The Purification and Characterisation of a Membrane Bound Pyroglutamyl Aminopeptidase Type-II from Bovine Brain

Thesis Submitted for the Degree of
Doctor of Philosophy
by

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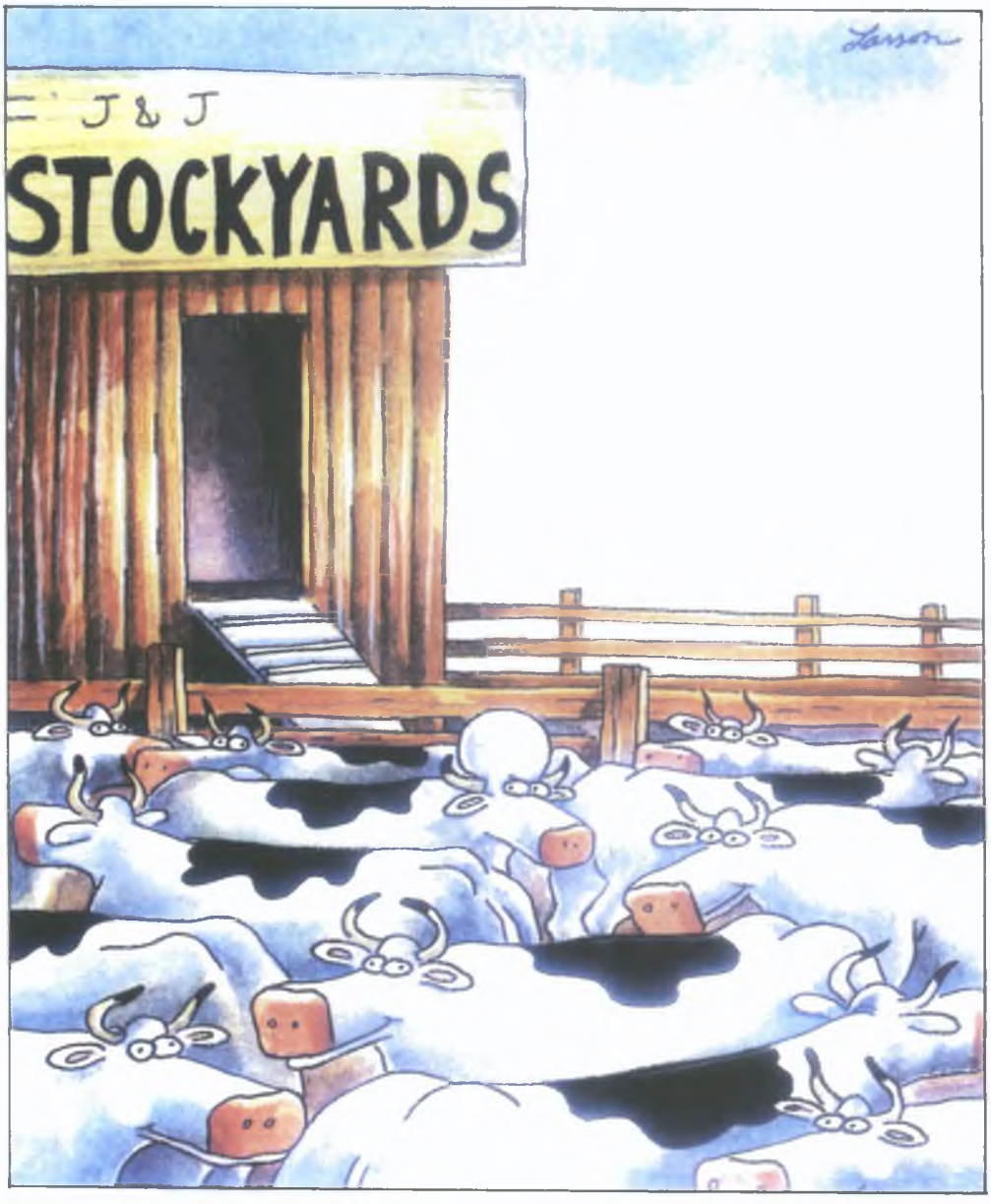
Declaration

I hereby certify that this material, which I now submit for assessment on the program of study leading to the award of Doctor of Philosophy 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 cited and acknowledged within the text of my work.

Signed: Seán Gallagher **Date:** 17/12/96

Seán P. Gallagher



Only Claire, with her oversized brain, wore an expression of concern.

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My most sincere thanks to the following

The wise one, Dr Brendan O'Connor, whose enthusiasm knows no bounds, for his support and friendship throughout this project and for showing that biochemistry is something to be enjoyed

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Abbreviations

The following abbreviations are used throughout this text.

AEBSF	4-(2-Aminoethyl)-benzenesulfonylfluoride
ATP	Adenosine 5'-triphosphate
BCA	Bicinchoninic acid
βNA	β-Naphthylamide
BSA	Bovine serum albumin
cDNA	copy Deoxyribonucleic acid
CDTA	1,2-Cyclohexanediaminetetraacetic acid
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
CNS	Central nervous system
CPHNA	N-1-carboxy-2-phenyl ethyl (N^{im}benzyl)-histidyl-ßNA
Cyclo His-Pro	His-Pro diketopiperazine
Da	Daltons
DLP	Direct linear plot
DMSO	Dimethylsulphoxide
DAPII	Dipeptidyl aminopeptidase II
DAPIV	Dipeptidyl aminopeptidase IV
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	[Ethylenebis (oxyethylenenitrilo)] tetraacetic acid
E-H	Eadie-Hofstee
fmoc-Pro-Pro-Nitrile	9-Fluorenylmethyloxycarbonyl-prolyl-pyrrolidine-2-nitrile
GH	Growth hormone
GPCR	GTP-binding protein-coupled receptor
GTP	Guanosine 5'-triphosphate
H-7	1-(5-isoquinolinylsulphonyl)-2-methyl piperazine
HPLC	High performance liquid chromatography
HPT	Hypothalamic pituitary thyroid
H-W	Hanes-Woolf
IgG	Immunoglobulin G
i.p	intraperitoneal
i v	Intraveneous
K_I	Inhibitor dissociation constant
K_m	Michaelis Menten constant
L-B	Lineweaver-Burk
LHRH	Luteinizing hormone releasing hormone
MCA	7-Amino-4-methyl-coumarin
M-M	Michaelis-Menten

MND	Motor neuron disease
mRNA	Messenger ribonucleic acid
2NA	2-Naphthylamide
NEM	N-Ethylmaleimide
NMDA	N-methyl-D-aspartate
PAGE	Polyacrylamide gel electrophoresis
PAPI	Pyroglutamyl aminopeptidase type-I
PAPII	Pyroglutamyl aminopeptidase type-II
PDMK	pGlu-diazomethylketone
PE	Prolyl endopeptidase
PEG	Polyethylene glycol
pGlu	Pyroglutamic acid
PMSF	Phenylmethylsulphonylfluoride
pNA	p-Nitroanilide
PTU	Propylthiouracil
Q	Quaternary anion
QSAR	Quantitative structure-activity relationship
R_f	Relative mobility
RNAase	Ribonuclease
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SP	Sulphopropyl
T_3	Triiodothyronine
T_4	Tetraiodothyronine
TEMED	N, N, N, N'-Tetramethyl ethylenediamine
TFA	Trifluoroacetic acid
TPA	12-0-tetradecanoyl phorbol 13-acetate
TRH	Thyrotropin releasing hormone
TRH-R	Thyrotropin releasing hormone receptor
TSH	Thyroid stimulating hormone
Tris	Tris(hydroxymethyl)amino methane
V_e	Elution volume
V_o	Void volume
v/v	Volume per volume
w/v	Weight per volume
Xaa-	Any amino acid
Z-	*N*-Benzyloxycarbonyl-

Amino Acid Abbreviations

Ala	Alanine	**Cys**	Cysteine	**His**	Histidine	**Met**	Methionine	**Thr**	Threonine
Arg	Arginine	**Gln**	Glutamine	**Ile**	Isoleucine	**Phe**	Phenylalanine	**Trp**	Tryptophan
Asn	Asparagine	**Glu**	Glutamate	**Leu**	Leucine	**Pro**	Proline	**Tyr**	Tyrosine
Asp	Aspartate	**Gly**	Glycine	**Lys**	Lysine	**Ser**	Serine	**Val**	Valine

2 MATERIALS AND METHODS

4 DISCUSSION

Abstract

Three new fluorimetric assays were developed for the detection of pyroglutamyl aminopeptidase type-II (PAPII) activity Two of these assays are based on hydrolysis of the TRH substrate analogue pGlu-His-Pro-MCA, while the third, a continuous assay, exploits the ability of the enzyme to hydrolyse the substrate pGlu-MCA

Following solubilisation of PAPII from the membrane fraction of bovine brain, using trypsin, the enzyme was purified 3,000-fold, with an overall yield of 24%, using a range of conventional chromatographic techniques

By gel filtration chromatography, a relative molecular mass of 214,000 Da was determined for the enzyme The purified PAPII was found to be relatively labile However, the presence of 1% v/v BSA was shown to greatly improve its stability Optimal enzyme activity was observed at 45°C A pH optimum of 6 8-7 6 was observed for the enzyme, with rapid inactivation occurring below pH 4 0 and above pH 9 2

Purified PAPII was strongly inhibited by the transition metals Cd^{2+}, Hg^{2+} and Zn^{2+}, while activation was observed in the presence of Co^{2+} The enzyme was identified as a metallopeptidase on the basis of its inhibition, in a time dependent manner, by metal-complexing agents and its subsequent reactivation in the presence of metal ions, including Zn^{2+}, Ni^{2+} and Co^{2+} Inhibition by cysteine protease inhibitors and activators and by the serine protease inhibitor AEBSF, was also observed

Substrate specificity studies revealed that, with the exception of pGlu-Phe-Pro-NH_2, pGlu-MCA and pGlu-ßNA, the purified enzyme cleaves N-terminal pyroglutamic acid from only tri- and tetrapeptides with a His residue in the penultimate position A number of N-terminal pyroglutamyl peptides of varying length were shown to competitively inhibit the enzyme Of these, LHRH and LHRH 1-5, although not substrates for the enzyme, were found to be potent inhibitors, with K_i values of 8µM and 11µM respectively

1. Introduction

1.1 Pyroglutamic Acid

Pyroglutamic acid (pGlu, Glp, <Glu), also known as pyrrolidone carboxylic acid (PCA, pyr) and 5-oxo-L-proline is a cyclical amino acid with unique properties Structurally, it may be considered to be an internally cyclised glutamic acid (see figure 1 1) The presence of the internal amide bond which forms between nitrogen-1 and carbon-5 chemically defines pyroglutamic acid as 2-carboxy γ-butyrolactam The internal linkage is neutral and functionally, acts as an amide (Abraham and Podell, 1981)

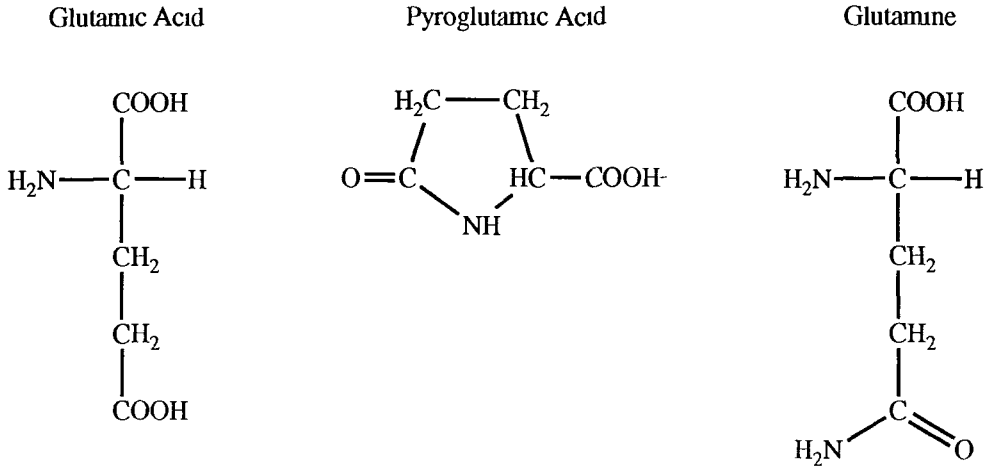


Figure 1.1 *Structures of pyroglutamic acid and its two possible amino acid precursors, glutamic acid and glutamine*

Pyroglutamic acid can be formed non-enzymatically by heating glutamic acid at 150°-160°C However, γ-derivatives of glutamic acid, for example glutamine, γ-esters of glutamic acid and γ-glutamyl peptides, cyclise much more rapidly than glutamic acid itself The non-enzymatic cyclisation of glutamine is substantial even at 37°C, 10% being converted to pyroglutamic acid after 24 hours (Orlowski and Meister, 1971)

The enzymatic synthesis of pyroglutamic acid, by a range of enzymes, has been demonstrated and is reviewed by Orlowski and Meister (1971) and Abraham and Podell (1981) The first indication of the enzymatic synthesis of pyroglutamic acid was the discovery of D-Glutamic acid cyclotransferase, an enzyme found in kidney and liver which is capable of converting D-glutamic acid to D-pyroglutamic acid Orlowski and Meister (1971) suggest that the role of this enzyme may be to detoxify D-glutamic acid, present in the diet or formed by bacterial metabolism in the intestine D-glutamic acid is one of the most abundant bacterial D-amino acids and is a very poor substrate for D-amino acid oxidase L-glutamic acid cyclotransferase (L-glutamic acid dehydrase) has been found in a variant of *Pseudomonas cruciviae* (Akida *et al*, 1959)

Glutamine synthetase, distributed widely in plants and animals, in the presence of ATP and Mg^{2+}, catalyses the synthesis of L-glutamine and D-glutamine from the respective optical isomers of glutamic acid γ-glutamylcysteine synthetase can also catalyse the formation of pyroglutamic acid in the presence

of ATP, L-cysteine and Mg^{2+}, through the formation of the intermediate L-γ-glutamyl-L-cysteine However, it is unlikely that this relatively slow reaction can be of any physiological significance (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 (γ-glutamyl transferase) and subsequently converted to pyroglutamic acid by γ-L-glutamyl cyclotransferase (γ-glutamyl lactamase), (Orlowski *et al*, 1969) This enzyme is widely distributed in animal tissues with relatively high levels of activity in kidney, liver, brain and skin In man, the highest levels of activity are found in the brain Orlowski and Meister (1971) suggest that this wide distribution implies metabolic significance for this enzyme A mammalian glutaminyl cyclase, capable of converting glutaminyl peptides into pyroglutamyl peptides was identified by Fischer and Spiess (1987)

It has been clearly demonstrated that exogenous pyroglutamic acid is not utilised in protein biosynthesis Instead, N-terminal pyroglutamic acid must be formed by cyclisation of the amino-terminal residue (i e glutamine or glutamic acid) It is most likely formed either late in protein translation or immediately prior to secretion of the completed protein from the cell (Abraham and Podell, 1981)

Pyroglutamic acid is present at the N-terminus of many proteins and bioactive peptides with diverse biological functions (see table 1 1) The activity of some bioactive peptides is dependent on the presence of this N-terminal residue (Abraham and Podell, 1981) As a free acid, pyroglutamic acid has been found in the tissue of patients with Hawkinsinuria disease (Borden *et al*, 1992) An increased level of the free acid has been shown in the plasma of patients with Huntington's disease (Uhlhass and Lange, 1988) It 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 physiological significance of pyroglutamic acid, its presence at the N-terminus of polypeptides may confer resistance to non-specific proteolytic degradation

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

Peptide / Protein	Sequence
TRH	pGlu-His-Pro-NH_2
TRH-Like Peptides	
Prostate (Fertilisation Promoting Peptide)	pGlu-Glu-Pro-NH_2
Testis	pGlu-Phe-Pro-NH_2
Alfalfa	pGlu-Tyr-Pro-NH_2
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-
Bombesin	pGlu-Gln-Arg-Leu-Gly-
Eledoisin	pGlu-Pro-Ser-Lys-
Anorexogenic Peptide	pGlu-His-Gly-OH
Fastigiatine	pGlu-Glu-Gln-OH
Fibrinopeptidase B	
Human	pGlu-Gly-Val-Asp(NH_2)-
Reindeer	pGlu-Leu-Ala-Asp-
Bovine	pGlu-Phe-Pro-Thr-Asp-
Physalaemin	pGlu-Ala-Asp(OH)-Pro-
Peptides From Snake Venoms	pGlu-Asp(NH_2)-Trp pGlu-Glu(NH_2)-Trp
Eiseine	pGlu-Glu-Ala-OH
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-
Heavy Chains of Rabbit IgG	pGlu-Ser-Val-Glu-Glu-
Mouse λ Chains	pGlu-Ala-Val-Val-
α2-CB1 of Rat Skin Collagen	pGlu-Tyr-Ser-Asp-Lys-
Human apoLp-Gln-II	pGlu-Ala-Lys-Glu-Pro-
Thymic Factor from Porcine Serum	pGlu-Ala-Lys-Ser-Gln-
Hypertrehalosaemic Neuropeptide	pGlu-Val-Asn-Phe-Ser-
Peptide Inhibiting Epidermal Mitosis	pGlu-Glu-Asp-Cys-Lys-OH
Colon Mitosis Inhibitory Peptide	pGlu-Glu-His-Gly-OH
Caerulein	pGlu-Gln-Asp-Tyr(SO_3H)-
Levitide	pGlu-Gly-Met-Ile-Gly-Thr-
Porcine Pancreatic Spasmolytic Polypeptide	pGlu-Lys-Pro-Ala-Ala-
Human Monocyte Chemoattractant	pGlu-Pro-Asp-Ala-Ile-
Growth Hormone From Tilipia	pGlu-Gln-Ile-Thr-Asp-

(Taken from Awadé *et al* (1994) and subsequently modified)

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 that is not attached to an α-carbon (McDonald and Barrett, 1986)

Pyroglutamyl Aminopeptidases (PAPs) represent one class of omega peptidases which specifically remove the L-pyroglutamyl residue from the amino-terminus of polypeptides by hydrolysis (see figure 1 2) Pyroglutamyl aminopeptidase has also been referred to as pyrrolidonyl peptidase, pyrrolidone carboxyl peptidase, pyrrolidone carboxylyl peptidase, pyrrolidonecarboxylate peptidase, pyroglutamyl arylamidase, pyroglutamate aminopeptidase, pyroglutamyl peptidase, L-pyroglutamyl peptide hydrolase, PYRase and 5-oxoprolyl-peptidase (Awadé *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 (PAPI), a soluble enzyme with biochemical characteristics similar to the bacterial PAPs The second class includes animal type-II pyroglutamyl aminopeptidase (PAPII), 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

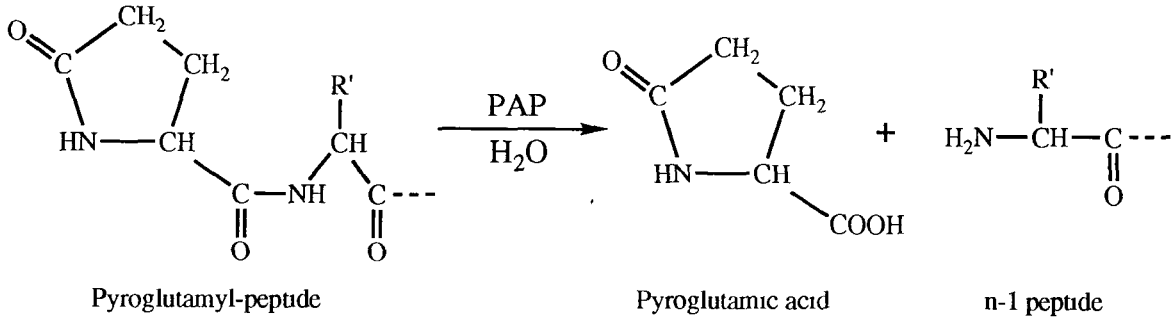


Figure 1.2 *Hydrolysis of pGlu from the N-terminus of L-pGlu-proteins or L-pGlu-peptides by PAP*

1.2.1 Class-I: Animal PAPI and Bacterial PAPs

PAP activity was originally discovered in a strain of *Pseudomonas fluorescens* by Doolittle and Armentrout (1968) and subsequently in a number of other species of bacteria including *Bacillus, Streptococcus, Staphlococcus, Micrococcus, Sarcinia, Citrobacter Klebsiella, Enterobacter, Arizona, Neisseria* and some strains of *Escherichia* (Szewczuk and Mulczyk, 1969) Shortly thereafter the enzyme was found to be present in a wide range of animal, plant and human tissues (Armentrout, 1969, Szewczuk and Kwiatkowska, 1970)

1.2.1.1 Biochemical and Biophysical Characteristics of Class-I PAPs

With the exception of the *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, Awadé *et al*, 1992) The hydrophobic character of four bacterial PAPs (based on the amino acid sequence deduced from cDNA) 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 PAPI (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 (Szewczuk and Kwiatkowska, 1970) The regional distribution of the enzyme in the supernatant fractions of rat brain reveals less than a two fold variation in regions from lowest to highest concentrations (Friedman and Wilk, 1986)

The mammalian PAPI is a monomeric enzyme with a molecular weight of 22,000 to 25,000 Daltons (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 These molecular weights represent multimeric forms of the enzyme with an average subunit molecular weight of approximately 25,000 Da, virtually the same as for the mammalian PAPI The genes for bacterial PAP from four sources are either 639 or 645 nucleotides long, encoding for 213 or 215 amino acids with deduced molecular weights of 22,441 to 23,777 Da (Awadé *et al*, 1994)

PAPI 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 active site cysteine has been identified by site directed mutagenesis following cloning of the gene from *Bacillus amyloliquefaciens* (Yoshimoto *et al*, 1993)

Both the animal PAPI and the bacterial PAPs are unstable and heat sensitive enzymes, being rapidly inactivated at temperatures above 50°C 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 purified enzyme (Cummins and O'Connor, 1996) Optimal enzyme activity is expressed at pH 8 5 for purified mammalian PAPI (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, Awadé *et al*, 1992)

A distinctive feature of PAPI and bacterial PAPs is a broad substrate specificity These enzymes have been shown to cleave N-terminal pGlu from a range of biologically active peptides including TRH, Acid TRH, LHRH, Neurotensin and Bombesin (Browne and O'Cuinn, 1983, Cummins and O'Connor, 1996) pGlu-Pro bonds are not normally hydrolysed by either bacterial or animal PAPI (Doolittle, 1970, 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-ßNA (Szewczuk and Kwiatowska, 1970), L-pGlu-pNA and L-pGlu-MCA (Fujiwara and Tsuru, 1978) resulted in PAP assays with greatly increased sensitivity

PAPI levels in mammals are developmentally regulated In mouse brain, the specific activity is highest on the 13th foetal day and declines thereafter until the 20th-22nd postnatal day with a plateau around birth (Faivre-Bauman *et al*, 1981) Similarly, in rat brain, lung and gut, highest levels of enzyme activity were observed in the late foetal stages with a subsequent decrease over the next two weeks to adult levels (Fuse *et al*, 1990)

There is clear evidence that PAPI is regulated in some cells and tissues The activity if PAPI in CH3 cells (a clonal line of rat anterior pituitary cells) was elevated following exposure for three days to 5-oxoprolinal (a specific PAPI inhibitor) This increase was due to an increase in V_{max} and was not blocked by a protein synthesis inhibitor This mode of inhibition may be due to a decrease in the degradation of PAPI (Friedman *et al*, 1986) The thyroid hormone L-3,5,3'-triiodothyronine (T_3) produced a dose-dependent increase in the specific activity of PAPI in CH3 cells following three days of exposure This increase was blocked by cycloheximide, indicating that the increase was due to enzymatic induction The increase was found to be cell specific since T_3 did not alter the specific activity of PAPI in the cell line AT-20 (Suen and Wilk, 1987) Chronic, but not acute administration of T_3 also elevated PAPI in selected brain regions of the rat (Suen and Wilk, 1989a) An effect similar to that produced by T_3 was also elicited by sodium butyrate This short chain fatty acid elevated PAPI three-fold in GH3 cells (Suen and Wilk, 1989b) However, a number of reports suggest that PAPI is not regulated by thyroid status PAPI levels in rat brain, adenohypophysis and liver have been shown to be unaffected by administration of T_3 or tetraiodothyromne (T_4) (Bauer, 1987a, Emerson *et al*, 1987, Scharfmann *et al*, 1990)

1.2 1.2 Inhibitors of Class-I PAPs

Potent and specific (both reversible and irreversible) inhibitors of PAPI 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 are highly potent irreversible inhibitors A three-fold molar excess of inhibitor to enzyme 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 protease forming hemiacetal adducts The aldehyde derivative of pyroglutamate, 5-oxoprolinal is a potent and specific competitive inhibitor *in vitro* with a K_I of 20-26nM 5-oxoprolinal is also active *in vivo* when administered to mice, however its potency is relatively weak *in vivo* , possibly due to rapid metabolism (Friedman *et al* , 1985)

pGlu-diazomethyl ketone (PDMK) is an irreversible PAPI inhibitor It is very potent *in vivo* as well as *in vitro* One hour following intraperitonal administration of 0 1 mg/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 PAPI As mentioned earlier, 2-pyrrolidone has been used to stabilise both the microbial and mammalian PAPI

1.2.1.3 Physiological Role and Current Uses of Class-I PAPs

As previously mentioned, PAPI has been shown to act on many different substrates having an N-terminal pGlu, including TRH Despite earlier interest in the role of PAPI 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) The role of PAPI 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 (Awadé *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 PAPI remains a possibility

As with the mammalian PAPI 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 fluorescens,* 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) Awadé *et al* (1994)

proposed that the high accumulation of peptides with an N-terminal pGlu may abnormally acidify the cell cytoplasm They therefore postulated that bacterial PAP may be involved in detoxification

The discovery of PAP was initially associated with the search for an enzyme capable of opening pyrrolidone rings (Doolittle and Armentrout, 1968), thus exposing an α-amino acid, in order to facilitate protein sequencing by conventional methods Nowadays, even though enzymatic and chemical methods are available to open pyrrolidone rings and mass spectrometry is available to overcome sequencing difficulties associated with N-terminal pGlu residues, researchers still use PAP (typically commercial calf liver PAPI or bacterial PAP) to confirm the presence of this residue Since PAP activity is present in some bacterial strains but is absent in others, specific PAPI assays have been used in bacterial diagnostic kits (Awadé *et al*, 1994)

1.2.2 Class-II: Animal PAPII and Serum PAP

In the late 1970s a pyroglutamyl aminopeptidase, capable of cleaving the pGlu-His bond of TRH was partially purified from rat serum (Taylor and Dixon, 1978) and porcine serum (Bauer and Nowak, 1979) Unlike PAPI which is inhibited by sulphydryl-blocking reagents (see section 1 2 1 1), the serum enzyme was not strongly affected by these substances While DTT and EDTA are necessary for expression of PAPI activity, the serum enzyme was inhibited in their presence The molecular weight of the enzyme was found to be approximately 260,000 Da, an order of magnitude greater than the soluble PAPI In addition to these differences, Bauer and Nowak (1979) observed that the serum enzyme catalysed the degradation of pGlu-ßNA extremely poorly while this is an excellent substrate for PAPI In 1981, Bauer *et al* reported that the serum PAP had a narrow substrate specificity cleaving only TRH or closely related peptides and thus proposed the name "Thyroliberinase" for the enzyme

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 to have a high molecular weight (approximately 180,000 Da) and to be optimally active only in the absence of DTT and EDTA, while the soluble PAPI required these substances for optimal activity This particulate enzyme has since become known as pyroglutamyl aminopeptidase type-II (PAPII, EC 3 4 19 6), to distinguish it from the soluble PAPI (EC 3 4 19 3) (McDonald and Barrett, 1986)

1.2.2 1 Regional Distribution of PAPII

Although PAPII has been shown to be present in many mammalian tissues, it appears to be mainly a CNS enzyme with highest levels of activity in the brain (see table 1 2 2 1) (Friedman and Wilk, 1986, Vargas *et al*, 1987, 1992a) Following cloning of a cDNA encoding the rat brain enzyme by Schauder *et al* (1994), the analysis of northern blots confirmed the heterogeneous distribution of PAPII mRNA

levels in rat Highest transcription levels were found in brain with lower levels in pituitary, lung and liver Kidney was not found to contain detectable levels of mRNA Within the brain the distribution of PAPII is heterogeneous In rabbit and rat brain highest levels of activity are found in the olfactory bulb while lowest levels are found in the adenohypophysis or hypophysis (see table 1 2 2 2) Although PAPII levels in rabbit spinal cord are lower than in brain, significant differences in enzyme distribution exist within this region The activity was found to be highest in the thoracic segments (in particular T1 and T2 regions) while PAPII activity was not detected in the sacral segments S3 and S4 (Vargas *et al*, 1992a) The specific activity of PAPII was found to be higher in rabbit retinal membranes than in any other non-CNS tissues and the specific activity in Y79 retinoblastoma cells was found to be greater than the highest activity found in other cell lines by approximately one order of magnitude (Wilk *et al*, 1988) Studies on primary cell cultures of rodent brain have shown that while a relatively high level of PAPII activity is found on neuronal cells, glial cells are almost devoid of activity In the pituitary, PAPII is localised preferentially on lactotrophs (Bauer *et al*, 1990, Cruz *et al*, 1991) The heterogeneous distribution of PAPII highlights another distinct difference between this enzyme and the cytosolic PAPI which is distributed quite homogeneously (see section 1 2 1)

Table 1.2.2.1 *Distribution of PAPII in Rabbit and Rat*

Organ	PAPII Specific Activity†	
	Rabbit[a]	Rat[b]
Brain	100	100
Testis	6 54	-
Spleen	2 88	2 7
Prostate	2 69	-
Adrenal	1 92	-
Heart	0 96	s a
Skeletal Muscle	0 77	s a
Lung	0 58	27
Serum	0 38	27
Kidney	0 38	s a
Liver	s a	2 5
Thymus	s a	-
Pancreas	s a	-

[a] Taken from Vargas *et al* (1992a) and subsequently modified
[b] Taken from Friedman and Wilk (1986) and subsequently modified
† Specific activity is presented as a percentage of that in brain
"s a " denotes sub-assay levels present, "-" indicates value not determined

Table 1.2.2.2 *Distribution of PAPII in Rabbit and Rat Brain Regions*

Region	PAPII Specific Activity†	
	Rabbit	Rat
Olfactory Bulb	100	100
Posterior part of Cerebral Cortex	99	-
Hippocampus	76	79 8
Cerebellar Vermis	71	-
Cerebellar Hemisphere	57	-
Nucleus Accumbens-lateral Septum	33	44 2
Mesencephalon	29	-
Median Eminence	23	-
Hypothalamus minus Median Eminence	19	-
Pons	17	-
Medulla Oblongata	14	17 7
Adenohypophysis	-	7 9
Hypophysis	3 5	-
Serum	0 3	1 7

