^0C. Stabilisation of the enzyme has been achieved using the reversible inhibitor 2-pyrrolidone (Armentrout and Doolittle, 1969; Szewczuk and Kwiatkowska, 1970; Mudge and Fellows, 1973) or by the addition of BSA to the partially purified or purified enzyme (Cummins and O'Connor, 1996). Enzyme stabilisation is discussed in further detail in section 1.3. Optimal enzyme activity is expressed at pH 8.5 for purified mammalian PAP-I (Lauffart *et al.*, 1988; Mantle *et al.*, 1991; Cummins and O'Connor, 1996) and at pH 7.0 to 9.0 for bacterial PAP (Doolittle and Armentrout, 1968; Kwiatkowska *et al.*, 1974; Awade *et al.*, 1992a). Interestingly, a thermostable PAP from *Pyrococcus furiosus* has been reported recently (Izu *et al.*, 1998). This enzyme retains its activity at 75-95^0C.

A distinctive biochemical feature of PAP-I is its broad pyroglutamyl-substrate specificity. This enzyme is capable of liberating the N-terminal pGlu residue from a range of biologically active peptides including TRH, acid TRH, LHRH, neurotensin, bombesin and anorexigenic (Browne and O'Cuinn, 1983; Cummins and O'Connor, 1996). Synthetic substrates such as pGlu-MCA, pGlu-pNA, pGlu-βNA, and isotopic TRH are also readily hydrolysed by PAP-I, as are synthetic dipeptides such as pGlu-Ala and pGlu-Val (Browne and O'Cuinn, 1983; Bauer and Kleinhoff, 1980;

Albert and Szewczuk, 1972). pGlu-Pro bonds are not normally hydrolysed by either bacterial or mammalian PAP-I (Dootittle, 1972; Mudge and Fellows, 1973; Browne and O'Cuinn, 1983; Cummins and O'Connor, 1996). However, the enzyme from the particulate fraction of *Klebsiella coacae* was found to be capable of hydrolysing the pGlu-Pro bond (Kwiatkowska *et al.*, 1974). Initial PAP assays were based on the cleavage of L-pyroglutamyl dipeptides such as L-pGlu-L-Ala and monitoring the amino acid release by the ninhydrin method (Doolittle and Armentrout, 1968). The development of the colourimetric and fluorimetric substrates L-pGlu-βNA (Szewczuk and Kwiatowska, 1970;), L-pGlu-pNA and L-pGlu-MCA (Fujiwara and Tsuru, 1978) resulted in PAP assays with greatly increased sensitivity.

Despite its broad substrate specificity, PAP-I is highly specific for N-terminal pGlu residues. A study by Capecchi and Loudon (1985) reports that minor alterations to the pGlu moiety of a given substrate has very deleterious effects on the ability of PAP-I to cleave this amino terminal group. More recently, Bundgaard and Moss (1989) have exploited this pGlu specificity as a means of developing potentially useful "prodrugs" which are resistant to PAP-I attack. These researchers demonstrated that altering the N-H group on the pGlu ring, results in a pGlu moiety that is completely resistant to cleavage by PAP-I (Moss and Bundgaard, 1989; 1992; Bundgaard and Moss, 1989).

PAP-I levels in mammals are developmentally regulated. One of the more recent studies on this measured developmental changes in PAP activities in rat brain cortex and cerebellum (de Gandarias et al., 1998). This study demonstrated that PAP-I activity is high in the perinatal period and decreases two or three fold subsequently, at a later stage in the cerebellum than in the brain cortex, reaching adult levels at the end of the first post natal month. The decrease in the activity of PAP-I coincides with increasing concentrations of brain TRH after the second postnatal week. It is suggested that PAP-I could play a part in the normal development of rat CNS.

1.2.1.2 Inhibitors of Class-I PAPs

Potent and specific (both reversible and irreversible) inhibitors of PAP-I have been described. Active site directed inhibitors were first synthesised by Fujiwara *et al.* (1981a, 1981b, 1982), for the *B. amyloliquefaciens* enzyme. These were pGlu-chloromethyl ketone (pGCK), Z-pGlu chloromethyl ketone (Z-pGCK) and Z-pGlu-diazomethyl ketone (Z-pGDK). The chloromethyl ketone derivatives were found to be highly specific, potent and irreversible inhibitors of this PAP. A three fold molar excess of inhibitor was sufficient to inactivate the enzyme by 95% after only one minute incubation. This inhibitor was found to be very unstable; therefore, the Z-pGCK derivative was prepared.

Although this inhibitor inactivated the enzyme at a ten-fold slower rate, it was, nonetheless, quite potent.

Aldehydes interact with cysteine proteases forming hemiacetal adducts. The aldehyde derivative of pyrogluamate, 5-oxoprolinal, is a potent and specific inhibitor of PAP-I *in vitro* with a K_i of 20-26nM. 5-oxoprolinal is also active but of low potency *in vivo* when administered to mice, possibly due to its rapid metabolism (Friedman *et al.*, 1985).

pGlu-diazomethyl ketone (PDMK) is an irreversible PAP-I inhibitor. It is very potent *in vivo* as well as *in vitro*. One hour following intraperitonal administration of 0.1mg/kg to mice, the brain enzyme was totally inhibited, and after 24 hours, the enzyme was still 50% inhibited. Since it is an irreversible inhibitor, restoration of enzyme activity requires the synthesis of new enzyme (Wilk *et al.*, 1985).

2-pyrrolidone is a pyroglutamyl substrate analogue which acts as a reversible, non-competitive inhibitor of PAP-I. As mentioned earlier, several studies have reported on the use of 2-pyrrolidone to stabilise PAP-I, either during purification or storage.

1.2.1.3 Physiological Role and current Uses of Class-I PAPs

As previously mentioned, PAP-I has been shown to act on many different substrates possessing an N-terminal pGlu, including TRH. Despite earlier interest in the role of PAP-I in TRH metabolism, it appears that this enzyme is not involved in the control of TRH levels *in vivo* (Mendez *et al.*, 1990; Salers *et al.*, 1991). This has been demonstrated by the exposure of hypothalamic cells to the PAP-I inhibitor, PDMK, which did not change TRH content or recovery of released TRH (Mendez *et al.*, 1990). The role of PAP-I still remains unclear. It has been proposed that it may contribute to the final stages of intracellular catabolism of peptides to free amino acids which are then released to the cellular pool (Mantle *et al.*, 1990; 1992). Thus the enzyme may, at least in part, be involved in the regulation of the cellular pool of free pGlu (Awade *et al.*, 1994). As described in section 1.1, free pGlu is known to have pharmacological properties, thus a specific pathway may exist to generate this molecule. The source of free pGlu that is associated with the aforementioned diseases remains unknown, but the involvement of PAP-I remains a possibility.

In some recent studies, researchers have found an increase in soluble PAP activity in certain physiological disorders. Soluble PAP was found to be increased the neurohypophysis and adrenal glands of hypertensive rats (Prieto *et al.*, 1998). Shaw *et al.* (1996) found that cytosolic PAP

activity was increased in patients with amylotrophic lateral sclerosis. They have suggested that an underlying abnormality of intracellular protein metabolism, in which PAP plays a role, may be responsible for the pathogenesis of the disease.

As with the mammalian PAP-I the role of the bacterial PAP remains unclear. This enzyme may be involved in the utilisation of peptides as a source of nutrients as suggested in the case of *Pseudomonas flourescens*, which was shown by Doolittle and Armentrout (1968) to be capable of growing with free pGlu as the sole source of carbon and nitrogen. Such a role for the enzyme is weakened by the observation that it is not commonly found in bacteria, and even within the same species some strains possess PAP activity while others lack the enzyme (Szewczuk and Mulczyk, 1969). Awade *et al.* (1994) proposed that the high accumulation of peptides with an N-terminal pGlu may abnormally acidify the cell cytoplasm, possibly indicating a role in detoxification.

The absence of an α-amino group in pGlu-peptides and proteins was, for many years, a major impediment in the characterisation of these materials, since amino terminal analysis, including stepwise degradation methods (Edman, 1950), could not be carried out. Consequently the initial impetus behind the discovery of PAP (Doolittle and Armentrout, 1968) was to isolate an enzyme activity that would render terminal amino groups accessible in pGlu-terminating peptides. In this regard, PAP (typically commercial calf liver PAP-I or bacterial PAP) has been extremely successful (Podell and Abraham, 1978). Indeed, despite the availability of enzymatic and chemical methods to open pGlu rings (Miyatake *et al.*, 1993; Van der Werf *et al.*, 1975), and physical methods such as mass spectrometry, which are available to overcome sequencing difficulties due to the N-terminal pGlu block (Khandke *et al.*, 1989), PAP still remains the de-blocking method of choice for many sequencers (Mozdzanowski *et al.*, 1998; Lu *et al.*, 1991)

Since PAP activity is present in some bacterial strains but is absent in others, specific PAP-I assays have been used in bacterial diagnostic techniques. For example, PAP activity has been exploited in diagnostic tests for the identification of *streptococcal* species (Wellstood, 1987; Panosian and Edberg, 1989; Dealler *et al.*, 1989) and more recently in the identification of *Salmonella* species (Inoue *et al.*, 1996).

1.2.2 Class-II: Animal PAP-II and Serum PAP

In the late 1970s a pyroglutamyl aminopeptidase which cleaved the pGlu-His bond of TRH was partially purified from rat (Taylor and Dixon, 1978) and porcine (Bauer and Nowak, 1979) serum. Unlike the previously characterised cytosolic PAP-I activity, this enzyme was not inhibited by

sulphydryl-blocking reagents such as 2-iodoacetamide and N-ethylmaleimide but could be inhibited by metal chelators such as EDTA and 1,10 phenanthroline. The molecular weight of the enzyme was found to be approximately 260,000 Da, an order of magnitude greater than the soluble PAP-I. Bauer *et al.* (1981) demonstrated that the selectivity of this enzyme was directed towards TRH or closely related peptides. Other pGlu substrates such as pGlu-βNA, LHRH and neurotensin were not cleaved. As a consequence of this narrow substrate specificity, the name "thyroliberinase" was subsequently proposed (Bauer *et al.*, 1981).

While several authors had reported that PAP activity was associated, at least in part, with the particulate fraction of brain (Schock, 1977; Hayes *et al.*, 1979; Griffiths *et al.*, 1979; Greaney *et al.*, 1980), Browne *et al.* (1981) were the first to demonstrate the existence of two distinct pyroglutamyl aminopeptidases in guinea pig brain. They found the particulate enzyme, in marked contrast to the soluble enzyme, was inhibited by DTT and EDTA. These observations were later confirmed by the findings of O'Connor and O'Cuinn (1984) who localised this particulate activity to the synaptosomal membrane preparations of guinea pig brain from which it could be solubilised by papain treatment. PAP-II has a molecular weight of 230,000 Da, consisting of two identical subunits of approximately 116,000 Da each (Bauer, 1994). Like serum "thyroliberinase", PAP-II has a substrate specificity restricted to TRH or closely related peptides.

This unprecedented degree of specificity for a particular peptide configuration, combined with the knowledge that (1) the active site of PAP-II appears to be extracellular (Charli *et al.*, 1988), (2) it is primarily located within neuronal elements of the CNS, (3) it has a differential distribution within the CNS and (4) the inhibition of PAP-II specifically increases recovery of TRH released from rat brain tissue (Charli *et al.*, 1989), would serve to indicate that the particulate PAP activity is responsible for specifically inactivating neuronally released TRH within the extracellular vicinity of target cells. Several studies reporting on the influence of thyroid hormones on PAP-II activity support this observation. Suen and Wilk (1989) and Bauer (1987, 1988) have clearly demonstrated the significantly increased TRH-degrading action of PAP-II in the anterior pituitary following acute treatment with triiodothyronine (T_3). Such tissue specific regulation of adenohypophyseal PAP-II by thyroid hormones suggests that it may serve an integrative function in modulating the response of adenohypophyseal target cells to TRH by terminating the biological activity of the tripeptide at this location, the increased TRH-degrading activity subsequently contributing to the negative feedback effect of thyroid hormones on the hypothalamic production of TRH. Wilk (1986) has subsequently proposed that the membrane bound PAP-II be considered the first characterised neuropeptide-specific peptidase.

1.3 Enzyme Stabilisation

" The term stability refers to an enzyme's resistance to adverse influences such as heat or other denaturants, that is, to the persistence of its molecular integrity or biological function in the face of extremes of temperature or other deleterious influences. A perfectly folded, fully functional monomeric enzyme can lose its biological activity *in vitro* by unfolding of its tertiary structure to a disordered polypeptide, in which key residues are no longer aligned closely enough for continued participation in functional or structure-stabilising interactions. Such unfolding is termed denaturation. It is usually cooperative and may be reversible if the denaturing influence is removed. An enzyme is also subject to chemical changes, which lead to an irreversible loss of activity or inactivation, particularly following unfolding " (O'Fagain, 1997).

" The use of enzymes is unfortunately quite restricted for a number of reasons. Notable among these is the loss of enzyme function with time. Recently, understanding of both inactivation processes and of stabilising interactions has increased, which has lead to improvements in enzyme stability. This has been achieved through protein engineering. This, however, can be quite a complex task and simpler stabilisation alternatives are available. These include the use of additives (usually solutes) and chemical modification. One of the main aims of these techniques is to reduce the lability of enzymes since enzymes with increased stability are often required for different applications, such as organic synthesis or for use in biosensors " (O'Fagain, 1997).

1.3.1 Use of Stabilising Additives

It has long been known that inclusion of low molecular weight substances can greatly stabilise the critical enzymes biological activity. A variety of compounds can increase the stability of enzymes in solution.

1.3.1.1 Types of Stabilising Molecules

The range of stabilising additives is very wide.

1.3.1.1.1 Use of Salts

Certain salts can significantly stabilise enzymes in solution. This effect varies with the constituent ions in the Hofmeister series, which relates to ionic effects on protein solubility. This series ranks both cations and anions in order of their stabilising effects. The most stabilising ions are on the left, whereas those on the right are actually destabilising.

$(CH_3)_4N^+ > NH^{4+} > K^+, Na^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$

$SO_4^{2-} > Cl^- > Br^- > NO^{3-} > ClO^{4-} > SCN^-$

The stabilising ions force enzyme molecules to adopt a tightly packed, compact structure by salting out hydrophobic residues. This helps prevent the unfolding, which is the initial event in any enzyme deterioration process. Certain ionic compounds such as glycine can also exert stabilising effects. These act by shielding charges and can stabilise enzymes at low concentrations; typical concentrations range from 20 to 400mM.

1.3.1.1.2 Use of Osmolytes

Osmolytes are a diverse group of substances comprising such compounds as polyols, sugars, polysaccharides, neutral polymers (such as PEG) and amino acids and their derivatives. In general, they affect water's bulk solution properties by forming many hydrogen bonds and aid formation of a 'solvent shell' around the enzyme molecule that is distinct from the bulk aqueous phase. They do not interact with the protein directly. Many of these compounds are found *in vivo* to control the osmotic pressure of eucaryotic and bacterial cells.

Sugars and polyols are used at high final concentrations: typical figures range from 10-40% w/v (Ó Fágáin, 1997). Sugars are thought to be the best stabilisers. They stabilise the lattice structure of water, thus increasing surface tension and viscosity. They stabilise hydration shells and protect against aggregation by increasing the molecular density of the solution (Schein, 1990).

Glycerol is a very widely used low molecular weight polyol. Its advantages include its ease of removal by dialysis and its non-interference with ion exchange chromatography. Its stabilisation effect appears to be due to the formation of tight hydrogen bonds between the polyol and the water resulting in a reduction of water activity and an increase in solvent structure. The protein is less able to unfold against a more structured polyol-water solvent than it is against water alone (Ó Fágáin *et al.*, 1988). However, its main drawback is that it is a excellent bacterial substrate. It has been suggested that the five-carbon sugar, xylitol, can be an effective alternative glycerol (Ó Fágáin, 1997). Xylitol can be recycled from buffers and it is not a convenient food source for bacteria.

Polymers such as PEG generally increase solvent viscosity and thereby help prevent aggregation. It has also been suggested that PEG brings about a change in the enzyme's solvent interactions resulting in a tightening of chain folding and progressive exclusion of water molecules from the relatively hydrophobic core of the enzyme, to which water had been previously able to gain access

(Mozhaev and Martinek, 1984). This results in a more compact hydrophobic core and an enzyme with improved stability (Ó Fágáin *et al.*, 1988). Typical final concentrations of PEG range from 1 to 15% w/v. Amino acids with no net charge, notably glycine and proline, can act as stabilisers if used in the range 20-500mM. They usually stabilise enzymes via weak electrostatic interactions.

1.3.1.1.3 Other Common Additives

Addition of specific substrates or competitive (reversible) inhibitors to purified enzymes can often exert great stabilising effects. Occupation of the enzyme's binding site(s) by these substances leads to minor, but significant conformational changes in the polypeptide backbone. The enzyme adopts a more tightly folded conformation, reducing any tendency to unfold and (sometimes) rendering it less prone to proteolytic degradation. Occlusion of the enzyme's active site(s) by a bound reversible inhibitor will protect those amino acid side chains that are critical for function. The reversible inhibitor, 2-pyrrolidone, has been used to stabilise PAP-I (see section 1.2.1.1).

The thiol group of cysteine is prone to destructive oxidative reactions. One can prevent or minimise these by using reducing agents such as 2-mercaptoethanol or dithiothreitol. Much of the oxidation of thiol groups is mediated by divalent metal ions, which can activate molecular oxygen. Complexation of free metal ions (where they are not themselves essential for activity) can prevent destructive oxidation of thiol groups. Chelating agents such as EDTA may be used to complex metal ions. Indeed both DTT and EDTA have been routinely used in the assay mixture for the determination of PAP-I activity and during PAP-I purification (Browne and O'Cuinn, 1983a; Cummins and O'Connor, 1996).