Taken from Vargas *et al* (1992a) and subsequently modified
† Specific activity is presented as a percentage of that in the olfactory bulb
- indicates value not determined

1.2.2.2 Subcellular Distribution of PAPII

Studies on the subcellular distribution of PAPII have demonstrated that, within the CNS, the enzyme is located on the synaptosomal membranes (O'Connor and O'Cuinn, 1984, Horsthemke *et al*, 1984, Torres *et al*, 1986, O'Leary and O'Connor, 1995a) It has been clearly demonstrated that PAPII is an integral (intrinsic) membrane protein which is not dissociated from the plasma membrane by successive washing with high or low ionic-strength buffers or by alkali treatment (O'Connor and O'Cuinn, 1984, Friedman and Wilk, 1986, Charli *et al*, 1988, O'Leary and O'Connor, 1995a) The topographical organisation of PAPII on the membrane was investigated by Charli *et al* (1988) using rat brain synaptosomes and primary cultures of foetal cortical cells These authors reported the following findings, (1) enzyme activity was detected on intact cells using a non-penetrating substrate, (2) the activity is not increased by homogenisation, solubilisation or permeabilisation and (3) PAPII activity is greatly decreased by trypsin treatment of intact cells Based on these findings they concluded that PAPII is an ectoenzyme (i e an integral membrane protein with an extracellularly localised active site) This classification was corroborated by the findings of Bauer *et al* (1990) and Cruz *et al* (1991) who found TRH to be degraded (at the pGlu-His bond) extracellularly by intact neuronal cells in culture Analysis of the amino acid sequence, deduced from the cDNA encoding PAPII from rat brain by Schauder *et al* (1994), revealed a stretch of 22 hydrophobic amino acids near the amino-terminus of the enzyme This is consistent with a

transmembrane-spanning domain, the aminoterminus of the enzyme located intracellularly and the bulk of the enzyme, including the active site, located extracellularly This confirms the classification of PAPII as an ectoenzyme

1.2.2.3 Biochemical and Biophysical Characteristics of Class-II PAPs

PAPII has been purified from a number of sources including rat liver and guinea pig, rat, porcine, rabbit and bovine brain Solubilisation of the enzyme from the plasma membrane has been achieved using the proteolytic enzymes papain (O'Connor and O'Cuinn, 1984, Scharfmann *et al*, 1989) and trypsin (Wilk and Wilk, 1989a,b, Bauer, 1994) Solubilisation was achieved using the non-ionic detergent Triton X-100 by O'Leary and O'Connor (1995a), although a detrimental effect of detergents on PAPII activity has been reported by a number of investigators (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994)

As mentioned earlier PAPII and serum PAP differ from the soluble PAPI with respect to their molecular weights Both enzymes are of relatively large molecular size with estimates of 230,000 - 240,000 Da for PAPII (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994, O'Leary and O'Connor, 1995a) and 260,000 Da for the serum enzyme (Taylor and Dixon, 1978, Bauer and Nowak, 1979) Suen and Wilk (1990) reported a 48,000 Da subunit of PAPII from Y-79 human retinoblastoma cells This was determined by immunoblot analysis of SDS PAGE gels using polyclonal antibodies raised to the enzyme This is consistent with a pentameric structure for the enzyme Following 200,000-fold purification of PAPII from porcine brain, Bauer (1994) demonstrated a single band with an approximate molecular weight of 116,000 Da by SDS PAGE under reducing or non-reducing conditions This indicates that, under native conditions, the enzyme exists as a dimer consisting of two identical subunits which are not linked covalently The enzyme was identified as a glycoprotein by lectin-binding analysis and by the reduction of the molecular mass to 97,000 Da upon treatment of the denatured enzyme with endoglycosidase-F/N-glycosidase F In its native form, however, the enzyme was found to be only partially deglycosylated and retained full enzymatic activity (Bauer, 1994) These findings are consistent with those of Schauder *et al* (1994), who calculated the mass of the enzyme to be 117,302 Da based on the amino acid sequence deduced from cDNA encoding rat brain PAPII These authors also identified 12 putative glycosylation sites m the extracellular domain of the enzyme

Maximal activity of serum PAP was observed at incubation temperatures between 40°C and 50°C Above 50°C the enzyme is rapidly and irreversibly inactivated (Bauer and Nowak, 1979) A broad pH optimum of 6 5 to 8 0 has been reported for the serum enzyme (Taylor and Dixon, 1978, Bauer and Nowak, 1979) while a slightly narrower pH optimum of 7 0 to 7 5 has been reported for PAPII (Wilk and Wilk 1989a,b)

Inhibition of PAPII and serum PAP by chelating agents such as EDTA, EGTA, 8-hydroxyquinoline, 1,10-phenanthroline, imidazole and sodium cyanide has been observed by all groups working on these

enzymes (Taylor and Dixon, 1978, Bauer and Nowak, 1979, Yamada and Mori, 1990, O'Connor and O'Cuinn, 1984, 1985, Garat *et al*, 1985, Wilk and Wilk, 1989a, Bauer, 1994, O'Leary and O'Connor, 1995a) The insensitivity of the enzymes to specific cysteine- and serine-protease inhibitors led to the assumption that PAPII and serum PAP are metallopeptidases Czekay and Bauer (1993) demonstrated a time dependent inhibition of PAPII with chelators and the subsequent reactivation of the EDTA-treated enzyme with Zn^{2+}, Ni^{2+} and Co^{2+} Modification studies on PAPII by these authors and by O'Connor and O'Cuinn (1987) have identified a tyrosine and a glutamic acid residue at the active site of the enzyme The basic amino acids histidine and arginine were also found to be essential for activity The presence of these amino acids is a common feature of metallopeptidases Furthermore, the amino acid sequence deduced by Schauder *et al* (1994) from cDNA encoding PAPII has been shown to contain the His-Glu-Xaa-Xaa-His consensus sequence of the zinc-dependent metallopeptidase family, in the extracellular domain of the enzyme These features are in complete agreement with the classification of PAPII as a zinc-metallopeptidase

1.2.2.4 Cloning of the PAPII Gene

Recently Schauder *et al* (1994) have reported the cloning of a cDNA encoding rat brain PAPII and the transient transfection of COS-7 cells with this DNA leading to the expression of an active ectopeptidase which displayed the characteristics of PAPII The data obtained at the molecular level were found to be totally compatible with data previously obtained by biochemical studies Some of these findings have been mentioned earlier including, the heterogeneous tissue distribution of the enzyme (section 1 2 2 1), its identification as an ectoenzyme (section 1 2 2 2), its molecular weight and identification as a glycoprotein and its identification as a zinc-metallopeptidase (section 1 2 2 3) The authors have identified putative sulphanation and phosphorylation sites which may be important for intracellular sorting and trafficking and for regulation

Southern blot analysis indicated that the enzyme is present as a single-copy gene This supports the proposal that serum PAP and PAPII (which exhibit almost identical chemical characteristics) are derived from the same gene (O'Connor and O'Cuinn, 1984, Wilk, 1986, Bauer, 1987b, Scharfmann *et al*, 1990, Yamada and Mori, 1990) It is possible that the serum enzyme is secreted from the liver and may be formed by alternative splicing (Schauder *et al*, 1994)

Significant homology was observed between the amino acid sequence of the rat PAPII and those of rat aminopeptidase N (34%) and mouse aminopeptidase A (32%), both of which have been identified as membrane-anchored zinc-metallopeptidases While the trans-membrane portions were found to be completely different, sequences in the extracellular domain displayed a high degree of homology Two stretches, each with seven amino acids are completely conserved indicating that these enzymes share a common ancestry (Schauder *et al*, 1994)

1.2.2.5 Substrate Specificity of Class-II PAPs

Probably the most interesting feature of PAPII and serum PAP is their extraordinarily high degree of substrate specificity Both enzymes have been shown to remove the N-terminal pGlu from only TRH or closely related peptides O'Connor and O'Cuinn (1984, 1985) and Elmore *et al* (1990) demonstrated that, of a range of N-terminal pGlu peptides tested, only TRH (pGlu-His-Pro-NH_2), Acid TRH (pGlu-His-Pro), LHRH 1-3 (pGlu-His-Trp), pGlu-His-Gly and pGlu-His-Pro-Gly were cleaved by the guinea pig brain enzyme Substitution of pGlu by Glu or of His by either Phe or N-Val in the TRH sequence abolished the susceptibility of the peptide to hydrolysis Some tolerance to substitution of the Pro-NH_2 residue was observed as indicated by the observation that -Trp, -Gly and -Pro-Gly in this position permitted enzyme activity Although the enzyme was found to be capable of hydrolysing pGlu-His-Pro-Gly, the extended substrates pGlu-His-Pro-Gly-NH_2 and pGlu-His-Pro-Gly-Lys were not hydrolysed Similarly, while LHRH 1-3 is hydrolysed, extending the sequence to LHRH 1-5 (pGlu-His-Trp-Ser-Lys) abolished enzyme activity (Elmore *et al* , 1990) Studies with rabbit brain PAPII, using various synthetic pyroglutamyl peptidyl naphtylamides were carried out by Wilk and Wilk (1989a,b) These authors found only pyroglutamyl-histidyl tripeptide derivatives to be hydrolysed When a benzyloxycarbonyl (Z, cpZ) group is added to the pyroglutamyl ring of the TRH analogue pGlu-His-Pro-NA the resulting peptide is not cleaved Other five-membered heterocyclic rings can substitute for the pyroglutamyl ring but hydrolysis is not observed if pGlu is substituted with a six-membered heterocyclic ring (Lanzara *et al* , 1989) Similar substrate specificity has been observed for PAPII from rat liver (Scharfmann *et al* , 1989) and bovine brain (O'Leary and O'Connor, 1995a) and for porcine serum PAP (Bauer *et al* , 1981) These observations suggest that the specificity of the enzyme is restricted to tripeptides, tripeptide amides and tetrapeptides with the pGlu-His sequence at the amino-terminus Due to its high degree of specificity for TRH, Bauer *et al* (1981) proposed the name "Thyroliberinase" for the serum enzyme Wilk (1986) proposed that PAPII may be the first true neuropeptide specific peptidase to be characterised

The affinity of PAPII and serum PAP for TRH is quite consistent among the various sources tested K_I/K_m values from 25μM to 56μM have been reported for TRH, with the majority of these falling within the range 40μM to 50μM (Taylor and Dixon, 1978, Bauer *et al* , 1981, O'Connor and O'Cuinn, 1985, Scharfmann *et al* , 1989, Yamada and Mori, 1990, Bauer, 1994, O'Leary and O'Connor, 1995a) With the exception of pGlu-His (which was shown to be a non-competitive inhibitor), all of the pyroglutamyl peptides tested by Elmore *et al* (1990) were found to be competitive inhibitors of PAPII It is interesting to note that the peptides pGlu-His-Pro, pGlu-His-Gly and pGlu-His-Pro-Gly each displayed higher K_I values than the corresponding amides, suggesting that the enzyme prefers an amide group at the carboxy-terminus of the peptide substrate (Elmore *et al* , 1990) The decapeptide LHRH, although not a substrate for the enzyme has been shown to be a strongly competitive inhibitor of PAPII, displaying a K_I value (20μM) lower than the enzymes K_I for TRH (40μM) (O'Connor and O'Cuinn, 1985) A similar finding was observed by Bauer *et al* (1981) for the serum enzyme However, in a recent report O'Leary and O'Connor (1995a) found the inhibition of bovine brain PAPII with LHRH to be non-competitive with a K_I of 820μM, considerably higher than the K_m of TRH (50μM)

1.2.2.4 Assays for Class-II PAPs

The determination of PAPII and serum PAP activity has been achieved by many researchers using radiolabelled TRH (for example, [^{3}H-Pro]TRH, [pGlu-^{14}C]TRH or [^{3}H-His]TRH) as a substrate A number of variations of the assay have been described in which, following hydrolysis of the substrate, the degradation products are separated by paper chromatography, thin layer chromatography, column chromatography, immunoaffinity or HPLC (Bauer, 1976, Taylor and Dixon, 1978, Garat *et al*, 1985, O'Connor and O'Cuinn, 1984,1985, Friedman and Wilk, 1986, Coggins *et al*, 1987a, Bauer *et al*, 1990) Following metabolite separation, the enzyme activity of the sample is quantified by measuring the radioactivity to determine either the amount of TRH remaining or the amount of pGlu, His-Pro-NH_2 or His-Pro diketopiperazine produced

In 1986, Friedman and Wilk developed a coupled enzyme assay for the detection of PAPII based on the cleavage of the synthetic substrate pGlu-His-Pro-2NA in the presence of excess dipeptidyl aminopeptidase type IV (DAPIV) The assay is based on the following reaction sequence

$$\text{pGlu-His-Pro-2NA} \xrightarrow{\textbf{PAPII}} \text{pGlu} + \text{His-Pro-2NA}$$

$$\text{His-Pro-2NA} \xrightarrow{\textbf{DAPIV}} \text{His-Pro} + \text{2NA}$$

The inclusion of Z-Pro-Prolinal, a specific prolyl endopeptidase inhibitor, in the assay mixture blocks the cleavage of the Pro-2NA bond and the inclusion of PDMK prevents the cleavage of the substrate by PAPI PAPII activity could thus be quantified by measuring the amount of 2NA released A modification of this procedure was described by O'Leary and O'Connor (1995a) who used the substrate pGlu-His-Pro-MCA Although DAPIV is not commercially available and must therefore be purified for use in the assay, this coupled enzyme assay is less labour-intensive and more economical than the radiolabelled assays It has the added advantage of avoiding the use of radioactivity

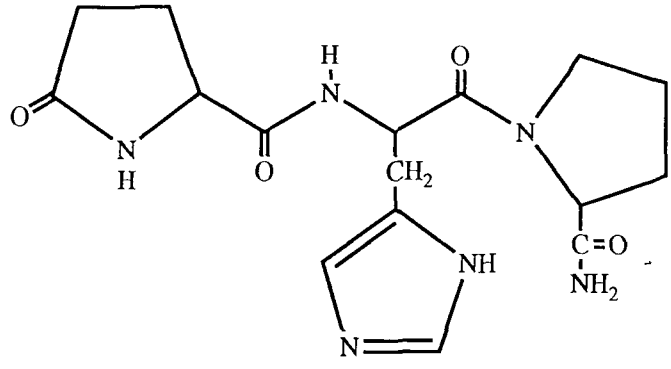
A number of researchers have reported that the PAPI substrates pGlu-ßNA, pGlu-pNA and pGlu-MCA are not hydrolysed by PAPII or serum PAP (O'Connor and O'Cuinn, 1985, Garat *et al*, 1985, Wilk and Wilk, 1989a) However, Bauer *et al* (1981) reported that the serum enzyme cleaves pGlu-ßNA, albeit at a rate 200-times slower than the calf liver PAPI Czekay and Bauer (1993) and Bauer (1994) demonstrated similar slow hydrolysis of the substrate with PAPII from porcine and rat brains The use of this fluorogenic substrate facilitates the continuous monitoring of PAPII activity in purified samples with high levels of activity

Due to the unique substrate specificity of class-II PAPs for only TRH or closely related peptides, a brief discussion on TRH is warranted

1.3 Thyrotropin-Releasing Hormone (TRH)

It has been recognised since the early 1950s that the hypothalamus exerts an important influence on the regulation of the pituitary-thyroid axis (Reichlin *et al*, 1978) In 1969, following rigorous chemical analysis of large numbers of ovine and porcine hypothalamic fragments, the structure of TRH was elucidated in the laboratories of Guillemin and Schally (Bøler *et al*, 1969, Burgus *et al*, 1969) Working independently, they reported that TRH was a weakly basic tripeptide with the amino acid sequence pyroglutamyl-histidyl-proline amide (pGlu-His-Pro-NH_2) (see figure 1 3 1) TRH has a strict conformational requirement for biological activity and almost any departure from its native structure results in substantial if not complete loss of activity (Guillemin and Burgus, 1972)

Figure 1.3 1 *Structure of Thyrotropin-Releasing Hormone (TRH)*



(pGlu ——— His —— Pro-NH_2)

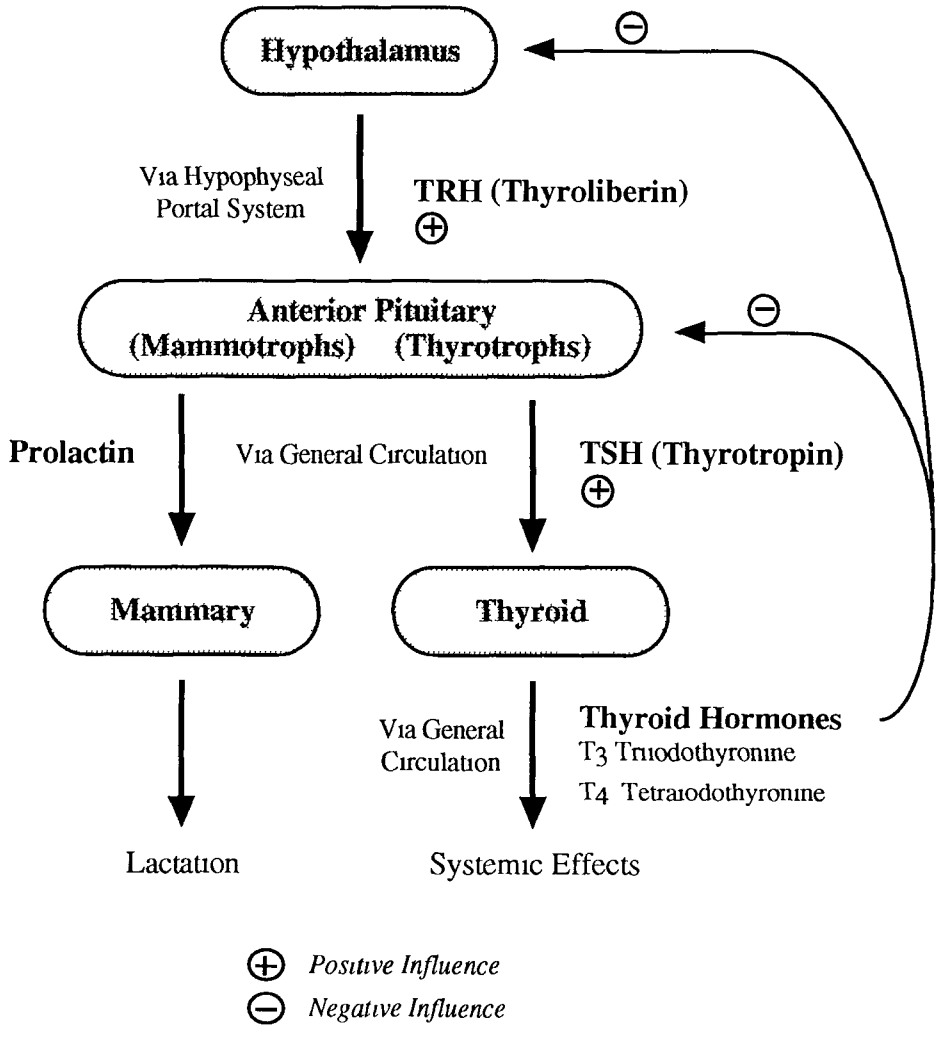
1.3.1 Neuroendocrine Role of TRH

The most obvious effect of TRH (also known as Thyrotropin-Releasing Factor or Thyroliberin), and that from which it derives its name(s), is the ability to stimulate the release of thyrotropin (Thyroid-Stimulating Hormone (TSH)) from the anterior pituitary (adenohypophysis) via binding to high affinity receptors (Jackson, 1982) TRH is released from the hypothalamic neurons into the hypothalamo-hypophyseal portal vessels that carry blood between the hypothalamus and the anterior pituitary The anterior pituitary contains several different types of endocrine cells TRH receptors are located on two pituitary cell types thyrotrophs (TSH-releasing cells) and mammotrophs (lactotrophs, prolactin-releasing cells) Binding of TRH to the receptors on these cells stimulates the release of TSH and prolactin (see figure 1 3 1 1)

TSH stimulates the thyroid gland to produce and release the thyroid hormones, tetraiodothyronine (T_4) and triiodothyronine (T_3) It is composed of two different, noncovalently associated glycoprotein subunits termed α-subunit and TSHß (Pierse and Parsons, 1981) Each subunit is encoded by a separate gene located on different chromosomes in humans and mice (Chin, 1986) Coordinate regulation of expression of the TSH subunit genes results in nearly balanced production of the subunit proteins However the α-

subunit is often synthesised and released in excess Only the heterodimer can act in the endocrine system (Chin *et al*, 1993) It is well known that TRH release at the hypothalamus and TSH synthesis by the pituitary thyrotroph are directly regulated by the thyroid hormones in a negative fashion (Hershman and Pekary, 1985) Thyroid hormones rapidly (within hours) decrease α-subunit and TSHß subunit mRNAs in pituitary tissue in a time- and dose- dependent fashion (Chin *et al*, 1985) The mechanism of thyroid hormone action proposed by Chin *et al* (1993) is as follows Thyroid hormones enter the cell via a passive mechanism (T_4 can be monodeiodinated to T_3 in certain cell types), and move from the cytoplasm to the nucleus where they interact with high affinity receptors (TRs) The TRs interact with target DNAs by association with specific receptors thus resulting in activation or repression of specific genes

Figure 1.3.1.1 *The Hypothalamic-Pituitary-Thyroid (HPT) Axis*



Rather than having a restricted endocrine function in stimulating the release of TSH and prolactin, TRH appears to have much more widespread effects on the release of other hormones such as growth hormone (GH), α-melanocyte-stimulating hormone, vasopressin, somatostatin, insulin and glucagon as well as noradrenaline and adrenaline (For review, see Griffiths, 1985 and 1987)

1.3.2 Regional Distribution of TRH

In its amino acid sequence and its biological activity, TRH exhibits no species specificity and it is readily active in all mammalian species studied including humans (Fleischer and Guillemin, 1976) TRH-like immunoreactivity or biological activity is widely distributed throughout the CNS, gastrointestinal tract and the body fluids of mammalian species (see table 1 3 2 1) (for review see Jackson, 1982) An investigation into the distribution of TRH in rat CNS showed that, while the highest concentration of TRH is in the hypothalamus, more than 70% of the total brain TRH is located extrahypothalamicly (Leppaluoto *et al*, 1978) where it has a wide distribution (see table 1 3 2 2)

Table 1.3.2.1 *Distribution of TRH in Tissues and Body Fluids of Various Animal Species*

Species	Tissue/Body Fluid	TRH Concentration (fmol/mg protein)
Human	Cerebellum	15
	Hypothalamus	1,093
	Placenta	54 6
	Pineal Gland	71 7
	Peripheral Blood	78 pg/ml
Rat	Cerebellum	31 5
	Hypothalamus	2,208
	Caecum	91
	Rectum	27 6
	Pancreas	93 8
	Retina	828
	Peripheral Blood	77 pg/ml
	Hypophyseal Portal Plasma	801 pg/ml
Frog	Brain	6 9
	Skin	2 6
	Blood	227 ng/ml
Monkey	Cerebellum, Anterior	2 3
	Hypothalamus, Anterior	151
Chicken	Hypothalamus	938 - 1,352
Snake	Hypothalamus	10,844 - 20,171
Salmon	Hypothalamus	5,188 - 7,284

Taken from Prasad (1985)

Table 1.3.2.2 *TRH Distribution in Rat Brain*

Brain Area	TRH Concentration (pg/mg tissue)
Extrahypothalamic Brain	
Brainstem	5
Cerebellum	2
Diencephalon	6
Olfactory Lobe	6
Cerebral Cortex	2
Hypothalamic-Pituitary Complex	
Dorsal Hypothalamus	49
Ventral Hypothalamus	64
Stalk Median Eminence	3,570
Posterior Pituitary	115
Anterior Pituitary	10

Taken from Jackson and Reichlin (1974)

1.3.3 Neuroregulatory Role of TRH

Information processing in the brain largely involves chemical communication among neurons through substances called neuroregulators These substances may be subdivided into those that convey information between adjacent nerve cells (neurotransmitters) and those that amplify or dampen neuronal activity (neuromodulators) Although TRH was first classified as a hormone through its effect on the pituitary, it possesses many characteristics which suggest that in addition to its neuroendocrine role, TRH may function as a neurotransmitter or neuromodulator The following features provide a formidable list of criteria consistent with such a neuronal role

- its extrahypothalamic location in the brain
- its localisation at the synaptic level
- its release at synaptic terminals
- its attachment to high affinity receptors that show a remarkable degree of anatomical localisation
- its specific effects on neuronal activity
- its stimulation of a wide range of centrally mediated behavioural effects (see table 1 3 3)
- the presence of brain peptidases capable of its inactivation

For a more complete review of this topic, see Griffiths (1985) and Prasad (1985)

Table 1.3.3 *Examples of CNS effects of TRH, resulting from direct application to specific brain regions or from intracerebroventricular injections*

- Reversal of narcotic / alcohol-induced sedation
- Reversal of natural sedation (hibernation)
- Thermoregulation induction of hypo- or hyper-thermia (depending on species)
- Wet-dog shaking activity - Symptoms similar to those produced by opiate withdrawal
- Locomotor activity
- Forepaw tremor (rats)
- Muscle tone improvement (antagonism of induced relaxation)
- Cardiovascular effects increased blood pressure and heart rate, increased cerebral blood flow
- Respiratory effects increased respiratory rate and minute volume
- Gastrointestinal effects increased or decreased motility (depending on species), increased gastric acid secretion and gastric emptying, increased pancreatic exocrine secretion
- Anorexic effects decreased food and water intake
- Antinociceptive action
- Increased catecholamine release from adrenal medulla
- Blood glucose hyperglycemia in rats, hypoglycemia in mice
- Anticonvulsant activity

Taken from Griffiths (1987)

1.3.4 Biosynthesis of TRH

Despite some initial confusion about the mechanism of TRH biosynthesis, the isolation of a cDNA from amphibian skin (a tissue rich in the tripeptide), showed that TRH is derived from a high molecular weight precursor (Richter *et al*, 1984) The rat preprohormone TRH precursor (prepro-TRH) contains five copies of the progenitor sequence Gln-His-Pro-Gly These are flanked by pairs of basic residues (typical prohormone processing signals), Lys-Arg at the amino end and Lys-Arg or Arg-Arg at the carboxyl end, and are separated from each other by intervening peptides that are not related to the TRH sequence (Lee *et al*, 1988) The human TRH cDNA encodes six copies of the TRH progenitor sequence, in contrast to the five copies present in the rat (Yamada *et al*, 1990) The intervening (connecting) sequences are numbered Ps1 to Ps6

Considerable effort has been devoted to elucidating the processing pattern of the TRH prohormone in various regions of the brain and in peripheral tissues Based on the deduced amino acid sequence of rat pro-TRH, proteolytic processing is expected to produce TRH plus several non-TRH peptides Although the major pathway of pro-TRH processing in the hypothalamus has been shown to involve nearly complete proteolytic cleavage at all of the basic residues that flank the TRH progenitor sequence, some studies have shown the presence of extended forms of TRH in the hypothalamus Peptides extended at the N-terminal

or C-terminal of TRH occur in similar concentrations in bovine hypothalamus, while only C-terminally extended forms of the tripeptide occur in rat hypothalamus Similarly, small amounts of C-terminally extended forms of TRH (such as TRH-Ps4) have been detected in spinal cord as a result of incomplete processing of the TRH precursor molecule Pro-TRH has been shown to be processed differently in the olfactory bulb with TRH-Ps4 and TRH-Ps5 as major end-products, demonstrating tissue-specific processing of the precursor (For a more complete review of this topic see Ladram *et al*, 1994)

It has recently been demonstrated that Ps4 possesses biological activities *in vitro* and *in vivo* (Bulant *et al*, 1990, Carr *et al*, 1992) It has been shown to potentiate TRH induced release of TSH and prolactin Specific binding sites for this TRH-potentiating peptide (distinct from TRH receptors) have been demonstrated in the pituitary A general property of prohormone proteins is that they have the potential to produce a number of regulatory peptides endowed with similar or different bioactivities The precise function(s) of intervening sequences within the precursor molecule is currently a matter of speculation It has long been assumed that they are functionally unimportant *throw away* peptides or that they may participate in the processing or intracellular routing of the prohormone Pro-TRH is an example of a prohormone which yields a *true* hormone TRH and a biologically active connecting peptide Ps4, that act in a synergistic manner on the same target cells to modulate hormone release For more extensive reviews on this topic, see Ladram *et al*, 1994 and O'Leary and O'Connor, 1995b

Mains *et al* (1990) have suggested that the maturation of pro-TRH is a sequential phenomenon starting in the endoplasmic reticulum after cleavage of the signal sequence Then, pro-TRH travels through the Golgi complex, where it undergoes enzymatic modifications such as phosphorylation, glycosylation, endoproteolysis, exoproteolysis and sulphation The terminal step, amidation, uses the glycine residue as an amide donor and is catalysed by peptidylglycine α-amidating monooxygenase (PAM) Cyclisation of the amino terminal glutamine residue to pyroglutamate is cyclised by a glutamine cyclase-like enzyme (Busby *et al*, 1987) Following processing in the Golgi complex, terminal maturation of TRH occurs in large dense core vesicles (secretory granules) The subsequent release of the mature TRH and prohormone related peptides at the synapse has been shown to involve voltage sensitive Ca^{2+} channels and protein kinase-C (Loudes *et al*, 1988)

1.3.5 TRH Receptors

The action of TRH on target cells, such as those of the anterior pituitary, is mediated through its interaction with highly specific membrane-bound receptors Recent cloning and sequencing of the TRH receptor (TRH-R) from anterior pituitary cells of different species has revealed that it is a member of the seven-transmembrane-spanning, GTP-binding protein-coupled family of receptors or GPCRs The mouse pituitary TRH-R mRNA encodes for a 393 amino acid GPCR (Straub *et al*, 1990) which bears significant homology with sequences observed for the cloned TRH-R from humans, 398 amino acids (Duthie *et al*, 1993, Matre *et al*, 1993) and for rats, 412 and 387 amino acids (two isoforms of this receptor, generated by alternative splicing, have been cloned and sequenced), (de la Pena *et al*, 1992a and

1992b) The major sequence variation between species and isoforms was seen to occur within the intracellular carboxy-terminal tail of the TRH-R (Morrison *et al*, 1994), a portion of the receptor sequence known to contain several serine and threonine residues which are potential sites for regulatory phosphorylation by kinase enzymes, including protein kinase C (Kikkawa *et al*, 1989)