Very dilute protein solutions are highly prone to inactivation and therefore should be concentrated as rapidly as possible. Where this is not possible, one can prevent inactivation by addition of an exogenous protein such as bovine serum albumin (BSA), typically to a final concentration of 1mg/mL. There are numerous reasons for the undoubted benefits of BSA addition (Scopes, 1994). BSA may act to prevent target protein adsorption to the container surfaces, it may prevent dissociation of subunits or it may even act in a chaperonin-like manner to prevent unfolding of the protein of interest. It is often included in reaction mixtures to stabilise dilute enzyme solutions. BSA has been used by researchers to stabilise PAP-I either during assay or storage (Browne and O'Cuinn, 1983a; Cummins and O'Connor, 1996). As well as a great number of polar and charged groups on the surface of enzymes, there are some nonpolar (hydrophobic) residues whose contact with water is thermodynamically unfavourable (Mozhaev and Martinek, 1984) and this can actually destabilise enzymes (Mozhaev *et al.*, 1988). BSA is a very hydrophobic molecule (Bigelow, 1967); therefore,

hydrophobic interactions with exposed hydrophobic groups on the enzyme of interest may lead to increased stability. Moshaev and Martinek (1984) noted that these interactions make an important, if not decisive, contribution to the maintenance of the native structure of enzymes. When the enzyme-BSA complex forms, the contact area of the nonpolar fragments with water decreases, the free energy of the system diminishes and hence the stabilisation takes place (Mozhaev and Martinek, 1984).

1.3.2 Use of Chemical Modifications

Chemical modification provides an alternative to the inclusion of the additives mentioned above. Because purified PAP-I is an unstable enzyme (section 1.2.1.1), the main aim of chemical modification would be to increase its stability. . Each of the 20 amino acids occurring in enzymes has a free R-group or side chain. Many of these have reactive functional groups such as the thiol group of cysteine or the amino group or lysine residues. At least 9 amino acid side chains (Cys, Lys, Asp and Glu, Arg, His, Trp, Tyr, Met) can react with specific reagents under mild conditions to yield chemically-modified protein derivatives, often with altered properties. Two distinct chemical modification strategies can benefit protein stability. These are (i) crosslinking, either intra- or intermolecular and (ii) surface group modification.

1.3.2.1 Use of Cross-Linking Reagents

Cross-linking of enzymes with bi(poly)-functional agents is widely used for protein stabilisation. The employment of external 'braces' prevents both the unfolding of the tertiary structure and the dissociation of oligomeric proteins into subunits (Mozhaev and Martinek, 1984). Inactivation is always a danger, since some of the amino acid side chains will be essential for function. For example reagents targetting cysteine groups should not be used with PAP-I since this residue has been found to be essential for activity (section 1.2.1.1). But careful choice of modifying agent(s) and conditions can often minimise activity losses.

Bifunctional crosslinking reagents are chemicals with two reactive functions, specific for a particular amino acid R-group, separated by an unreactive middle portion. They are either homobifunctional, in which both reactive groups are the same, or heterobifunctional, in which both the functional groups are different. Homobifunctional reagents include imidoesters and bismaleimides that are amino- and thiol-group specific respectively. Heterobifunctional reagents include derivatives of N-hydroxysuccinimide that are specific for both amino and thiol groups. Means and Feeney (1990) and Ji (1983) give a comprehensive list of homo- and hetero-bifunctional crosslinking reagents. Thiol and amino groups are frequently targeted because they are reactive and occur frequently in protein

primary structures. Other groups or side chains that can be targeted include carboxyl groups on aspartyl and glutamyl residues, guanidino groups of arginine residues and imidazole groups of histidine residues. Molecular rigidification is achieved by crosslinking, i.e., by the reaction of both functional groups of the bifunctional reagent with the enzyme backbone. Thus the reagents allow free, soluble enzymes to be stabilised against denaturation.

The success or failure in increasing enzyme stability by treatment with bifunctional reagents largely depends on the length of the bifunctional molecule, and hence on the distance between the molecular centres to be crosslinked (Torchilin *et al.*, 1978). Thus, a crosslinking reagent may form molecular bridges in one type of enzyme molecule and not in another. When successful crosslinking occurs, it implies that the targeted enzyme has molecular distances that suited the crosslinking reagent employed. Certain workers have quite successfully stabilised enzymes by arranging intra- or inter-molecular cross-links of defined lengths using these reagents (Ó Fágáin *et al.*, 1988).

1.3.2.1.1 Crosslinking with N-Hydroxysuccinimide Esters

N-Hydroxysuccinimide (NHS) esters are among the most popular of the modifying reagents, because of their mild chemical reaction conditions coupled to their commercial availability and ease of synthesis. They can be either homo- or hetero-bifunctional (Ji, 1983). Reaction of NHS-esters occurs most efficiently at pH 7-8 in most buffers that do not contain free amino groups. Reaction is rapid, with most accessible protein amino groups attacked within 10 to 20 minutes at temperatures ranging from 4-25^{0}C.

Unfortunately NHS-esters are poorly soluble in aqueous buffer and need to be dissolved in a minimal volume of an organic solvent such as DMSO, prior to enzyme addition. This may cause problems if high concentrations of modifier are required as high solvent concentrations may lead to some inactivation. Therefore, at the very least, appropriate controls must be included to account for this possibility.

The NHS reaction scheme is shown in figure 1.3.3. The principle product of reaction with an amine is an amide; thus the positive charge of the original amino group is lost. The reaction involves the nucleophilic attack of an amine on the acid carboxyl of an NHS-ester to form an amide, releasing the NHS.

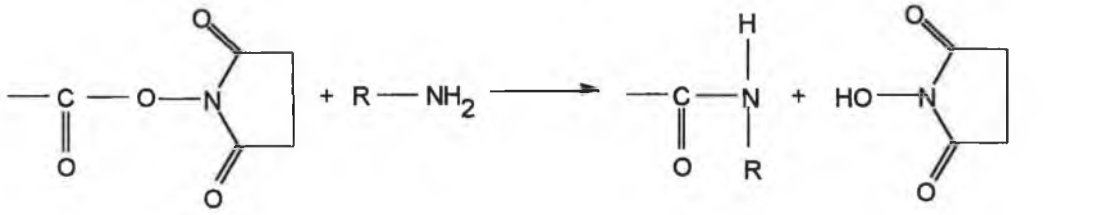


Figure 1.3.3 ***N-Hydroxysuccinimide Ester Reaction Scheme***

1.3.2.2 Surface Group Modification

Crosslinking is not a prerequisite for stabilisation. Dramatic increases in stabilisation can result merely from the alteration of surface groups. As with crosslinking inactivation is a possibility but this can be avoided through careful choice of modifier. But a number of distinct advantages also exist. Relatively little structural information is required concerning the target protein, as is the case with PAP-I. The experiments are often quick and simple to carry out and protocols may be readily implemented.

Ó Fágáin (1997) has described some of the chemical modification reagents that have been used in the past with varying degrees of success with numerous enzymes. Mozhaev *et al.*, 1988 has achieved considerable stabilisation via hydrophilisation of the protein surface, which reduces unfavourable surface hydrophobic contacts with water. The modified enzyme, in which the non-polar surface area was reduced, was shown to be more stable against irreversible thermoinactivation than the native enzyme. Through chemical modification it has also been possible to change the pH optima of enzymes, the relative reactivity towards substrates and the pattern of substrate and product inhibition.

Many of the reagents used in alteration of surface groups have also been of great value in the investigating the nature of the active site residues. They can distinguish, for example, serine from cysteine proteases by the use of appropriate reagents. It can identify those amino acids that participate in catalysis and those that are important in substrate binding. Few, if any, chemical modifiers are absolutely specific for a given type of functional group. The outcome of the modification can be influenced, to some degree, by every type of experimental variable (Cohen,

1970). Table 1.3.2 lists some reactive amino acid R-groups and some of the compounds used for their modification.

Amino acid	Side Chain	Reagents	Reaction	Ref
Cysteine	Thiol	N-ethylmaleimide	Alkylation	1.
Lysine	Amino	2,4,6 Trinitrobenzene Sulphonic acid	Addition	1.
Arginine	Guanidino	Phenylglyoxal	-	2.
Histidine	Imidazole	Diethylpyrocarbonate	Addition	3.
Aspartic acid	Carboxyl	Carbodiimides	Amidation	4.
Glutamic acid	Carboxyl	Carbodiimides	Amidation	4.
Tryosine	Phenol	Tetranitromethane	Nitration	5.

Table 1.3.2 ***Amino Acid Side Chains and Reagents for their modification***

1. Ó Fágáin, 1997; 2. Takahashi, 1977; 3. Dickenson and Dickenson, 1975; 4. Carraway and Koshland, 1972; 5. Riordan and Vallee, 1972b.

There are also a variety of other methods by which stabilisation can be achieved. These are based on the strengthening of hydrophobic interactions by non-polar reagents and the introduction of new polar or charged groups that give additional ionic or hydrogen bonds to the enzyme molecule (Ó Fágáin and O'Kennedy, 1991).

2. Materials and Methods

2.1 Materials

Sigma Chemical Company (Poole, Dorset, England):

AA-NHS
Acetaldehyde
Ammonium persulphate
Bovine Serum Albumin
Diethylpyrocarbonate
2,2'-dithiopyridine
EDTA
EG-NHS
EDC
Glycine
2-Iodoacetamide
Iodoacetic acid
Laurel sulphate
2-Mercaptoethanol
MCA
N-acetylimidazole
N-ethylmaleimide
N,N'-Methylene-Bisacrylamide
Phenylglyoxal
Potassium phosphate (dibasic)
Potassium phosphate (monobasic)
Proline
Pyridoxal phosphate
Rose bengal
Sephadex G-25
Silver stain high MW kit
Silver stain kit
Sodium chloride
Sucrose
TEMED
Tetranitromethane
2,4,6 trinitrobenzene sulphonic acid
Xylitol

Bachem Feinchemikalein AG (Bubendorf, Switzerland):

pGlu-MCA
Thyroliberin

BDH Chemicals Ltd. (Poole, Dorset, England):

Acrylamide
Bromophenol blue
Citric acid
Dimethylsulphoxide
Glacial acetic acid
Glycerol
Hydrochloric acid
Polyethylene Glycol 6000

Merck Chemical Company (Frankfurt, Germany):

Ammonium sulphate
Potassium chloride
Sodium hydroxide

Aldrich Chemical Company (Poole, Dorset, England):

2-Pyrrolidone

Pharmacia Fine Chemical Company (Uppsala, Sweden):

Activated Thiol Sepharose 4B
Phenyl Sepharose CL-4B
DEAE-Sepharose Fast Flow
Sephadex G-100

Melford Laboratories (Chelsworth, Ipswich, Suffock, England)

Tris buffer
Dithiothreitol

Bio-Rad Laboratories (Hercules, California, USA):

Bio-Rad protein assay dye reagent concentrate

Kepak Meats (Clonee, Co.Meath, Ireland):

Bovine whole brain

Pierce Chemical Company (Illinois, USA):

BCA reagent

2.2 Determination of Enzyme Activities

2.2.1 Cytosolic Pyroglutamyl Aminopeptidase in Crude Samples

Cytosolic pyroglutamyl aminopeptidase (PAP-I) activity was determined according to a modification of the method of Cummins and O'Connor (1996) (see Fig 2.2.1). 100µL of sample was incubated for 1 hour at 37°C with 400µl of 0.1mM pGlu-MCA in 50mM potassium phosphate buffer pH 7.4 containing 10mM DTT, 10mM EDTA and 0.75% v/v DMSO. The reaction was terminated by the addition of 1mL of 1.5M acetic acid. All assays were performed in triplicate and suitable negative controls were prepared by adding acetic acid to the enzyme prior to the substrate. Liberated MCA was determined using a Perkin-Elmer LS50 Fluorescence Spectrophotometer with excitation and emission wavelengths of 370nm and 440nm respectively. Excitation slit widths were maintained at 10nm while emission slit widths were adjusted as appropriate for the level of flourescence produced. Samples containing particulate material were centrifuged at 13000 rpm for 10 minutes, using a Heraeus Sepatech Biofuge A prior to flourescence reading.

2.2.2 Cytosolic Pyroglutamyl Aminopeptidase in Purified Samples

Purified preparations of the enzyme (i.e. pooled post gel filtration and affinity chromatography samples) were assayed as outlined in section 2.2.1 with the following exception. 80µL of sample was incubated for 1 hour at 37°C with 20µL of 10mg/mL protease free BSA and 400µL of 0.1mM pGlu-MCA in 50mM potassium phosphate buffer, pH7.4 containing 10mM DTT, 10mM EDTA and 0.75% v/v DMSO. Liberated MCA was determined as outlined in section 2.2.1.

2.2.3 Microplate Assay

In addition to the assays outlined in sections 2.2.1 and 2.2.2, a non-quantitative microplate assay was devised to facilitate the detection of cytosolic PAP in the large number of fractions generated by column chromatography. Two assays were employed in order to detect enzyme activity. In the first method (used for post ion-exchange fractions) 100µL of substrate (0.1mM pGlu-MCA + 10mM DTT + 10mM EDTA as in section 2.2.1) was added to 50µL of sample. After incubation of this mixture at 37°C for 30 minutes, the reaction was terminated by the addition of 100µL of 1.5M acetic acid. The second method (used for post gel filtration and post affinity chromatography fractions) involved adding 100µL of substrate to a mixture of 40µL of sample and 10µL of 10mg/mL protease free BSA. This mixture was incubated at 37°C for 30 minutes after which the reaction was terminated by the addition of 100µL of 1.5M acetic acid. Suitable negative controls for both methods were included on each plate by adding acid to the sample prior to the substrate.

Liberated MCA was detected in the same manner as outlined in section 2.2.1 using a Perkin-Elmer LS50 Fluorescence Spectrophotometer fitted with a microplate reader.

Assay protocol:

1. (a) 100μL of sample + 400μL of 0.1mM pGlu-MCA or
   (b) 80μL of sample + 20μL of BSA + 400μL of 0.1mM pGlu-MCA
2. React at 37°C for 60 minutes
3. Stop reaction with 1mL of 1.5M acetic acid
4. Read fluorescence at Ex: 370nm and Em: 440nm

Assay schematic:

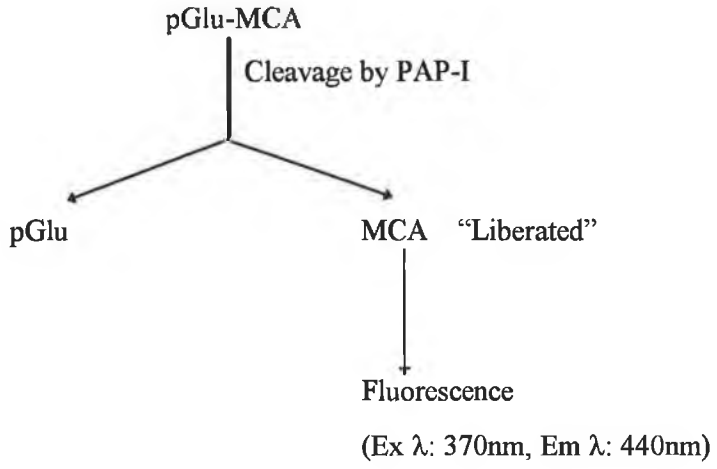


Fig 2.2.1 ***Assay of cytosolic PAP activity using pGlu-MCA***

2.3 7-Amino-4-Methyl-Coumarin Standard Curves

In order to quantify the activity of cytosolic PAP using "quenched" fluorometric substrates, 7-Amino-4-Methyl-Coumarin (MCA) standard curves were constructed. A 200μM MCA stock in 2% v/v DMSO was prepared in 50mM potassium phosphate buffer, pH 7.4, at 37°C. This stock solution was stored in the dark at 4°C. Lower concentrations of MCA were achieved using 50mM potassium phosphate buffer, pH 7.4 as diluent. Standard curves were constructed by substituting the appropriate MCA standard for substrate under the assay conditions described in sections 2.2.1 and 2.2.2. Ranges of 0-1.6μM, 0-8μM and 0-80μM MCA were prepared. Fluorometric intensity was measured using a Perkin-Elmer LS50 Fluorescence Spectrophotometer with excitation and emission

wavelengths of 370nm and 440nm respectively. Excitation slit widths were maintained at 10nm while emission slit widths were adjusted to produce fluorimetric intensities appropriate for the range being analysed.

A unit of enzyme activity was defined as that which liberates one nanomole of MCA per minute at 37°C. Conversion of fluorometric intensities to units of enzyme activity was achieved using the following formula:

$$\text{Units per mL} = \frac{\text{Fluorometric Intensity}}{\text{Slope}} \times 0.0667$$

The 'slope' is obtained from an appropriate MCA standard curve. The factor 0.0667 considers the method of preparation of the standard curve, the conversion of μM to nanomoles, the conversion of hours to minutes and the conversion from "per 80μL" or "per 100μL" to "per mL" of sample.

2.4 Protein Determination

2.4.1 Absorbance at 280nm

The absorbance of proteins based on the λ_{max} of tryptophan and tyrosine residues at 280nm was used as a non-quantitative method of determining protein concentrations in post column chromatography fractions. A Shimadzu UV 160A Spectrophotometer was used to determine this absorbance.

2.4.2 Standard Bicinchoninic Acid Protein Assay

The Bicinchoninic Acid (BCA) protein assay, based on the method of Smith et al., (1985), was used for the determination of protein concentration in crude homogenate, supernatant (S_2) and pooled post anion exchange samples. These samples were dialysed for 20 hours against 50mM potassium phosphate buffer, pH 7.4 to remove interfering substances such at Tris/HCl buffer, DTT and EDTA. Before dialysis, the homogenate was rehomogenised to remove particulate material using a hand operated homogeniser and subsequently diluted in 50mM potassium phosphate buffer, pH 7.4. Samples with a protein concentration outside the limits of the BCA assay (0.02-1.8mg/mL) were diluted as appropriate in 50mM potassium phosphate buffer, pH 7.4. 200μL BCA working reagent was added to 10μL of dialysed and/or diluted sample in triplicate in a 96 well microplate and incubated for 30 minutes at 37°C, after which the absorbance at 560nm was measured using a

TiterTek Multiscan PLUS spectrophotometric plate reader. A 0-1.8mg/mL BSA standard curve was prepared and assayed in parallel with samples each time it was performed.

2.4.2.1 Enhanced Bicinchoninic Acid Protein Assay

This assay was used to monitor the protein in post affinity chromatography fractions. The assay was performed as described in section 2.4.2 with the following exceptions: fractions were dialysed extensively against distilled water and an incubation temperature of 60°C was used.

2.4.3 Biorad Protein Assay

The Biorad protein assay, based on the method of Bradford (1976), was used to determine protein concentration in pooled post gel filtration and affinity chromatography samples. These samples were dialysed for 20 hours against distilled water in order to remove interfering substances. 0.8mL of dialysed sample was incubated with 0.2mL of Biorad working reagent in triplicate at room temperature for 5 minutes after which, the absorbance at 595nm was read on a Shimadzu UV 160A spectrophotometer. A 0-24μg/mL BSA standard curve was prepared in parallel with the assay each time it was performed.

2.5 Purification of PAP-I from Bovine Brain Cytosol

Cytosolic pyroglutamyl aminopeptidase was purified according to a modification of the method of Cummins and O'Connor (1996). All steps were performed at 4^0C unless otherwise stated.

2.5.1 Tissue Preparation and Centrifugation

25g of bovine brain was homogenised in 100mL of 50mM Tris/HCl buffer at pH 8.0 containing 2mM DTT and 2mM EDTA with the aid of a Sorvall Omni Mixer. Tissue was disrupted by three 7 second pulses at a speed setting of five with a 20 second pause between pulses. 80mL of the crude homogenate was then centrifuged for 45 minutes at 15,000 rpm (27,200g) using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor. The supernatant (S_1) was retained while the pellet was resuspended in 40mL distilled water (osmotic shock) using four up and down strokes of a 40mL Glas-Col hand held pestle and tube homogeniser. The resuspended pellet was centrifuged as before. The resulting supernatant was combined with S_1 to form S_2 while the new pellet was discarded.

2.5.2 DEAE Sepharose Fast Flow Anion-Exchange Chromatography

An 18mL DEAE Sepharose column (2.5cm × 3.7cm) was equilibrated with 100mL of 50mM Tris/HCl buffer at pH 8.0 containing 2mM DTT and 2mM EDTA. 40mL of supernatant (S_2) was applied to the column, after which the column was washed with 120mL of equilibration buffer. The column was then eluted with a 60mL linear 0-0.75M NaCl gradient in equilibration buffer. 5mL fractions were collected during sample application and wash stages and 3mL fractions were collected during elution. A flow rate of 2mL/min was maintained throughout this procedure. All fractions were assayed for cytosolic PAP activity and protein as outlined in sections 2.2.3 and 2.4.1 respectively. Fractions containing the highest PAP-I activity were pooled.

2.5.3 Sephadex G100 Gel Filtration

The post DEAE Sepharose PAP-I was concentrated six-fold via reverse osmosis using polyethylene glycol. Glycerol was added to the concentrated sample to a final concentration of 10% v/v. A 230mL Sephadex G100 gel filtration column (2.5cm × 47cm) was equilibrated with 350mL of 50mM Tris/HCl buffer at pH 8.0 containing 2mM DTT, 2mM EDTA and 100mM KCl. 1mL of concentrated sample was loaded under the buffer head, after which the column was washed with 220mL of equilibration buffer. After 50mL had passed through the column, 5mL fractions were collected. A flow rate of 0.5mL/min was maintained throughout this procedure. All fractions were assayed for cytosolic PAP activity and protein as outlined in sections 2.2.3 and 2.4.1 respectively. Fractions containing the highest PAP-I activity were pooled.

2.5.4 Activated Thiol Sepharose 4B Affinity Chromatography

A 13mL Activated Thiol Sepharose 4B column (1.5cm × 7.2cm) was equilibrated with 100mL of 50mM Tris/HCl at pH 8.0 containing 2mM EDTA and 0.3M NaCl. 20mL of pooled post gel filtration PAP-I was dialysed for 3 hours against 1L of 50mM Tris/HCl buffer at pH 8.0 containing 2mM EDTA (this buffer was changed after 1.5 hours) before being applied to the column at a flowrate of 0.25mL/min. The column was washed with 20mL of equilibration buffer at 0.25mL/min, followed by a further 20mL at a flowrate of 0.5mL/min. Elution was achieved isocratically with 50mL of 10mM DTT in equilibration buffer. A flow rate of 0.5mL/min was maintained during the equilibration and elution stages. 5mL fractions were collected during sample application and wash stages and 3mL fractions collected thereafter. All fractions were assayed for cytosolic PAP activity and protein as outlined in sections 2.2.3 and 2.4.2.1 respectively. Fractions containing the highest PAP-I activity were pooled. Protease free BSA, to a final concentration of 0.2% w/v, was added to this purified enzyme, which was subsequently stored at -80°C. Unless otherwise indicated, this was the form of the purified enzyme used for characterisation and stabilisation studies.

2.6 Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS PAGE) was carried out on the various fractions generated throughout the purification procedure, from crude cytosol (S_2) to purified PAP-I (post affinity chromatography), to assess the efficiency of the purification procedure, according to the method of Laemmli (1970). Deionised water was used throughout this procedure.

2.6.1 Sample Preparation

Samples to be electrophoresed were dialysed extensively for 24 hours against 1L 62.5mM Tris/HCl, pH 6.8, at room temperature with buffer changes after 3, 6, and 18 hours. Dialysed samples were diluted appropriately in 62.5mM Tris/HCl at pH 6.8 and subsequently mixed with an equal volume of sample solubilisation buffer which consisted of 20% v/v glycerol, 8% w/v SDS, 10% v/v 2-mercaptoethanol and 0.01% w/v bromophenol blue in 62.5mM Tris/HCl, pH 6.8. Table 2.6.1 lists the silver stain molecular weight standards used. All samples were incubated for 1.5 minutes in a boiling water bath and placed on ice prior to loading onto the gel.

Molecular Weight Standard	Source	Molecular Weight (Daltons)
β-Galactosidase	*E. Coli*	116,000
Phosphorylase B	Rabbit muscle	97,000
Albumin	Bovine serum	66,000
Fumarase	Porcine heart	48,500
Carbonic anhydrase	Bovine erythrocytes	29,000

Table 2.6.1. ***Molecular Weight Markers used for SDS PAGE***

2.6.2 Gel Preparation and Electrophoresis

The following stock solutions were prepared:

Resolving gel buffer	3M Tris/HCl at pH 8.8
Stacking gel buffer	0.5M Tris/HCl at pH 6.8
Acryl/Bisacryl stock	30% w/v acrylamide, 0.8% w/v bisacrylamide
Ammonium persulphate	1.5% w/v, freshly prepared
SDS	10% w/v
Electrode buffer	0.192M Glycine, 0.1%w/v SDS and 0.025M Tris/HCl at pH 8.3

A 160mm × 160mm × 1mm, 10% resolving gel overlayed with a 3.75% stacking gel was cast in an Atto vertical electrophoresis Midi system. The volumes of the various solutions used to prepare the gels are shown in table 2.6.2. 20μL of the appropriately prepared samples were loaded onto the gel which was electrophoresed in the elecrode buffer. Electrophoresis proceeded for approximately 4 hours at 25mA per gel.

2.6.3 Silver Staining

A Sigma AG25 silver stain kit was employed to perform the silver staining of SDS PAGE gels according to the method of Heukeshoven and Dernick (1985). Table 2.6.3 outlines the staining procedure. This procedure was followed for the staining of gels with the exception of the reducing step which was deemed unnecessary. Digital images of gels were obtained using a UVP ImageStore 7500. This incorporated a UVP White/UV Transilluminator/Camera unit driven by ImageStore 7500 software. Images acquired were either stored digitally on disk, or printed using a Sony Videographics Printer UP-860 CE.

Solution	Volume Required for 10% Resolving gel	Volume Required for 3.75% Stacking Gel
Acryl/Bisacryl stock	10mL	2.5mL
Resolving gel buffer	3.75mL	-
Stacking gel buffer	-	5mL
SDS	0.3mL	0.2mL
Deionised water	14.45mL	11.3mL
Ammonium persulphate	1.5mL	1mL
TEMED	15µL	15µL

Table 2.6.2. ***SDS Gel Electrophoresis Gel Preparation***

Step	Reagent	Volume	Duration
1. Fixing	30% v/v EtOH, 10% v/v Glacial Acetic Acid	300mL × 3	20 min × 3
2. Rinsing	Deionised Water	300mL × 3	10 min × 3
3. Silver Staining	Silver Nitrate	300mL	30 min
4. Rinsing	Deionised Water	300mL	10-20 sec.
5. Developing	Sodium Carbonate, Formaldehyde	150mL × 2	5-8 min ×3
6. Development Stop	1% v/v Acetic Acid	300mL	5 min
7. Rinsing	Deionised Water	300mL × 3	10 min × 3
8. Reducing	Potassium Ferricyanide, Sodium Thiosulphate, Sodium Carbonate	300mL	10-30 sec.
9. Rinsing	Tap Water	Running Water	1 min
10. Rinsing	Deionised Water	300mL × 3	10min × 3

Table 2.6.3. ***Recommended Silver Staining Procedure for SDS PAGE Gels***

2.7 Development of the pGlu-MCA Based Assay

2.7.1 Linearity of the pGlu-MCA Assay with Respect to Time

The linearity of the pGlu-MCA based assay with respect to time was investigated. This study was performed on the various fractions generated throughout the purification procedure, from the crude cytosol (S_2) to purified PAP-I (post affinity chromatography). These samples were assayed as outlined in section 2.2.1 except that the assays were terminated with 1.5M acetic acid after 10, 20, 30, 40, 50 and 60 minutes.

2.7.2 Effect of BSA on a Purified Preparation of Cytosolic PAP during Assay

The effect of BSA on a purified preparation (post affinity chromatography) of cytosolic PAP during assay was investigated. The purified sample was added to an equal volume of protease-free BSA to give a final concentration of 0 to 3 mg/mL in the enzyme sample. Cytosolic PAP activity was determined as outlined in section 2.2.1.

2.7.3 Linearity with Respect to Time in the Presence of BSA

The linearity of the pGlu-MCA based assay with respect to time in the presence of 2 mg/mL BSA was investigated. This study was performed on the post gel filtration and post affinity chromatography samples. These samples were assayed as outlined in section 2.2.2 except that the assays were terminated with 1.5M acetic acid after 10, 20, 30, 40, 50, and 60 minutes.

2.8 Active Site Studies

The effect of a range of protein modifying reagents on purified PAP-I activity was investigated in order to identify the amino acids at the active site of PAP-I that are necessary for the expression of activity.

2.8.1 Effect of Modifying Agents on PAP-I Activity

Stock solutions of a range of protein modifying reagents were prepared as outlined in table 2.8.1. Lower concentrations of each were achieved by dilution with 50mM potassium phosphate buffer or 50mM Tris/HCl, both at the appropriate pH's. The pH of the stock solutions were adjusted to a suitable pH using 50mM monobasic or dibasic potassium phosphate buffer as appropriate or by the addition of 1M HCl when Tris buffer was used.

Aliquots of purified PAP-I containing 0.2% w/v BSA were dialysed against the appropriate buffer used in reagent preparation as outlined in table 2.8.1. For the sulphydryl-blocking reagents, the dialysis buffer contained 20μM DTT, 20μM EDTA and 0.1M NaCl and proceeded for 3 hours. For the remaining reagents, the dialysis buffer contained 2mM DTT, 2mM EDTA and 0.1M NaCl and proceeded for 15 hours.

320μL of the dialysed enzyme was incubated for 15 minutes at 37°C with 80μL of modifying reagent in an appropriate buffer, as indicated in table 2.8.1, and 80μL of the same buffer at the same pH. Appropriate controls were set up by substitution of 80μL of the corresponding buffer for the modifying reagent. Some of the modifiers were removed by Sephadex G25 gel filtration as outlined in section 2.8.2. Samples (either post incubation or post G25 gel filtration) were assayed for PAP-I activity as outlined in section 2.2.1. It should be noted that the samples modified by the sulphydryl-blocking reagents were assayed with substrate containing 20μM DTT and 20μM EDTA. When the enzyme was incubated with 2,4,6 trinitrobenzene sulphonic acid, pyridoxal phosphate or acetaldehyde, parallel incubations were set up with each of these modifying reagents in sodium borohydride such that the final concentration of sodium borohydride was 2mM. The effect of each of the modifiers not removed by gel filtration on fluorescence was determined as outlined in section 2.3.

Modifier	Concentration (mM)	Preparation
Tetranitromethane	6	50mM Tris/HCl, pH 8.0 [a]
2,4,6 trinitrobenzene sulphonic acid	6	50mM Tris/HCl, pH 8.0
Phenylglyoxal	6	50mM Tris/HCl, pH 8.0
Pyridoxal phosphate	60	50mM Tris/HCl, pH 8.2 [b]
Acetaldehyde	60	50mM Tris/ HCl, pH 8.0
1-Ethyl-3-(3-Dimethylamino-propyl)carbodiimide	120	50mM potassium phosphate buffer, pH 5.0
	120	50mM potassium phosphate buffer, pH 5.5
	60	50mM potassium phosphate buffer, pH 6.0
	60	50mM potassium phosphate buffer, pH 7.0
	60	50mM Tris/HCl, pH 8.0
Diethylpyrocarbonate	60	50mM potassium phosphate buffer,pH 6.0 [c]
Sulphydryl-blocking reagents		
Rose bengal	60	50mM Tris/HCl, pH 7.7
Iodoacetamide	60	50mM Tris/HCl, pH 8.0
Iodoacetic acid	60	50mM Tris/HCl, pH 8.0
N-Ethylmaleimide	60	50mM Tris/HCl, pH 8.0 [b]
N-Acetylimidazole	300	50mm Tris/HCl, pH 8.0

Table 2.8.1 ***Preparation of Protein Modifying Reagents***

[a] 60mM stock prepared in 100% v/v ethanol

[b] dissolution was aided by heating in a boiling water bath

[c] 600mM stock freshly prepared in 100% v/v ethanol

2.8.2 Modifier Removal Using Sephadex G25 Gel Filtration

Modifiers were removed from the sample by the method of Helmerhorst and Stokes (1980). Sephadex G25 was swollen by constant agitation for 3 hours. 10mL columns (1.5cm × 5.7cm) were packed and equilibrated with the dialysis buffer indicated in section 2.8.1. The columns were allowed to run dry by centrifugation at 1600rpm for 3 minutes using a Heraeus Sepatech Megafuge 1.0. 450μL of post incubation sample was applied to the top of the column while 450μL of the control sample was applied to another column. The columns were centrifuged at 1600rpm for 2 minutes. The eluant from each column was collected. The resin was unpacked, swelled and washed before use again.

2.8.3 Protection of the Active Site by the Addition of the Competitive Inhibitor pGlu-His-Pro-NH_2 (TRH).

80μL of 3.75mM TRH in the appropriate buffer was added to 320μL of dialysed enzyme. 80μL of modifying reagent in suitable buffer was then added and the mixture was incubated for 15 minutes at 37°C. Controls were set up by replacement of 80μL of modifying reagent with 80μL of appropriate buffer. The treated samples were assayed for PAP-I activity as described in section 2.8.1.

2.8.4 Time Course Inhibition Studies

This study was carried out in order to determine the rapidity with which certain modifiers act on the enzyme. This was carried out as outlined in sections 2.8.1 and 2.8.3 except that the enzyme was incubated at 37°C with the modifier in the presence and absence of TRH for 0, 2, 5, 10 and 15 minutes. Controls were set up by substitution of the 80μL of modifier or TRH with 80μL of either 50mM potassium phosphate or 50mM Tris/HCl buffers at the appropriate pH. Samples were subsequently assayed for residual activity.

2.9 Stability studies

In an attempt to find an alternative stabilising compound to BSA, numerous other potential stabilising compounds were tested in order to assess their effectiveness as stabilising compounds during assay and during storage. The reasoning behind this was to attempt to further stabilise the enzyme using some simple chemical modifying reagents. If the enzyme was stored in protein, the modifying reagents could well react with the high concentration of protein before it could react with the enzyme. Therefore an alternative(s) to BSA was sought.

2.9.1 Effect of Potential Stabilisers on PAP-I Activity

Stocks of various potential stabilisers were prepared as outlined in table 2.9.1. Two parallel assays were set up, in order to test two concentrations of each stabiliser. One consisted of adding 125µL of post ATS pool (with no BSA added) to 125µL of stabiliser stock and 250µL of 50mM Tris/HCl pH 8.0. The other consisted of adding 125µL of post ATS affinity pool (with no BSA added) to 250µL of stabiliser stock and 125µL of 50mM Tris/HCl pH 8.0. Appropriate controls were set up by substitution of the stabiliser stock with the appropriate buffer. 100µL aliquots of each of these were assayed as outlined in section 2.2.1. The effect of each of the reagents on fluorescence was determined as outlined in section 2.3.