TRH-Rs have been shown to be widely distributed throughout the CNS and peripheral tissues Virtually all brain tissues tested have been shown to contain detectable levels of TRH-Rs However, major species differences have been observed in both absolute and relative TRH-R binding in different brain regions Several brain regions have been shown to exhibit binding equal to or exceeding that in the anterior pituitary gland of the same species including the amygdala, hypothalamus and nucleus accumbens (Taylor and Burt, 1982) A study of the distribution of mRNA encoding the TRH-R in rat brain and pituitary by *in situ* hybridisation has shown even distribution of the TRH-R mRNA throughout the anterior lobe of the pituitary, thyrotrophs and mammotrophs, but no hybridisation was detected in the posterior lobe In virtually every area demonstrating TRH-R mRNA expression, prohormone or TRH immumoreactivity has previously been described The presence of TRH-Rs in the same regions which synthesise TRH precursors suggests a local action of TRH (Zabavnik *et al*, 1993) Recently, the direct TRH precursor, TRH-Gly, has been shown to interact directly with the TRH-R *in vitro*, causing the release of TSH and prolactin (Yamada *et al*, 1995)

TRH-R mRNA has also been shown to be widely distributed throughout rat peripheral tissues, albeit at a considerably lower concentration than in the pituitary, as is TRH itself The rank order of the expression in these tissues (quantified by Northern blot analysis) was brain, uterus, thymus, testis, submandibular gland, heart and adrenal gland Although TRH is distributed widely in the digestive organs such as liver, intestine and pancreas, strong signals of TRH-R mRNA were not detected in these tissues The wide distribution of TRH and TRH-Rs in peripheral tissues suggests a role for TRH not only in the CNS but also in these tissues (Fukusumi *et al*, 1995)

Down regulation of receptors is an important mechanism for diminishing cell responsiveness to many hormones, neurotransmitters and growth factors The number of receptors on the surface membrane of cells may be decreased by the action of the receptor ligand (homologous down regulation) or by the action of other extracellular regulatory factors that interact with other cell receptors (heterologous down regulation) Homologous down regulation of TRH-Rs by TRH as well as heterologous down regulation by other extracellular molecules such as thyroid hormones, epidermal growth factor and vasoactive intestinal peptide have been reported Importantly, the decrease in the number of TRH-Rs causes adenohypophyseal cells to exhibit a less marked response to TRH Down regulation caused by TRH is not due to rapid receptor internalisation, but rather to a slow decrease in TRH-Rs with a half-time of 12 hours It has been proposed that homologous down regulation is caused by a TRH induced increase in the rate of mRNA turnover due to decreased mRNA stability and to increases in the degrading activity of an RNAase (for review, see Gershengorn, 1993)

1.3.6 Therapeutic Applications of TRH

Diagnostically, intravenous (i v) administration of TRH has been used to assess the integrity of the hypothalamic-pituitary-thyroid axis in clinical conditions associated with disturbances in the thyroid hormone levels However, with the introduction of sensitive assays for TSH, the need for the TRH test has declined, except in cases where the capacity of the pituitary to secrete TSH is being examined Interestingly, the potential therapeutic applications of TRH that have attracted the most attention are not based on its endocrine properties, but on its broad spectrum of stimulatory actions within the CNS (see table 1 3 3) (Griffiths, 1987) These CNS-mediated effects provide the rationale for the use of TRH in the treatment of brain and spinal injury and certain CNS disorders such as Alzheimer's disease and motor neuron disease (MND) The endocrine actions of TRH such as stimulation of the pituitary-thyroid axis, resulting in hyperthyroidism, are regarded as adverse side effects in the treatment of CNS-related conditions The beneficial effects of TRH on CNS disorders and trauma appear to be due partly to its ability to potentiate other neurotransmitter systems and to reverse or attenuate certain actions of secondary injury factors that occur as a result of CNS trauma The exact mechanism by which TRH improves these conditions is still not fully understood (Kelly, 1995)

TRH was initially used to treat brain and spinal injury because of its ability to antagonise the damaging actions of endogenous opioids (which contribute to secondary tissue damage after CNS trauma), without reducing opioid analgesia The basis of the neuroprotective actions of TRH has now been extended to include its ability to antagonise the actions of other secondary injury factors such as leukotrines and platelet-activating factor, as well as to restore magnesium homeostasis, improve bioenergetic status and decrease tissue oedema after brain injury (Faden and Salzman, 1992)

The use of TRH in the treatment of certain kinds of memory dysfunction, including Alzheimer's disease, is based on its ability to potentiate the activity of several neurotransmitter systems that are involved in memory, including cholinergic, noradrenergic, serotonergic and dopaminergic systems (Horita *et al* , 1986) Intraperitoneal (i p) administration of TRH has been shown to protect against disruption of memory in animals (Stwertka *et al* , 1991) In a pilot study involving Alzheimer's disease patients, i v administration of TRH significantly improved arousal and modestly improved semantic memory (Mellow *et al* , 1989) Recently, TRH has been shown to increase memory retention and retrieval in rats through activation of N-methyl-D-aspartate (NMDA) -receptor mediated processes (Kasparov and Chizh, 1992)

MND is characterised by progressive loss of voluntary motor function and is invariably fatal Gradual degradation of the large motor neurons in the cerebral cortex, brain stem and cervical and lumbar spinal cord occurs Death usually occurs from respiratory failure due to bulbar or diaphragmatic involvement Evidence showing that TRH has neurotrophic (neuron-growth promoting) and neuroregulatory effects in the motor neurons, together with its apparent beneficial effects in the human spinocerebellar degenerations, would seem to indicate a possible role for TRH in the management of MND Clinical trials using TRH in the treatment of MND patients have showed promising results (For review see Kelly, 1995)

There are a number of other conditions for which TRH may have potential beneficial effects, including cerebral ischaemia (stroke), Down's syndrome, schizophrenia, depression, epilepsy, reversal of narcotic overdose and haemorrhagic, endotoxic and anaphylactic shock For more comprehensive reviews on these and other clinical applications of TRH see Griffiths (1987), Holaday *et al* (1989) and Kelly (1995)

1.3.7 TRH Analogues

In addition to its hormonal actions, the main drawbacks to using TRH as therapeutic agent in the treatment of CNS disorders are (i) its rapid enzymatic degradation and hence its relatively short half-life (see section 1 3 8) and (ii) its relative inability to cross the blood-brain barrier (only about 0 2% of an i v injected dose has been detected in cerebrospinal fluid) (Nagai *et al*, 1980) Several strategies are currently being investigated in an attempt to overcome these disadvantages and to capitalise on the neuroregulatory effects of TRH These include the following (i) TRH analogues, (ii) peptide mimetics, (iii) microspheres, (iv) intrathecal drug delivery systems and (v) inhibitors of TRH-specific peptidases For a review of these topics, see Kelly (1995)

In an attempt to overcome the susceptibility of TRH to enzymatic degradation, various analogues have been prepared and evaluated The chemical structures of some of these analogues are shown in figure 1 3 7 2 All of the analogues shown are agonists of TRH that are capable of binding to and activating TRH-Rs Studies *in vitro* have shown that methylation of prolinamide in TRH, as seen in RX77368 and RX74355, protects against deamidation by prolyl endopeptidase Alternatively, replacement of the pyroglutamyl residue with, for example, a six-membered ring as seen in the case of CG3509, CG3703 and TA-0910, confers resistance to the action of the PAPs (Griffiths *et al*, 1989)

The ability of TRH or its analogues to bind to central or pituitary receptors is dependent on the three-dimensional conformation of the peptide and the interplay of a variety of forces such as electrostatic interactions Comparative conformation-activity relationships for hormonally and centrally acting TRH analogues now indicate that certain conformations are preferred at the CNS receptor, while others are preferred at the pituitary receptor This is not to suggest that pituitary and CNS TRH-Rs are, intrinsically, chemically different It could be that factors closely associated with TRH-Rs in the membrane, influence the binding of ligands to the receptor It is also a possibility that more than one type of TRH-R exists (Ward *et al*, 1987)

The TRH analogue [3-MeHis] TRH is the most potent analogue in stimulating the release of TSH from the pituitary However, although it has a very high affinity for central TRH-Rs, it is experimentally less active in the brain than TRH (Ward *et al*, 1987) In contrast, several analogues that have a much lower affinity for TRH-Rs, but that are much more stable to degradation by brain peptidases show enhanced central activity over TRH The analogue TA-0910, administered either orally or intravenously exerts CNS effects 100 or 30 times, respectively, as potent as TRH In contrast, its TSH-releasing action is only

$^{1}/_{50}{}^{th}$ that of TRH, indicating a successful dissociation between the CNS and hormonal actions Its resistance to degradation by serum PAP also contributes to its pharmacological potency (Chishima, 1994) RX77368, the most enzymatically stable analogue, is excreted 60% unchanged and would be expected to have greatest activity by virtue of this fact RX77368 generally varies in potency between 2 and 220 times that of TRH despite a relatively low affinity for TRH-Rs compared with that of [3-MeHis] TRH (Metcalf, 1982)

Quantitative structure-activity relationship (QSAR) studies reveal that TRH and its analogues can be fitted into three well defined classes (P,C and Y) depending on the relative position of the rings (see figure 1 3 7 1) The P conformer has the rings orientated like the three blades of a propeller, in C conformers they are positioned like a cup The Y class has the two rings in close proximity (the subscripts describe which ones) Hence there are three subclasses, $Y_{1,2}$, $Y_{1,3}$ and $Y_{2,3}$ (Ward *et al*, 1987, Griffiths *et al*, 1989) The $Y_{2,3}$ confirmation (that predicted for RX77368) is the most active in the brain while the P conformation (that predicted for [3-MeHis] TRH) is the most active in the pituitary The distinction between the two accounts for the fact that [3-MeHis] TRH is more potent than TRH in the pituitary but less potent in the brain (Griffiths *et al*, 1989)

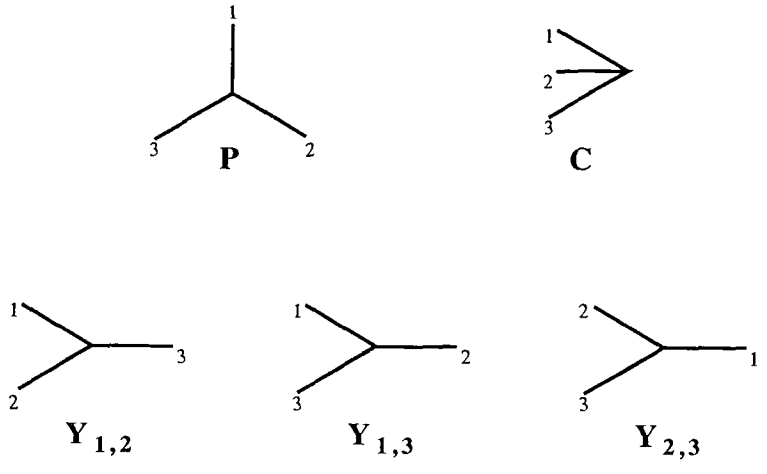
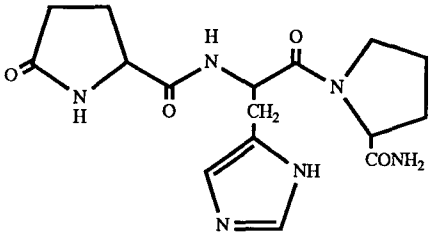
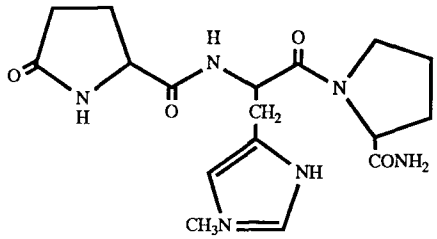


Figure 1.3 7 1 *Conformational preferences of TRH and its analogues, showing the relative positions of the rings* 1=pGlu, 2=His, 3=Pro-NH_2

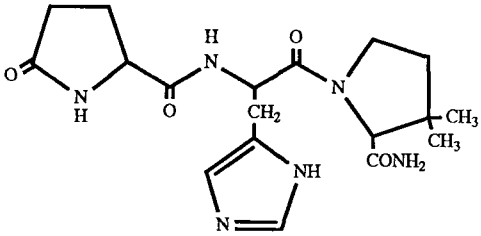
Figure 1.3.7.2 *Chemical structures of TRH and some of its analogues, showing various modifications to the tripeptide pGlu-His-Pro-NH_2*



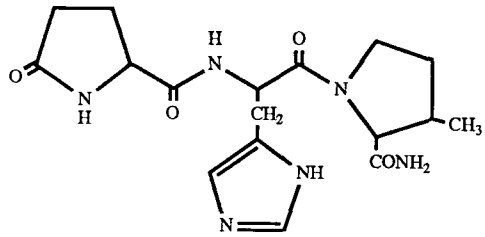
TRH



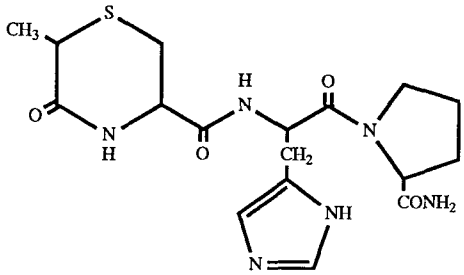
[3-MeHis] TRH



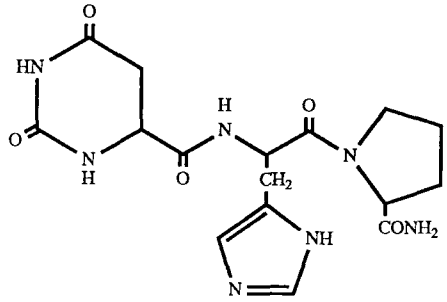
RX77368



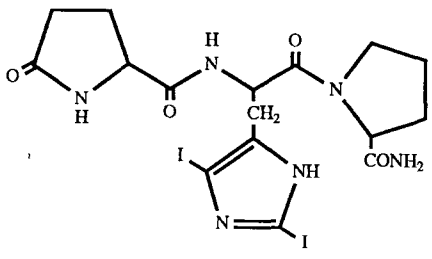
RX74355



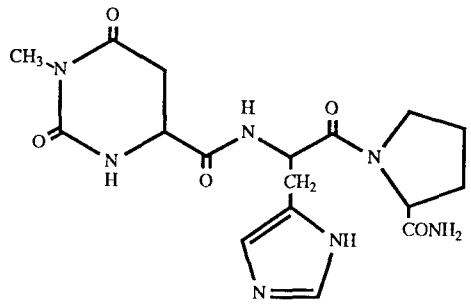
CG3703



CG3509



2,4-diiodo(Im)-TRH



TA-0910

In addition to the susceptibility of TRH to enzymatic degradation, the low lipophilicity of TRH may also be a reason for the limited ability of the peptide to penetrate the blood-brain barrier A possible approach to diminish these delivery problems is derivatisation of the peptide to produce prodrugs or transport forms which are more lipophilic than the parent peptide and more resistant to degradation by enzymes present at the mucosal barrier or in the blood At the same time, the prodrug derivatives should be capable of releasing the parent peptide spontaneously or enzymatically in the blood following their absorption (Bundgaard and Moss, 1989 and 1990, Moss and Bundgaard, 1992) N-acylation of the imidazole group of the histidine residue of TRH has resulted in derivatives which are much more lipophilic than TRH itself These derivatives are totally resistant to degradation by serum PAP, thus prolonging their half-life *in vivo* On the other hand, they are readily bioreversible as the parent TRH is formed quantitatively from the derivatives by spontaneous hydrolysis or by plasma esterase-catalysed hydrolysis (Bundgaard and Moss, 1990)

For more extensive reviews on TRH analogues and their therapeutic uses, see Griffiths (1987), Griffiths *et al* (1989) and Sharif (1993)

1.3.8 Enzymatic Inactivation of TRH

As for other biologically active substances, highly efficient and specific degradation and / or elimination mechanisms must exist in order to terminate the action of TRH Inactivation of TRH through energy dependent uptake mechanisms has not been demonstrated, while in contrast, its rapid *in vitro* inactivation has been reported by a number of investigators In general, degradative enzymes may play an important part in controlling hormone action Initially by regulating the amount of the hormone available for release at its site of production or the amount actually reaching its site of action, then by controlling the duration of action through inactivation at the receptor sites in particular target tissues and finally by removal of the hormone from the general circulation so that too large a response to the hormone is prevented (Griffiths and Kelly, 1979)

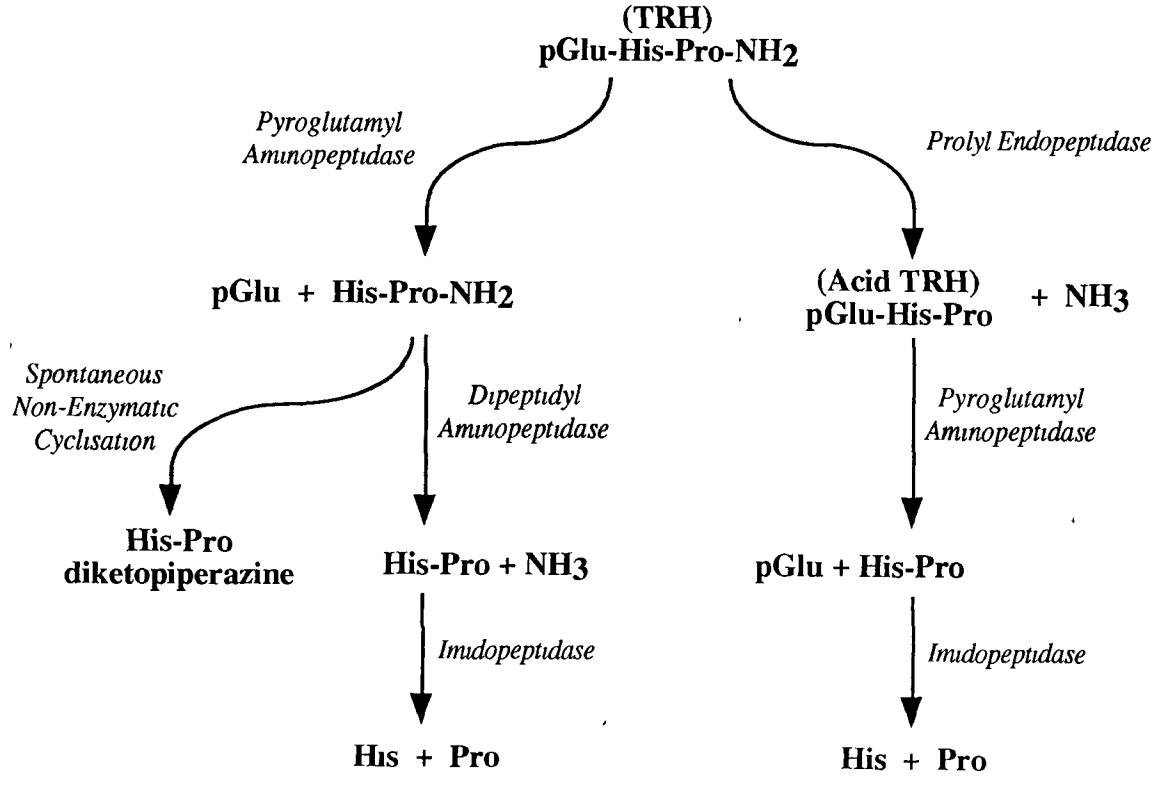
Due to its unique structural features such as the blocked N- and C- termini, combined with the internal proline residue, TRH is resistant to general proteolytic attack (Burgus and Guillemin, 1970) Two pathways exist for the primary inactivation of TRH *in vitro*, (a) cleavage of the pGlu-His bond by pyroglutamyl aminopeptidases (PAPs) and (b) cleavage of the C-terminal amide by prolyl endopeptidase (PE) (see figure 1 3 8)

Following primary inactivation of TRH by PAP, the metabolite His-Pro-NH_2 is formed Spontaneous and non-enzymatic cyclisation of this metabolite to His-Pro diketopiperazine (cyclo His-Pro), has been reported at neutral and alkaline pH (Peterkofsky *et al*, 1982, Prasad *et al*, 1982) Cyclo His-Pro, which does not appear to be further degraded by any enzymatic mechanism, is itself reported to posses endocrine activity (Brabant *et al*, 1981, Melmed *et al*, 1982) as well as numerous CNS actions (Peterkofsky *et al*, 1982, Prasad *et al*, 1982) The conversion of TRH to cyclo His-Pro thus represents an instance of the *biotransformation* of a peptide (Griffiths and McDermott, 1984) Any enzyme capable of converting His-Pro-NH_2 to a metabolite other than cyclo His-Pro must therefore be considered to be a regulator of its formation, by competing with the biotransformation process Dipeptidyl aminopeptidases (DAPs) have been described (type-II, EC 3 4 14 2, in the soluble fraction and type-IV, EC 3 4 14 5, in the particulate fraction), which are capable of competing with the spontaneous cyclisation process to produce His-Pro from His-Pro-NH_2 (Bauer and Kleinkauf, 1980, Browne and O'Cuinn, 1983, O'Connor and O'Cuinn, 1986) His-Pro itself has been shown to modify behavioural activity affecting motor output and emotional responses Binding to high- and low- affinity sites in fresh rat brain slices has also been demonstrated (Coggins *et al*, 1986 and 1987a,b) An imidopeptidase capable of cleaving His-Pro-NH_2 at the His-Pro bond has also been reported (Matsui *et al*, 1979, Torres *et al*, 1986) The proposed subcellular localisation of this enzyme in the particulate fraction has not been definitively demonstrated

PE (EC 3 4 21 26), also known as prolyl oligopeptidase and post-proline-cleaving enzyme, has been described predominantly as a soluble, cytoplasmic enzyme However, its presence in serum and in the particulate fraction of tissue homogenates, including synaptosomal membranes, has also been demonstrated (Dalmaz *et al*, 1986, Maes *et al*, 1994, O'Leary *et al*, 1996) PE has been identified as a serine protease (Yoshimoto *et al*, 1977), with a sulphydryl group necessary for expression of enzyme

activity (Browne and O'Cuinn, 1983) The enzyme has been shown to have a broad substrate specificity In addition to its ability to deamidate TRH to acid TRH, PE has been shown to hydrolyse a wide range of naturally occurring peptides at the carboxyl side of proline, including oxytocin, angiotensin, bradykinin, substance P, neurotensin, vasopressin and LHRH (for review, see O'Cuinn *et al*, 1990) The cleavage of TRH by PE may also be considered to be a biotransformation since acid TRH is reported to produce a "wet dog shaking" effect following intraventricular injection (Boschin *et al*, 1980) Acid TRH may be further metabolised by PAP and subsequently to its substituent amino acids by the action of imidopeptidase For a more comprehensive review of TRH metabolism see O'Cuinn *et al* (1990)

Figure 1.3.8 *Possible Degradation Pathways of TRH*



1.4 Physiological Role of Class-II PAPs

1.4.1 *In Vivo* Degradation of TRH

Given that many putative neurotransmitters can be degraded by more than one peptidase and that many peptidases are capable of hydrolysing more than one neuropeptide, some guidelines are necessary for the identification of the inactivating peptidase for each neuropeptide Schwartz *et al* (1981) and Loh *et al* (1984) have provided the following guidelines (slightly modified by O'Cuinn *et al*, 1990) for the identification of neuropeptide-inactivating peptidases

- that the enzyme should be active at physiological pH
- that the hydrolysis products should be biologically inactive or at least should not exhibit the same biological activity as the parent molecule in the context of neurotransmission
- that the inactivating enzyme should be strategically located to hydrolyse synaptically released neuropeptides (application of this criterion may confer anatomical specificity on the peptidase where only one neuropeptide is acting as a neurotransmitter)
- that enzymatic inhibition would protect synaptically released neuropeptides
- that enzyme inhibition would produce the biological actions of neuropeptides
- that enzyme activity might exhibit adaptive change to modified neurotransmission
- that substrate specificity would account for increased biological activity of synthetic analogues

Due to their intracellular localisation, neither PAPI nor cytosolic PE can be important for the inactivation of TRH after its release Moreover, since uptake of TRH by energy-dependent processes has not been demonstrated, these enzymes are apparently not even important for the inactivation of TRH at secondary control levels (Bauer, 1987b) Charli *et al* (1987) showed that TRH levels were unaffected by *in vitro* treatment of rat hypothalamic slices with the PE inhibitor Z-Pro-Pro-Prolinal and the PAPI inhibitor PDMK or by the *in vivo* administration of these inhibitors This suggests that PAPI and PE are not involved in regulating intracellular levels of TRH

The identification of PAPII as an ectoenzyme localised on the synaptosomal membrane of neuronal cells, combined with its unique substrate specificity, suggests a role for this enzyme in the termination of TRH mediated neurotransmission Support for this possibility is provided by the observation that ^{3}H-TRH degradation by neuronal cells in primary culture was not inhibited by PAPI or PE inhibitors while LHRH, a competitive inhibitor of PAPII, reduced the ability of these cells to degrade ^{3}H-TRH (Bauer *et al*, 1990) Further support was provided by the observation that N-1-carboxy-2-phenyl ethyl (N^{im} benzyl)-histidyl-ßNA (CPHNA), a reversible inhibitor of PAPII (K_i=8µM) and only slightly inhibitory to PAPI, effected a 2-fold recovery of TRH released basally or by K^+ stimulation of rat hypothalamic slices The involvement of PAPII in the termination of TRH neurotransmission is further supported by the observation that the effect of CPHNA was found to be greater in regions with higher PAPII activity (a 5-fold increase in TRH recovery was observed for olfactory bulb slices) It is also noteworthy that these significant effects were observed despite only a 40% inhibition of the enzyme (Charli *et al*, 1989)

1.4.2 Regulation of PAPII Activity *In Vivo*

A mandatory prerequisite for a regulatory function of PAPII is that the enzyme itself is regulated by some other mechanism

1.4.2.1 Short Term Regulation of PAPII by Phosphorylation

As mentioned previously in section 1 2 2 4, cloning of a cDNA encoding rat brain PAPII has revealed a number of putative phosphorylation sites which the authors proposed may be important for regulation of the enzyme (Schauder *et al*, 1994) Suen and Wilk (1990 and 1991) proposed that PAPII is subject to short-term regulation by protein kinase C-mediated phosphorylation 12-0-tetradecanoyl phorbol 13-acetate (TPA) directly activates the Ca^{2+} and phospholipid dependent protein kinase C The authors observed that exposure of Y-79 human retinoblastoma cells to TPA decreased the activity of the enzyme in a time- and concentration- dependent manner Enzyme activity was shown to decrease to 10% of control levels within 15 minutes and subsequently return to 70% of control levels in 1 hour Pretreatment of cells with the protein kinase C inhibitor H-7 [1-(5-isoquinolinylsulphonyl)-2-methyl piperazine dihydrochloride] or sphingosine was shown to prevent the inactivation of PAPII by TPA The inactivation of the enzyme was shown to follow the same time-course as translocation and activation of protein kinase C It was demonstrated that inactivation of PAPII was not due to dissociation or internalisation of the enzyme but due to a time-dependent phosphorylation of a 48,000 Da subunit of the enzyme (Suen and Wilk, 1990)

A second phase of decreasing levels of PAPII activity has been observed after longer exposure of cells to TPA After 1 hour, enzymatic activity has been shown to slowly and progressively decline, eventually reaching 15% of the control after 7 hours Cotreatment of cells with H-7 prevented the second phase of inactivation It has been demonstrated that this second phase of PAPII inactivation after exposure to TPA is due to the inhibition of enzyme synthesis (Suen and Wilk, 1991)

1.4.2.2 Regulation of PAPII by Thyroid Hormones

Considering the implication of PAPII in TRH metabolism, the most obvious question would be whether or not the enzyme is regulated by thyroid hormones As previously mentioned, TRH release at the hypothalamus and TSH synthesis by the pituitary thyrotroph are regulated by thyroid hormones (T_3 and T_4) in a negative fashion (see section 1 3 1) In addition, heterologus down-regulation of TRH-Rs by these hormones has been reported (see section 1 3 5) The activities of PAPII and serum PAP are also stringently controlled by thyroid hormones A number of investigators have studied the effect of these hormones by monitoring enzyme levels following the induction of hypo- or hyper- thyroidism in animals Hypothyroidism can be induced by treatment with the mild goitrogenic agent propylthiouracil (PTU), by feeding the animals on a low iodine diet or by thyroidectomy Hyperthyroidism can be induced by treatment with T_3 or T_4 At this point it should be noted that *in vitro*, T_3 itself does not affect the activity of PAPII (Bauer, 1987a)

The effect of thyroid hormones on serum PAP levels has been clearly demonstrated by a number of investigators Induction of hypothyroidism causes a slow decrease, over several weeks, in serum PAP levels while induction of hyperthyroidism causes a more rapid, dose-related increase in the level of the enzyme, after a 24 hour lag phase (Bauer, 1976, Emerson and Wu, 1987, Ponce *et al* , 1988, Scharfmann *et al* , 1990) A significant sex difference was also observed, with euthyroid female rats showing only 85% of the enzymatic activity of euthyroid males (Bauer, 1976) Yamada and Mori (1990) reported that in human serum, neither hypo- nor hyper- thyroidism caused a significant change in serum PAP activity The difference between human and rat serum remains to be elucidated

The regulation of membrane bound PAPII by thyroid hormones has been shown to be tissue specific While, like serum PAP, the adenohypophyseal enzyme is regulated by thyroid hormones and a significant sex difference is observed, no such hormonal regulation is observed for PAPII from kidney, lung, hypothalamus and various brain regions (Bauer, 1987a,1988, Emerson and Wu, 1987, Ponce, 1988, Suen and Wilk, 1989) However, Scharfmann *et al* (1990) showed that liver PAPII is regulated in a similar manner to the serum enzyme, providing further evidence that serum PAP may be a secreted form of the liver enzyme Suen and Wilk (1989) reported that PAPII levels in rat brain frontal cortex are elevated following chronic T_3 treatment, an effect which may have been obscured in previous studies using whole brain homogenates

Schomburg and Bauer (1995) carried out an extensive investigation into the effects of thyroid hormones on the mRNA levels of the TRH-R, PAPII and TSH in rat pituitary TRH-R mRNA was found exclusively in the adenohypophysis, with none detected in posterior pituitary including the intermediate lobe Transcripts of PAPII were found in both parts of the pituitary but T_3-induced alterations were observed only in the anterior pituitary While transcripts of preproTRH, TRH-R and PAPII were all detected in the hypothalamic tissue preparations, T_3-induced alterations in these mRNA levels were not observed by Northern blot analysis

In agreement with previous reports, the authors observed a reduction in length of the TSHß mRNA transcripts 4 hours after a single injection of T_3, followed by a slow decrease in the TSHß mRNA levels, reaching minimum levels after 48 hours Newly synthesised TSHß transcripts of the original size were detected after 96 hours although the concentration was still below control levels indicating that euthyroid conditions were not yet reached Under these conditions, no profound changes in the α-subunit mRNA levels were observed

The steady state mRNA levels of the TRH-R changed much more rapidly under these conditions A decrease to 60% of control levels was observed 2 hours after injection with minimal levels (35% of control) being observed after a further 2 hours and basal levels being regained only 24 hours after T_3 injection

Steady state mRNA levels of PAPII also changed rapidly in response to T_3 injection with a 5-fold increase being observed after 4 hours and maximal levels (>10-times above control levels), being observed after 6-24 hours Basal levels were observed 96 hours after injection Previous investigations by this group demonstrated a rapid increase in the activity of PAPII when pituitary cell aggregates were cultured in the presence of T_3 They noted that the observed lag phase of 5 hours is remarkably short in view of the considerable cellular trafficking that is required before the enzyme is finally expressed on the cell surface (Bauer *et al*, 1990)

Schomburg and Bauer (1995) also studied the response to an induced hypothyroid state by treating euthyroid rats with PTU After 1 day of treatment, adenohypophyseal transcript levels of PAPII decreased to 40% of control values and declined further to 15% within 3-4 days TRH-R mRNA levels increased slowly After 1 day of PTU treatment no change was observed but after 4 days, transcript levels were elevated to 200% of the control value TSHß transcript concentrations changed in parallel with TRH-R levels, reaching 330% of control values within 4 days of PTU treatment

Analysis of T_3 induced changes suggests that the relatively slow decline in TSHß levels is secondary in time to the acute pretranslational regulation of the TRH-R and PAPII The fast alterations of the transcript concentrations occur in mirror image to, and precede the corresponding changes in TRH-R and PAPII activity, suggesting that the controlled balance of these factors is of critical importance for an adequate adenohypophyseal response (Schomburg and Bauer, 1995)

1.4.2.3 Regulation of PAPII by Estrogens

As mentioned earlier, a significant sex difference was observed in the levels of PAP in rat serum (Bauer, 1976) Similarly, adenohypophyseal PAPII levels in male rats was found to be 3-fold higher than in female rats (Bauer, 1988) Following ovariectomy, the activity rapidly increases within a few days, reaching the value of male rats within 3 weeks Subsequent treatment with estradiol benzoate causes the PAPII levels to return to basal levels Likewise, the activity of the adenohypophyseal enzyme of normal male rats decreases as a result of estradiol benzoate treatment The inverse effect of thyroid hormones and estradiol was demonstrated by administering T_3 alone, or in combination with estradiol benzoate to female rats With T_3 alone, a 36-fold increase in enzyme activity was observed within 3 days, whereas for the same time period, only a 16-fold increase was observed when both hormones were given These effects are specific for estradiol since other steriods have no effect on PAPII activity (Bauer, 1988) Northern blot analysis has shown that male rats contain more transcripts for adenohypophyseal PAPII than female rats, therefore the effect of estrogens is probably exerted at the level of transcription Since an inverse relationship between TRH-R levels and PAPII levels is expected, it is not surprising that the adenohypophysis of female rats contains considerably more transcripts for the TRH-R than those of males (Schomburg *et al*, 1993)

Serum PAP (which is moderately regulated by thyroid hormones and estrogens) might modulate the adenohypophyseal TRH levels by controlling the amount of TRH reaching this region via the hypothalamo-hypophyseal portal blood system Alternatively, this enzyme may serve a scavenger function to ensure that TRH released into the peripheral circulation or produced peripherally does not reach the TRH responsive target of the anterior pituitary (Bauer, 1995) At the pituitary level, the specific location of PAPII on lactotrophs (Bauer *et al*, 1990) and the stringent control by estradiol and thyroid hormones might indicate that the adenohypophyseal enzyme could modulate the stimulatory effect of TRH on prolactin release by determining the availability and / or the duration of action of TRH on these target cells Prolactin secretion might be modulated by an alternative *feedback mechanism* through the integrative function of PAPII according to the hormonal conditions of the body (Bauer, 1995) In agreement with the proposed function of brain PAPII in the inactivation of synaptically released TRH (in order to clear the target site for the transmission of the next TRH signal), the activity of the enzyme should not be a limiting factor, and therefore it is not surprising that the activity of the brain enzyme is not influenced by hormonal manipulation of the animals (Bauer, 1988)

1.4.3 Ontogenesis of Class-II PAPs

The developmental pattern of PAPII occurs in a region specific manner In rat serum, PAP activity is absent at birth and appears on the 20th postnatal day, increasing thereafter, reaching adult levels on day 45 Rat liver PAPII follows the same ontogenic pattern as the serum enzyme, once again suggesting that the serum enzyme may be a secreted form of the liver enzyme (Scharfmann and Aratan-Spire, 1991) The developmental pattern of the adenohypophyseal enzyme in rat has been shown to be somewhat similar to that of the liver and serum enzymes Enzyme activity was shown to be absent at birth, first appearing on the 8th postnatal day, increasing until day 28 and subsequently declining until adult levels were reached (Vargas *et al*, 1992b) This ontogenic pattern correlates well with that of TSH (peak serum and pituitary levels between postnatal days 8 and 28) and thyroid hormones (peak serum values between postnatal days 12 and 28) (Gayo *et al*, 1986) The parallelism between PAPII and T_3 or T_4 ontogenesis suggests that these hormones are involved in the development of PAPII activity in these regions because of their known stimulatory actions on enzyme activity in these regions (see section 1 4 2 2) (Vargas *et al*, 1992b) Bauer *et al* (1990) reported that adenohypophyseal PAPII is preferentially if not exclusively localised on prolactin cells The coincident developmental patterns of prolactin cells and adenohypophyseal PAPII is in agreement with PAPII being synthesised in these cells (Vargas *et al*, 1992b)

Adenohypophyseal PAPII development is clearly delayed as compared to the brain enzyme In rat hypothalamus, PAPII activity has been detected on the 16th foetal day A sharp increase (7-fold) occurs between foetal day 22 and postnatal day 8 with a subsequent decrease thereafter until adult levels are reached PAPII in cortex tissue shows a similar developmental pattern to that in hypothalamus (Vargas *et al*, 1992b, de Gandarias *et al*, 1994) In contrast, olfactory bulb has been shown to present two peaks of PAPII activity A 2-fold increase from days 1-3 followed by a decrease at day 5 and a 2 4-fold increase on

day 22 followed by a subsequent decrease to adult levels has been observed A biphasic ontogeny in this area has also been observed for TRH and TRH mRNA levels (with a first peak at postnatal day 5 and a second one between days 20-30) (Vargas *et al*, 1992b) and also for cyclo His-Pro (Lamberton *et al*, 1984)

Vargas *et al* (1992b) surmised that the ontogenesis of adenohypophyseal PAPII occurs in parallel to neurogenesis of the median eminence and development of the hypothalamic-adenohypophyseal axis PAPII development in brain occurs before or during synaptogenesis in a region specific manner, leading to the ultimate regional distribution of the enzyme attained in the adult animal

2. Materials and Methods

2.1 Materials

Sigma Chemical Company (Poole, Dorset, England):

AG-25 Silver Stain Kit
Ala-MCA
7-Amino-4-Methyl-Coumarin (MCA)
Ammonium Persulphate
Bacitracin
Benzamidine
Blue Dextran
Bovine Serum Albumin (BSA)
Bowman-Birk, trypsin / chymotrypsin inhibitor
Calcium Sulphate ($CaSO_4$ $2H_2O$)
Cadmium Sulphate ($CdSO_4$ $8/3H_2O$)
CDTA
Cellulose Type 50
CHAPS
Cobalt Sulphate ($CoSO_4$ H_2O)
5,5-Dithio-bis 2-nitrobenzoic Acid
Dithiothreitol (DTT)
EDTA
EGTA
Eledoisin
N-Ethylmaleimide
Glycine
8-Hydroxyquinoline
Imidazole
2-Iodoacetamide
Iodoacetate
Lauryl Sulphate (SDS)
Luliberin (LHRH)
Magnesium Sulphate ($MgSO_4$ $7H_2O$)
Manganese Sulphate ($MnSO_4$ H_2O)
2-Mercaptoethanol
Mercuric Sulphate ($HgSO_4$)
N,N'-Methylene-Bisacrylamide
MW-GF-200 Marker Kit
Nickel Sulphate ($NiSO_4$ $6H_2O$)
Neurotensin
pGlu-ßNA
pGlu-Glu-Pro-NH_2
pGlu-His-Gly
pGlu-His-Gly-NH_2
pGlu-His-Pro
pGlu-pNA
1,10-Phenanthroline
Phenylmethylsulphonylfluoride(PMSF)
Phospholipase C
Potassium Phosphate (Monobasic)
Potassium Phosphate (Dibasic)
Pro-MCA
Puromycin
Pyroglutamic Acid
Saponin
Sephadex G-25
Silver Stain SDS PAGE High MW Standard -Kit
Sodium Chloride (NaCl)
Sodium Dexoycholate
TEMED
Thioglycolic Acid
Trizma Base
Trypsin
Trypsin Inhibitor from Soybean
Zinc Sulphate ($ZnSO_4$ $7H_2O$)

Bachem Feinchemikalein AG (Bubendorf, Switzerland):

Cyclo(His-Pro)
Gly-Pro-MCA
His-Pro-OH
pGlu-Ala-OH
pGlu-Gly-OH
pGlu-His-Pro-MCA
pGlu-His-Pro-Gly-OH
pGlu-MCA
pGlu-Phe-OH
pGlu-Val-OH
Thyroliberin (TRH)
Z-Gly-Pro-MCA

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

Acetic Acid
Acetone
Acetonitrile
Acrylamide
Ammonia Solution
Biuret Reagent
Bromophenol Blue
Calcium Chloride ($CaCl_2$)
Citric Acid
Copper Sulphate ($CuSO_4$)
Dimethylsulphoxide (DMSO)
Glacial Acetic Acid
Glycerol
Hydrochloric Acid
Iron (III) Chloride ($FeCl_3$ $6H_2O$)
Polyethylene Glycol 6,000
Triton X-100
Urea
Zinc Chloride ($ZnCl_2$)

Merck Chemical Company (Frankfurt, Germany):

Ammonium Sulphate
Di-Sodium Hydrogen Phosphate (Na_2HPO_4)
Potassium Chloride (KCl)
Sodium Hydroxide (NaOH)

Pharmacia Fine Chemical Company (Uppsala, Sweden).

Chelating Sepharose Fast Flow
Phenyl Sepharose CL-4B
Sephacryl S-200 HR
SP-Sepharose Fast Flow
Q-Sepharose High Performance

Aldrich Chemical Company (Poole, Dorset, England):

1,7-Phenanthroline
4,7-Phenanthroline
2-Pyrrolidone
Trifluoroacetic Acid (TFA)

Calbiochem-Novabiochem (UK) Ltd. (Nottingham, England):

LHRH 1-5
LHRH 1-6
LHRH 1-7
pGlu-Pro-NH_2

Peninsula Laboratories (Belmont, CA., USA):

pGlu-His
pGlu-His-Pro-Gly-NH_2

Pierce Chemical Company (Illinois, USA):

BCA Reagent

Boehringer Mannheim (UK) Ltd (East Sussex, England):

4-(2-Aminoethyl)-benzenesulfonyl fluoride (Pefabloc SC)
Papain
Trizma Base

Mount Sinai School of Medicine (New York - Courtesy of Dr. S. Wilk):

Fmoc-Pro-Pro-Nitrile
Z-Pro-Prolinal

Trinity College (Dublin - Courtesy of Dr. J. Kelly):

pGlu-Phe-Pro-NH_2

Kepak Ltd., Clonee, Dublin

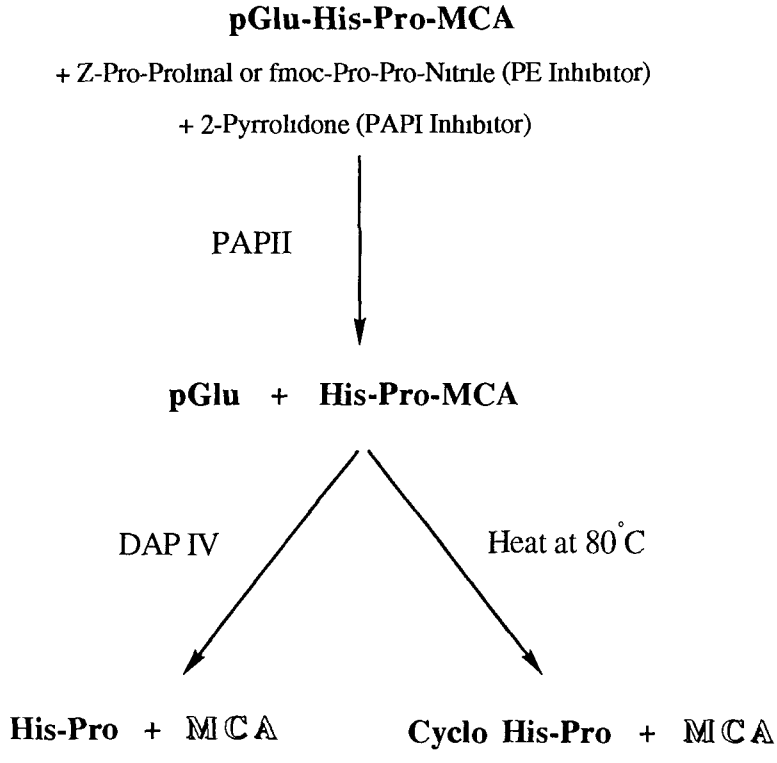
Bovine Brain and Blood

2.2 Determination of Enzyme Activities

2.2.1 Pyroglutamyl Aminopeptidase Type II

Two fluorimetric assays were employed for the detection of Pyroglutamyl Aminopeptidase Type II (PAPII) activity (see figure 2 2 1) Both assays are based on the cleavage of the substrate pGlu-His-Pro-MCA in the presence of a specific prolyl endopeptidase (PE) inhibitor, Z-Pro-Prolinal (Wilk and Orlowski, 1983) or fmoc-Pro-Pro-Nitrile (Li *et al* , 1996) and 2-pyrrolidone, a reversible pyroglutamyl aminopeptidase type I (PAPI) inhibitor (Armentrout and Doolittle, 1969)

Figure 2.2.1 *Fluorimetric Assays of PAPII*



Fluorescence of "Liberated" MCA Measured (Ex 370nm, Em 440nm)

2.2.1.1 A Fluorimetric Coupled Enzyme Assay for PAPII

PAPII activity was determined by a modification of the method of Friedman and Wilk (1986) 400µl of 0 1mM pGlu-His-Pro-MCA in 20mM potassium phosphate buffer, pH 7 5 was added to a mixture of, 100µl of sample, 20µl of 100µM Z-Pro-Prolinal or 26µM fmoc-Pro-Pro-Nitrile, 20µl of 1 3M 2-Pyrrolidone and 20µl of partially purified Dipeptidyl Aminopeptidase Type IV (DAPIV) (see section 2 5) The mixture was incubated for 1 hour at 37°C before the reaction was terminated by the addition of 940µl of 1 5M acetic acid All assays were performed in triplicate and suitable negative controls were prepared by incubating substrate separate from the other components of the incubation mixture for the hour, followed by the addition of acetic acid to the incubation mixture prior to the addition of substrate Liberated MCA was determined using a Perkin-Elmer LS-50 fluorescence spectrophotometer with

excitation and emission wavelengths of 370nm and 440nm respectively Excitation and emission slit widths were adjusted as appropriate for the level of fluorescence produced Samples containing particulate material were centrifuged at 13,000 rpm for 10 minutes, using a Heraeus Sepatech Biofuge A, prior to fluorescence reading

In addition to the above assay, a non-quantitative microplate assay was devised to facilitate the detection of PAPII in the large number of fractions generated by column chromatography 100µl of 0 1mM pGlu-His-Pro-MCA in 20mM potassium phosphate buffer, pH 7 5 was added to a mixture of 50µl of sample and 20µl of a "cocktail" containing equal volumes of 100µM Z-Pro-Prolinal or 26µM fmoc-Pro-Pro-Nitrile, 1 3M 2-Pyrrolidone and DAPIV The mixture was incubated for 1 hour at 37°C before the reaction was terminated by the addition of 150µl of 1 5M acetic acid A suitable negative control was included on each plate by replacing the enzyme sample with buffer Liberated MCA was detected in the same manner as above, using a Perkin-Elmer LS-50 fluorescence spectrophotometer fitted with a microplate-reader

2.2.1.2 A Non-Enzymatic Cyclisation Fluorimetric Assay for PAPII

This assay was carried out as described in section 2 2 1 1 with the following exceptions DAPIV was omitted from the incubation mixture and the volume of 1 5M acetic acid added was increased to 960µl, thus bringing the final volume to 1 5ml Following addition of the acetic acid, the reaction mixture was incubated at 80°C for 30 minutes to facilitate the non-enzymatic cyclisation of His-Pro-MCA to His-Pro diketopiperazine (cyclo His-Pro) and free MCA Liberated MCA was determined as outlined in section 2 2 1 1

2.2.2 Pyroglutamyl Aminopeptidase Type I

Pyroglutamyl Aminopeptidase Type I (PAPI) activity was determined according to the method of Cummins and O'Connor (1996), a modification of the original procedure of Fujiwara and Tsuru (1978) 100µl of sample was incubated for 1 hour at 37°C with 400µl of 0 1mM pGlu-MCA in 20mM potassium phosphate buffer, pH 7 5 containing 2% v/v DMSO, 2mM DTT and 2mM EDTA 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 incubating substrate and sample separately for the hour, followed by the addition of acetic acid to the incubation mixture prior to the addition of substrate Liberated MCA was determined as outlined in section 2 2 1 1

2.2.3 Prolyl Endopeptidase

Prolyl endopeptidase (PE) activity was determined according to a modification of the original procedure of Yoshimoto *et al* (1979) 100µl of sample was incubated for 1 hour at 37°C with 400µl of 0 1mM Z-Gly-Pro-MCA in 20mM potassium phosphate buffer, pH 7 5 containing 4% v/v DMSO, 2mM DTT and 2mM EDTA 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 incubating substrate and sample

separately for the hour followed by the addition of acetic acid to the incubation mixture prior to the addition of substrate Liberated MCA was determined as outlined in section 2 2 1 1

A non-quantitative microplate assay was also developed in order to facilitate the detection of PE in the large number of fractions generated by column chromatography 100µl of sample was incubated for 1 hour at 37°C with 100µl of 0 1mM Z-Gly-Pro-MCA in 20mM potassium phosphate buffer, pH 7 5 containing 4% v/v DMSO, 2mM DTT and 2mM EDTA The reaction was terminated by the addition of 100µl of 1 5M acetic acid A suitable negative control was included on each plate by replacing the enzyme sample with buffer Liberated MCA was determined as outlined in section 2 2 1 1

2.2.4 Dipeptidyl Aminopeptidase Type IV

Dipeptidyl aminopeptidase type IV (DAPIV) activity was determined according to a modification of the original procedure of Kato *et al* (1978) 100µl of sample was incubated for 1 hour at 37°C with 400µl of 0 1mM Gly-Pro-MCA in 20mM potassium phosphate buffer, pH 7 5 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 incubating substrate and sample separately for the hour followed by the addition of acetic acid to the incubation mixture prior to the addition of substrate Liberated MCA was determined as outlined in section 2 2 1 1

A non-quantitative microplate assay using the substrate Gly-Pro-MCA (0 1mM) was used to facilitate the detection of DAPIV in the large number of fractions generated by column chromatography as described in section 2 2 3

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

In order to quantify the activity of enzymes on "quenched" fluorimetric substrates, 7-Amino-4-Methyl-Coumarin (MCA) standard curves were constructed 10mM MCA in 100% DMSO was diluted to a concentration of 200µM using 20mM potassium phosphate buffer, pH 7 5, at 37°C This stock solution was stored at 4°C Lower concentrations of MCA were achieved using 20mM potassium phosphate buffer, pH 7 5 as diluent Standard curves were constructed by substituting the appropriate MCA standard for substrate under the assay conditions described in section 2 2, 2 9 7 and 2 10 11 2 Ranges of 0-0 3µM, 0-2µM, 0-10µM and 0-40µM MCA were prepared Fluorimetric intensity was measured using a Perkin-Elmer LS-50 fluorescence spectrophotometer with excitation and emission wavelengths of 370nm and 440nm respectively The excitation slit width was maintained at 10nm while the emission slit width was adjusted to produce fluorimetric intensities appropriate for the range being analysed

A unit of enzyme activity was defined as that which liberates one picomole of MCA per minute at 37°C Conversion of fluorimetric intensities to units of enzyme activity was achieved using the following formulae

$$\text{Units per ml} = \frac{\text{Fluorimetric Intensity}}{\text{Slope}} \times 66\,667$$

$$\text{Units per mg} = \frac{\text{Fluorimetric Intensity}}{\text{Slope x Protein}} \times 66\,667$$

where "Slope" is the slope of an appropriate MCA standard curve, "Protein" is the protein concentration, in mg/ml, of the sample being assayed The factor "66 667" considers the method of preparation of the standard curve, the conversion of µM to picomoles, the conversion of hours to minutes and the conversion from "per 100µl" to "per ml" of sample

2.4 Protein Determination

Three methods were employed for the determination of protein concentration in samples Due to the difference in sensitivity of each of the assays and their susceptibility to interfering substances, no single method was suitable for all applications

2.4.1 Biuret Protein Assay

The biuret protein assay was used for the determination of protein concentration in samples containing high levels of protein such as serum and post cation exchange serum samples 50µl of sample was incubated with 200µl of Biuret reagent for 30 minutes at 37°C after which the absorbance at 560nm was measured using a TiterTek Multiscan PLUS spectrophotometric plate reader A 0-10mg/ml BSA standard curve was prepared in parallel with the assay each time it was performed

2.4.2 Bicinchoninic Acid (BCA) Protein Assay

The Bicinchoninic Acid (BCA) protein assay, based on the original method of Smith *et al* (1985), was used for the determination of protein concentration in samples containing relatively low levels of protein Samples with protein concentrations above the range of the assay were diluted as appropriate while samples with protein concentrations below the range of the assay were concentrated by reverse-osmosis using polyethylene glycol (PEG) 6,000 Samples containing interfering substances such as imidazole, glycerol or PEG were dialysed extensively against distilled water prior to assay Two distinct protocols were used as appropriate for the samples being assayed

2.4.2.1 BCA assay Standard Protocol

10µl of sample was incubated with 200µl of BCA working reagent for 30 minutes at 37°C after which the absorbance at 560nm was measured using a TiterTek Multiscan PLUS spectrophotometric plate reader A 0-2mg/ml BSA standard curve was prepared in parallel with the assay each time it was performed

2.4.2.2 BCA assay. Enhanced Protocol

20µl or 50µl of sample was incubated with 200µl of BCA working reagent for 30 minutes at 60°C after which the absorbance at 560nm was measured using a TiterTek Multiscan PLUS spectrophotometric plate reader An appropriate BSA standard curve (0-0 5mg/ml BSA for assays incorporating 20µl of sample and 0-0 018mg/ml BSA for assays incorporating 50µl of sample) was prepared in parallel with the assay each time it was performed

2.4.3. Absorbance at 280nm

The absorbance of proteins based on the λ_{max} of tryptophan 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 using quartz cuvettes This method is only suitable for samples containing relatively high levels of protein

2.5 Partial Purification of Dipeptidyl Aminopeptidase Type IV From Bovine Serum

Due to the fact that this enzyme is not commercially available, DAPIV was partially purified from bovine serum for use in the PAPII coupled enzyme assay described in section 2 2 1 1

2.5.1 Bovine Serum Production

Bovine whole blood was collected from a freshly killed animal The whole blood was transported to a 4°C cold room and the clot allowed to shrink for 24 hours The remaining unclotted whole blood was then decanted and centrifuged at 6,000 rpm (4,100g) for 1 hour using a Beckman J2-MC refrigerated centrifuge fitted with a JA-21 rotor at 4°C The serum thus produced was divided into 20mL aliquots and stored at -20°C

2.5.2 SP Sepharose Fast Flow Cation-Exchange Chromatography

A 20ml SP Sepharose Fast Flow cation-exchange column (2 5cm x 4 1cm) was equilibrated with 100ml of 100mM potassium phosphate buffer, pH 5 5 20ml of bovine serum was dialysed for 12 hours at 4°C into 4L of 100mM monobasic potassium phosphate buffer (KH_2PO_4) and was subsequently centrifuged for 30 minutes at 15,000 rpm (27,200g) using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor at 4°C, to remove post-dialysis precipitate The resulting supernatant was applied to the column which was then washed with 40ml of the equilibration buffer Bound protein was eluted with 50ml of 500mM NaCl in 100mM potassium phosphate buffer, pH 7 5 4ml fractions were collected throughout the procedure which was carried out at 4°C at a flowrate of 2ml/min (24 4cm/hour) Fractions were assayed for PE and DAPIV activity using the microplate assays outlined in sections 2 2 3 and 2 2 4 respectively Protein determinations for each fraction were achieved using the biuret assay as described in section 2 4 1 Fractions containing the highest DAPIV activity were pooled

2.5.3 Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography

A 20ml Phenyl Sepharose CL-4B hydrophobic interaction column (2 5cm x 4 1cm) was equilibrated with 100ml of 1M ammonium sulphate in 100mM potassium phosphate buffer, pH 7 0 6 607g of dry ammonium sulphate was added to the post-SP Sepharose DAPIV pool and the pH adjusted using 1M NaOH, resulting in a final volume of 50ml containing 1M ammonium sulphate in 100mM potassium phosphate buffer at pH 7 0 Following the application of this sample, the column was washed with 60ml of the equilibration buffer Bound protein was eluted with 60ml of 5mM potassium phosphate buffer, pH 7 5 5ml fractions were collected throughout the procedure which was carried out at 4°C at a flowrate of 2ml/min (24 4cm/hour) Fractions were assayed for PE and DAPIV activity using the microplate assays outlined in sections 2 2 3 and 2 2 4 respectively Protein determinations for each fraction were achieved using absorbance readings at 280nm as described in section 2 4 3 Fractions containing the highest DAPIV activity were pooled

2.5.4 Concentration of Partially Purified DAPIV

Post-Phenyl Sepharose CL-4B DAPIV (partially Purified DAPIV) was dialysed for 18 hours into 2L of 100mM potassium phosphate buffer, pH 7 5 at 4°C, with buffer changes at 3 and 6 hours, to remove the ammonium sulphate The post-dialysis sample was concentrated by reverse osmosis with polyethylene glycol 6,000 The concentrated sample was dialysed for a further 9 hours into 2L of 100mM potassium phosphate buffer, pH 7 5 at 4°C with buffer changes at 3 and 6 hours to remove polyethylene glycol which passed through the dialysis membrane during concentration Glycerol was added to the sample to a final concentration of 10% v/v and 1ml aliquots were stored at -20°C

2.5.5 Investigation of Enzymatic Purity of Partially Purified DAPIV

The enzymatic purity of the partially purified DAPIV was investigated in order to determine the suitability of this material for use in the PAPII coupled enzyme assay, described in section 2 2 1 1 The activities of PAPII, PAPI and PE in the sample were determined as outlined in sections 2 2 1 2, 2 2 2 and 2 2 3 respectively In addition, the sample was assayed for aminopeptidase activity using the substrates Pro-MCA and Ala-MCA The assay procedures are described in section 2 10 10 1

2.6 Determination of Optimal Conditions for the Solubilisation of PAPII From the Particulate Fraction of Bovine Brain

2.6.1 Preparation of Washed Membranes from Bovine Brain

25g of bovine brain was homogenised in 100ml of 20mM potassium phosphate buffer, pH 7 5 with the aid of a Sorvall Omni Mixer Tissue was disrupted by three 5 second pulses at speed setting four with a 10 second pause between pulses The crude homogenate was centrifuged for 1 hour at 15,000 rpm (27,200g) using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor The supernatant (S1) was discarded while the pellet (P1) was resuspended to a volume of 125ml in 1M NaCl, 20mM potassium phosphate buffer at pH 7 5, using 5 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 (S2)

was discarded while the pellet (P2) was resuspended as above, to a volume of 125ml in distilled water and recentrifuged as before Once again the supernatant (S3) was discarded The washed membranes, thus obtained in the pellet (P3), were resuspended to a final volume of 95ml with 20mM potassium phosphate buffer, pH 7 5 as previously described All procedures were carried out at 4°C or on ice

2.6.2 Investigation of Various Membrane Solubilisation Regimes

In order to determine the optimum method of PAPII solubilisation from the membranes, a range of detergents and proteases were tested

10% stock solutions of the detergents CHAPS, saponin, sodium deoxycholate and Triton X-100 were prepared in 20mM potassium phosphate buffer at pH 7 5 A range of concentrations from 0 to 10% of each detergent were prepared using 20mM potassium phosphate buffer, pH 7 5 as diluent. 300µl of each detergent concentration being tested was added to 2 7ml of the resuspended washed membranes, (prepared as outlined in section 2 6 1), resulting in final concentrations of 0-1% detergent Following incubation for 1 hour on ice with constant shaking, 500µl of the incubation mixture was retained while the remainder was centrifuged at 13,000 rpm for 15 minutes using a Heraeus Sepatech Biofuge A

1mg/ml stock solutions of phospholipase C and the proteases papain (30 units/mg), chymotrypsin (90 units/mg) and trypsin (10,500 umts/mg) were prepared in 20mM potassium phosphate buffer at pH 7 5 A range of concentrations from 0 to 1mg/ml of each was prepared using 20mM potassium phosphate buffer, pH 7 5 as diluent 300µl of each test concentration was added to 2 7ml of the resuspended washed membranes in duplicate, resulting in final concentrations of 0-100µg/ml Incubations were for 1 hour on ice or at 25°C with constant shaking, following which the action of chymotrypsin and trypsin was terminated by the addition of 100µl of 1mg/ml Bowman-Birk, trypsin/chymotrypsin inhibitor in 20mM potassium phosphate buffer, pH 7 5 (32 3µg/ml final concentration) The action of papain was terminated by the addition of 100µl of 31mM iodoacetamide in 20mM potassium phosphate buffer, pH 7 5 (1mM final concentration) 500µl of each incubation mixture was retained while the remainder was centrifuged at 13,000 rpm for 15 minutes using a Heraeus Sepatech Biofuge A

Following centrifugation the pellets were discarded and the supernatants retained The coupled enzyme assay, described in section 2 2 1 1, was used to determine PAPII activity in the supernatants and in the pre-centrifugation homogenates