Potential Stabiliser	Concentration	Preparation
BSA	2 mg/mL	50mM Potassium Phosphate pH 7.4
Glycerol	20 %v/v	50mM Tris/HCl pH 8.0
Xylitol	20 %w/v	50mM Tris/HCl pH 8.0
Sucrose	20 %w/v	50mM Tris/HCl pH 8.0
PEG	15 %w/v	50mM Tris/HCl pH 8.0
Glycine	500mM	50mM Tris/HCl pH 8.0
Citrate	500mM	NaOH pH 7.0
Proline	500mM	50mM Tris/HCl pH 8.0

Table 2.9.1. ***Preparation of potential stabilising reagents***

2.9.2 Stability of PAP-I During Assay

The linearity of the pGlu-MCA based assay with respect to time was investigated in the presence of any of the above reagents that showed potential as stabilisers. This was carried out as outlined in section 2.7.3 with the exception that the BSA was replaced with the appropriate concentration of PEG. The assay was also carried out in the presence of both BSA and PEG. Suitable controls were included by replacing both BSA and PEG with an appropriate buffer.

2.9.3 Stability of PAP-I During Storage

The stability of purified PAP-I stored at -80^{0}C in the presence of various compounds was investigated. The enzyme was stored in 2mg/mL BSA, 1mg/mL BSA, 15% v/v glycerol and finally with no additions. Aliquots of enzyme stored in the presence of these compounds were removed at intervals and assayed for residual PAP-I activity. Samples containing BSA were assayed as outlined in section 2.2.1 while samples containing no BSA were assayed as outlined in section 2.2.2.

2.10 Initial Stabilisation of PAP-I via Chemical Modification

2.10.1 Modification with EG-NHS and AA-NHS

10mg/mL Ethylene Glycol-bis (succinic acid N-hydroxylsuccinimide ester) (EG-NHS) and Acetic Acid N-hydroxysuccinimide ester (AA-NHS) stock in 25% v/v DMSO were prepared in 10mM potassium phosphate buffer, pH 7.6. Purified PAP-I containing **1 mg/mL** BSA was dialysed against 10mM potassium phosphate buffer, pH 7.6, containing 2mM DTT, 2mM EDTA and 0.1M KCl. Dialysed enzyme was added to the modifier stock such that the enzyme was exposed to 1mg/mL, 2mg/mL, 3mg/ml or 4mg/mL of modifier. Suitable controls of unmodified enzyme were included by substitution of the modifier with 10mM potassium phosphate buffer, pH 7.6, containing the appropriate concentration of DMSO. The modifier was left to react with PAP-I at room temperature for 20 minutes. The reaction was terminated by removal of the modifier on a Sephadex G25 column pre-equilibrated with 10mM potassium phosphate buffer, pH 7.6, containing 2mM DTT, 2mM EDTA and 0.1M KCl, as outlined in section 2.8.2.

2.10.2 Thermostability of Native and Modified Enzyme

Native and modified enzyme was preheated to 37^0C for 3 minutes followed by heating at 55^0C for 10 minutes. The native and modified enzymes were removed and placed on ice, before assaying for residual activity as outlined in section 2.2.1

3. Results

3.1 Preparation of MCA Standard Curves

As outlined in section 2.2.1 the specific fluorimetric substrate pGlu-MCA, was used to assay for cytosolic PAP activity. The enzymatic release of MCA was monitored fluorimetrically at excitation and emission wavelengths of 370 and 440nm respectively. MCA release alone could then be quantitated by using a standard curve prepared with the latter compound under corresponding assay conditions and read at the corresponding slit widths. Plots of fluorimetric intensity versus MCA concentration are presented in figures 3.1.1, 3.1.2, 3.1.3 and 3.1.4. The inner filter effect observed when crude homogenate or S_2 were used as the enzyme sample, is demonstrated in figures 3.1.1 and 3.1.2 respectively. It should be noted that the MCA concentration expressed on the x-axis of these figures represents the concentration present in the 400μL of standard used to construct the curves.

3.2 Protein Standard Curves

Protein standard curves were prepared using BSA as outlined in section 2.4. Plots of absorbance at 560nm and 595nm versus BSA concentration are presented. Figure 3.2.1 shows the standard curve for the BCA assay, while figure 3.2.2 shows the standard curve obtained for the Biorad assay.

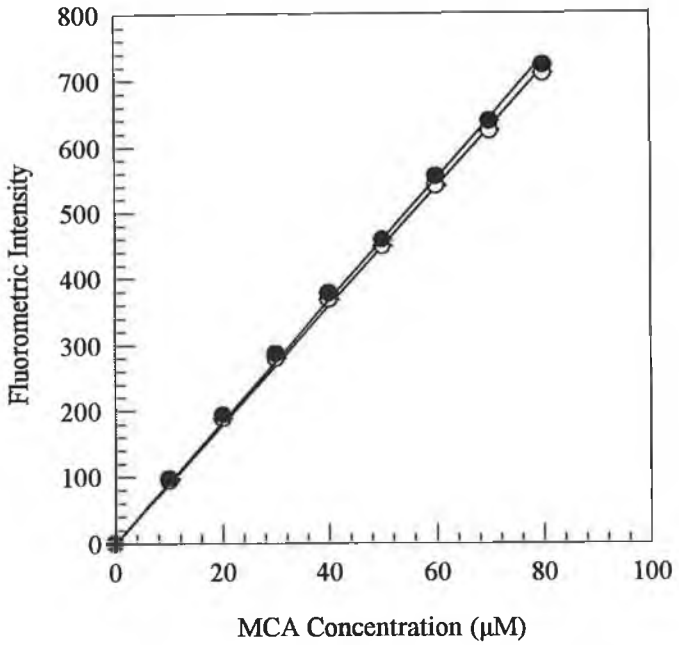


Figure 3.1.1 MCA Standard curves. Plots of fluorimetric intensity versus MCA concentration. 100μL of buffer (•—•) or 100μL of crude homogenate (o—o) was combined with 400μL of a standard concentration of MCA and 1mL of 1.5M acetic acid before being analysed fluorimetrically as outlined in section 2.3. The emission slit width was set at 2.5nm. Error bars represent the SD of triplicate readings.

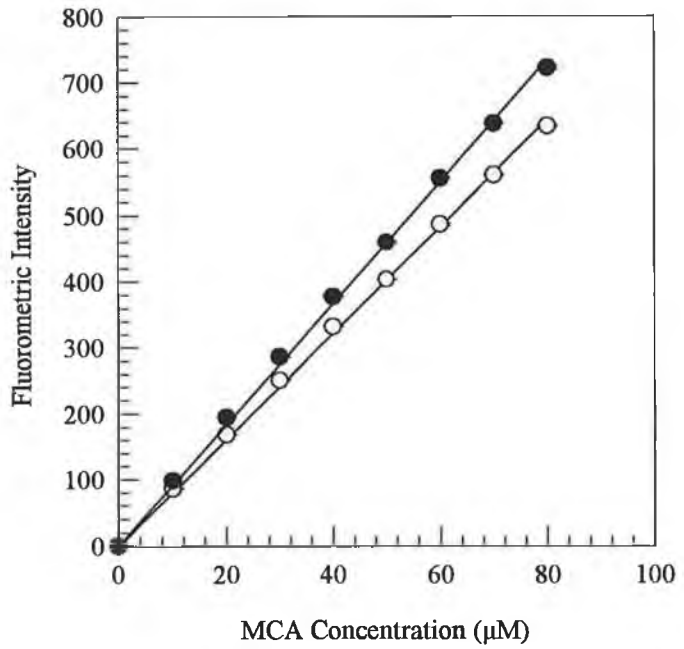


Figure 3.1.2 MCA Standard Curves. Plots of fluorimetric intensity versus MCA concentration. 100μL of buffer (•—•) or 100μL of S_2 (o—o) was combined with 400μL of a standard concentration of MCA and 1mL of 1.5M acetic acid before being analysed fluorimetrically as outlined in section 2.3. The emission slit width was set at 2.5nm. Error bars represent the SD of triplicate readings.

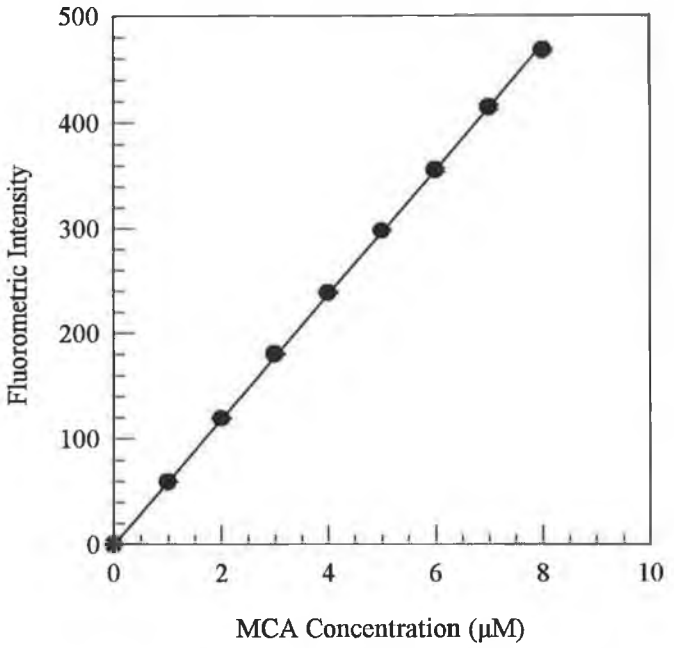


Figure 3.1.3 MCA Standard Curves. Plot of fluorimetric intensity versus MCA concentration. 100μL of buffer was combined with 400μL of a standard concentration of MCA and 1mL of 1.5M acetic acid before being analysed fluorimetrically as outlined in section 2.3. The emission slit width was set at 5nm. Error bars represent the SD of triplicate readings.

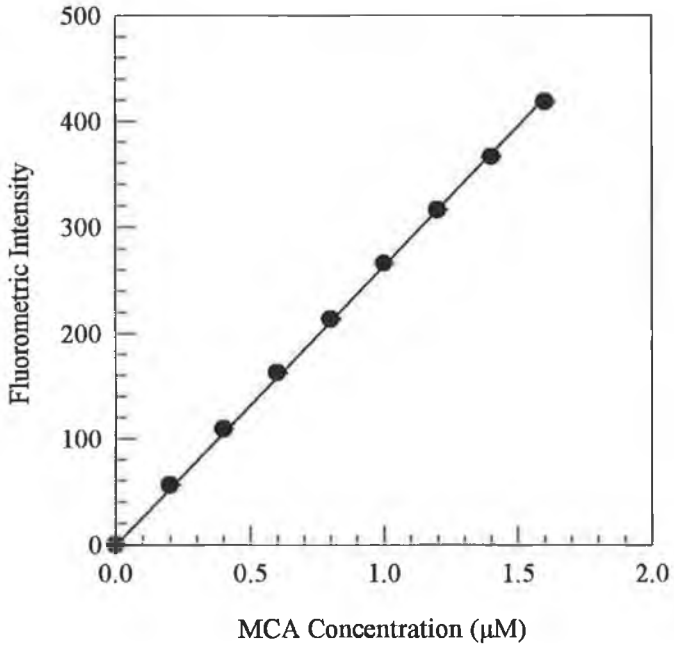


Figure 3.1.4 MCA Standard Curve. Plot of fluorimetric intensity versus MCA concentration. 100μL of buffer was combined with 400μL of a standard concentration of MCA and 1mL of 1.5M acetic acid before being analysed fluorimetrically as outlined in section 2.3. The emission slit width was set at 10nm. Error bars represent the SD of triplicate readings.

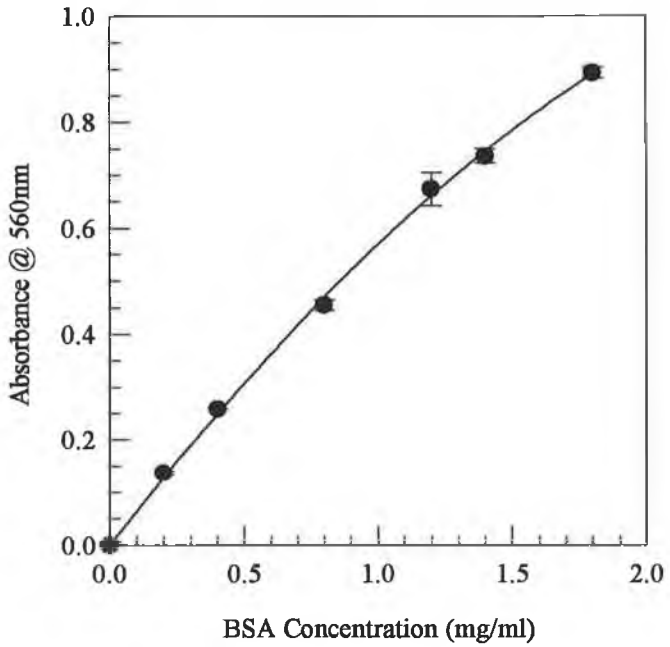


Figure 3.2.1 BSA Standard Curve. Plot of absorbance at 560nm versus BSA concentration obtained using the standard BCA assay as outlined in section 2.4.2. Error bars represent the SD of triplicate readings.

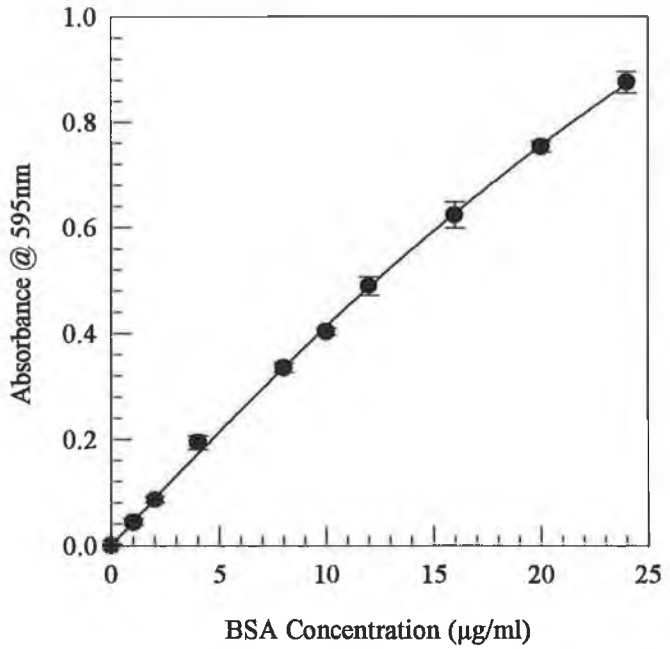


Figure 3.2.2 BSA Standard Curve. Plot of absorbance at 595nm versus BSA concentration obtained using the Biorad assay as outlined in section 2.4.3. Error bars represent the SD of triplicate readings.

3.3 Purification of PAP-I from the Cytosolic Fraction of Bovine Brain

3.3.1 Tissue Preparation and Centrifugation

80mL of crude homogenate was centrifuged and from this a combined supernatant volume (S_2) of 91mL was obtained. This represented an active recovery of 89.2% of the initial PAP-I activity.

3.3.2 Partial Purification of Cytosolic PAP by Anion-Exchange Chromatography

The ion exchange column was equilibrated as outlined in section 2.5.2. Under these conditions, cytosolic PAP bound to the column whilst unbound contaminants were washed through the column with approximately 6 column volumes of equilibration buffer. Following the application of the linear NaCl gradient, bound PAP-I activity eluted from the column, emerging between fractions 41 and 47 to give a final volume of 19.9mL when pooled. The active recovery of applied PAP-I activity was 87.2%. The elution profile of this column is illustrated in Fig 3.3.1.

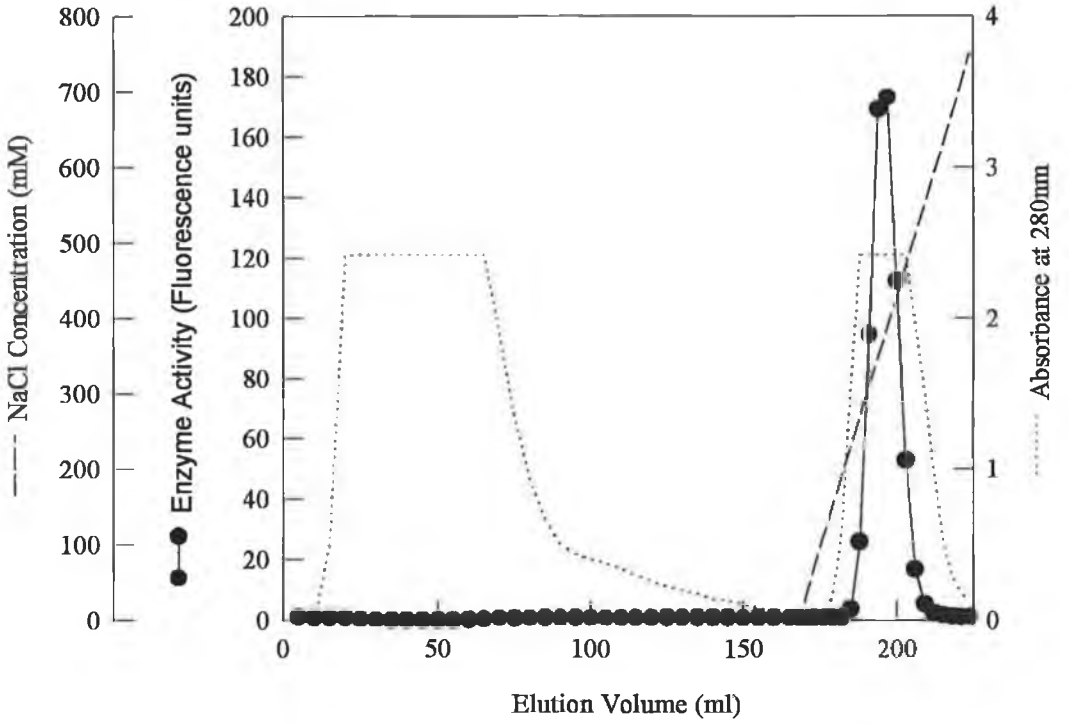


Figure 3.3.1 Elution Profile of PAP-I Activity from DEAE Sepharose Fast Flow Anion Exchange Chromatography. 40mL of supernatant was applied to an 18mL DEAE Sepharose column as outlined in section 2.5.2. The column was eluted with a linear NaCl gradient(----------). Fractions were analysed for PAP-I activity (•—•) as outlined in section 2.2.3 and for protein (……..)as outlined in section 2.4.1. Fractions 41-47 were combined to form the post DEAE Sepharose pool (19.9mL).