2.6.3 Comparison of the Specific Activity of PAPII Released by the Various Solubilisation Regimes

20ml of washed membranes, (prepared as outlined in section 2 6 1), were treated with 0 4% w/v CHAPS, 0 4% v/v Triton X-100, and 0 4% w/v sodium deoxycholate, for 1 hour on ice Similarly, 20ml of washed membranes were incubated with 5µg/ml papain and 5µg/ml trypsin, for 1 hour at 25°C, using the same ratios as outlined in section 2 6 2 Samples were centrifuged for 1 hour at 15,000 rpm, (27,200g), at 4°C using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor The non-enzymatic cyclisation fluorimetric assay, described in section 2 2 1 2, was used to determine the PAPII activity in the resulting supernatants MCA standard curves were constructed for each sample in which standards were substituted for substrate in order to determine the effect of the samples on fluorescence The protein concentration of each sample was determined using the BCA assay described in section 2 4 2 1, thus allowing the specific activity of PAPII in each sample to be determined

2.6.4 Stability of PAPII Released by Various Solubilisation Regimes

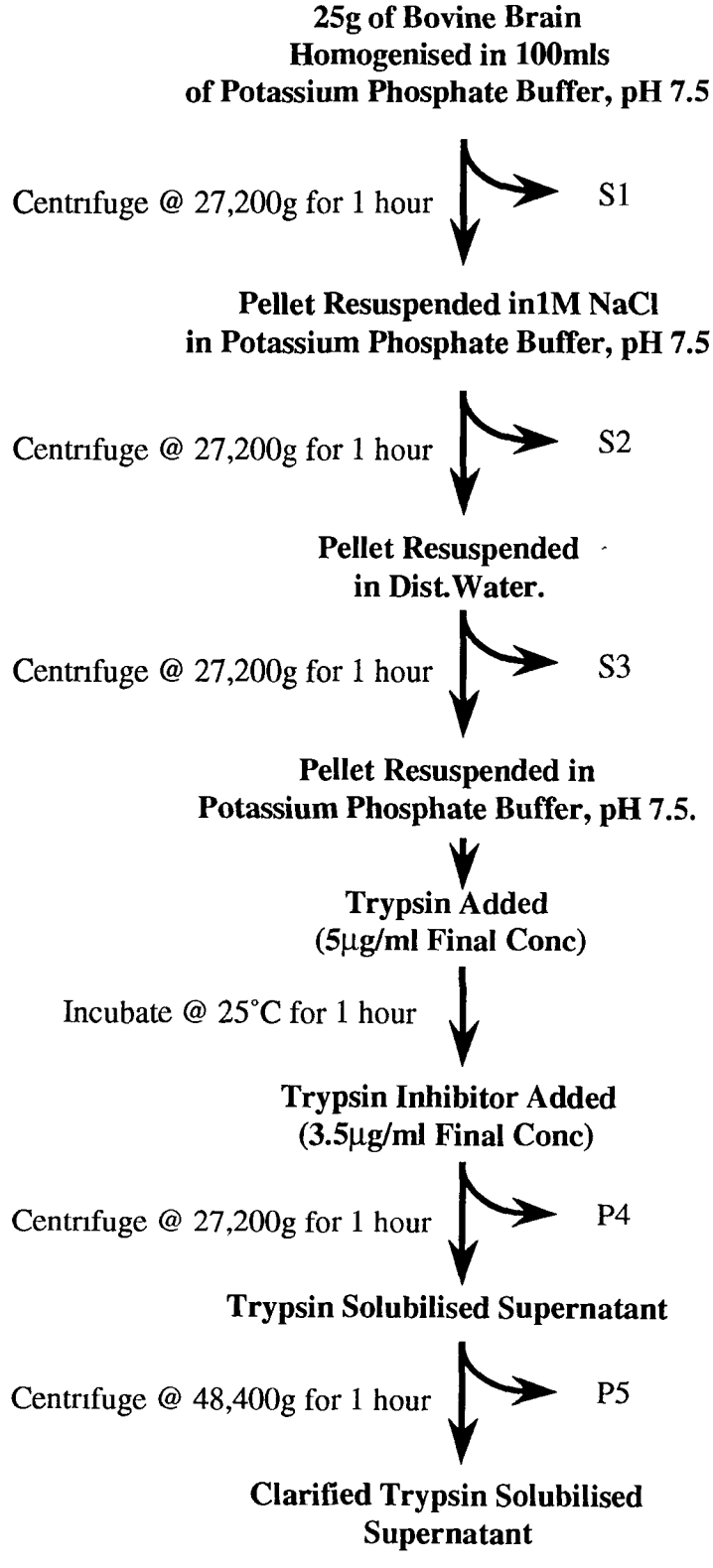
500µl aliquots of each of the supernatants obtained by the procedure outlined in section 2 6 3 were stored at 4°C and at -20°C Over a fourteen day period samples were removed and assayed for PAPII activity as described in section 2 4 2 1

2.7 Purification of PAPII from the Membrane Fraction of Bovine Brain

2.7.1 Solubilisation of PAPII from the Washed Membranes

To 95ml of resuspended washed membranes, prepared as outlined in section 2 6 1, 5ml of a 0 1mg/ml stock solution of trypsin in 20mM potassium phosphate buffer, pH 7 5 was added (final concentration 5µg/ml) Following incubation at 25°C for 1 hour, the reaction was terminated by the addition of 3ml of a 0 12mg/ml stock solution of soybean trypsin inhibitor (final concentration 3 5µg/ml) The suspension was centrifuged for 1 hour at 20,000 rpm, (48,400g), at 4°C using a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor The resulting pellet was discarded while the supernatant was recentrifuged as above to remove any remaining particulate matter This clarified trypsin solubilised supernatant was stored on ice for further use The entire membrane washing and solubilisation procedure is outlined in figure 2 7 1

Figure 2.7.1 *Washing and Solubilisation of Bovine Brain Membranes*



2.7.2 Q-Sepharose High Performance Anion-Exchange Chromatography

78 5ml of clarified trypsin solubilised supernatant was applied to a 10ml Q-Sepharose column (2 5cm x 2cm) which was equilibrated with 100ml of 20mM potassium phosphate buffer, pH 7 5 Following a 50ml wash with equilibration buffer the bound protein was eluted isocratically with 50ml of 100mM NaCl in 20mM potassium phosphate buffer at pH 7 5 4 5ml fractions were collected throughout the procedure which was carried out at 4°C at a flowrate of 1ml/min (12 2cm/hour) Fractions were assayed for PAPII activity using the non-enzymatic cyclisation fluorimetric assay, described in section 2 2 1 2 Protein determinations for each fraction were achieved using the BCA assay as described in section 2 4 2 1 Fractions containing the highest PAPII activity were pooled

2.7.3 Chelating-Sepharose Fast Flow Immobilised Metal Ion Chromatography

Two chelating Sepharose columns were connected in series Zn^{2+} was immobilised on the first column, 14ml bed volume (1 5cm x 8cm), by passing 30ml of 0 3M $ZnCl_2$ through the column and followed by 75ml of distilled water The second column, 5ml bed volume (1 5cm x 3cm), was not charged with metal ions Both columns were equilibrated with 150mM NaCl in 20mM potassium phosphate buffer at pH 7 5

The volume of the post-Q-Sepharose PAPII pool was increased to 110% of its original volume by the addition of 650mM NaCl in 20mM potassium phosphate buffer at pH 7 5, thus bringing the final NaCl concentration to 150mM All of this sample was applied to the pre-equilibrated chelating-Sepharose columns Following a 45ml wash with equilibration buffer the bound protein was eluted with a 50ml linear gradient of 0-45mM imidazole in equilibration buffer at pH 7 5 4ml fractions were collected throughout the procedure which was carried out at 4°C A flowrate of 0 5ml/min (17cm/hour) was employed for regeneration, equilibration, loading and washing while a flowrate of 1ml/min (34cm/hour) was employed for the elution step Fractions were assayed for PAPII activity using the non-enzymatic cyclisation fluorimetric assay, described in section 2 2 1 2 Protein determinations for each fraction were achieved usmg 20µl of sample in the enhanced BCA assay described in section 2 4 2 2 Fractions containing the highest PAPII activity were pooled

2.7.4 Preparation of Calcium Phosphate-Cellulose Resin

Calcium phosphate gel was prepared by a modification of the methods of Barranger (1976) and Donlon and Kaufman (1980) This procedure was carried out at room temperature Into 1 5L of 0 5M $CaCl_2$, a volume of 1L of 0 5M Na_2HPO_4 was added at a flowrate of 10ml/min, while being stirred continuously After mixing the final volume for a further 15 minutes, 1 5ml of concentrated ammonia solution was added and stirring continued for 10 minutes The precipitated gel was allowed to settle and the supernatant decanted The precipitate was then washed in 1L of distilled water and stirred for 5 minutes before allowing the gel to settle and again decanting the supernatant This washing procedure was repeated eight times to remove all traces of ammonia, which was monitored using Nessler's reagent The washed gel was stored at 4°C in 1L of degassed, distilled water

10g of Sigmacell cellulose type-50 powder, to be used as a filter aid, was soaked overnight in 100ml of 150mM KCl in 500mM potassium phosphate buffer at pH 6 8 Following extensive washing, and removal of fines, with distilled water, the cellulose was dried overnight in a 70°C oven

Prior to column packing, 1g of washed, dried cellulose was suspended in 10ml of 20mM potassium phosphate buffer, pH 7 5 12ml of an even suspension of calcium phosphate in water was added to the cellulose in buffer suspension and the mixture was stirred gently until an even suspension was obtained

2.7.5 Calcium Phosphate-Cellulose Chromatography

The post-chelating-Sepharose PAPII pool was dialysed for 12 hours into 2L of 10mM potassium phosphate buffer, pH 7 5, with one buffer change at 10 hours All of this dialysed sample was applied to a 9ml calcium phosphate-cellulose column (1 5cm x 5cm), prepared as outlined in section 2 7 4, which was pre-equilibrated with 60ml of 10mM potassium phosphate buffer, pH 7 5 Following a 30ml wash with equilibration buffer, the bound protein was eluted with a 40ml linear gradient from 10-100mM potassium phosphate buffer at pH 7 5 4ml fractions were collected throughout the procedure which was carried out at 4°C A flowrate of 0 3ml/min (10 2cm/hour) was employed for regeneration, equilibration, loading and washing while a flowrate of 0 5ml/min (17cm/hour) was employed for the elution step Fractions were assayed for PAPII activity using the non-enzymatic cyclisation fluorimetric assay, described in section 2 2 1 2 Protein determinations for each fraction were achieved using 20µl of sample in the enhanced BCA assay described in section 2 4 2 2 Fractions containing the highest PAPII activity were pooled

2.7.6 Sephacryl S-200 HR Gel-Filtration Chromatography

The post calcium phosphate-cellulose PAPII pool was concentrated to 1 7ml using an Amicon Centriplus-50 concentrator, at 4°C, in a Beckman J2-MC refrigerated centrifuge fitted with a JA-20 rotor 300µl of glycerol was added to the concentrated sample, thus bringing the final concentration to 15% v/v This sample was applied, by submergence below the buffer head, to a 230ml Sephacryl S-200 HR gel filtration column (2 5cm x 47cm), which was pre-equilibrated with 400ml of 150mM KCl in 20mM potassium phosphate buffer at pH 7 5 The column was eluted with 250ml of equilibration buffer After 87ml had passed through the column, 3 7ml fractions were collected A flowrate of 1ml/min (12 2cm/hour) was employed throughout the procedure which was carried out at 4°C Fractions were assayed for PAPII activity using the non-enzymatic cyclisation fluorimetric assay, described in section 2 2 1 2 Protein determinations for each fraction were achieved using 50µl of sample in the enhanced BCA assay described in section 2 4 2 2 Fractions containing the highest PAPII activity were pooled Unless otherwise indicated, an equal volume of 2% w/v protease free BSA was added to this purified enzyme and 3ml aliquots were stored at -20°C, for use in characterisation studies

2.8 Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS PAGE) was carried out on the purified PAPII (post Sephacryl S-200) sample, to assess the efficiency of the purification procedure, according to the method of Laemmli (1970) Deionised water was used throughout this procedure

2.8.1 Sample Preparation

Purified PAPII was concentrated by reverse osmosis, using PEG 6,000 Aliquots of purified PAPII concentrated 10-fold, 5-fold and un-concentrated were subsequently dialysed into 2L of 62 5mM Tris-HCl, pH 6 8, for 24 hours with buffer changes at 6 and 18 hours Dialysed samples were 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 at pH 6 8 Silver stain SDS molecular weight standards were prepared according to the manufacturers recommendations and subsequently diluted 1/2, 1/5 and 1/10 with solubilisation buffer Table 2 8 1 lists the molecular weight markers used All samples were incubated for 2 minutes in a boiling water bath and allowed to cool to room temperature prior to loading onto the gel

Table 2.8.1 *Molecular Weight Markers Used for SDS PAGE*

Molecular Weight Standard	Source	Molecular Weight (Daltons)
ß-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

2.8.2 Gel Preparation and Electrophoresis

A 16cm x 16cm x 1mm, 7 5% 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 8 2 20µl of each sample or molecular weight marker was loaded onto the gel which was electrophoresed in an electrode buffer consisting of 1 92M Glycine, 1% w/v SDS and 0 25M Tris at pH 8 3 Electrophoresis proceeded for approximately 3 hours at 25mA per gel

Table 2.8.2 *SDS PAGE Gel Preparation*

Solution	Volume Required for 7.5% Resolving Gel (ml)	Volume Required for 3.75% Stacking Gel (ml)
30% Acrylamide, 0.8% Bisacrylamide	7.5	2.5
3M Tris-HCl pH 8.8	3.75	-
0.5M Tris-HCl pH 6.8	-	5
10% v/v SDS	0.3	0.2
Deionised Water	17	11.3
1.5% w/v Ammonium Persulphate	1.5	1
TEMED	0.015	0.015

2.8.3 Silver Staining

A Sigma AG-25 Silver Stain kit was employed to perform the silver staining of SDS PAGE gels based on the method of Heukeshoven and Dernick (1985). Table 2.8.3 outlines the recommended staining procedure. This procedure was followed for the staining of gels with the exception that the development time was extended to 20 minutes and the reducing step was deemed to be unnecessary. The relative mobility (R_f) of each of the molecular weight markers was determined by dividing the distance migrated by the standard by the distance migrated by the tracking dye. A plot of Log_{10} of molecular weight versus R_f was constructed. This calibration curve facilitated the determination of the molecular weight of unknown protein bands. An image of the stained gel was recorded using black and white photography of the gel.

Table 2.8.3 *Recommended Silver Staining Procedure for SDS PAGE Gels*

Step	Reagent	Volume	Duration
1. Fixing	30% v/v EtOH / 10% v/v Glacial Acetic Acid	300ml x 3	20 min. x 3
2. Rinsing	Deionised Water	300ml x 3	10 min. x 3
3. Silver Staining	Silver Nitrate	300ml	30 min.
4. Rinsing	Deionised Water	300ml	10-20 sec.
5. Developing	Sodium Carbonate / Formaldehyde	150ml x 2	5-8 min. x 2
6. Development Stop	1% v/v Glacial Acetic Acid	300ml	5 min.
7. Rinsing	Deionised Water	300ml x 3	10 min. x 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 x 3	10 min. x 3

2.9 Development of pGlu-His-Pro-MCA Based PAPII Assays

2.9.1 Inhibition of PE by Z-Pro-Prolinal and fmoc-Pro-Pro-Nitrile

16mM Z-Pro-Prolinal in 100% methanol was diluted to a final concentration of 100µM using 20mM potassium phosphate buffer, pH 7 5 A range of dilutions from 0-100µM Z-Pro-Prolinal were prepared using the same buffer as diluent 20µl of each test concentration was added to 100µl of the cytosolic fraction of bovine brain (S1 fraction), obtained as outlined in section 2 6 1 2 4mM fmoc-Pro-Pro-Nitrile in 100% DMSO was diluted to a final concentration of 26µM using 20mM potassium phosphate buffer, pH 7 5 A range of dilutions from 0-26µM fmoc-Pro-Pro-Nitrile were prepared using the same buffer as diluent. 20µl of each test concentration was added to 100µl of the post-Q-Sepharose PAPII pool, obtained as outlined in section 2 7 2 The PE assay described in section 2 2 3 was carried out on all samples to determine the residual enzyme activity The final concentration ranges, under assay conditions, were 0-3 85µM Z-Pro-Prolinal and 0-1µM fmoc-Pro-Pro-Nitrile

2.9.2 Inhibition of PAPI by 2-Pyrrolidone

2-pyrrolidone was incubated at 37°C until a homogeneous solution was obtained A range of dilutions from 0-100% 2-pyrrolidone (0-13 16M) was prepared using 20mM potassium phosphate buffer, pH 7 5 as diluent 20µl of each test concentration was added to 100µl of the cytosolic fraction of bovine brain (S1 fraction), obtained as outlined in section 2 6 1 The PAPI assay described in section 2 2 2 was carried out to determine the residual enzyme activity The final 2-pyrrolidone concentration range, under assay conditions, was 0-0 51M

2.9.3 The Effect of DAPIV on MCA Release

The amount of partially purified DAPIV required to catalyse the liberation of MCA from all of the His-Pro-MCA produced by the action of PAPII, on the substrate pGlu-His-Pro-MCA, was investigated This study was performed on both a crude sample and a purified sample

100µl of crude homogenate (see section 2 6 1), 20µl of 26µM fmoc-Pro-Pro-Nitrile and 20µl of 1 32M 2-Pyrrolidone was incubated for 1 hour at 37°C with 400µl of 0 1mM pGlu-His-Pro-MCA The reaction was terminated by incubating the mixture at 80°C for 2 minutes When reaction mixture had cooled to 37°C, a range of volumes from 0-150µl of partially purified DAPIV (see section 2 5 4) was added The final volume of the incubation mixture was adjusted to 690µl by the addition of 20mM potassium phosphate buffer, pH 7 5 Following further incubation for 1 hour at 37°C the reaction was terminated by the addition of 810µl of 1 5M acetic acid Liberated MCA was determined as outlined in section 2 2 1 1

80µl of purified PAPII containing 1% w/v BSA (see section 2 7 6), and 20µl of 26µM fmoc-Pro-Pro-Nitrile was incubated for 1 hour at 37°C with 400µl of 0 1mM pGlu-His-Pro-MCA The reaction was terminated by incubating the mixture at 80°C for 2 minutes When the reaction mixture had cooled to 37°C, a range of volumes from 0-300µl of partially purified DAPIV (see section 2 5 4) was added The final volume of the incubation mixture was adjusted to 800µl by the addition of 20mM potassium

phosphate buffer, pH 7 5 Following further incubation for 1 hour at 37°C the reaction was terminated by the addition of 700µl of 1 5M acetic acid Liberated MCA was determined as outlined in section 2 2 1 1

All assays were performed in triplicate A suitable negative control for each sample was prepared by incubating substrate separate from the other components of the incubation mixture for 1 hour at 37°C, and subsequently for 2 minutes at 80°C Following the addition of substrate, the appropriate volumes of DAPIV and buffer were added and following 1 hour further incubation at 37°C, acetic acid was added

2.9.4 The Effect of Time at 80°C on MCA Release

The length of time at 80°C required for the liberation of MCA from all of the His-Pro-MCA produced by the action of PAPII, on the substrate pGlu-His-Pro-MCA, was investigated This study was performed on both a crude sample and a purified sample

100µl of crude homogenate (see section 2 6 1), 20µl of 26µM fmoc-Pro-Pro-Nitrile and 20µl of 1 32M 2-Pyrrolidone was incubated for 1 hour at 37°C with 400µl of 0 1mM pGlu-His-Pro-MCA The reaction was terminated by the addition of 960µl of 1 5M acetic acid The reaction mixture was incubated at 80°C for a range of times from 0-50 minutes, following which the samples were cooled by incubation in an ice-water bath Liberated MCA was determined as outlined in section 2 2 1 1

80µl of purified PAPII containing 1% w/v BSA (see section 2 7 6), and 20µl of 26µM fmoc-Pro-Pro-Nitrile was incubated for 1 hour at 37°C with 400µl of 0 1mM pGlu-His-Pro-MCA The reaction was terminated by the addition of 1ml of 1 5M acetic acid The reaction mixture was incubated at 80°C for a range of times from 0-180 minutes, following which the samples were cooled by incubation in an ice-water bath Liberated MCA was determined as outlined in section 2 2 1 1

All assays were performed in triplicate A suitable negative control for each sample was prepared by incubating substrate separate from the other components for the hour at 37°C, followed by the addition of acetic acid prior to the addition of substrate and subsequent incubation for the appropriate length of time at 80°C

2.9.5 Linearity of PAPII Activity With Respect To Time

The linearity of PAPII activity, against the substrate pGlu-His-Pro-MCA, with respect to time was investigated This study was performed on both a crude sample and a purified sample

100µl of crude homogenate (see section 2 6 1) or 100µl of purified PAPII containing 1% w/v BSA (see section 2 7 6), 20µl of 26µM fmoc-Pro-Pro-Nitrile and 20µl of 1 32M 2-Pyrrolidone was incubated for a range of times from 0-120 minutes at 37°C with 400µl of 0 1mM pGlu-His-Pro-MCA The reaction was terminated by the addition of 960µl of 1 5M acetic acid The reaction mixture was incubated at 80°C for 30 minutes, following which the samples were cooled by incubation in an ice-water bath Liberated MCA was determined as outlined in section 2 2 1 1

2.9.6 Linearity of the Non-Enzymatic Cyclisation PAPII Assay With Respect to Enzyme Concentration

A range of dilutions of purified PAPII (see section 2 7 6) were prepared using 20mM potassium phosphate buffer, pH 7 5 as diluent Each dilution was assayed for PAPII activity as outlined in section 2 2 1 2 A range of dilutions of purified PAPII containing 1% w/v BSA was prepared using 1% w/v BSA in 20mM potassium phosphate buffer at pH 7 5 as diluent Each dilution was assayed for PAPII activity as outlined in section 2 2 1 2 This study was also carried out using a modification of the assay described in section 2 2 1 2 in which the incubation time at 80°C was increased to 2 hours In each case, a standard curve of enzyme concentration versus fluorescence was constructed

2.9.7 A Quantitative Assay for the Determination of PAPII Activity

A modification of the assay described in section 2 2 1 2, in which the incubation time at 80°C was increased to 2 hours, was employed for the quantitative determination of PAPII activity in crude or partially purified samples A further modification of this procedure, in which 2-pyrrolidone and Z-Pro-Pro-Prolinal or fmoc-Pro-Pro-Nitrile were omitted, and the volume of acetic acid added to terminate the reaction was increased to 1ml, was used for the characterisation of the purified enzyme Characterisation studies which required absolute values for PAPII activity employed a 2 hour incubation time at 80°C, while those requiring only relative values employed a 30 minute incubation time at 80°C

2.10 Characterisation of PAPII

2.10.1 Relative Molecular Mass Determination via Gel-Filtration Chromatography

A 230ml Sephacryl S-200 HR gel filtration column (2 5cm x 47cm) was employed for the determination of the relative molecular mass of PAPII under non-denaturing conditions A flowrate of 1ml/min (12 2cm/hour) was employed throughout the procedure which was carried out at 4°C

2.10.1.1 Void Volume Determination

Blue dextran was prepared at a concentration of 2mg/ml in 20mM potassium phosphate buffer, pH 7 5 containing 15% v/v glycerol 2ml of this solution was applied, by submergence below the buffer head, to the Sephacryl S-200 HR gel filtration column which was pre-equilibrated with 400ml of 150mM KCl in 20mM potassium phosphate buffer at pH 7 5 The column was eluted with 150ml of the equilibration buffer 2ml fractions were collected throughout the run The presence of blue dextran in the post-column fractions was detected by measuring the absorbance at 620nm using a TiterTek Multiscan PLUS spectrophotometric plate reader, and the void volume (V_0) of the column was thus determined

2.10.1.2 Elution Volume Determination for Protein Standards

Five known molecular mass standards, provided by a Sigma MW-GF-200 Kit, were dissolved individually in 20mM potassium phosphate buffer, pH 7 5 containing 15% v/v glycerol The standards used and the

concentrations at which they were prepared are shown in table 2 10 1 The standards were applied individually in a 2ml volume, by submergence below the buffer head, to the Sephacryl S-200 HR gel filtration column which was pre-equilibrated with 400ml of 150mM KCl in 20mM potassium phosphate buffer at pH 7 5 The column was eluted with 200ml of equilibration buffer 2ml fractions were collected throughout each run The presence of cytochrome C in the post-column fractions was detected by measuring the absorbance at 405nm using a TiterTek Multiscan PLUS spectrophotometric plate reader while the other standards were detected using the BCA protein assay described in section 2 4 2 1

The elution volume (V_e) of each standard was determined and a plot of Log_{10} of molecular mass versus V_e/V_0 was constructed Using this calibration curve, it is possible to estimate the relative molecular mass of PAPII since its elution volume (V_e) has already been determined as outlined in section 2 7 6

Table 2.10.1 *Molecular Weight Standards Used for Gel Filtration*

Molecular Weight Standard	Source	Approx Molecular Weight (Daltons)	Concentration (mg/ml)
ß-Amylase	Sweet Potato	200,000	5
Alcohol Dehydrogenase	Yeast	150,000	5
Albumin	Bovine Serum	66,000	5
Carbonic Anhydrase	Bovine Erythrocytes	29,000	5
Cytochrome C	Horse Heart	12,400	2

2.10.2 Stability of Purified PAPII Under Various Storage Conditions

Purified PAPII (see section 2 7 6), was diluted with an equal volume of 40% v/v glycerol in 20mM potassium phosphate buffer at pH 7 5, 2% w/v BSA in 20mM potassium phosphate buffer at pH 7 5 or 20mM potassium phosphate buffer, pH 7 5 500µl aliquots of each were stored at 4°C, -20°C and -80°C Over a three week period aliquots were removed and assayed for PAPII activity as outlined in section 2 9 7 A plot of enzyme activity versus time was constructed for each storage condition

2.10.3 The Effect of Assay Temperature on PAPII Activity

Purified PAPII containing 1% w/v BSA was assayed at 25, 37, 45, 50 and 60°C for 15 minutes, by a modification of the assay described in section 2 9 7 Both the enzyme and substrate were preincubated for 2 minutes at the corresponding temperature, prior to assay A plot of enzyme activity versus assay temperature was constructed The linearity of enzyme activity with respect to time at 45°C was investigated by performing the assay over a range of times from 0-60 minutes

2.10.4 The Effect of pH on PAPII Activity

2.10.4.1 pH Activity Profile

7ml of purified PAPII containing 1% w/v BSA was dialysed for 12 hours into 4L of distilled water 50µl of dialysed enzyme was preincubated for 15 minutes at 37°C, with 50µl of 40mM buffer at a range of pH values from 2 6 to 10 5 The buffers used and their respective pH ranges are shown in table 2 10 4 Each sample was assayed for PAPII activity as described in section 2 9 7 The substrate used for each assay was prepared by diluting 0 2mM pGlu-His-Pro-MCA, prepared in distilled water, with an equal volume of the corresponding buffer at a concentration of 40mM, at the corresponding pH Hence the enzyme was preincubated and assayed in the same buffer and at the same pH in each case A plot of enzyme activity versus pH was constructed

MCA standard curves were prepared, as outlined in section 2 3, for each buffer at each pH in order to determine the effect of these conditions on fluoresence Due to the large number of samples, each curve was constructed using only two MCA standard concentrations and 1% w/v BSA, in 20mM potassium phosphate buffer at pH 7 5, was used in the place of purified enzyme

2.10.4.2 pH Inactivation Curve

7ml of purified PAPII containing 1% w/v BSA was dialysed for 12 hours into 4L of distilled water 50µl of dialysed enzyme was preincubated for 15 minutes at 37°C, with 50µl of 40mM buffer at a range of pH values from 2 6 to 10 5 The buffers used and their respective pH ranges are shown in table 2 10 4 Each sample was assayed for PAPII activity as described in section 2 9 7 The substrate used for each assay was prepared by diluting 0 2mM pGlu-His-Pro-MCA, prepared in distilled water, with an equal volume of the 40mM potassium phosphate buffer Samples which were preincubated in the pH range 2 6-4 0 were assayed using substrate at pH 8 0 Those which were preincubated in the pH range 9 0-10 5 were assayed using substrate at pH 7 2 while all others were assayed using substrate at pH 7 5 Hence the enzyme was preincubated at a wide range of pH values but all assays proceeded within the pH range 6 8-7 6 A plot of enzyme activity versus preincubation pH was constructed

MCA standard curves were prepared, as outlined in section 2 10 4 1 in order to determine the effect of these conditions on fluoresence

Table 2 10 4 *Buffering Systems Used to Determine the Effect of pH on PAPII Activity*

Buffer	pH Range
Citric Acid / Di Basic Potassium Phosphate	2 6 - 7 5
Potassium Phosphate	6 0 - 8 0
Tris / HCl	7 0 - 9 0
Glycine / NaOH	8 5 - 10 5

2.10.5 The Effect of Functional Reagents on PAPII Activity

Stock solutions of a range of functional reagents were prepared as outlined in table 2 10 5 Lower concentrations of each were achieved using 20mM potassium phosphate buffer, ± 5% v/v acetone, at pH 7 5 as appropriate The pH of the stock solutions were adjusted to pH 7 5 using 20mM monobasic or dibasic potassium phosphate buffer as appropriate or by the addition of 1M NaOH where necessary

10ml of purified PAPII containing 1% w/v BSA was dialysed into 2L of 20mM potassium phosphate buffer at pH 7 5 for 18 hours with one buffer change at 6 hours 50µl of dialysed PAPII was preincubated for 15 minutes at 37°C with an equal volume of each functional reagent to be tested The residual PAPII activity was then determined as outlined in section 2 9 7 A positive control for each buffer system (± 5% v/v acetone) was prepared in which the appropriate buffer replaced functional reagents

MCA standard curves were prepared, as outlined in section 2 3, for each functional reagent in order to determine the effect of these conditions on fluoresence Due to the large number of samples, each curve was constructed using only two MCA standard concentrations and 1% w/v BSA, in 20mM potassium phosphate buffer at pH 7 5, was used in the place of purified enzyme

2.10.6 The Effect of Metal-Complexing Agents on PAPII Activity with Respect to Time

The effect of a range of metal-complexing agents and the non-chelating 1,7- and 4,7-phenanthroline on PAPII activity over time was investigated 1 5ml of purified PAPII containing 1% w/v BSA, dialysed as outlined in section 2 10 5, was incubated on ice with an equal volume of each reagent to be tested at a concentration of 2mM (see section 2 10 5) A positive control for each buffer system (± 5% v/v acetone) was prepared in which purified PAPII was incubated with an equal volume of the appropriate buffer Over a 24 hour period aliquots were removed and the residual PAPII activity in each sample was determined as outlined in section 2 9 7 A plot of residual PAPII activity versus time for each reagent was prepared

MCA standard curves, which were prepared earlier (see section 2 10 5), were used to determine the effect of these conditions on fluoresence

Table 2.10.5 *Preparation of Functional Reagents*

Compound Class	Compound Name	Concentration	Preparation[a]
Cysteine Protease Inhibitors	Iodoacetamide	20mM	Buffer
	Iodoacetate	20mM	Buffer
	N-Ethylmaleimide (NEM)	20mM	Buffer
	Dithiobisnitrobenzoic acid (DTNB)	2mM	Buffer[b]
Cysteine Protease Activators	Dithiothreitol (DTT)	20mM	Buffer
	2-Mercaptoethanol	20mM	Buffer
	Thioglycolic acid	20mM	Buffer
Serine Protease Inhibitors	AEBSF (Pefabloc SC)	20mM	Buffer
	Phenylmethanesulfonylfluoride (PMSF)	2mM	5% v/v Acetone[c]
Metallopeptidase Inhibitors	Imidazole	20mM	Buffer
	CDTA	20mM	Buffer
	EDTA	20mM	Buffer
	EGTA	20mM	Buffer
	8-Hydroxyquinoline	20mM	5% v/v Acetone[b]
	1,10-Phenanthroline	20mM	5% v/v Acetone
	1,7-Phenanthroline	20mM	5% v/v Acetone
	4,7-Phenanthroline	20mM	5% v/v Acetone[b]
Other Substances	2-Pyrrolidone	100mM	Buffer
	Bacitracin	2mg/ml	Buffer
	Puromycin	2mM	Buffer
	Benzamidine	20mM	Buffer

[a] Functional reagents were prepared in 20mM potassium phosphate buffer, or in 5% v/v acetone (initial dissolution in 100% acetone and subsequent addition of 20mM potassium phosphate buffer) The pH of the stock solution was adjusted to pH 7 5 using 20mM monobasic and dibasic potassium phosphate buffer as appropriate or by the addition of 1M NaOH where necessary

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

[c] PMSF was freshly prepared immediately prior to use

2.10.7 The Effect of Metal Ions on PAPII Activity

Stock solutions of a range of metal salts were prepared at a concentration of 2mM, as outlined in table 2 10 7 Lower concentrations of each were achieved by dilution with 20mM potassium phosphate buffer, pH 7 0 or 20mM tris/HCl, pH 7 0 as appropriate The pH of the stock solutions was adjusted to pH 7 0 using 20mM monobasic or dibasic potassium phosphate buffer as appropriate or by the addition of 1M HCl when tris buffer was used

5ml aliquots of purified PAPII containing 1% w/v BSA were dialysed into either 2 5L of 20mM potassium phosphate buffer at pH 7 0 or 2 5L of 20mM tris/HCl buffer at pH 7 0 for 18 hours, with one buffer change at 6 hours 50µl of dialysed PAPII was incubated for 15 minutes at 37°C with an equal volume of each metal salt to be tested The residual PAPII activity was then determined as outlined in section 2 9 7 A positive control for each buffer system (potassium phosphate buffer and tris/HCl) was prepared in which the appropriate buffer replaced the metal salt The substrate used in the assay was prepared in 20mM potassium phosphate buffer at pH 7 0 or 20mM tris/HCl at pH 7 0 as appropriate

MCA standard curves were prepared, as outlined in section 2 3, for each metal salt in order to determine the effect of these conditions on fluoresence Due to the large number of samples, each curve was constructed using only two MCA standard concentrations 1% w/v BSA, in 20mM potassium phosphate buffer at pH 7 0 or tris/HCl pH 7 0, was used in the place of purified enzyme

Table 2.10.7 *Preparation of Metal Salts*

Metal Salt	Concentration (mM)	Preparation[a]
$CuSO_4$	2	20mM potassium phosphate buffer
$MgSO_4$	2	20mM potassium phosphate buffer
$FeCl_3$	2	20mM potassium phosphate buffer
$CaSO_4$	2	20mM tris / HCl
$CdSO_4$	2	20mM tris / HCl
$CoSO_4$	2	20mM tris / HCl[b]
$HgSO_4$	2	20mM tris / HCl[c]
$MnSO_4$	2	20mM tris / HCl
$NiSO_4$	2	20mM tris / HCl
$ZnSO_4$	2	20mM tris / HCl

[a] The pH of the stock solution was adjusted to pH 7 0 using 20mM monobasic and dibasic potassium phosphate buffer as appropriate or by the addition of 1M HCl when tris buffer was used

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

[c] Dissolved initially in a small volume of 1M HCl followed by the gradual addition of tris base

2.10.8 Reactivation of EDTA-Inactivated PAPII by Metal Ions

2.10.8.1 EDTA-Inactivation of PAPII

Purified PAPII containing 1% w/v BSA was dialysed into 2 5L of 20mM tris/HCl buffer at pH 7 0 as outlined in section 2 10 7 500µl of 50mM EDTA in 20mM tris buffer, adjusted to pH 7 0 with 1M NaOH, was added to 4 5ml of dialysed PAPII thus resulting in a final concentration of 10mM EDTA The mixture was incubated for 7 hours at 25°C A positive control was prepared in which tris/HCl at pH 7 0 was added in place of EDTA Aliquots of the control and the EDTA-treated sample were assayed for PAPII activity, as outlined in section 2 9 7, using substrate which was prepared in 20mM tris/HCl at pH 7 0

2.10.8.2 EDTA Removal Using Sephadex G-25 Gel Filtration

EDTA was removed from the sample by a modification of the method of Salvesen and Nagase (1989) Four 10ml Sephadex G-25 columns (1 5cm x 5 7cm) were equilibrated with 50ml of tris/HCl at pH 7 0 The columns were allowed to run dry under gravity before being centrifuged at 1,000 rpm (184g) for 30 seconds using a Heraeus Sepatech Megafuge 1 0 1ml of EDTA-treated sample was applied to the top of each of three of the columns while 1ml of the control sample was applied to the top of the other The columns were centrifuged at 1,200 rpm (265g) for 1 minute The eluant from each of the columns was collected and that from the three EDTA-treated samples combined Aliquots of the control and the EDTA-treated sample were assayed for PAPII activity as outlined in section 2 10 8 1

2.10.8.3 Reactivation Using Metal Salts

50µl of post-gel filtration, EDTA-treated PAPII was preincubated for 15 minutes at 37°C with an equal volume of each metal salt to be tested The PAPII activity in each sample was then determined as outlined in section 2 10 8 1 A positive control was prepared in which 50µl of tris/HCl was added to 50µl of the post-gel filtration control sample

MCA standard curves, which were prepared earlier (see section 2 10 7), were used to determine the effect of these conditions on fluoresence

2.10.9 The Effect of DMSO on PAPII Activity

3ml of purified PAPII, containing 1% w/v BSA, was dialysed into 2 5L of 20mM potassium phosphate buffer at pH 7 5 for 18 hours at 4°C, with one buffer change at 6 hours PAPII activity was determined as outlined in section 2 9 7 using 0 1mM pGlu-His-Pro-MCA prepared in 20mM potassium phosphate buffer at pH 7 5 containing DMSO at a range of concentrations from 0-10% v/v MCA standard curves were prepared as outlined in section 2 3 to determine the effect of these conditions on fluoresence A plot of PAPII activity versus DMSO concentration in the substrate was constructed

2.10.10 Investigation of the Substrate Specificity of PAPII

2.10.10.1 The Activity of PAPII Against a Range of Quenched Fluorimetric Substrates

The activity of purified PAPII against a range of quenched fluorimetric substrates was tested in order to assess the enzymatic purity of the sample and its substrate specificity The substrates tested, their method of preparation and the enzymes which they are used to detect are listed in table 2 10 10 1 100µl of purified PAPII was incubated with 400µl of each substrate, at a concentration of 0 1mM, for 1 hour at 37°C 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 incubating the substrate and the enzyme separately for the hour, followed by the addition of acetic acid to the incubation mixture prior to the addition of substrate Liberated MCA was determined as outlined in section 2 2 1 1

Table 2.10.10.1 *Preparation of Quenched Fluorimetric Substrates*

Substrate	Preparation[a]	Used to Detect	Reference
Z-Gly-Pro-MCA	4% v/v DMSO	PE	Yoshimoto *et al* (1979)
Gly-Pro-MCA	Buffer	DAPIV	Kato *et al* (1978)
Pro-MCA	Buffer	Proline Aminopeptidase	Sanderink *et al* (1988)
Ala-MCA	Buffer	Alanine Aminopeptidase	Mantle *et al* (1983)
pGlu-MCA	2% v/v DMSO	PAPI	Fujiwara and Tsuru (1978)

[a] Substrates were prepared in 20mM potassium phosphate buffer at pH 7 5 or by initial dissolution in 100% DMSO with subsequent addition of 20mM potassium phosphate buffer at pH 7 5

2.10.10.2 The Activity of PAPII Against pGlu-MCA, pGlu-ßNA and pGlu-pNA

The PAPI substrates pGlu-MCA, pGlu-ßNA and pGlu-pNA were prepared at a concentration of 0 2mM in 2% v/v DMSO by initial dissolution in 100% DMSO and subsequent addition of 20mM potassium phosphate buffer at pH 7 5 60µl of 1 3M 2-pyrrolidone and 240µl of purified PAPII, containing 1% w/v BSA, was premcubated to 37°C in a glass cuvette within the heated cuvette block of a Perkin-Elmer LS-50 fluorescence spectrophotometer or a Unicam uv/vis Spectrometer UV2, as appropriate The reaction was initiated by the addition of 1 2ml of the substrate being tested The contents of the cuvette were mixed and the liberation of MCA, ßNA or pNA was measured continuously over a period of 1 hour Appropriate negative controls were prepared for each substrate by including 20mM potassium phosphate buffer at pH 7 5 in place of PAPII MCA was measured fluorimetrically using excitation and emission wavelengths of 370nm and 440nm respectively with excitation and emission slit widths of 10nm and 20nm respectively ßNA was measured fulorimetrically using excitation and emission wavelengths of 340nm and 410nm respectively with excitation and emission slit widths of 10nm and 20nm respecuvely pNA was determined by monitoring the absorbance at 410nm

2 10.10.3 Investigation of the Substrate Specificity of PAPII by HPLC

The use of HPLC facilitated the separation and identification of cleavage products resulting from the incubation of PAPII with a range of pyroglutamyl substrates The HPLC system used consisted of a Beckman System Gold™ Programmable Solvent Module 126 (dual pump), Diode Array Detector Module 168 and Autosampler 507 A Beckman Ultrasphere™ (Octyl) C-8 reverse phase column (4 6mm x 20cm) fitted with a Beckman Ultrasphere™ C-18 reverse phase guard-column (4 6mm x 4 5cm) was used with the above system The buffers used were, buffer A 0 2% v/v TFA in deionised water and buffer B 0 2% v/v TFA + 70% v/v acetonitrile in deionised water

Purified PAPII containing 1% w/v BSA was dialysed as outlined in section 2 10 9 150µl of dialysed enzyme was incubated for 18hours at 37°C with an equal volume of the peptide under investigation, prepared at a specific concentration in 20mM potassium phosphate buffer at pH 7 5 The reaction was terminated by the addition of 12 5µl of 5% v/v TFA An appropriate negative control for each reaction was prepared by incubating substrate and enzyme separately over the 18 hours and adding the TFA immediately after the addition of substrate

20µl of sample was injected onto the column which was pre-equilibrated for 5 minutes in buffer A Elution was achieved using buffer A for 5 minutes followed by a linear gradient of 0-80% buffer B A flowrate of 1ml/min was employed throughout the procedure The absorbance of the eluant was measured at 206nm in all cases with the exception of MCA containing peptides which were monitored at 342nm

Standard preparations of His-Pro-OH, Cyclo His-Pro and pyroglutamic acid, were run under the above conditions in order to facilitate the identification of the metabolites resulting from the incubation of peptides with PAPII

2 10.10.4 Monitoring the Cleavage of Substrates Over Time by PAPII, Using HPLC

Pyroglutamic acid, TRH, pGlu-His-Gly-OH and pGlu-His-Pro-Gly-NH_2 were prepared at a concentration of 0 5mM in 20mM potassium phosphate buffer, pH 7 5 + 0 2% v/v TFA Acid TRH was prepared at a concentration of 0 35mM in the same buffer Dilutions of each were prepared and run on the HPLC under the conditions described in section 2 10 10 3 Standard curves of peak area versus peptide concentration were constructed

1ml of dialysed PAPII (see section 2 10 10 3) was incubated with an equal volume of TRH, acid TRH, pGlu-His-Gly-OH and pGlu-His-Pro-Gly-NH_2, which were prepared at double the concentrations outlined above, in 20mM potassium phosphate buffer at pH 7 5 Over a 20 hour period 300µl aliquots of each were removed and the reaction was terminated by the addition of 5% v/v TFA Samples were run on the HPLC as outlined in section 2 10 10 3 The concentrations of peptide and pyroglutamic acid in the incubation mixtures were determined using the standard curves A plot of concentration versus incubation time was constructed for each peptide

2.10.11 Kinetic Studies

2.10.11.1 K_m Determination for pGlu-His-Pro-MCA

The Michaelis Menten constant (K_m) was determined for the reaction of PAPII with the fluorimetric substrate pGlu-His-Pro-MCA A range of concentrations of the substrate from 1-200µM, was prepared in 20mM potassium phosphate buffer at pH 7 5 Purified PAPII containing 1% v/v BSA, dialysed into 20mM potassium phosphate buffer at pH 7 5 as outlined in section 2 10 9, was assayed using this substrate concentration range as outlined in section 2 9 7 The K_m and V_{max} values for the substrate were determined using Michaelis-Menten, Eadie-Hofstee, Lineweaver-Burk, Hanes-Woolf and Direct Linear Plot analysis

2.10.11.2 K_m Determination for pGlu-MCA

The Michaelis Menten constant (K_m) was determined for the reaction of PAPII with the fluorimetric substrate pGlu-MCA Post Sephacryl S-200 PAPII (see section 2 7 6), diluted 1/5 with 5% v/v BSA in 20mM potassium phosphate buffer at pH 7 5 (final concentration 1% w/v BSA), was dialysed into 20mM potassium phosphate buffer at pH 7 5 as outlined in section 2 10 9

750µM pGlu-MCA was prepared in 20mM potassium phosphate buffer at pH 7 5 containing 4% v/v DMSO by initial dissolution in 100% DMSO and subsequent addition of buffer which was warmed to 37°C A range of concentrations of the substrate from 50-750µM, was prepared in the same buffer The activity of PAPII across this substrate range was tested 250µl of dialysed enzyme was added to 1ml of substrate Following incubation for 1 hour at 37°C the reaction was terminated by the addition of 250µl of 6M acetic acid All assays were performed in triplicate and suitable negative controls were prepared by incubating substrate and sample separately for the hour and adding acetic acid to the incubation mixture prior to the addition of substrate Liberated MCA was determined as outlined in section 2 2 1 1 The K_m and V_{max} values for the substrate were determined using a range of kinetic models

2.10.11.3 K_i Determination for Pyroglutamyl Peptides

The inhibitor dissociation constant (K_i) for a range of pyroglutamyl peptides with PAPII was determined using the substrate pGlu-His-Pro-MCA A range of concentrations of pGlu-His-Pro-MCA, from 2-400µM, was prepared in 20mM potassium phosphate buffer at pH 7 5 1ml of each substrate dilution was mixed with an equal volume of the pyroglutamyl peptide being tested (prepared to a specific concentration in 20mM potassium phosphate buffer pH 7 5) Purified PAPII containing 1% v/v BSA, dialysed into 20mM potassium phosphate buffer at pH 7 5 as outlined in section 2 10 9, was assayed using this substrate concentration range as outlined in section 2 9 7 The K_i values for the peptides and the type of inhibition, were determined using the kinetic models described in section 2 10 11 2

3. Results

3.1 7-Amino-4-Methyl-Coumarin Standard curves

MCA standard curves were prepared as outlined in section 2 3 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 serum or crude homogenate of bovine brain are used as the enzyme sample, is demonstrated in figures 3 1 1 and 3 1 2 It should be noted that the MCA concentration expressed on the X-Axis of these figures represents the concentration present in the 400µl or 1ml 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 versus BSA concentration are presented Figure 3 2 1 shows the standard curve for the Biuret assay, while figures 3 2 2 and 3 2 3 show standard curves obtained for the standard and enhanced protocols of the BCA assay, respectively

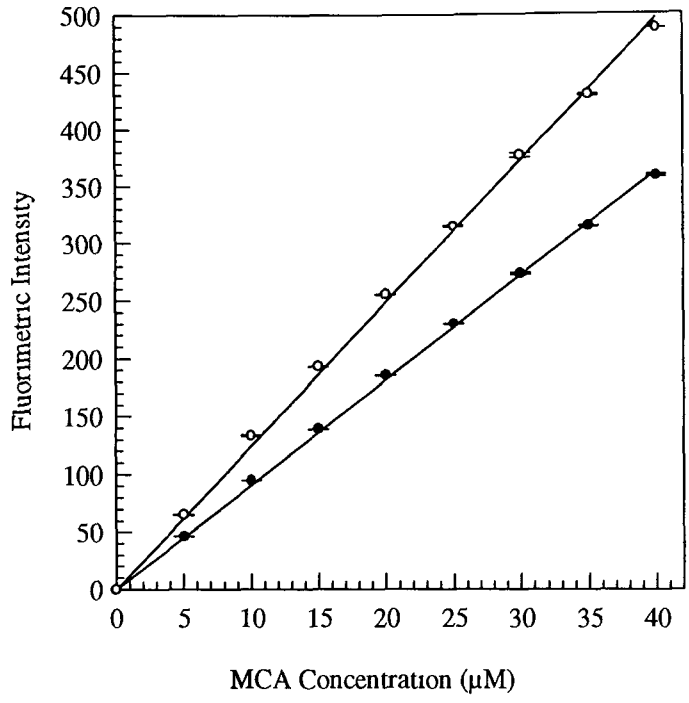


Figure 3.1.1 MCA Standard Curves. Plots of fluorimetric intensity versus MCA concentration 100μl of buffer (o—o) or 100μl of serum(•—•) was combined with 400μl of a standard concentration of MCA and 1ml of 1 5M acetic acid before being analysed fluorimetrically as outlined in section 2 3 The emission slit width was set at 2 5nm Error bars represent the SEM of triplicate readings

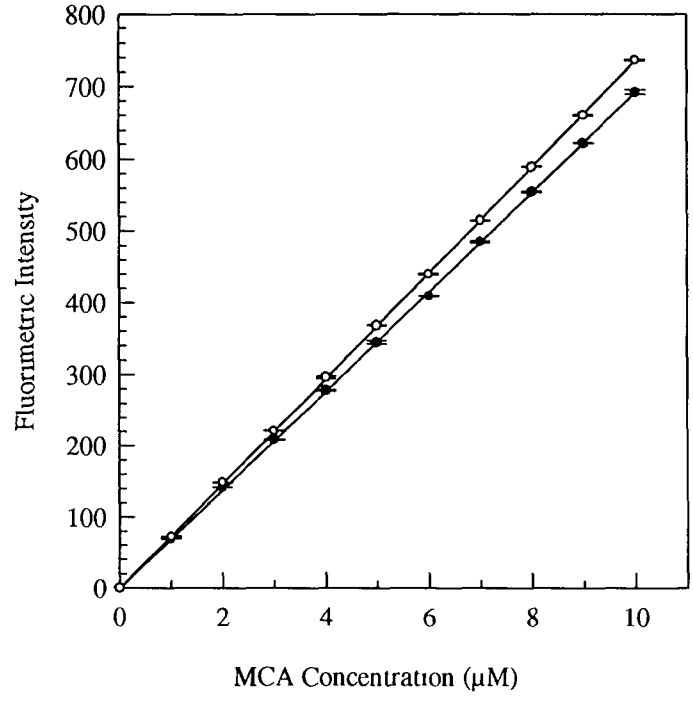


Figure 3.1.2 MCA Standard Curves Plots of fluorimetric intensity versus MCA concentration 100μl of buffer (o—o) or 100μl of crude homogenate of bovine brain (•—•) was combined with 400μl of a standard concentration of MCA, 20μl of 100μM Z-Pro-Prolinal, 20μl of 1 3M 2-pyrrolidone and 960μl of 1 5M acetic acid Samples were analysed fluorimetrically as outlined in section 2 3 The emission slit width was set at 5 0nm Error bars represent the SEM of triplicate readings

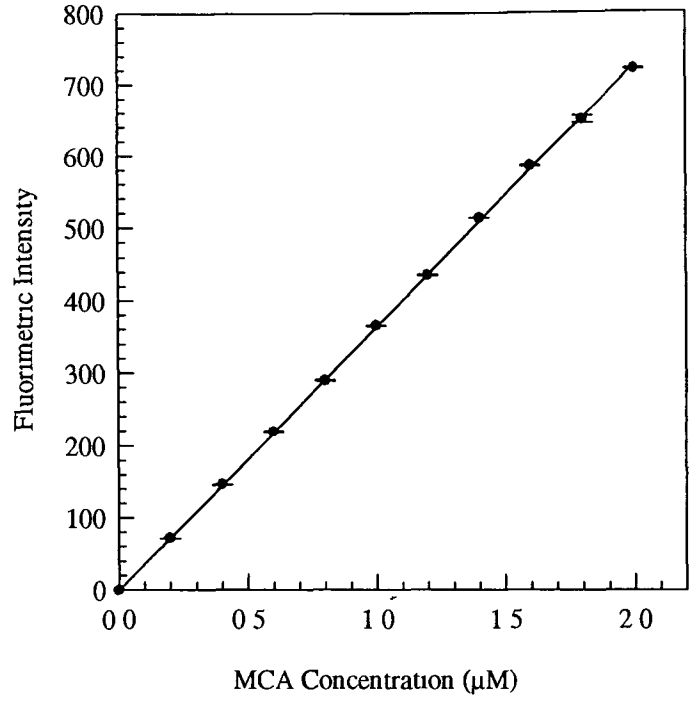


Figure 3.1.3 MCA Standard Curve. Plot of fluorimetric intensity versus MCA concentration 100μl of buffer was combined with 400μl of a standard concentration of MCA and 1ml of 1 5M acetic acid before being analysed fluorimetrically as outlined in section 2 3 The emission slit width was set at 10nm Error bars represent the SEM of triplicate readings

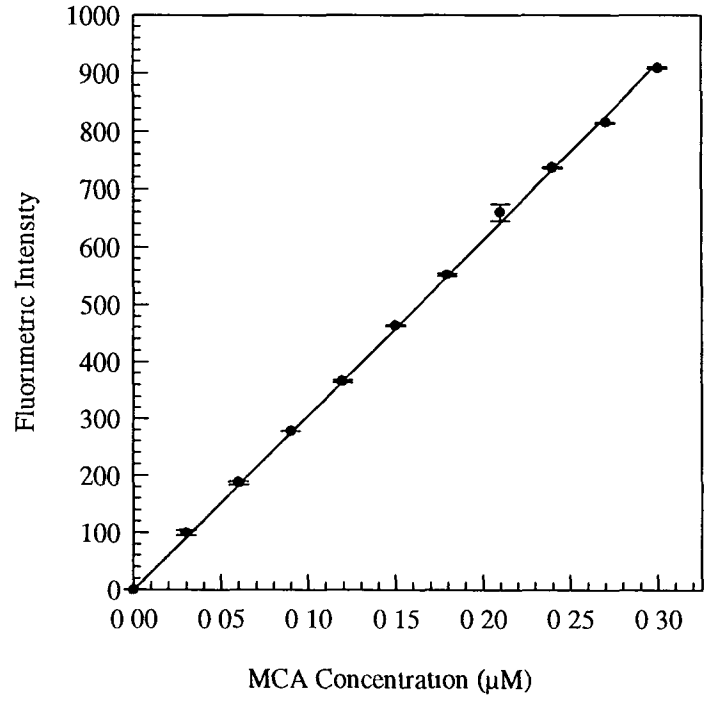


Figure 3.1.4 MCA Standard Curve. Plot of fluorimetric intensity versus MCA concentration 250μl of 1% w/v BSA in 20mM potassium phosphate buffer was combined with 1ml of a standard concentration of MCA and 250μl of 6M acetic acid before being analysed fluorimetrically as outlined in section 2 3 The emission slit width was set at 20nm Error bars represent the SEM of triplicate readings This standard curve relates to the conditions used for the determination of the K_m value for the reaction of PAPII with the substrate pGlu-MCA, as outlined in section 2 10 11 2

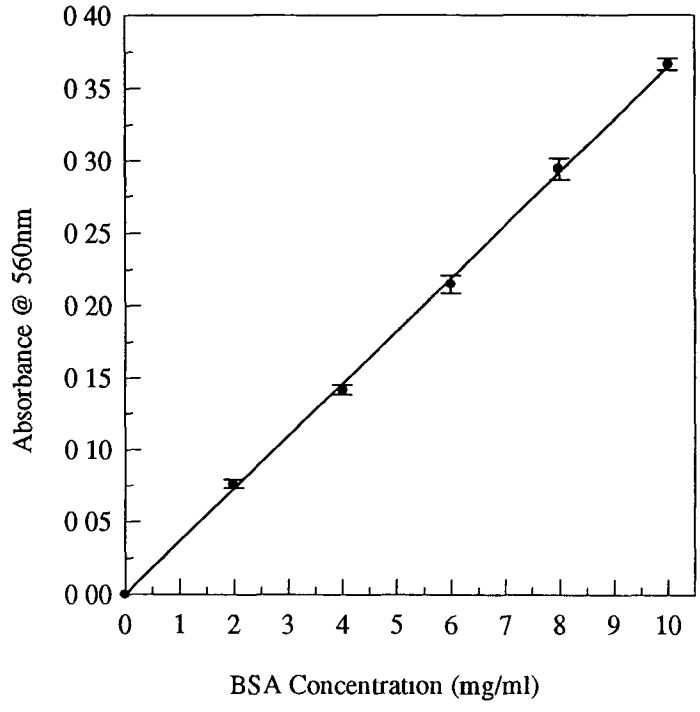


Figure 3.2.1 BSA Standard Curve. Plot of absorbance at 560nm versus BSA concentration obtained using the Biuret assay as outlined in section 2 4 1 Error bars represent the SEM of triplicate readings

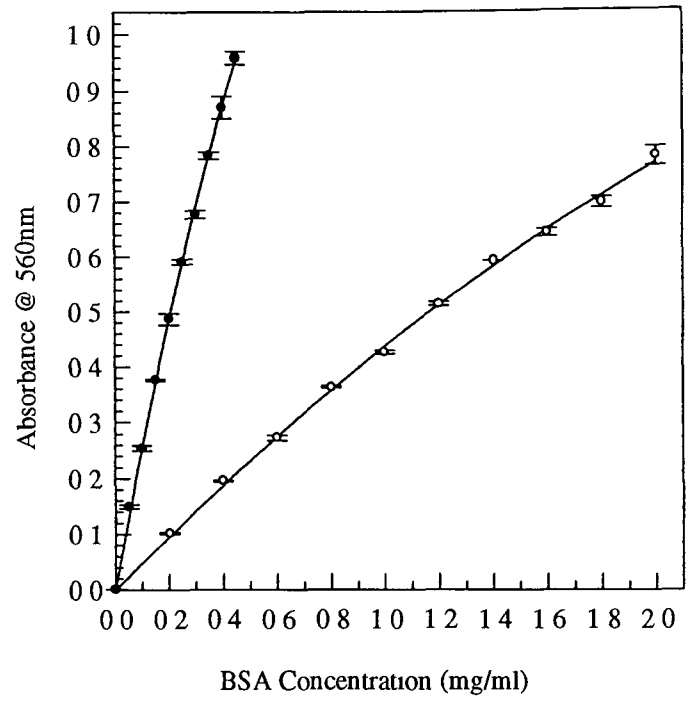


Figure 3.2.2 BSA Standard Curves. Plot of absorbance at 560nm versus BSA concentration obtained using the BCA assay as outlined in section 2 4 2 10µl of a standard concentration of BSA was incubated for 30 minutes at 37°C with 200µl of BCA working reagent (o—o) as outlined in section 2 4 2 2 or 20µl of a standard concentration of BSA was incubated for 30 minutes at 60°C with 200µl of BCA working reagent (•—•) as outlined in section 2 4 2 2 Error bars represent the SEM of triplicate readings

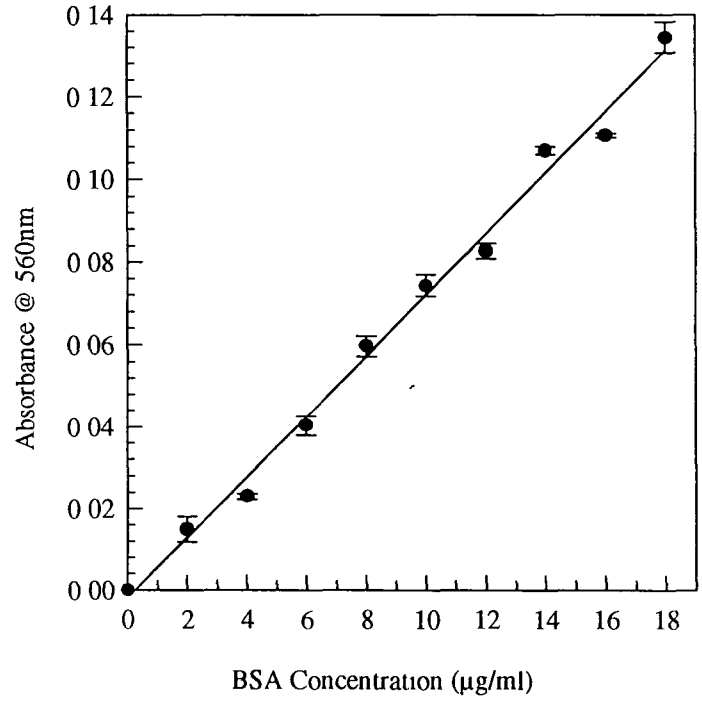


Figure 3.2.3 BSA Standard Curve. Plot of absorbance at 560nm versus BSA concentration obtained using the BCA assay as outlined in section 2 4 2 50µl of a standard concentration of BSA was incubated for 30 minutes at 60°C with 200µl of BCA working reagent as outlined in section 2 4 2 2 Error bars represent the SEM of triplicate readings

3.3 Partial Purification of DAPIV From Bovine Serum

3.3.1 Bovine Serum Production

Serum was produced as outlined in section 2 5 1 From 4 5L of whole blood collected, 1L of unclotted blood was obtained after 24 hours at 4°C Following centrifugation, 800ml of serum was obtained