3.3.3 Further Purification of Cytosolic PAP by Gel Filtration Chromatography

Following concentration by reverse osmosis and the addition of glycerol to a final concentration of 10% v/v, the post ion exchange pool was applied to a Sephadex G100 column as outlined in section 2.5.3. Fractions 21-29 were combined to form the post Sephadex G100 pool (43.5mL). 54.6% of PAP-I activity was recovered from this step. The elution profile can be seen in Fig 3.3.2.

3.3.4 Affinity Chromatography of Cytosolic PAP on Activated Thiol Sepharose 4B

20mL of dialysed post gel filtration PAP-I was applied to an Activated Thiol Sepharose 4B column as outlined in section 2.5.4. Under these conditions, PAP-I bound to the column whilst unbound contaminants were washed through with equilibration buffer. The bound PAP-I was subsequently eluted by the inclusion of 10mM DTT in the equilibration buffer, emerging between fractions 19-24 to give a final volume of 17mL when pooled. The active recovery of applied PAP-I activity was 33.9%.

The release of 2-thiopyridone from the column was also monitored by absorbance spectrophotometry at 343nm. Two distinct peaks of 2-thiopyridone were observed within the elution profile. The first peak emerged after sample application, and results from the binding of thiol enzymes to the column functional groups. A second peak emerged immediately after elution commenced. The elution profile obtained can be seen in Fig 3.3.4

Table 3.3.1 summarises the overall purification scheme

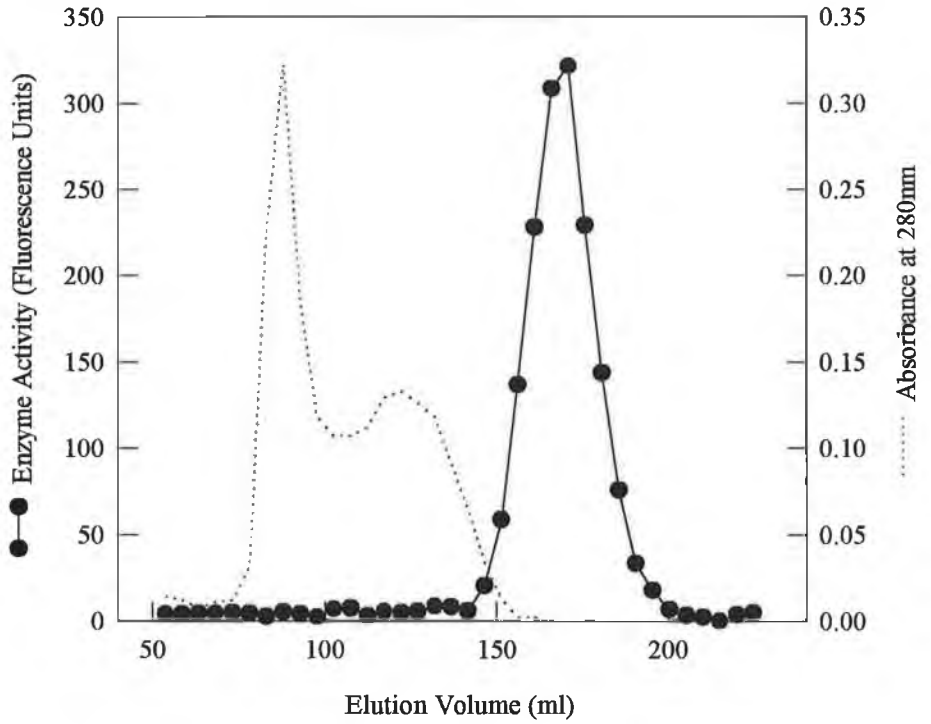


Figure 3.3.2 Elution Profile of PAP-I Activity from Sephadex G100 Gel Filtration Chromatography. Following concentration by reverse osmosis and the addition of glycerol to a final concentration of 10% v/v, 1mL of concentrated post DEAE Sepharose pool was applied to a 230mL Sephadex G100 column as outlined in section 2.5.3. Fractions were assayed for PAP-I activity (•—•) as outlined in section 2.2.3 and for protein (.....) as outlined in section 2.4.1. Fractions 21-29 were combined to form the post Sephadex G100 pool (43.5mL).

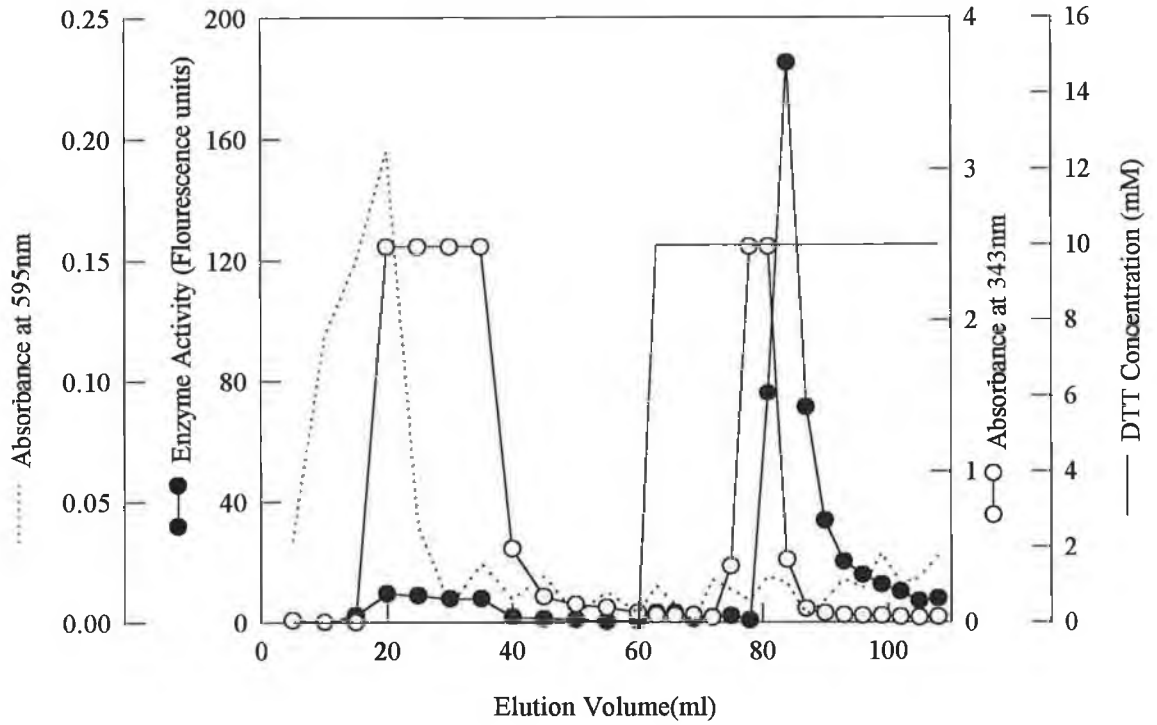


Figure 3.3.4 Elution Profile of PAP-I Activity from ATS 4B Affinity Chromatography. 20mL of dialysed post gel filtration pool was applied to a 13mL ATS 4B column as outlined in section 2.5.4. The column was eluted isocratically with 10mM DTT (—). Fractions were assayed for PAP-I activity (•—•) as outlined in section 2.2.3, for protein (……) as outlined in section 2.4.2.1 and for 2-thiopyridone (o—o) as outlined in section 3.3.4. Fractions 19-24 were combined to form the post ATS 4B affinity chromatography pool (17mL).

Purification stage	Total Protein (mg)	Total Activity (units*)	Specific Activity (units/mg)	Purification Factor	Recovery (%)
Crude Homogenate	1713.6	260.0	0.15	1.00	100
Crude Cytosol (S_2)	484.5	232.0	0.48	3.16	89.2
DEAE Sepharose	180.8	202.2	1.12	7.37	77.8
Sephadex G100	10.4	110.3	10.61	69.94	42.4
ATS 4B	1.0	37.4	37.44	246.90	14.4

Table 3.3.1 ***Purification of Pyroglutamyl Aminopeptidase from Bovine Brain Cytosol*** . *1 unit of enzyme activity is defined as that amount of enzyme which releases 1 nanomole of MCA per minute at 37^0C

3.4 Polyacrylamide Gel Electrophorseis

Polyacrylamide gel electrophoresis was carried out on samples from each step of the purification scheme. A photograph of the gel obtained following electrophoresis and silver staining (as outlined in section 2.6.3) is shown in figure 3.4.1. One distinct band was visualised in the lane containing post Activated Thiol Sepharose PAP-I. This band corresponded to a molecular weight of approximately 24,680 Da.

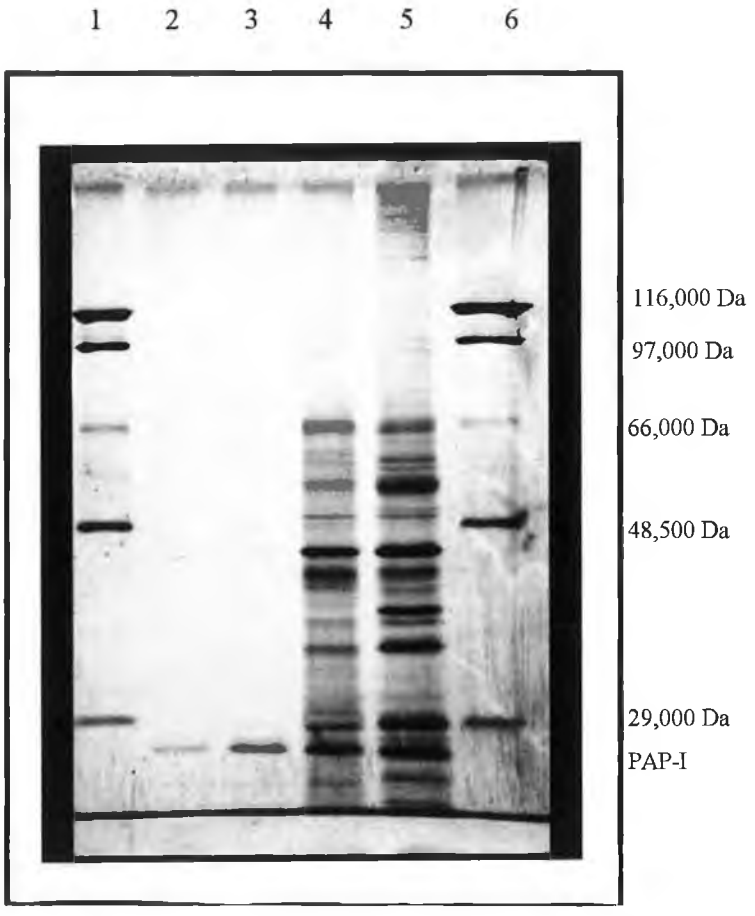


Fig 3.4.1 SDS PAGE Silver Stained Gel. A 16cm × 16cm × 1mm, 10% resolving gel, overlayed with a 3.5% stacking gel, was electrophoresed as outlined in section 2.6.2. Samples which were prepared as outlined in section 2.6.1, were loaded in the following order:

Lane 1. Molecular weight markers

Lane 2. Post affinity chromatography

Lane 3. Post gel filtration

Lane 4. Post ion exchange

Lane 5. Crude cytosol (S_2)

Lane 6. Molecular weight markers

3.5 Development of the pGlu-MCA Based Assay

The linearity of the pGlu-MCA based assay with respect to time was investigated as outlined in section 2.7.1. The release of MCA following incubation of pGlu-MCA with crude cytosol (S_2) and post ion-exchange PAP-I for varying lengths of time is shown in figures 3.5.1 and 3.5.2 respectively. In both cases, the release of MCA with respect to time is linear over 1 hour.

The effect of BSA on a purified preparation of cytosolic PAP was investigated. The release of MCA following incubation of pGlu-MCA with purified PAP-I in the presence of various concentrations of protease-free BSA can be seen in figure 3.5.3. A minimum protein concentration of 1 to 2 mg/mL BSA in the 100μL of purified preparation is required for optimum pGlu-MCA hydrolysis.

The linearity of the pGlu-MCA based assay with respect to time was investigated as outlined in sections 2.7.1 and 2.7.3. The release of MCA, in the presence and absence of BSA, following incubation of pGlu-MCA with post gel filtration and post affinity chromatography PAP-I is shown in figures 3.5.4 and 3.5.5 respectively. In both cases the release of MCA with respect to time is **not** linear in the **absence** of BSA. The presence of 2mg/mL BSA in the 100μL of PAP-I is required to keep the release of MCA linear with respect to time over the hour.

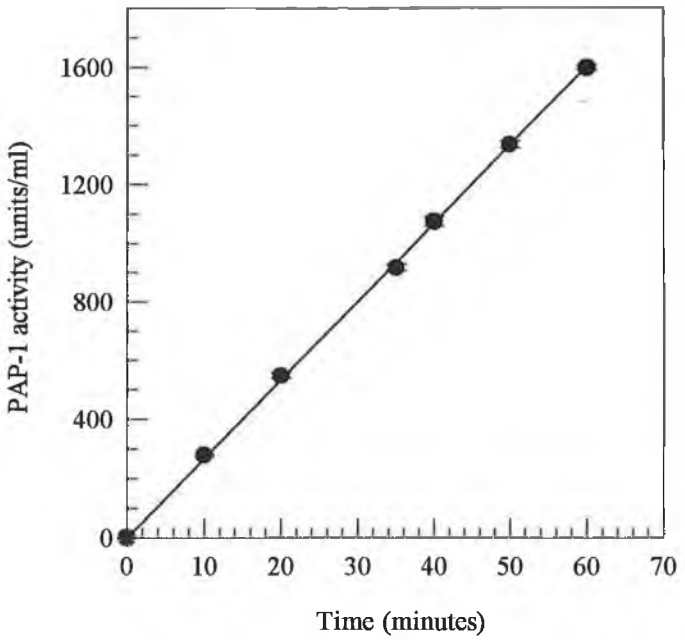


Figure 3.5.1 Linearity of PAP-I Activity from the Supernatant with Respect to Time. Supernatant (S_2) was assayed over a range of times at 37°C as outlined in section 2.7.1. Liberated MCA was determined as outlined in sections 2.2.1 and 2.3. Error bars represent the SD of triplicate readings.

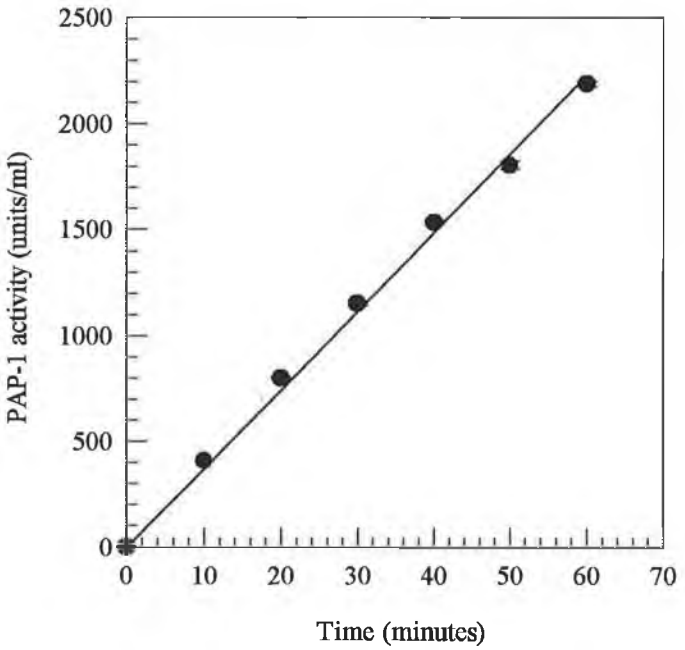


Figure 3.5.2 Linearity of PAP-I Activity from the Post Ion Exchange Pool with Respect to Time. Post DEAE ion exchange pool was assayed over a range of times at 37°C as outlined in section 2.7.1. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

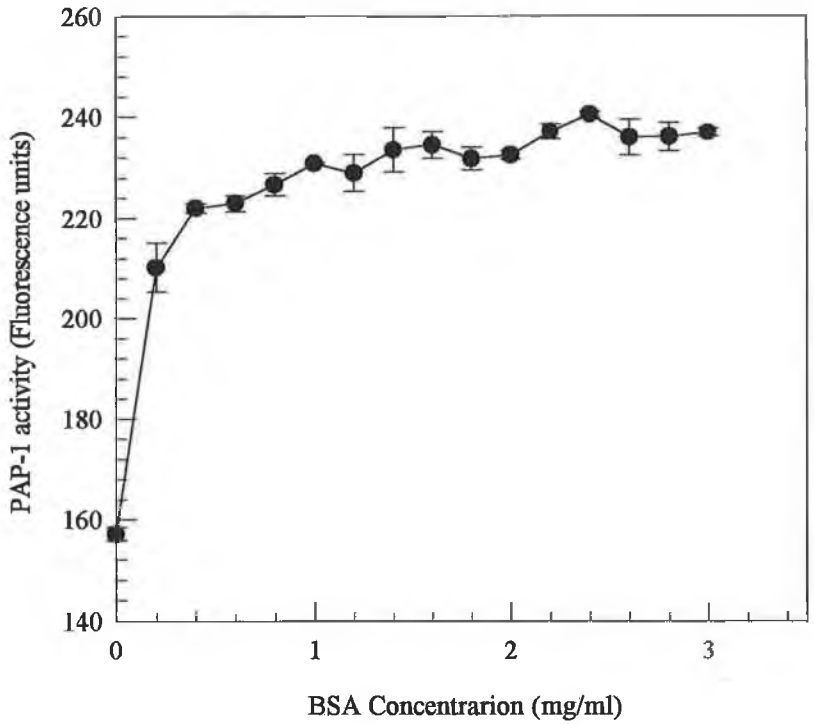


Figure 3.5.3 The Effect of BSA on a Purified Preparation of PAP-I. Purified PAP-I (post affinity chromatography) was combined with various concentrations of BSA as outlined in section 2.7.2. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