3.3.2 SP Sepharose Fast Flow Cation-Exchange Chromatography

Following dialysis of 20ml of bovine serum, the post-dialysis precipitate was removed by centrifugation as outlined in section 2 5 2, resulting in 21 5ml of clear supernatant at pH 5 3 The supernatant was applied to an SP Sepharose column as outlined in section 2 5 2 Bound protein was eluted isocratically with 500mM NaCl Fractions 3-11 were combined to form the post-SP Sepharose pool (35ml) The elution profile of this column is illustrated in figure 3 3 1

3.3.3 Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography

Dry ammonium sulphate was added to the post-SP Sepharose pool as outlined in section 2 5 3 Following the addition of the ammonium sulphate a precipitate formed, however, the precipitate dissolved as the pH approached 7 0 with the addition of 1M NaOH The sample (50ml adjusted volume) was applied to a phenyl sepharose column as outlined in section 2 5 3 Bound protein was eluted isocratically with 5mM potassium phosphate buffer, pH 7 5 Fractions 11-20 were combined to form the post-phenyl Sepharose pool (45ml) The elution profile of this column is illustrated in figure 3 3 2

3.3.4 Concentration of Partially Purified DAPIV

As outlined in section 2 5 4, the post-Phenyl Sepharose pool was dialysed into 100mM potassium phosphate buffer, pH 7 5 and concentrated to a final volume of 26ml by reverse osmosis with PEG 6,000 Following further dialysis into 100mM potassium phosphate buffer at pH 7 5, glycerol was added to a final concentration of 10% v/v

Table 3 3 1 summarises the purification scheme

3.3.5 Investigation of Enzymatic Purity of Partially Purified DAPIV

The enzymatic purity of the partially purified DAPIV was investigated as outlined in section 2 5 5 No PAPII, PAPI or PE activity was detected in the sample No aminopeptidase activity was detected using the substrates Pro-MCA and Ala-MCA

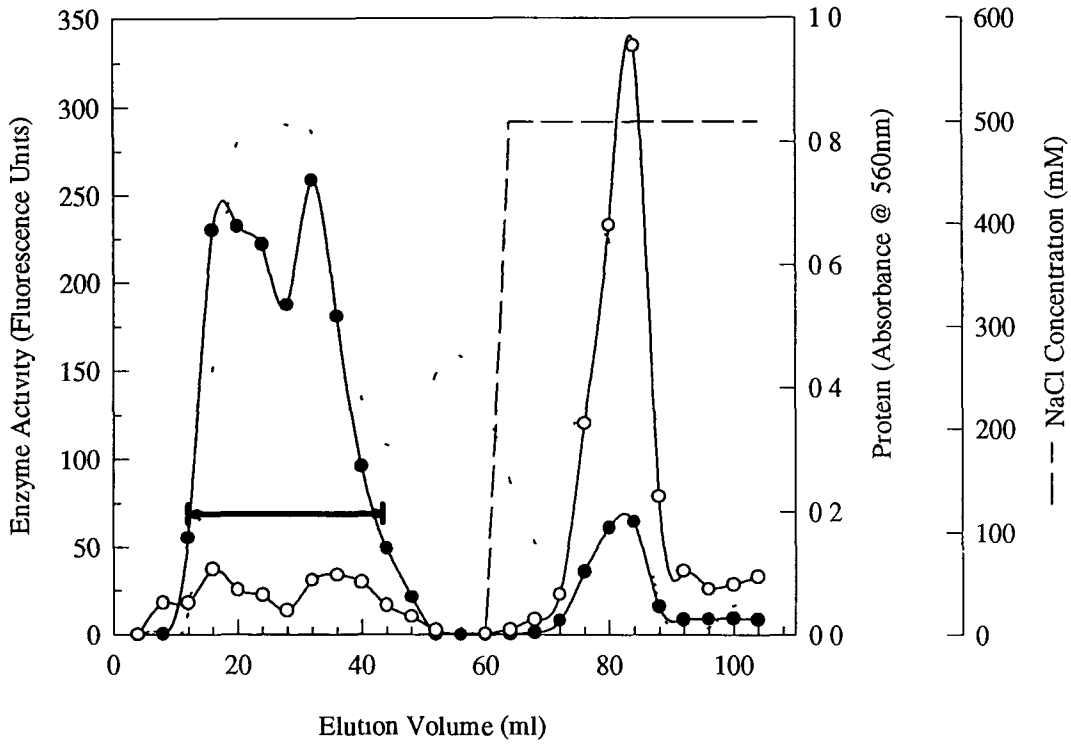


Figure 3.3.1 Elution Profile of DAPIV Activity and Z-Gly-Pro-MCA Degrading Activity from SP Sepharose Cation-Exchange Chromatography. 21 5ml of dialysed serum was applied to a 20ml SP Sepharose column as outlined in section 2 5 2 The column was eluted isocratically with 500mM NaCl (– – –) Fractions were assayed for protein ( ) using the Biuret assay as outlined in section 2 4 1, Z-Gly-Pro-MCA degrading activity (o—o) as outlined in section 2 2 3 and DAPIV activity (•—•) as outlined in section 2 2 4 Fractions 3-11 were combined to form the post-SP Sepharose pool (35ml) as indicated (↔)

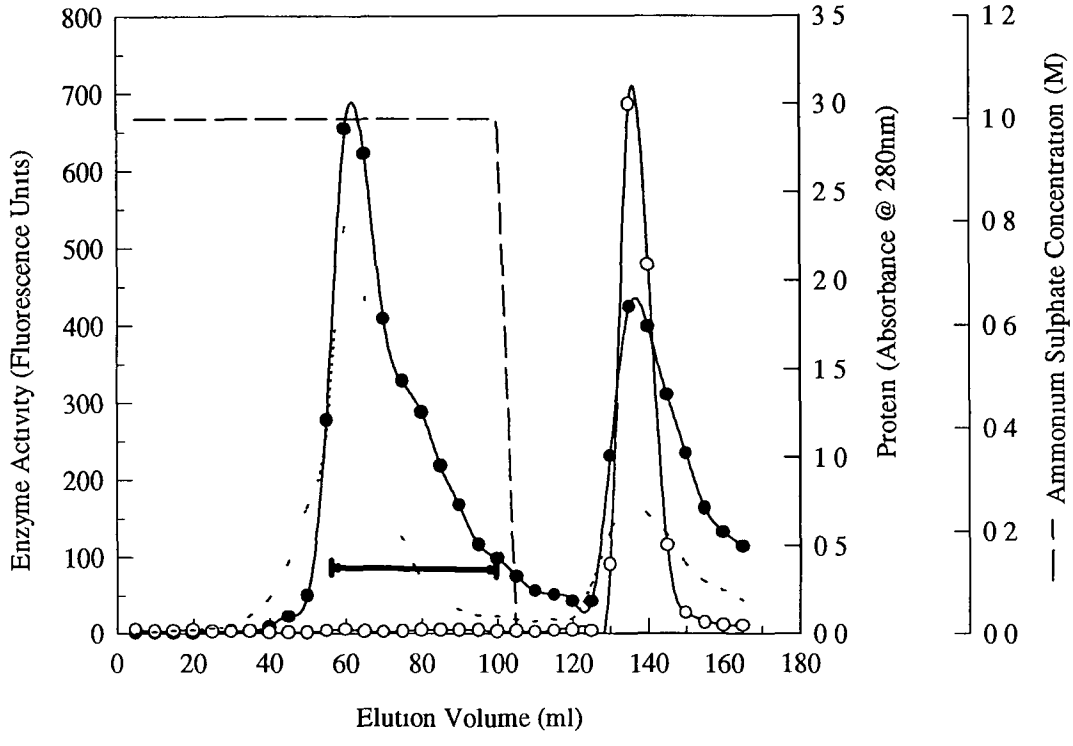


Figure 3.3.2 Elution Profile of DAPIV Activity and Z-Gly-Pro-MCA Degrading Activity from Phenyl Sepharose Hydrophobic Interaction Chromatography. Post-SP Sepharose DAPIV, adjusted to 1M ammonium sulphate in a total volume of 50ml, was applied to a 20ml Phenyl Sepharose column as outlined in section 2 5 3 The column was eluted isocratically with 5mM potassium phosphate buffer at pH 7 5 containing no ammonium sulphate (– – –) Fractions were assayed for protein ( ) using the absorbance at 280nm as outlined in section 2 4 3, Z-Gly-Pro-MCA degrading activity (o—o) as outlined in section 2 2 3 and DAPIV activity (•—•) as outlined in section 2 2 4 Fractions 11-20 were combined to form the post-phenyl Sepharose pool (45ml) as indicated (↔)

Table 3.3.1 *Partial purification of DAPIV from bovine serum*

Sample	Total Activity (Units*)	Total Protein (mg)	Specific Activity (Units/mg)	Purification Factor (Fold)	Yield (%)
Serum	116,389	1,616	72 02	1	100
Post-Phenyl Sepharose (Concentrated)	73,253	205 7	356 12	4 9	62 9

* 1 Unit of enzyme activity is defined as that which releases 1 picomole of MCA per minute from 80μM Gly-Pro-MCA at 37°C

3.4 Determination of Optimal Conditions for the Solubilisation of PAPII From the Particulate Fraction of Bovine Brain

The optimal conditions for the solubilisation of PAPII were determined as outlined in section 2 6 Washed membranes from bovine brain were obtained as outlined in section 2 6 1 and were treated with a range of detergents and proteases as outlined in section 2 6 2 Phospholipase C and the detergent saponin had no solubilisation effect and are therefore not discussed further The PAPII activity in the supernatants represents the amount of enzyme released from the membranes while the activity in the pre-centrifugation homogenates represents the total activity remaining after treatment with the detergent / protease It should be noted that the non-quantitative, coupled enzyme assay described in section 2 2 1 1 was used to determine PAPII activity In addition, MCA standard curves were not prepared for each sample to account for the inner-filter effect

The effectiveness of the detergents Triton X-100, CHAPS and sodium deoxycholate in releasing PAPII is illustrated in figure 3 4 1 Triton X-100 and CHAPS solubilised 100% and 90% of enzyme activity respectively at a concentration of 1% No loss of total enzyme activity in the homogenates was observed with these detergents Sodium deoxycholate solubilised 73% of PAPII activity at a concentration of 0 4% w/v At sodium deoxycholate concentrations above 0 2% w/v a decrease in the total enzymatic activity in the homogenate was observed

The effectiveness of the proteases trypsin, chymotrypsin and papain at 25°C and on ice are illustrated in figures 3 4 2 and 3 4 3 respectively Trypsin was found to be the most effective protease for the solubilisation of PAPII Following incubation for 1 hour at 25°C and on ice, maximal amounts of the enzyme was released by 5µg/ml and 10µg/ml trypsin respectively and by 5µg/ml and 50µg/ml papain respectively 100µg/ml chymotrypsin released maximal amounts of the enzyme when incubated for 1 hour at both temperatures At protease concentrations above 1µg/ml a loss of total enzyme activity in the homogenates was observed

The PAPII activity, protein concentration and PAPII specific activity of supernatants, produced by the solubilisation regimes described in section 2 6 3, are presented in figure 3 4 4 Supernatants were obtained following 1 hour incubation at 25°C with 5µg/ml papain and trypsin, and following 1 hour incubation on ice with 4% (v/v or w/v) Triton X-100, CHAPS and sodium deoxycholate In each case the figures are presented relative to the highest value obtained It should be noted that the quantitative, non-enzymatic cyclisation assay described in section 2 2 1 2 was used for the determination of PAPII activity In addition, MCA standard curves were prepared as outlined in section 2 3 to account for the inner-filter effect of the samples

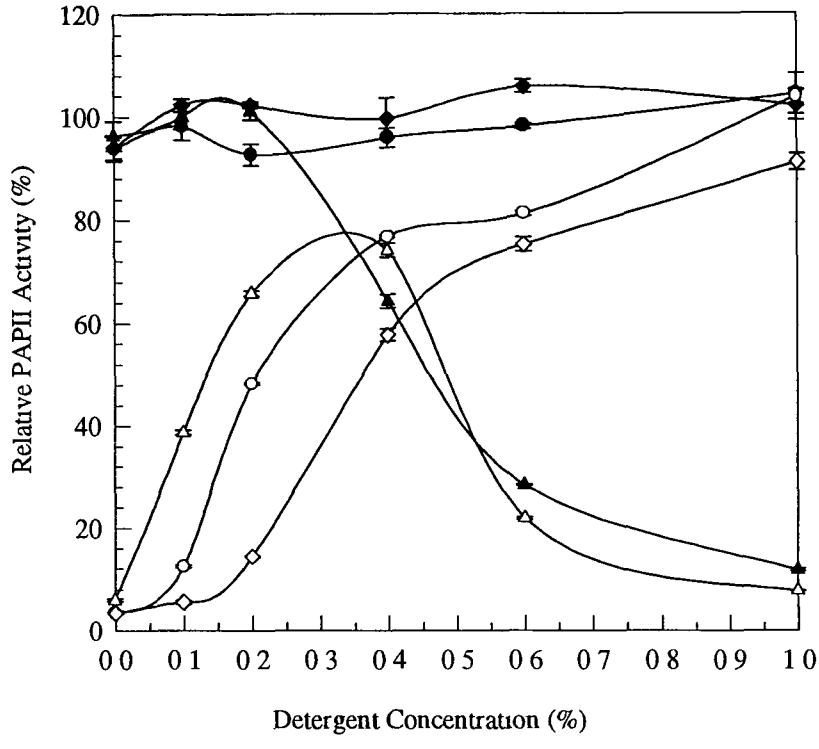


Figure 3.4.1 Solubilisation of PAPII by Detergents. 2 7ml of resuspended washed membranes from bovine brain was incubated on ice for 1 hour with 300µl of 20mM potassium phosphate buffer at pH 7 5 containing a range of concentrations of CHAPS (◇—◇), sodium deoxycholate (Δ—Δ) and Triton X-100 (○—○) as outlined in section 2 6 2 PAPII activity was determined as outlined in section 2 2 1 1 The pre-centrifugation homogenates (**filled symbols**) were assayed to determine the total activity remaining after treatment and the supernatants (**hollow symbols**) were assayed to determine the amount of activity solubilised by the detergent Error bars represent the SEM of triplicate readings

Figure 3.4.2

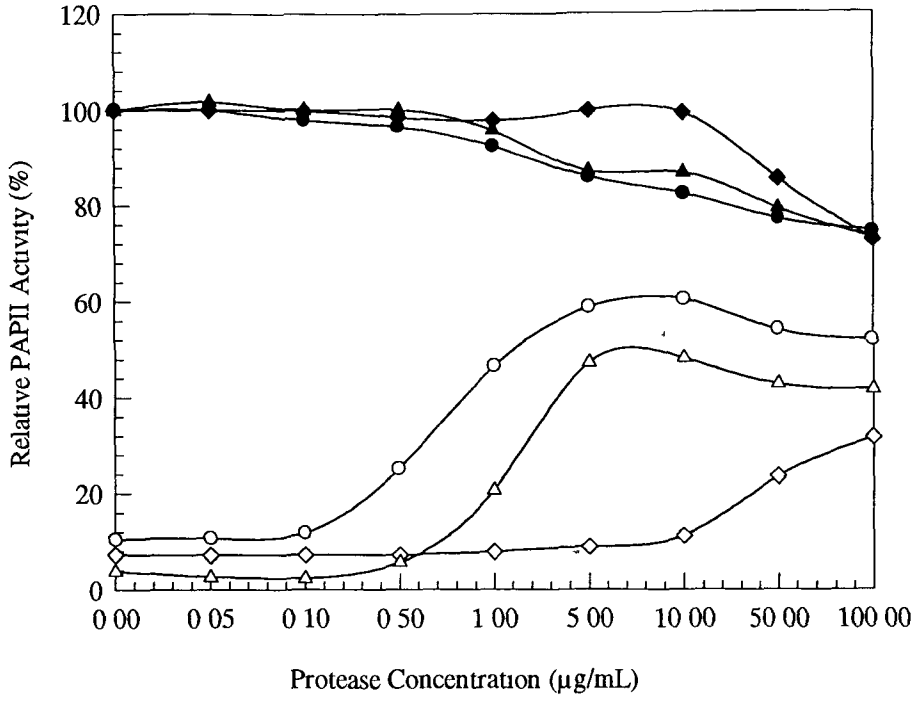
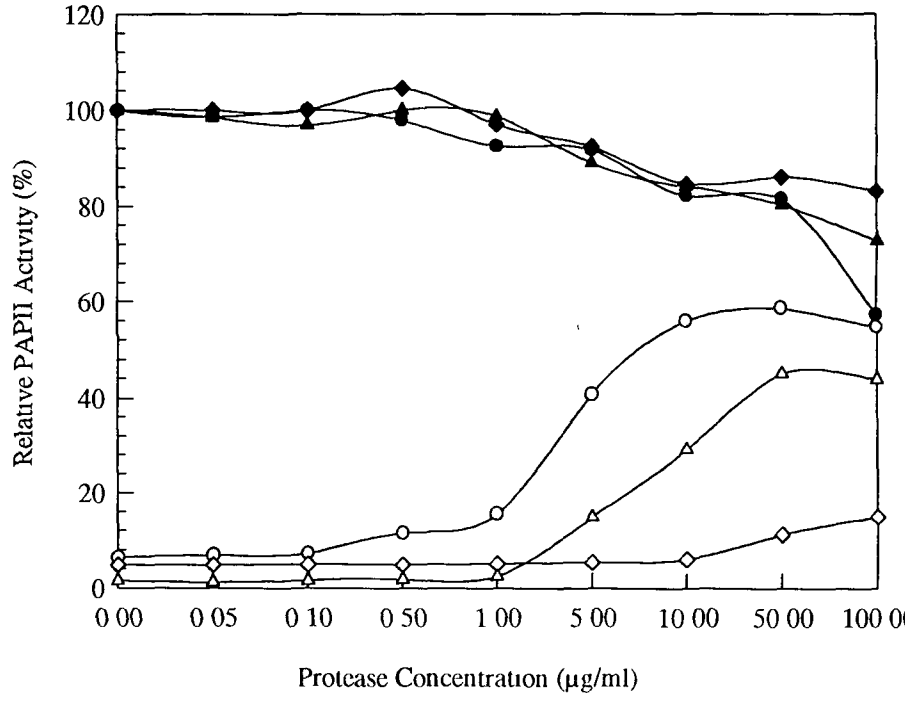


Figure 3.4.3



Figures 3 4.2 and 3 4.3 Solubilisation of PAPII by Proteases 2 7ml of resuspended washed membranes from bovine brain was incubated for 1 hour at 25°C (figure 3 4 2) or on ice (figure 3 4 3) with 300µl of 20mM potassium phosphate buffer at pH 7 5 containing a range of concentrations of chymotrypsin (◇—◇), papain (Δ—Δ) and trypsin (○—○) as outlined in section 2 6 2 PAPII activity was determined as outlined in section 2 2 1 1 The pre-centrifugation homogenates (**filled symbols**) were assayed to determine the total activity remaining after treatment and the supernatants (**hollow symbols**) were assayed to determine the amount of activity solubilised by the protease

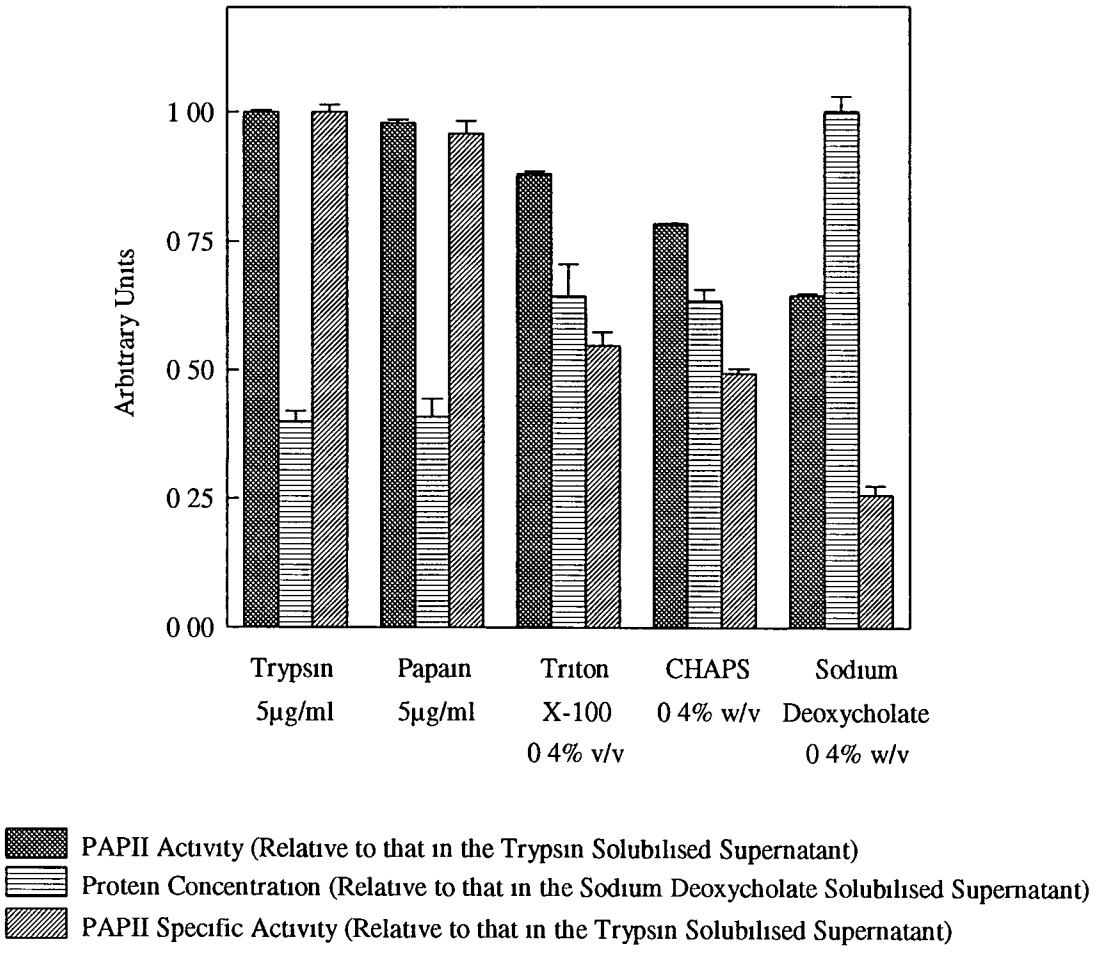


Figure 3.4.4 Comparison of PAPII Activity, Protein Concentration and PAPII Specific activity in Supernatants Obtained by Various Solubilisation Regimes Washed membranes from bovine brain were solubilised as outlined in section 2 6 3 The resulting supernatants were assayed for PAPII activity as outlined in section 2 2 1 2 and for protein as outlined in section 2 4 2 1 Error bars represent the SEM of triplicate readings

3.4.1 Stability of PAPII Released by Various Solubilisation Regimes

The stability of PAPII solubilised under the conditions outlined in section 2 6 3, was investigated as outlined in section 2 6 4 Plots of PAPII activity (relative to the initial activity), versus time stored at 4°C and -20°C are presented in figures 3 4 5 and 3 4 6 respectively The enzyme activity in the Triton X-100 and sodium deoxycholate solubilised supernatants, stored at 4°C, was not monitored beyond 3 and 6 days respectively, due to microbial contamination

When stored at 4°C, no loss of PAPII activity was observed in the trypsin solubilised supernatant over a 14 day period 90% and 33% enzyme activity remained after 14 days in the papain and CHAPS solubilised supernatants respectively 53% and 52% enzyme activity remained after 24 hours in the Triton X-100 and sodium deoxycholate solubilised supernatants respectively

When stored at -20°C for 14 days, the PAPII activity in all of the supernatants was abolished with the exception of the trypsin solubilised supernatant which retained 77% of the initial enzyme activity After 24 hours at -20°C, 5% and 35% of the initial PAPII activity remained in the supernatants solubilised with Triton X-100 and sodium deoxycholate respectively After 3 days at -20°C, less than 50% of the initial PAPII activity remained in the supernatants solubilised with papain and CHAPS

Figure 3.4.5

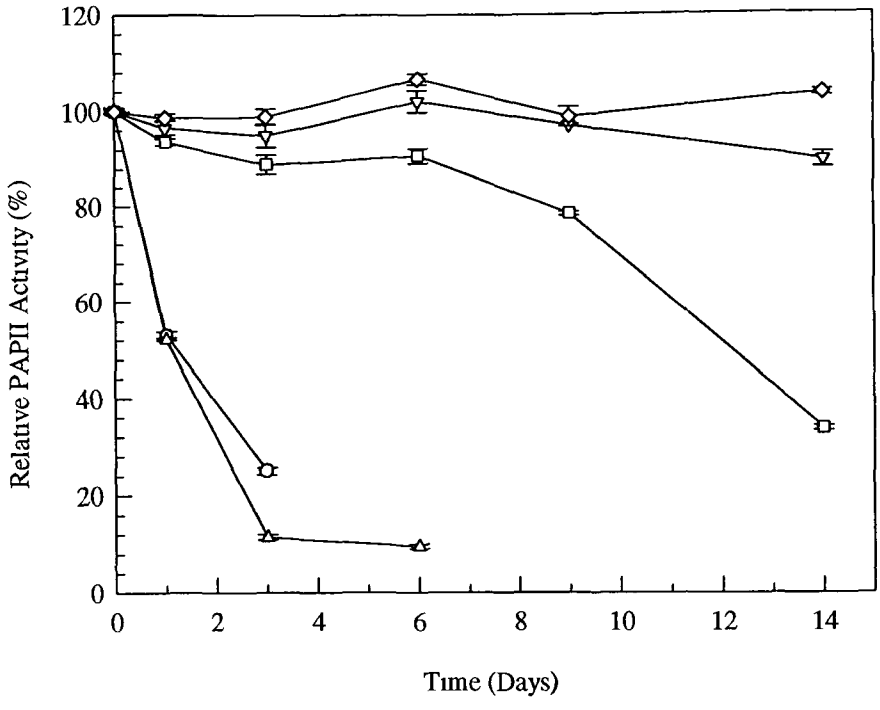
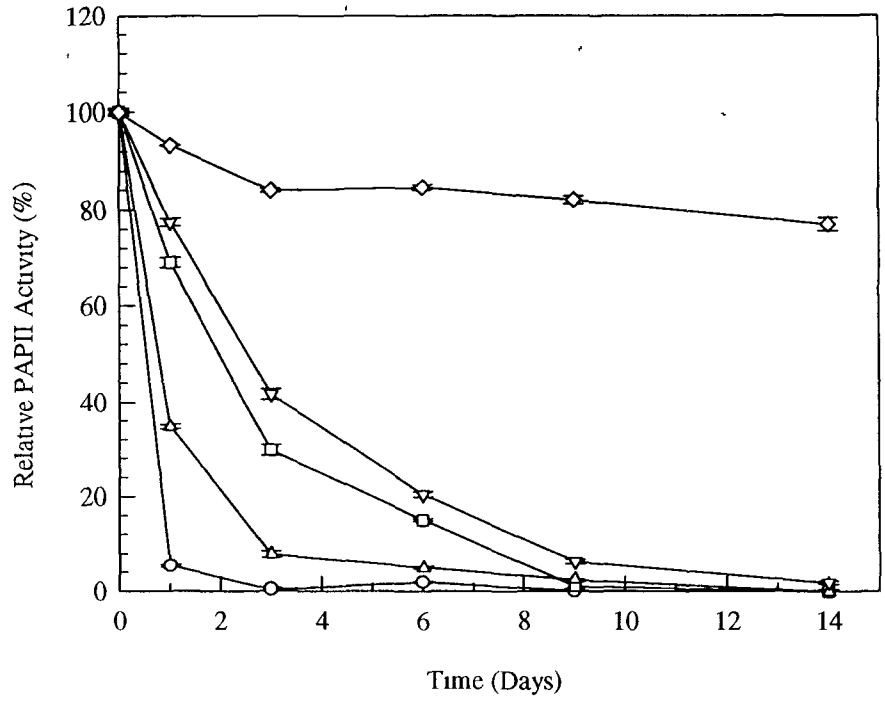


Figure 3.4.6



Figures 3.4.5 and 3 4.6 The Stability of PAPII Released by Various Solubilisation Regimes. Figures 3 4 5 and 3 4 6 illustrate the stability of PAPII stored at 4°C and -20°C respectively Washed membranes from bovine brain were solubilised, as outlined in section 2 6 3, by incubation with 5µg/ml trypsin (◇—◇), 5µg/ml papain (▽—▽), 0 4%w/v CHAPS (□—□), 0 4%v/v Triton X-100 (○—○) or 0 4%w/v sodium deoxycholate (△—△) Aliquots of the resulting supernatants were stored at 4°C and -20°C as described in section 2 6 4 Over a fourteen day period, aliquots were withdrawn and assayed for PAPII activity as outlined in section 2 2 1 2 Error bars represent the SEM of triplicate readings

3.5 Purification of PAPII From the Membrane Fraction of Bovine Brain

3.5.1 Solubilisation of PAPII from the Washed Membranes of Bovine Brain

Washed membranes from 25g of bovine brain were prepared in a final volume of 95ml as outlined in section 2 6 1 72% of the total PAPII activity was recovered in this fraction Following solubilisation with 5µg/ml trypsin for 1 hour at 25°C, as outlined in section 2 7 1, 79ml of clarified trypsin solubilised supernatant was obtained 500µl of this sample was retained for enzyme activity and protein determinations This solubilisation procedure released 92% of PAPII activity and 14 6% of protein from the washed membranes into the supernatant

3.5.2 Q-Sepharose High Performance Anion-Exchange Chromatography

78 5ml of clarified trypsin solubilised supernatant was applied to a Q-Sepharose column as outlined in section 2 7 2 Bound protein was eluted isocratically with 100mM NaCl in 20mM potassium phosphate buffer at pH 7 5 Fractions 33-37 were combined to form the post-Q-Sepharose pool (22ml) 500µl of this pool was retained for enzyme activity and protein determinations 95% of PAPII activity was recovered from this step The elution profile of this column is illustrated in figure 3 5 1

3.5.3 Chelating-Sepharose Fast Flow Immobilised Metal Ion Chromatography

21 5ml of 650mM NaCl in 20mM potassium phosphate buffer at pH 7 5 was added to the post-Q-Sepharose pool resulting in a final volume of 23 65ml, containing 150mM NaCl in 20mM potassium phosphate buffer at pH 7 5 This material was applied to two chelating-Sepharose columns connected in series, as outlined in section 2 7 3 Bound protein was eluted using a linear gradient from 0-45mM imidazole in 20mM potassium phosphate buffer at pH 7 5 Fractions 26-29 were combined to form the post-chelating-Sepharose pool (15 5ml) 500µl of this pool was retained for enzyme activity and protein determinations 86% of PAPII activity was recovered from this step The elution profile of this column is illustrated in figure 3 5 2