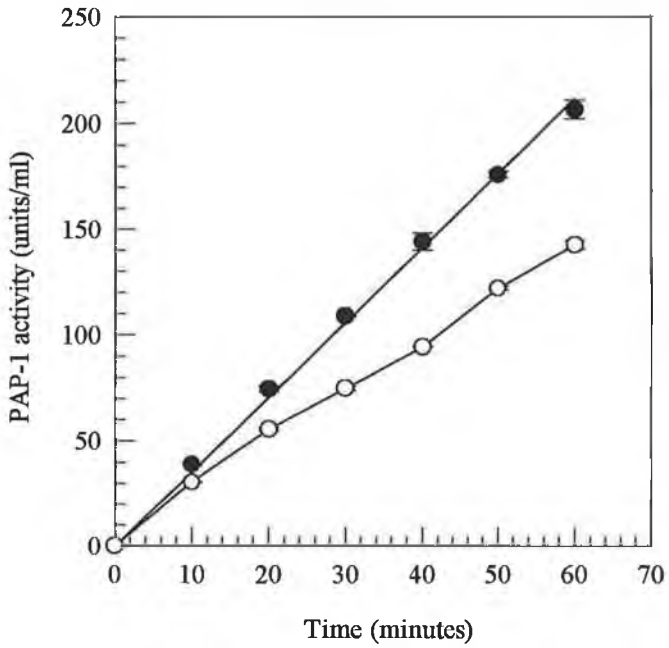


Figure 3.5.4 Linearity of PAP-I Activity from the Post Gel Filtration Pool with Respect to Time. Post G100 gel filtration PAP-I was assayed over a range of times at 37^{0}C in the presence (•—•) and absence (o—o) of 2mg/mL BSA as outlined in sections 2.7.1 and 2.7.3. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

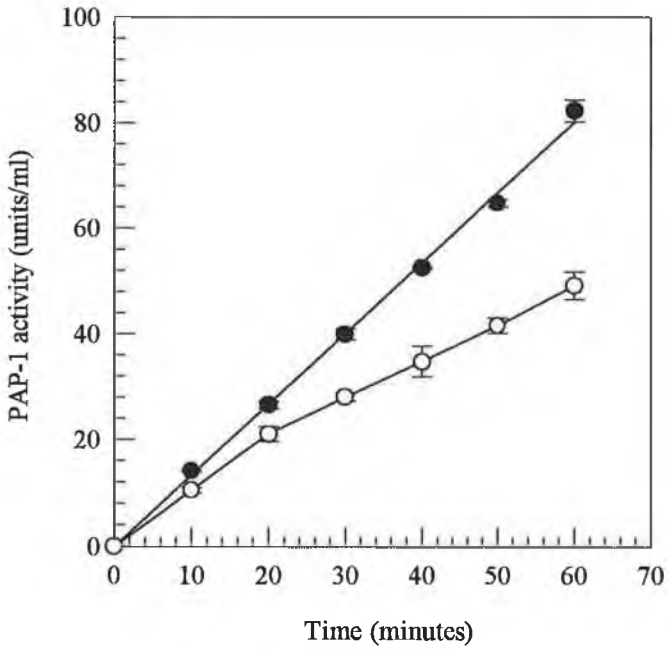


Figure 3.5.5 Linearity of PAP-I Activity from the Post Affinity Chromatography Pool with Respect to Time. Post ATS 4B affinity chromatography PAP-I was assayed over a range of times at 37^{0}C in the presence (•—•) and absence (o—o) of 2mg/mL BSA as outlined in sections 2.7.1 and 2.7.3. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings

3.6 Active Site Studies

3.6.1 The Effect of Modifying Reagents on PAP-I Activity

The effect of a range of modifying reagents on PAP-I activity was carried out as outlined in section 2.8.1. The effect of these reagents on PAP-I activity is summarised in table 3.6.1. Significant inhibition was observed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, diethylpyrocarbonate, rose bengal, iodoacetamide, iodoacetic acid and N-Ethylmalemimide

3.6.2 Protection of the Active Site by the Addition of pGlu-His-Pro-NH_2 (TRH)

The effect of protecting the active site with the competitive inhibitor TRH was carried out as outlined in section 2.8.3. The effect of this protection can be seen in table 3.6.2. It is evident, when TRH was present during the incubations, significant protection was afforded to the enzyme from inactivation by EDC, DEPC, rose bengal, iodoacetamide, IAA and NEM.

Reagent	Final Conc. (mM)	Activity as a % of untreated samples ±SD
Tetranitromethane	1	89.1 ± 2.3 [b]
Phenylglyoxal	1	102.9 ± 0.5 [b]
2,4,6 Trinitrobenzene sulphonic acid[a]	0.5	92.8 ± 1.7 [b]
Pyridoxal phosphate[a]	5	94.8 ± 1.0 [b]
Acetaldehyde[a]	5	99.5 ± 4.4 [b]
1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide	20 @ pH 5.0	98.7 ± 3.7 [c]
	20 @ pH 5.5	97.9 ± 1.5 [c]
	10 @ pH 6.0	94.3 ± 1.0 [b]
	10 @ pH 7.0	102.1 ± 0.8 [c]
	10 @ pH 8.0	5.0 ± 0.7 [c]
Diethylpyrocarbonate	2.5	36.8 ± 0.3 [c]
	10	1.7 ± 0.5 [c]
Rose bengal	1	7.1 ± 0.2 [c]
Sulphydryl blocking reagents		
Iodoacetamide	0.1	17.0 ± 0.8 [c]
Iodoacetic acid	0.1	42.6 ± 0.5 [c]
N-Ethylmalemimide	1	42.2 ± 0.8 [c]
N-Acetylimidazole	50	100.1 ± 5.5 [c]

Table 3.6.1 ***The Effect of Various Protein-Modifying Reagents on PAP-I Activity.*** The effect of protein modifying reagents on PAP-I activity was determined as outlined in section 2.8.1. and residual activity determined as outlined in section 2.2.1

[a] All assayed in the presence of 2mM sodium borohydride

[b] Modifier removal prior to assay as outlined in section 2.8.2

[c] Modifier not removed prior to assay

Modifying reagent	1mM TRH	Activity as a % of untreated samples ±SD
None	-	100
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (@ pH 8.0)	-	5.0 ± 0.7
	+	18.9 ± 0.8
Diethylpyrocarbonate	-	36.8 ± 0.3
	+	78.5 ± 7.8
Rose bengal	-	7.1 ± 0.2
	+	14.6 ± 1.3
Iodoacetamide	-	17.0 ± 0.8
	+	63.8 ± 1.6
Iodoacetic acid	-	42.6 ± 0.5
	+	82.4± 11.6
N-Ethylmalemimide	-	42.2 ± 0.8
	+	92.3 ± 2.5

Table 3.6.2 ***Protective Effect of the Competitive Inhibitor TRH on the Inactivation of Purified PAP-I by Modifying Reagents.*** The effect of protein modifying reagents on PAP-I activity in the presence (+) and absence (-) of 3.75mM TRH was determined as outlined in section 2.8.3. Residual activity was determined as outlined in section 2.2.1.

3.6.3 Time course inhibition studies

The speed with which certain modifiers act on purified PAP-I was determined as outlined in section 2.8.4. The effect of EDC, IAA, NEM and DEPC over time on PAP-I activity can be seen in figures 3.6.1, 3.6.2, 3.6.3 and 3.6.4 respectively. An immediate inhibition was observed for all four modifiers tested. In the case of IAA and NEM, TRH afforded significant protection immediately. In the case of DEPC the protective effect of TRH did not take effect until after 2 minutes, whereas with the EDC, TRH did not start protecting the enzyme until after approximately 10 minutes.

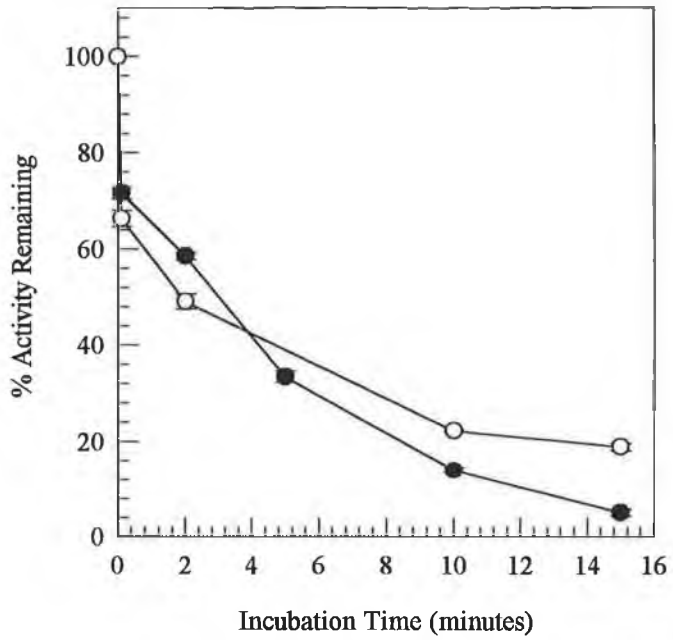


Figure 3.6.1 Effect of EDC on PAP-I Activity over Time. Plot of residual PAP-I activity versus incubation time. Purified PAP-I was incubated over a range of times at 37°C with 10mM EDC in the presence (o—o) and absence (•—•) of 0.625mM TRH as outlined in section 2.8.4. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

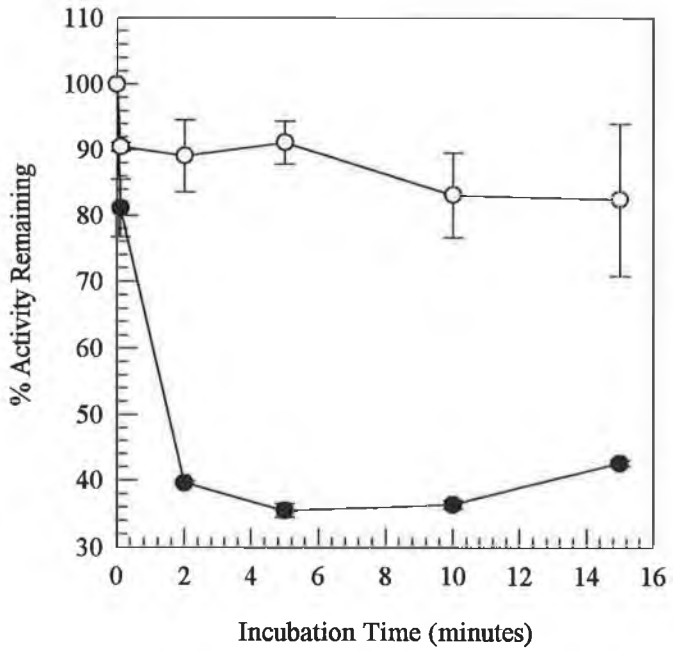


Figure 3.6.2 Effect of IAA on PAP-I Activity over Time. Plot of residual PAP-I activity versus incubation time. Purified PAP-I was incubated over a range of times at 37°C with 0.1mM IAA in the presence (o—o) and absence (•—•) of 0.625mM TRH as outlined in section 2.8.4. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

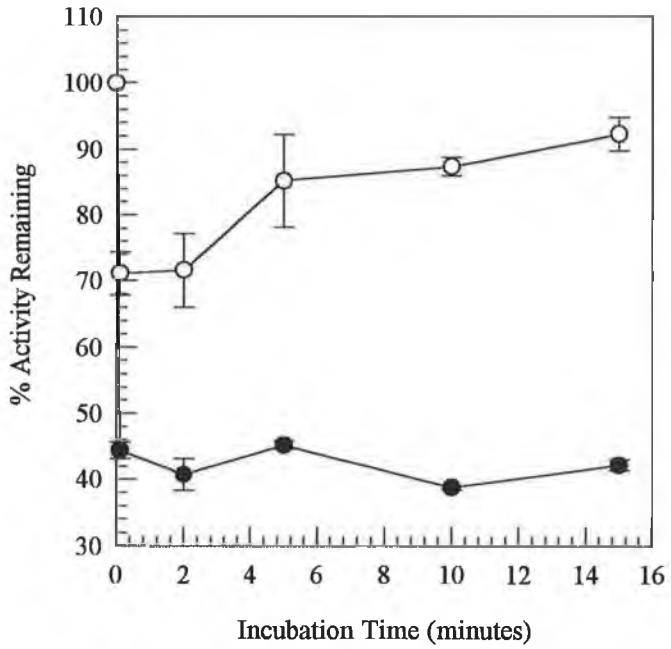


Figure 3.6.3 Effect of NEM on PAP-I Activity over Time. Plot of residual PAP-I activity versus incubation time. Purified PAP-I was incubated over a range of times at 37°C with 1mM NEM in the presence (o—o) and absence (•—•) of 0.625mM TRH as outlined in section 2.8.4. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

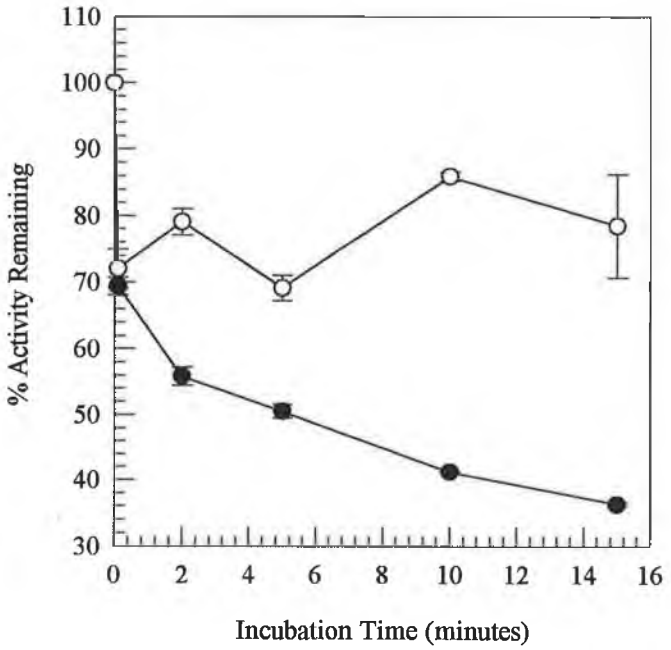


Figure 3.6.4 Effect of DEPC on PAP-I Activity over Time. Plot of residual PAP-I activity versus incubation time. Purified PAP-I was incubated over a range of times at 37°C with 2.5mM DEPC in the presence (o—o) and absence (•—•) of 0.625mM TRH as outlined in section 2.8.4. Liberated MCA was determined as outlined in section 2.2.1. Error bars represent the SD of triplicate readings.

3.7 Stability Studies

3.7.1 Effect of Potential Stabilisers on PAP-I Activity

The effect of various stabilisers on PAP-I activity was examined as outlined in section 2.9.1. Table 3.7.1 shows the effect of these stabilisers on PAP-I activity.

Potential stabiliser	Residual PAP-I activity as a percentage of the control ± SD					
	Concentrations (% v/v, % w/v or mM) [a]					
	5%	3.75%	125mM	10%	7.5%	250mM
Glycerol	93.3 ± 5.7			88.3 ± 0.4		
Xylitol	94.8 ± 2.6			76.6 ± 2.3		
Sucrose	87.4 ± 3.8			87.0 ± 6.7		
PEG		131.5 ± 4.4			131.0 ± 11.0	
Glycine			107.3 ± 6.0			124.4 ± 8.1
Citrate			108.7 ± 1.1			100.5 ± 3.4
Proline			98.5 ± 4.9			81.8 ± 5.8
Control	100					
BSA (2mg/mL)	161.7 ± 4.0					

Table 3.7.1 ***Effect of Various Potential Stabilisers on PAP-I Activity.*** The effect of potential stabilisers on PAP-I activity was determined as outlined in section 2.9.1. Residual activity was determined as outlined in section 2.2.1.

[a] See table 2.9.1 Note these are the concentrations in the 100µL aliquots prior to assay

It is evident that, other than BSA and PEG, none of the reagents showed any potential as stabilisers.

3.7.2 Stability of PAP-I During Assay

The linearity of the pGlu-MCA based assay with respect to time in the presence of PEG was compared to that in the presence of BSA, as outlined in section 2.9.2. Figure 3.7.1 shows that PEG is not nearly as effective as BSA in maintaining assay linearity. Even a combination of BSA and PEG is not as effective as BSA alone.

3.7.3 Stability of PAP-I During Storage

The stability of PAP-I during storage was assessed as outlined in section 2.9.3. Figure 3.7.2 shows that the presence of 1 or 2 mg/mL BSA stabilises the enzyme, with over 80% of the original activity remaining after one month. Glycerol is not nearly as effective at stabilising PAP-I. 65% of the initial activity remains after one month at -80^{0}C. When purified PAP-I is stored with no additions, only 40% of the original activity remains after one day. This residual activity remains relatively constant throughout the month.

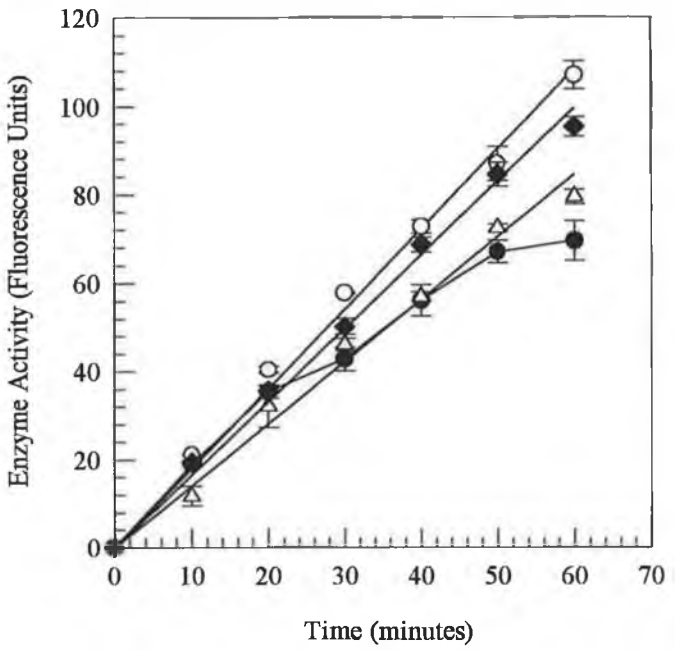


Figure 3.7.1 The Linearity of Purified PAP-I Activity with Respect to Time. Post ATS 4B affinity chromatography PAP-I was assayed over a range of times at 37^{0}C in the presence of BSA (o—o), PEG (Δ—Δ), BSA/PEG combination (♦—♦) and without any additions (•—•), as outlined in section 2.9.2. Error bars represent the SD of triplicate readings.

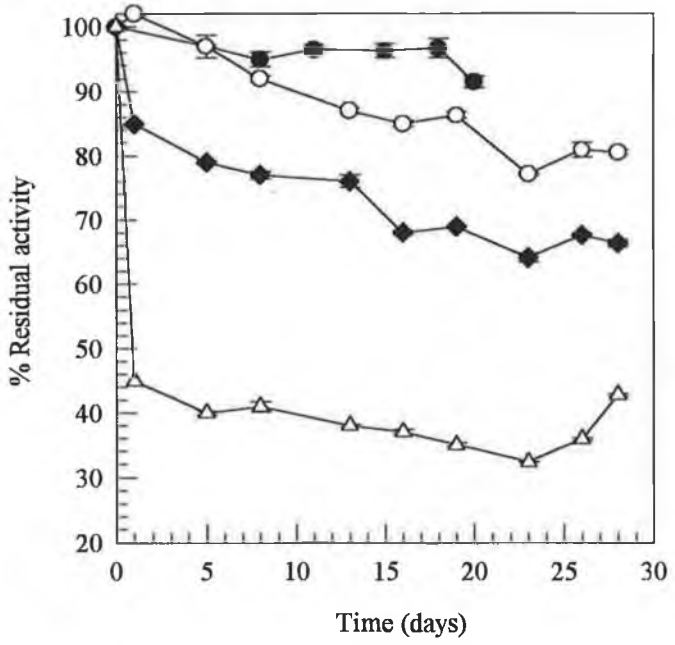


Figure 3.7.2 Stability of Purified PAP-I. Plot of residual PAP-I activity versus time stored at -80^{0}C. Post ATS 4B affinity chromatography was stored in the presence of 0.1% w/v BSA (•—•), 0.2% w/v BSA (o—o), 15% v/v glycerol (♦—♦) and without any additions (Δ—Δ) as outlined in section 2.9.3. Error bars represent the SD of triplicate readings.

3.8 Initial Stabilisation of PAP-1 via Chemical Modification

3.8.1 Modification with EG-NHS and AA-NHS

Table 3.8.1 shows the effect of various concentrations of EG-NHS and AA-NHS on PAP-I activity.

Modifier concentration (mg/mL)	Activity as a percentage of the control ± SD	
	EG-NHS	AA-NHS
Control	100	100
1	108.6 ± 3.0	N.D.
2	21.8 ± 0.4	42.4 ± 0.8
3	17.3 ± 4.2	N.D.
4	18.7 ± 4.4	24.9 ± 0.8

Table 3.8.1 ***Effect of EG-NHS and AA-NHS on PAP-I Activity***. The effect of NHS esters on PAP-I activity was determined as outlined in section 2.10.1. Activities are expressed as a percentage of the control (100%) in the absence of any modifier. N.D Not determined due to lack of material.

Concentrations of modifier higher than 1mg/mL significantly inhibit the enzyme.

3.8.2 Thermostability of Native and Modified Enzyme

The thermostability of native and modified enzyme was determined as outlined in section 2.10.2. The result of this is shown in table 3.8.2. EG-NHS modified shows almost a twofold increase in thermostability over native (unmodified) PAP-I.

Modifier concentration (mg/mL)	Activity as a percentage of the control	
	EG-NHS	AA-NHS
Control	100	100
1	170.4	N.D.
2	105.5	81.4
3	22.7	N.D.
4	39.3	95.4

Table 3.8.2 ***The Thermostability of Native and Modified Enzyme.*** Native and modified PAP-I were heated at 55^{0}C for 10 minutes. The activities are expressed as a percentage of the control (100%) in the absence of any modifier after heating at 55^{0}C for 10 minutes. N.D. Not determined due to lack of material.

4. Discussion

4.1 MCA Standard Curves: The Inner Filter Effect

Fluorimetric assays offer increased sensitivity over colourimetric assays (Blackmon *et al.*, 1992; Lloyd, 1981; Rendell 1987). Due to the fact that all of the enzyme assays described in section 2.2 are based on the release of MCA from specific substrates, a brief discussion on fluorescence spectrometry is warranted. Liberated MCA was quantified by reference to standard curves, prepared as outlined in section 2.3. Figures 3.1.1 to 3.1.4 are examples of standard curves obtained. The inclusion of biological samples such as crude homogenate or S_2 of bovine brain in the standards resulted in a decrease in the slope of the curve, as illustrated in figures 3.1.1 and 3.1.2. This decrease is primarily due to two factors; the *inner filter effect* and *quenching*. It is not always clear from the literature, whether or not these phenomena have been taken into consideration by other investigators using fluorimetric enzyme assays.

The inner filter effect occurs whenever there is a compound present in the sample with an absorption band which overlaps either the excitation or emission band of the fluorescent analyte. Radiation at the excitation wavelength (370nm for MCA) may be absorbed before it can excite the fluorescent species. There may also be absorption of emitted radiation (440nm for MCA) before it leaves the cell. Other terms used to describe this effect include *self absorption*, *prefilter* and *pastfilter effects* and *the trivial effect*. It becomes a problem when the absorption is high or when the concentration of the absorbing species, and therefore its absorbance, varies from sample to sample.

Quenching involves the removing of energy from a molecule in the excited state, by another molecule, usually as the result of a collision. This can be important in fluorescence spectrometry since the fluorescence of the analyte may be quenched by the molecules of other compounds present in the incubation mixture. Compounds containing heavy atoms (especially the heavier halogens) are effective quenching agents. Compounds containing unpaired electrons, such as molecular oxygen in aerated buffers, can also act as efficient quenching agents. However, it is apparent that the most common source of error and misinterpretation is due to the inner filter effect (Lloyd, 1981).

The purification of PAP-I from bovine brain is an example of how failure to consider the inner filter effect, could lead to misinterpretation of results. If a standard curve incorporating S_2 (see figure 3.1.2) had not been prepared, the total PAP-I activity in the supernatant would have been calculated to be 204 units, 12% lower than the actual value of 232 (see table 3.3.1). Similarly, if a standard curve incorporating crude homogenate had not been prepared (see figure 3.1.1) the total PAP-I activity in the starting sample would have been calculated to be 255 units, 2% lower than the actual

value of 260 (table 3.3.1). As a result of this, an erroneous final yield of PAP-I would have been obtained. The quenching effect was not observed with any of the post column enzyme fractions.

Preparation of a standard curve, consisting of nine MCA concentrations, each in triplicate and incorporating enzyme, requires 2.7mL of enzyme sample. Although the presence of biological sample causes a decrease in slope, the standard curve remains linear over the MCA concentration range (figures 3.1.1 to 3.1.4). Therefore, in order to minimise the amount of sample required, mini-standard curves were prepared which incorporated only two MCA concentrations (0μM MCA and a top standard). This reduced the amount of sample required to 600μL per curve. In addition, it was found that 0.2% w/v BSA in 50mM Tris/HCl buffer at pH 8.0 could be used instead of purified PAP-I containing 0.2% w/v BSA. This greatly reduced the amount of purified enzyme required for standard curve preparation during characterisation studies.

It should be noted that compounds other than biological samples were found to contribute to the inner filter/quenching effects. Some of the modifying reagents also contributed to the inner filter effect. This necessitated the preparation of quenched standard curves for each modifier as outlined above. For example, a decrease of 17.5% was observed in the slope when sample containing 1mM rose bengal was incorporated into the standard curve.

The final factor to be discussed in relation to fluorescence spectrophotometry is the geometry of the sample cell. The magnitude and characteristics of inner filter effects depends strongly on sample cell geometry (Lloyd, 1981). The cuvette based assays (1.5mL final volume) employ perpendicular geometry. In this case, the fluorescence emitted is collected along an axis at right angles to the excitation beam. Because of its freedom from the effects of scattered and transmitted excitation, this is the preferred arrangement (Lloyd, 1981). All quantitative assays were performed in this manner, using glass cuvettes. The microplate assays described in section 2.2.3, employ front surface geometry. In this arrangement, the fluorescence is collected and measured through the illuminated surface. Front surface geometry is particularly prone to inner filter effects and a high level of scattered light is diverted to the detector. As a result of these factors, coupled with the loss in sensitivity, due to the optical fibres contained in the microplate reader accessory, microplate enzyme assays were used only for non-quantitative determinations.

4.2 Purification of Cytosolic Pyroglutamyl Aminopeptidase from Bovine Brain.

The purification procedure used was a modification of that used by Cummins and O'Connor (1996). The main aim of the modification was to increase the yield of purified PAP-I. Yields from the original purification protocol were less than 7%. The entire purification procedure was carried out at 4^0C and the pH was maintained at 8.0 in order to minimise the loss of activity. An adsorption process was used as the first step due to its high capacity while gel-filtration, which has a much lower capacity, was used as an intermediate step. The sequence in which the chromatographic steps were arranged also served to minimise the length of time spent purifying the enzyme. The complete purification required approximately 30 hours.

Following the tissue preparation and centrifugation procedure outlined in section 2.5.1, 89% of the PAP-I activity was observed in the soluble fraction. This was an improvement on the original procedure, which had a yield of 80%. The main difference was the use of distilled water instead of buffer, in the resuspension of the first pellet. Researchers had suggested the use of an osmotic shock technique, using distilled water, in order to lyse entrapped vesicles formed during the homogenisation procedure, which may, and indeed does, harbour occluded enzyme activity (Van Amsterdam *et al.*, 1983). The use of a further osmotic shock step may yield more of the occluded activity but it will result in further dilution of the sample. It should also be noted that Mudge and Fellows (1973) have completely localised a pGlu-Ala hydrolysing activity from bovine pituitary homogenates to the soluble fraction following an ultracentrifugation procedure (100,000g).

Anion exchange chromatography using DEAE Sepharose Fast Flow proved an ideal first step in the purification of PAP-I from bovine brain. As can be seen from figure 3.3.1, most of the contaminating protein runs through the column under the conditions used. Application of a salt gradient results in elution of PAP-I. An excellent active recovery of 87% was achieved using this technique.

Gel filtration of post anion-exchange PAP activity on a Sephadex G100 column enabled excellent separation of PAP-I from other cytosolic components (figure 3.3.2). The main reason for the apparently low overall yield obtained using the original procedure was that only 18% active recovery was obtained using Sephacryl S200 HR gel filtration. Initial attempts to improve this yield centred around finding a column more suitable for the molecular weight of PAP-I and also finding a potential stabilising agent for the enzyme. It was decided that Sephadex G100 gel filtration would be more

appropriate for the relatively low (24,000 daltons) molecular weight of PAP-I. Initial yields from this column appeared to be approximately the same as those from the Sephacryl S200 HR gel filtration column.

The reversible inhibitor and stabiliser, 2-pyrrolidone, has been previously used by researchers to stabilise PAP-I (Armentrout and Doolittle, 1969; Szewczuk and Kwiatkowska, 1970; Mudge and Fellows, 1973). Addition of 2-pyrrolidone into the sample prior to application to the gel filtration column, its inclusion in the running buffer and its subsequent removal by dialysis proved unsuccessful in stabilising PAP-I. Glycerol and BSA are both widely used as enzyme stabilisers (see section 1.3.1.1.2 and 1.3.1.1.3). Glycerol and BSA were added to the fraction collection tubes (in two different purification runs) prior to the gel filtration run in an attempt to find out if they would stabilise the enzyme as it comes of the column. The glycerol did not stabilise the PAP-I while the BSA resulted in much higher yields (almost 55%).

Further investigation of this result lead to the discovery that during the assay of PAP-I from the G100 column, pGlu-MCA hydrolysis does not proceed in a linear fashion with respect to time (see figure 3.5.4). When BSA was incorporated into the assay, the hydrolysis of the substrate proceeds in a linear fashion (for further discussion of this topic, see section 4.4). Development of the assay allows for a more accurate estimate of recovery from the G100 gel filtration column (approximately 55%).

Affinity chromatography of post gel filtration PAP-I activity exploited the sulphydryl nature of this enzyme and subsequently proved to be an efficient 'clean-up' step. Using an Activated Thiol Sepharose 4B column, PAP-I activity could be specifically bound to the column through the formation of disulphide linkages between the matrix functional groups and intact thiol groups located within the active site of the enzyme. The recovery of cytosolic PAP from this column was low (34% of total applied activity) but the excellent resolution of PAP-I from contaminating proteins (see figure 3.3.4) and a 10-fold reduction in protein justified its inclusion in the overall purification procedure. The low recovery is partly due to the fact that some of PAP-I does not bind to the column. Looking at figure 3.3.4, unbound activity appears minimal but the peak is masked somewhat by the 2-thiopyridone capping group coming off the column which quenches fluorescence during the microplate assay. The reason why all of the PAP-I does not bind may be explained as follows. The reducing agent, DTT, must be removed in a short time period. The enzyme thiol groups must be reduced, with all the disulphide groups converted to thiols. The reducing agent must be removed quickly in order to mimimise reversion of thiol groups to disulphide groups prior to

chromatography. This may have occurred with some of the PAP-I molecules, but a balance must be struck in the dialysis time, between removal of most of the DTT and reversion of the thiol groups. The exact mechanism of affinity chromatography using Activated Thiol Sepharose 4B is briefly illustrated in Fig 4.3.3. For a more in depth review of this technique, see Brocklehurst et al. (1974).

An overall purification factor of 247-fold with a 14.4% recovery was obtained for the purification of the enzyme from bovine brain (see table 3.3.1). This represents a significant improvement on the original purification procedure, which achieved a 91-fold purification factor and a 6.7% recovery (Cummins and O'Connor, 1996).

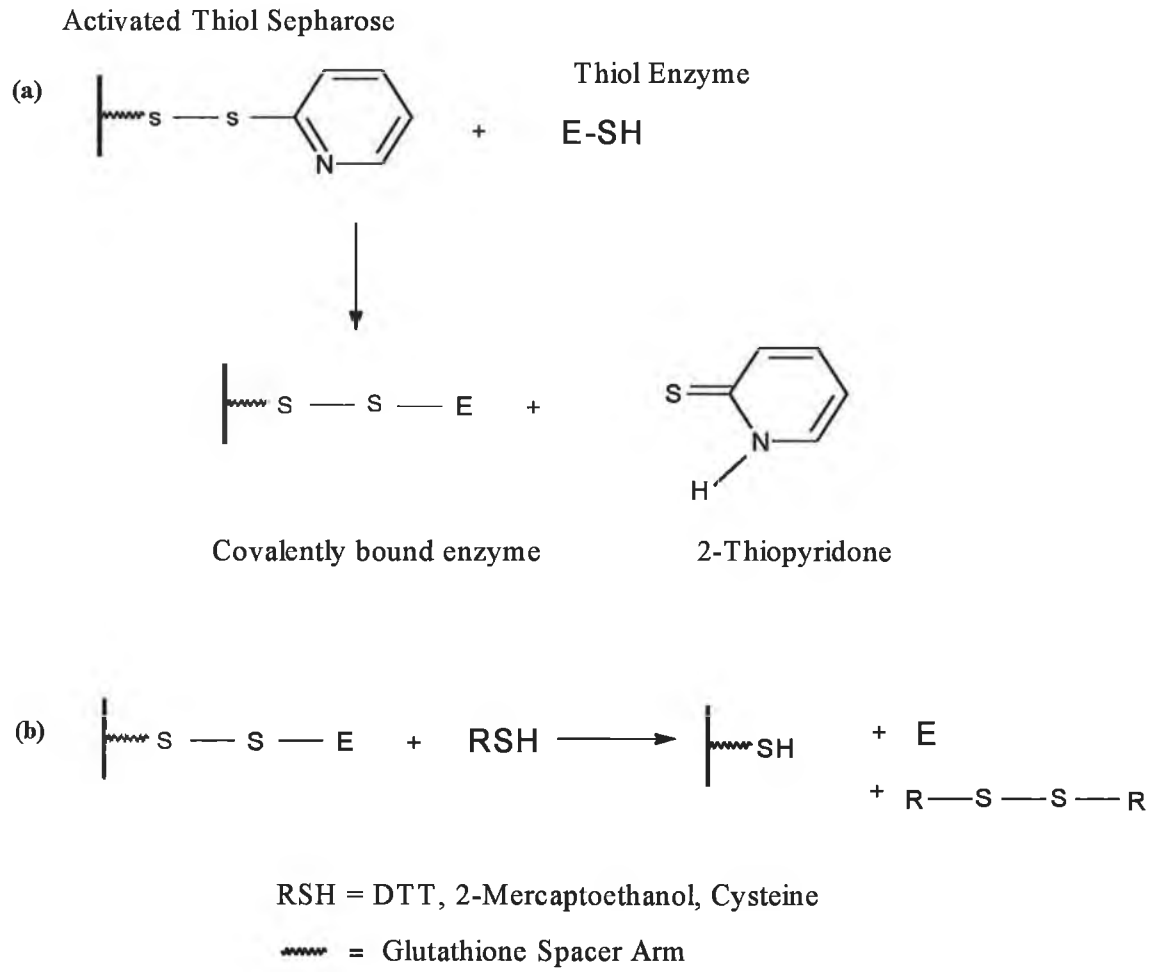


Figure 4.3.3. *Protein purification strategy using Activated Thiol Sepharose (ATS).* ***(a)*** *Protein in reduced form is applied to ATS resin that is capped with 2-Thiopyridone linked to Sepharose via a glutathione spacer arm. The protein binds covalently via a disulphide bond to the resin, replacing the capping group (2-Thiopyridone).* ***(b)****. Application of reducing agent (RSH) such as DTT, reduces the disulphide bond, eluting the bound protein from the resin.*

4.3 Assessment of the Effectiveness of the PAP-I Purification Procedure using Polyacrylamide Gel Electrophoresis

The photographic evidence presented in figure 3.4.1 demonstrates that the gel filtration in particular effectively separates soluble PAP activity from the bulk of the proteins. This fact is corroborated by the 10-fold increase in specific activity obtained (see table 3.3.1). The inclusion of the affinity chromatography step is further justified by comparing lanes 2 and 3 in figure 3.4.1. ATS 4B affinity chromatography clearly removes several of the bands faintly visible in lane 3.

The silver stained band corresponding to purified PAP-I activity is clearly displayed in lane 2 of figure 3.4.1. Some additional bands are also faintly visible in this lane. As to whether these bands are contaminating protein or artefactual bands remains unclear. Bauer (1994) has suggested that aretfaetual bands can arise during silver staining when 2-mercaptoethanol is used in the sample buffer (as in this case). It can be concluded that the enzyme has been purified to near homogeneity using this purification scheme. From the gel, the purified PAP-I can be seen to migrate slightly further than the carbonic anhydrase marker molecular (29,000 daltons) to a position representing a relative molecular mass of approximately 24,680. It should be noted that several workers have previously reported that soluble PAP activity of similar molecular mass. Mantle et al. (1990, 1991) have reported a native molecular mass of 22,000 daltons for soluble PAP activity isolated from human kidney and skeletal muscle. It also compares favourable with the molecular mass of 24,000, obtained from the same source by other workers in our laboratory (Cummins and O'Connor, 1996).

4.4 Development of the pGlu-MCA based assay

Once PAP-I was purified it was necessary to re-evaluate the assay procedure and determine the optimal conditions for PAP-I activity and assay linearity. For an enzyme assay to be reproducible and quantitative, substrate hydrolysis must be linear with respect to time and enzyme concentration. As mentioned in section 4.2, the PAP-I assay from post gel filtration samples was non-linear (see fig 3.5.4). Therefore, it was necessary to perform assay development studies on post gel-filtration PAP-I as well as on purified PAP-I. When the assay for both was found to be optimal, all subsequent assays were performed as outlined in section 2.2.2. All figures presented in table 3.3.1 were obtained using the optimised assays.

The fluorogenic substrate pGlu-MCA was first used by Fujiwara and Tsuru (1978), who noted that the release of MCA could be followed with high sensitivity. The assay used throughout this project is

a modification of the method used by Cummins and O'Connor (1996). The concentration of DTT in present in the substrate solution was increased from 2mM to 10mM in order to ensure full expression of PAP-I activity. It has been found that 6 to 8mM DTT is required for full expression of enzyme activity (unpublished results from our laboratory). EDTA was included in the assay mixture to chelate any metal ions, which can cause oxidation of thiol groups (see section 1.3.1.1.3).

The progress curves of most enzyme reactions are of the form shown in figure 4.4.

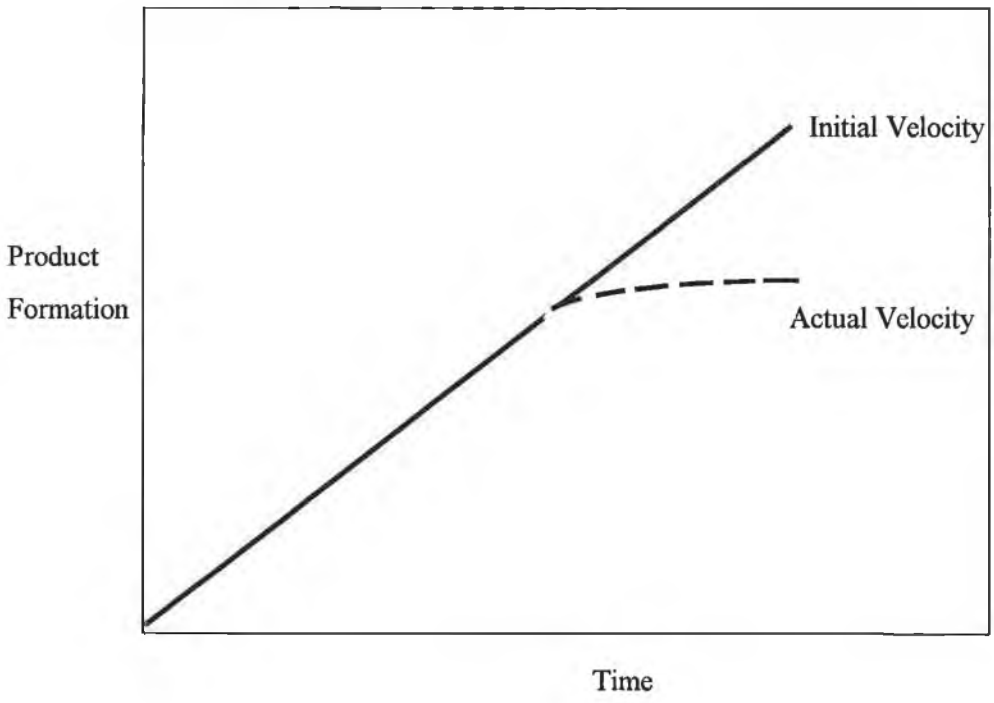


Figure 4.4 ***Linearity of Product Formation with Respect to Time***

The time course is linear to begin with but the rate of product formation decreases with time. Various factors may contribute to this decrease and primary among these, in the case of PAP-I, is thought to be the enzyme's instability during assay. This is highlighted in the progress curves of PAP-I activity against pGlu-MCA shown in figures 3.5.1, 3.5.2, 3.5.4 and 3.5.5. These graphs illustrate that when supernatant (S_2) or post ion exchange pool is used as the enzyme source, the reaction proceeds in a linear fashion over the one hour time period. When post gel filtration or post-affinity chromatography samples are used, the reaction proceeds in a non-linear fashion, with the reaction rate departing from linearity in less than ten minutes. The inclusion of BSA in the assay mixture restores linearity.

As mentioned in section 1.3.1.1.3, BSA is often included in reaction mixtures to stabilise dilute enzyme mixtures. Post gel filtration PAP-I is very dilute with a protein concentration of 19μg/mL in a volume of 46mL. The binding of an enzyme to resins coupled with hydrophobic groups, like phenyl sepharose is indicative of hydrophobic residues on the enzyme surface (Schein, 1990). Indeed PAP-I does bind to phenyl sepharose when applied in 1M ammonium sulphate (results not shown). The hydrophobic interactions PAP-I makes with the highly hydrophobic molecule, BSA, will, as outlined in section 1.3.1.1.3, play a significant part in the stabilisation of PAP-I. As can be seen from figure 3.5.3, approximately 1-2mg/mL BSA is required for optimal stabilisation, and therefore activity, during assay.

4.5 Active Site Studies

To date, mammalian PAP-I has not been sequenced. Therefore, some knowledge of its active site would be useful, for example, in the synthesis of PAP-I inhibitors. A range of modifying reagents were used, as outlined in section 2.8, in order to identify the amino acids at the active site of the enzyme. These results are presented in table 3.6.1.

Diethylpyrocarbonate inhibited PAP-I at the two concentrations tested (2.5mM and 10mM). DEPC is a specific modifier of histidine residues (Dickenson and Dickenson, 1975). This suggests that a histidine residue is essential for activity. TRH is known to be a competitive inhibitor of PAP-I (Cummins and O'Connor, 1996). The presence of TRH offered significant protection against inactivation of the enzyme by DEPC (see table 3.6.2), a finding suggesting the essential nature of histidine in events at the active site. However, as can be seen from figure 3.6.4, this protective effect did not occur immediately, but did so after approximately 2 minutes. The effect lasts throughout the fifteen minute experiment, with the 'degree' of protection even increasing slightly. The fact that a His residue may be essential in catalysis is further substantiated by the fact that the enzyme is inhibited by rose bengal, which is reported to catalyse photooxidation of histidine residues (Cohen, 1970). Again, significant protection is afforded to the enzyme by the presence of TRH. It is worth mentioning at this point that His residues are known to have proton donor or acceptor function in many peptidase active sites.

N-Ethylmalemimide, iodoacetic acid and iodoacetamide all significantly inhibited PAP-I (see table 3.6.1). Therefore, a cysteine residue is required for activity. This correlates with the previously known fact that PAP-I is a member of the cysteine protease family (Browne and O'Cuinn, 1983a; Cummins and O'Connor, 1996). When TRH was included in the assay with these modifiers,

significant protection was afforded to the enzyme, indicative of the fact that cysteine plays an essential role in catalysis at the active site. As with the DEPC, TRH does not exert its affect in the presence of IAA until approximately 2 minutes (see figure 3.6.2). When NEM is used, the protective effect is almost instantaneous and the 'degree' of protection actually increases slightly throughout the course of the experiment. Cysteine also forms part of the catalytic site of PAP-I from some bacterial sources (Awade *et al.*, 1992b; Gonzales and Robert-Boudouy, 1994).

PAP-I is also significantly inhibited by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Carbodiimides are bivalent coupling reagents with the general formula R-N=C=R'. These water-soluble reagents have been used for the modification of carboxyl groups in proteins (Carraway and Koshland, 1972) to produce O-acylisoureas which in turn react with nucleophiles such as amino groups (Ji, 1983). Acidic conditions have been used in order to minimise cross-linking of carboxyls with lysine (a nucleophile) residues (Carraway and Koshland, 1972). Here, conditions of neutral to alkaine pH were required for inactivation. TRH affords the enzyme significant protection (see figure 3.6.1); therefore, aspartic acid and/or glutamic acid, in all probability, are essential for enzyme activity or substrate binding. Because of the pH required for inactivation, the possibility that lysine may be at the active site cannot be discounted.

2,4,6 Trinitrobenzene sulphonic acid, pyridoxal phosphate and acetaldehyde all fail to inhibit PAP-I, both in the presence and absence of sodium borohydride. All these compounds are specific for lysine residues. This, therefore, eliminates the possibility that lysine forms part of the active site, as was suggested above.

Failure of N-acetylimidazole to inhibit PAP-I indicates that a tryosine residue is not essential for activity. N-acetylimidazole has been used primarily for the acetylation of tyrosyl residues (Riordan and Vallee, 1972a). This is further confirmed by the tetranitromethane result. As mentioned in section 1.3.2, TNM is involved in the nitration of tyrosine residues. Therefore, tyrosine is not required for enzyme activity. Arginine does not form part of the active site either because phenylglyoxal has no effect on the enzyme (Takahashi, 1977).

Therefore, along with Cys and His residues, Asp and/or Glu are necessary for enzymatic activity and they are all probably located at the active site. Interestingly, a similar study with PAP-I from *Pseudomonas fluorescens* came to a similar conclusion, that Cys and His residues are essential for enzyme activity and that Asp, Glu, Asn and/or Gln complete the catalytic triad or tetrad (Gonzales and Robert-Baudouy, 1994). A more recent study by the same researchers on PAP from the same

source came to the conclusion that Cys and His constitute the nucleophilic and imidazole residues of the active site, while Asp or Glu might constitute the third part of the active site (Le Saux et al., 1996). This may also be true of PAP-I from a mammalian source. Tyr, Lys and Arg are not involved in the expression of catalytic activity of PAP-I from a mammalian source. Serine is also not involved in the expression of activity due to its lack of inhibition by phenylmethylsulphonylfluoride (Cummins and O'Connor, 1996).

4.6 Stability Studies

PAP-I is known to be an unstable enzyme (sections 1.2.1.1 and 4.4). BSA has been found to stabilise the enzyme (section 4.4). However, an alternative to BSA was sought. As mentioned in section 1.3.1, numerous low molecular weight substances are known to stabilise enzymes. A range of these were prepared as outlined in table 2.9.1. The reason for the preparation of these reagents can be explained as follows. Chemical modification of PAP-1 was to be attempted at a later date. Storage of PAP-I in BSA would almost certainly interfere with these modifications, by 'mopping up' all, or most, of the modifier before it has a chance to act on PAP-I.

As can be seen from table 3.7.1, none of the additives tested were as effective as BSA at increasing PAP-I activity. The only one which showed any promise was PEG. It was decided to test the effectiveness of PEG in stabilising PAP-I during assay. From figure 3.7.1, it can be seen that, while PEG increases the activity of PAP-I, it is not effective in maintaining assay linearity. Even a combination of BSA and PEG was not as effective as BSA alone. Once these initial stability studies proved unsuccessful, it was concluded that it is a protein to protein interaction (most likely the hydrophobic interactions mentioned in section 4.4) that stabilises PAP-I. The only alternative was to attempt some initial chemical modifications with PAP-I stored in a lower concentration of BSA. It was already established that 1mg/mL BSA was as effective as 2mg/mL BSA in attaining optimum activity (figure 3.5.3). From figure 3.7.2, it can also be concluded that PAP-I is as stable, if not more so, in 1mg/mL BSA as it is in 2mg/mL BSA when stored at -80^{0}C. Glycerol has been used regularly in the storage of enzymes; as can be seen from figure 3.7.2, it is not nearly as effective as BSA in maintaining PAP-I activity during storage. O'Fagain, 1997 stated that while , it may seem foolish to add an exogenous, contaminating protein, such as BSA deliberately to a pure protein preparation, occasionally this may be the price to be paid to avoid instability and inactivation. Therefore, purified PAP-I stored in 1mg/mL BSA was used for chemical modification studies.

4.7 Stabilisation of PAP-I via Chemical Modification

PAP-I stabilisation was attempted using the N-hydroxysuccinimide esters, due to their mild chemical reaction conditions, their rapid rate of reaction and their specific targeting of amino groups, which have been found to be non-essential for catalytic activity (section 4.5). Despite this, concentrations of EG-NHS and AA-NHS higher than 1mg/mL proved detrimental to PAP-I activity (table 3.8.1). Nevertheless, the thermostability of both native (unmodified) and modified PAP-I was assessed by heating both for 10 minutes at 55^0C. Table 3.8.2 shows that the EG-NHS modified enzyme shows almost a twofold increase in thermostability over unmodified enzyme. AA-NHS modified enzyme at the concentrations used did not show any increase in thermostability. The possibility of increased thermostability with AA-NHS modified enzyme, at a concentration of 1mg/mL, cannot be discounted. Crosslinking with agents such as NHS esters introduces 'braces' to the enzymes primary structure and this prevents the unfolding of the tertiary structure. Cross-linking was almost certainly successful in the case of EG-NHS. The cross-link(s) introduced probably prevented the enzyme structure from unfolding upon exposure to heat. Further work could focus on examining the resistance of this 'stabilised' enzyme to, for example, organic solvents or denaturants. Careful choice of other cross-linkers, like the amino-specific bisimidates, could also lead to stabilisation. In conclusion, preliminary modification studies do show some promise although substantial work needs to be done in order to test the limits of this 'stabilised' enzyme.

4.8 Summary

Pyroglutamyl aminopeptidase was purified from the cytosolic fraction of bovine whole brain by chromatography with DEAE sepharose, G100 gel filtration and ATS 4B affinity chromatography. This resulted in a 247-fold purification and a 14.4% recovery of enzyme activity.

Prior to further work, the PAP-I fluorimetric activity assay was optimised. This assay was found to be linear over an assay period of one hour, using 100μM pGlu-MCA prepared in 0.75% v/v DMSO. Because PAP-I is a cysteine protease, DTT was included in the assay at a concentration of 10mM. EDTA was also incorporated to chelate any inhibitory metal ions. The partially purified and purified enzyme was found to be unstable during assay. The inclusion of 1 or 2 mg/mL BSA served to stabilise the enzyme, during assay and also during storage. Other potential stabilisers tested were not as effective as BSA at increasing or maintaining PAP-1 activity (see table 3.7.1). The relative molecular mass of PAP-I, determined by SDS PAGE, was found to be 24,680 Da.

PAP-I was confirmed to be a cysteine protease based on its sensitivity to IAA, NEM and iodoacetamide. The enzyme was also inhibited by DEPC and EDC, suggesting that His and Glu and/or Asp are also involved in binding and/or catalytic activity at the active site. These results were confirmed by the protective effect of the competitive inhibitor TRH on inactivation of PAP-I by these modifying reagents. Cys and His may constitute the nucleophilic and imidazole residues of the active site, while Asp or Glu might constitute the third part of the active site. Lys, Tyr, Arg, and Ser are not involved in expression of activity and, therefore, do not take part in events at the active site.

Initial stabilisation via chemical modification demonstrated that EG-NHS-modified PAP-I showed almost a two-fold increase in thermostability over the native, unmodified enzyme. At the concentrations tested, AA-NHS modified enzyme did not show any increase in thermostability.

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