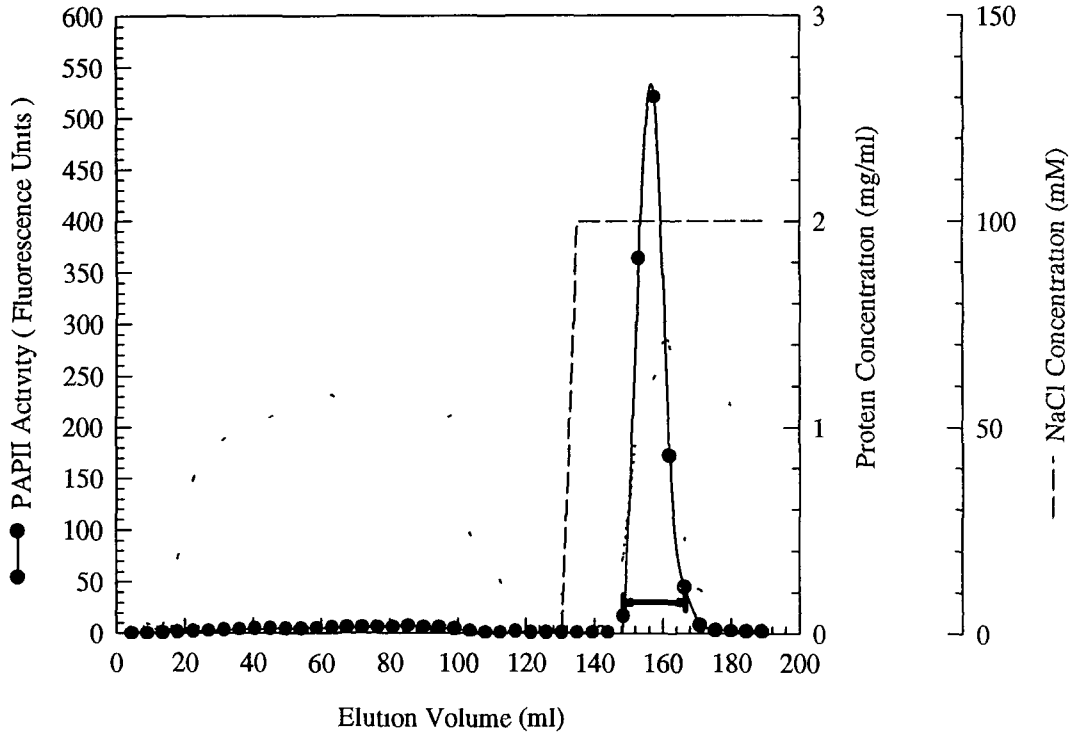


Figure 3.5.1 Elution Profile of PAPII Activity from Q-Sepharose High Performance Anion-Exchange Chromatography. 78 5ml of clarified trypsin solubilised supernatant was applied to a 10ml Q-Sepharose column as outlined in section 2 7 2 The column was eluted isocratically with 100mM NaCl (– – –) Fractions were assayed for PAPII activity (•—•) as outlined in section 2 2 1 2 and for protein ( ) using the standard BCA assay as outlined in section 2 4 2 1 Fractions 33-37 were combined to form the post-Q-Sepharose pool (22ml) as indicated (↔)

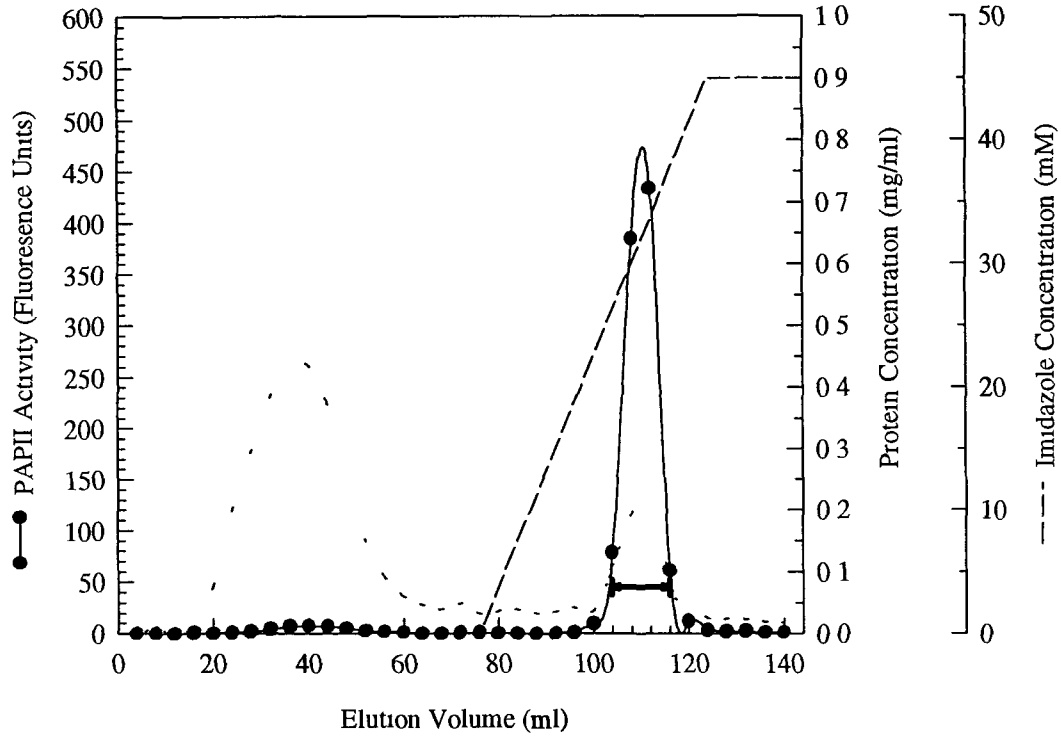


Figure 3.5.2 Elution Profile of PAPII Activity from Chelating-Sepharose Fast Flow Immobilised Metal Ion Chromatography. 2 15ml of 650mM NaCl in 20mM potassium phosphate buffer at pH 7 5 was added to the post-Q-Sepharose pool resulting in a final volume of 23 65ml, containing 150mM NaCl in 20mM potassium phosphate buffer at pH 7 5 This material was applied to two chelating Sepharose columns connected in series (19ml total volume) as outlined in section 2 7 3 The column was eluted using a linear gradient from 0-45mM imidazole (– – –) Fractions were assayed for PAPII activity (•—•) as outlined in section 2 2 1 2 and for protein ( ) using 20µl of sample in the enhanced BCA assay as outlined in section 2 4 2 2 Fractions 26-29 were combined to form the post-chelating-Sepharose pool (15 5ml) as indicated (↔)

3.5.4 Calcium Phosphate-Cellulose Chromatography

Following dialysis into 10mM potassium phosphate buffer at pH 7 5, the post-chelating Sepharose pool (18ml) was applied to a calcium phosphate-cellulose column as outlined in section 2 7 5 Bound protein was eluted by the application of a linear gradient from 10-100mM potassium phosphate buffer at pH 7 5 Fractions 15-20 were combined to form the post-calcium phosphate-cellulose pool (24 5ml) 500µl of this pool was retained for enzyme activity and protein determinations 61% of PAPII activity was recovered from this step The elution profile of this column is illustrated in figure 3 5 3

3.5.5 Sephacryl S200 HR Gel-Filtration Chromatography

Following concentration by ultrafiltration and the addition of glycerol to a final concentration of 15% v/v, the post-calcium phosphate-cellulose pool (2ml) was applied to a Sephacryl S200 column as outlined in section 2 7 6 Fractions 12-17 were combined to form the post-Sephacryl S200 pool (22ml) 66% of PAPII activity was recovered from this step The elution profile of this column is illustrated in figure 3 5 4

Table 3 5 1 summarises the purification scheme

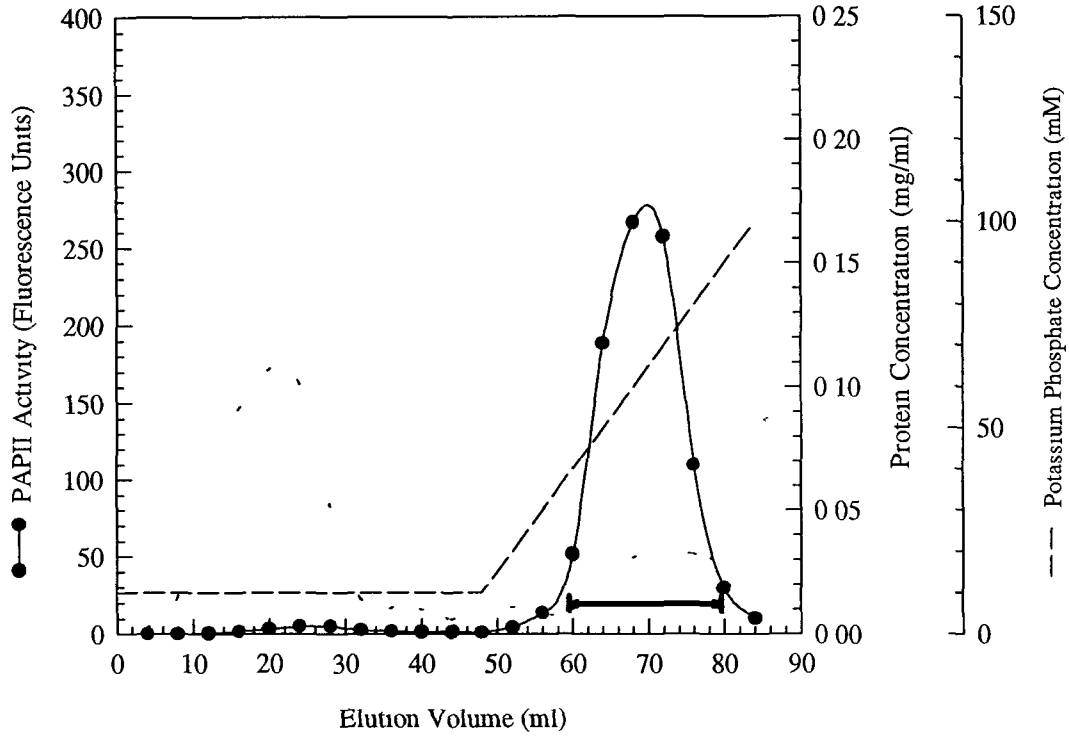


Figure 3.5.3 Elution Profile of PAPII Activity from Calcium Phosphate-Cellulose Chromatography. Following dialysis into 10mM potassium phosphate buffer at pH 7 5 the post-chelating Sepharose pool (18ml) was applied to a 9ml calcium phosphate-cellulose column as outlined in section 2 7 5 The column was eluted using a linear gradient from 10-100mM potassium phosphate buffer at pH 7 5 (– – –) Fractions were assayed for PAPII activity (•—•) as outlined in section 2 2 1 2 and for protein ( ) using 20µl of sample in the enhanced BCA assay as outlined in section 2 4 2 2 Fractions 15-20 were combined to form the post-calcium phosphate-cellulose pool (24 5ml) as indicated (↔)

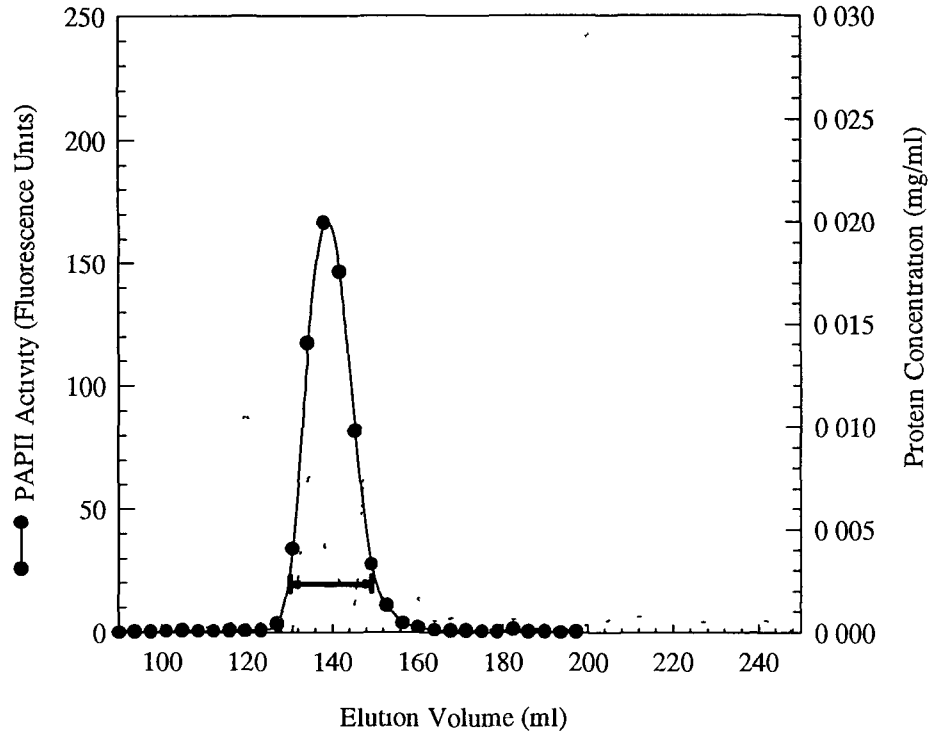


Figure 3 5.4 Elution Profile of PAPII Activity from Sephacryl S200 HR Gel-Filtration Chromatography. Following concentration by ultrafiltration and the addition of glycerol to a final concentration of 15% v/v, the post-calcium phosphate-cellulose pool (2ml) was applied to a 230ml Sephacryl S200 column as outlined in section 2 7 6 The column was eluted using 150mM KCl in 20mM potassium phosphate buffer at pH 7 5 Fractions were assayed for PAPII activity (•—•) as outlined in section 2 2 1 2 and for protein ( ) using 50µl of sample in the enhanced BCA assay as outlined in section 2 4 2 2 Fractions 12-17 were combined to form the post-Sephacryl S200 pool (22ml) as indicated (↔)

Table 3.5.1 *Purification of PAPII from Bovine Brain*

Purification Step	Volume (ml)	Total protein (mg)	Total activity (Units*)	Specific Activity (Units*/mg)	Purification Factor (Fold)	Yield (%)	For each individual step	
							Purification Factor (Fold)	Yield (%)
Homogenate	125	1937 5	13290	6 86	1	100	—	—
Particulate fraction	125	1450 0	12213	8 42	1 23	91 9	1 23	91 9
Salt washed pellet	125	1087 5	11504	10 58	1 54	86 6	1 26	94 2
Water washed pellet	95	1026 0	10442	10 18	1 48	78 6	0 96	90 8
Trypsin solubilised supernatant	79	150 1	9601 6	63 97	9 3	72 2	6 28	92 0
Post-Q-Sepharose chromatography	22	20 46	9156 9	447 55	65 2	68 9	7 00	95 4
Post-chelating-Sepharose chromatography	15 5	3 10	7901 5	2548 86	371 6	59 5	5 70	86 3
Post-calcium phosphate-cellulose chromatography	24 5	0 49	4847 4	9892 57	1442	36 5	3 88	61 3
Post-Sephacryl S200 chromatography	22 0	0 154	3218 1	20896 75	3047	24 2	2 11	66 4

* 1 Unit of enzyme activity is defined as that which releases 1 picomole of MCA per minute from 74μM pGlu-His-Pro-MCA at 37°C PAPII activity was determined using the quantitative, non-enzymatic cyclisation assay, described in section 2 9 7 with a 2 hour incubation period at 80°C

3.6 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out on post-Sephacryl S200 PAPII as outlined in section 2.8. A photograph of the gel obtained following electrophoresis and silver staining (as outlined in section 2.8.3) is shown in figure 3.6.1. Figure 3.6.2 shows a calibration curve of Log_{10} of molecular weight versus R_f, constructed as outlined in section 2.8.3 Seven distinct bands, varying in molecular weight from 55-141 kDa, were visualised in the lanes containing the concentrated post-Sephacryl S200 PAPII. Two bands with molecular weights of 55 kDa and 70 kDa were visualised in the lane containing the un-concentrated sample.

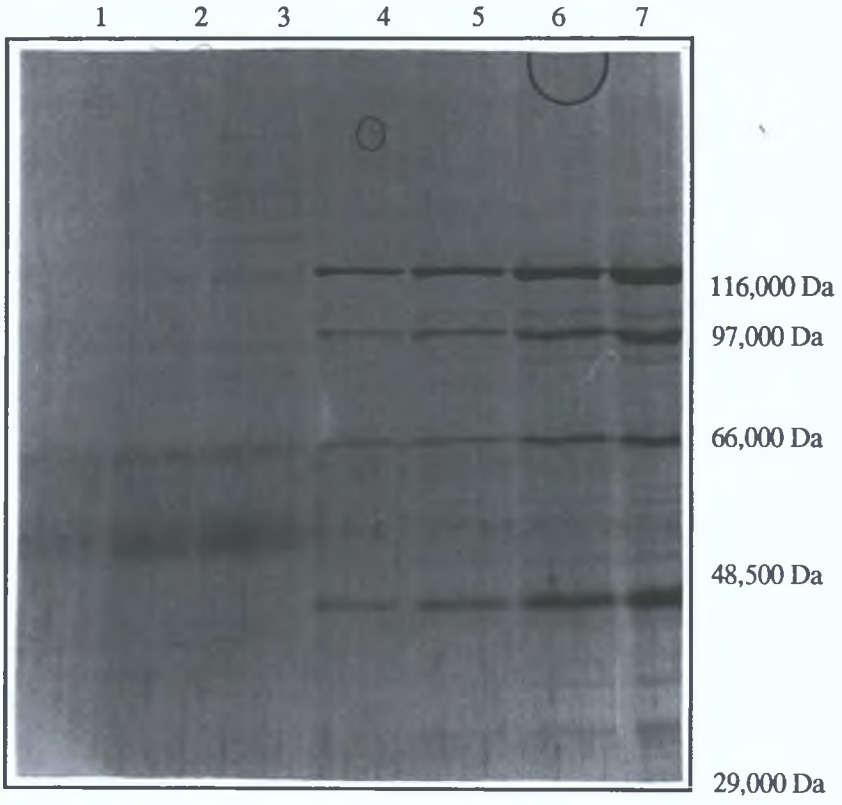


Figure 3.6.1 SDS PAGE Silver Stained Gel. A 16cm x 16cm x 1mm, 7.5% resolving gel, overlayed with a 3.5% stacking gel, was electrophoresed as outlined in section 2.8.2. Silver staining was carried out as outlined in section 2.8.3. Samples which were prepared as outlined in section 2.8.1, were loaded in the following order:

Lane 1. Post-Sephacryl S200 PAPII (un-concentrated)
Lane 2. Post-Sephacryl S200 PAPII (concentrated 5-fold)
Lane 3. Post-Sephacryl S200 PAPII (concentrated 10-fold)
Lane 4. Molecular weight markers (diluted 1/10)
Lane 5. Molecular weight markers (diluted 1/5)
Lane 6. Molecular weight markers (diluted 1/2)
Lane 7. Molecular weight markers (neat)

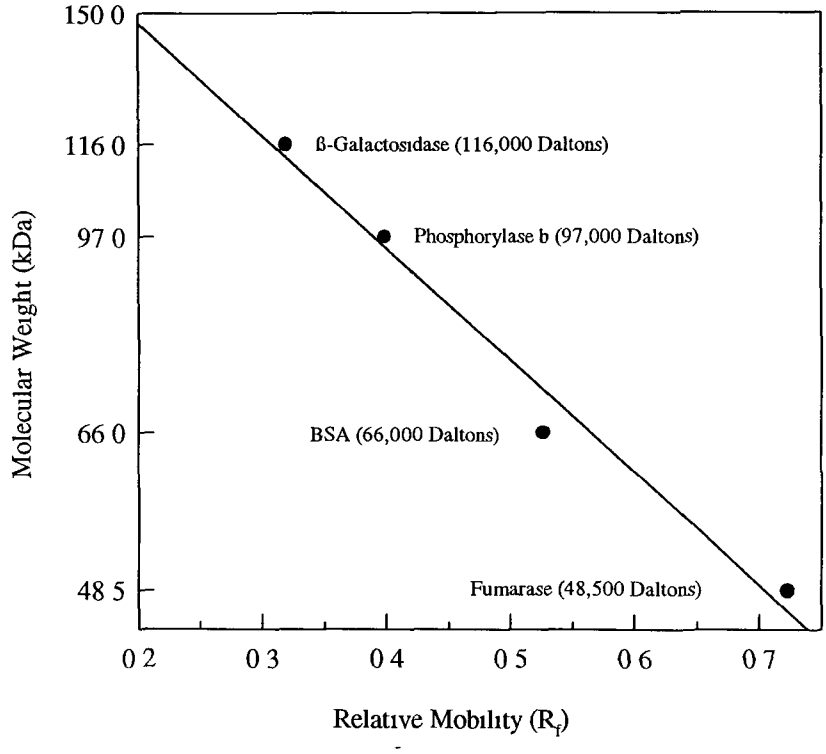


Figure 3.6.2 SDS PAGE Calibration Curve. Plot of Log_{10} of molecular weight versus relative mobility (R_f) A 16cm x 16cm x 1mm, 7 5% resolving gel, overlayed with a 3 5% stacking gel, was electrophoresed as outlined in section 2 8 2 Silver staining was carried out as outlined in section 2 8 3 Molecular weight standards were prepared as outlined in section 2 8 1

3.7 Development of pGlu-His-Pro-MCA Based PAPII Assays

3.7.1 Inhibition of PE by Z-Pro-Prolinal and fmoc-Pro-Pro-Nitrile

The inhibition of PE by Z-Pro-Prolinal and fmoc-Pro-Pro-Nitrile was investigated as outlined in section 2 9 1 The inhibition of PE in bovine brain cytosol by Z-Pro-Prolinal is illustrated in figure 3 7 1 1 100% inhibition was achieved by the addition of 20µl of 100µM Z-Pro-Prolinal (final concentration 3 85µM under assay conditions) The inhibition of PE in the post-Q-Sepharose PAPII pool by fmoc-Pro-Pro-Nitrile is illustrated in figure 3 7 1 2 The addition of 20µl of 26µM fmoc-Pro-Pro-Nitrile (final concentration 1µM under assay conditions) resulted in 100% inhibition of the enzyme

3.7.2 Inhibition of PAPI by 2-Pyrrolidone

The inhibition of PAPI in bovine brain cytosol by 2-pyrrolidone, investigated as outlined in section 2 9 2, is illustrated in figure 3 7 2 Maximal inhibition (95%) was achieved by the addition of 20µl of 1 316M 2-pyrrolidone (final concentration 51mM under assay conditions)

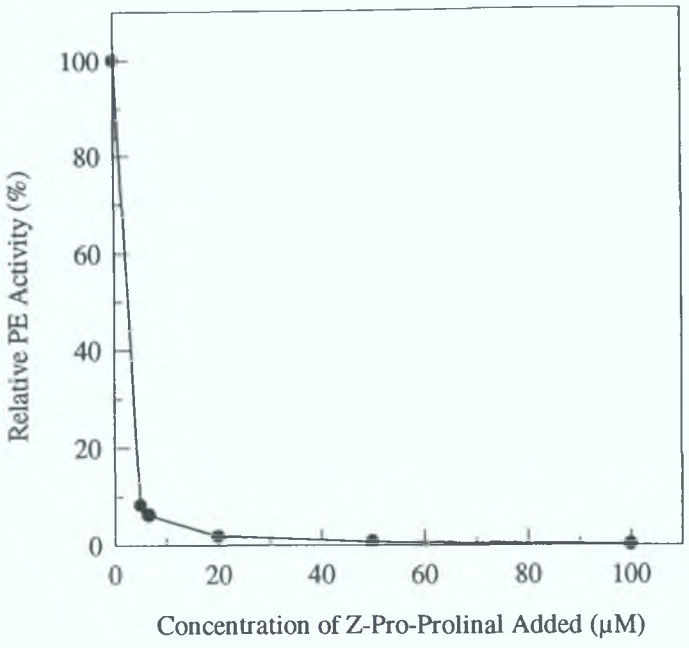


Figure 3.7.1.1 Inhibition of PE by Z-Pro-Prolinal. 20μl of Z-Pro-Prolinal, at a range of concentrations from 0-100μM, was added to 100μl of bovine brain cytosol as outlined in section 2.9.1, resulting in a range of concentrations of Z-Pro-Prolinal from 0-3.85μM under assay conditions. Samples were assayed for residual PE activity as described in section 2.2.3.

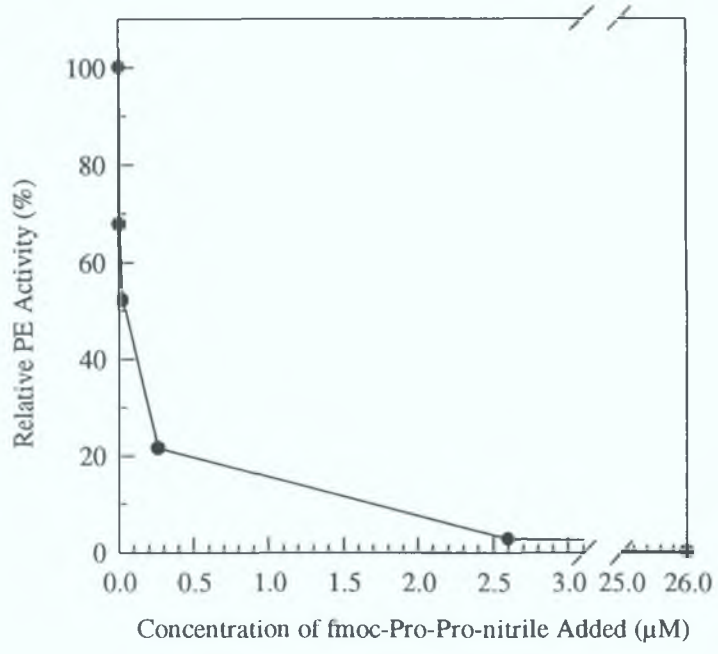


Figure 3.7.1.2 Inhibition of PE by fmoc-Pro-Pro-Nitrile. 20μl of fmoc-Pro-Pro-Nitrile, at a range of concentrations from 0-26μM, was added to 100μl of the post-Q-Sepharose PAPII pool as outlined in section 2.9.1, resulting in a range of concentrations of fmoc-Pro-Pro-Nitrile from 0-1μM under assay conditions. Samples were assayed for residual PE activity as described in section 2.2.3.

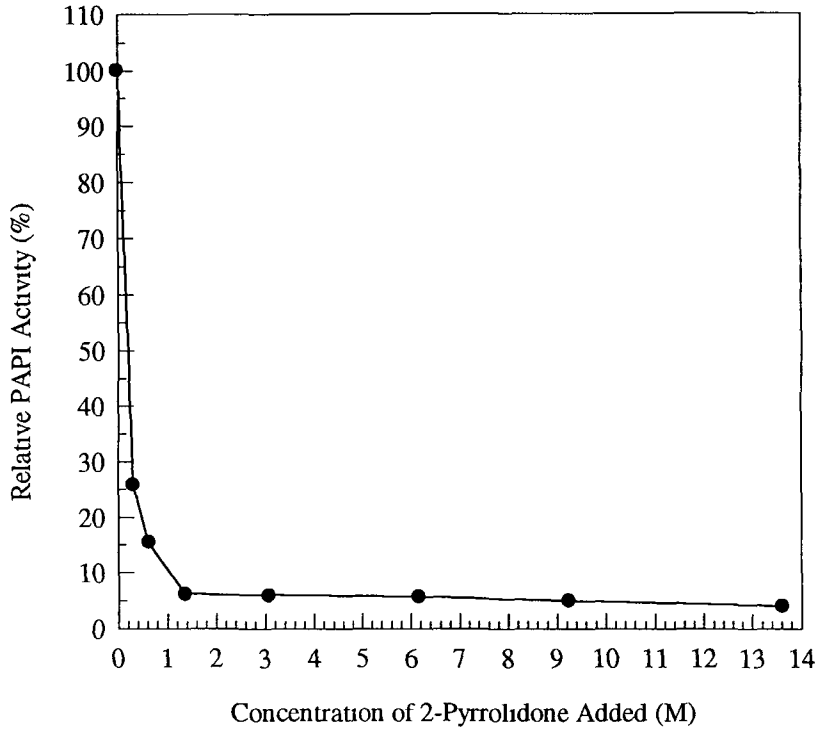


Figure 3.7.2 Inhibition of PAPI by 2-Pyrrolidone. 20µl of 2-Pyrrolidone, at a range of concentrations from 0-13 16M, was added to 100µl of bovine brain cytosol as outlined in section 2 9 2, resulting in a range of concentrations of 2-Pyrrolidone from 0-0 51M under assay conditions Samples were assayed for residual PAPI activity as described in section 2 2 2

3.7.3 The Effect of DAPIV on MCA Release

The amount of partially purified DAPIV (see section 2 5) required to catalyse the liberation of MCA from all of the His-Pro-MCA produced by the action of PAPII, on the substrate pGlu-His-Pro-MCA, was investigated as outlined in section 2 9 3 The effect of increasing volumes of DAPIV on the release of MCA following the incubation of pGlu-His-Pro-MCA with crude homogenate and purified PAPII is illustrated in figures 3 7 3 1 and 3 7 3 2 respectively Maximal amounts of MCA are released following incubation for 1 hour at 37°C with 20µl and 250µl of DAPIV in the assays containing crude homogenate and purified PAPII respectively

3.7.4 The Effect of Time at 80°C on MCA Release

The incubation time at 80°C required to catalyse the liberation of MCA from all of the His-Pro-MCA produced by the action of PAPII, on the substrate pGlu-His-Pro-MCA, was investigated as outlined in section 2 9 4 The effect of time at 80°C on the release of MCA following the incubation of pGlu-His-Pro-MCA with crude homogenate and purified PAPII is illustrated in figures 3 7 4 1 and 3 7 4 2 respectively Maximal amounts of MCA are released following incubation for 20 minutes and 120 minutes at 80°C of the assays containing crude homogenate and purified PAPII respectively

3.7.5 Linearity of PAPII Activity with respect to Time

The linearity of PAPII activity with respect to time was investigated as outlined in section 2 9 5 The release of MCA following the incubation of pGlu-His-Pro-MCA with crude homogenate and purified PAPII for varying lengths of time, followed by a 30 minute incubation at 80°C, is illustrated in figures 3 7 5 1 and 3 7 5 2 respectively In both cases, the release of MCA with respect to time is linear over 2 hours

Figure 3.7 3.1

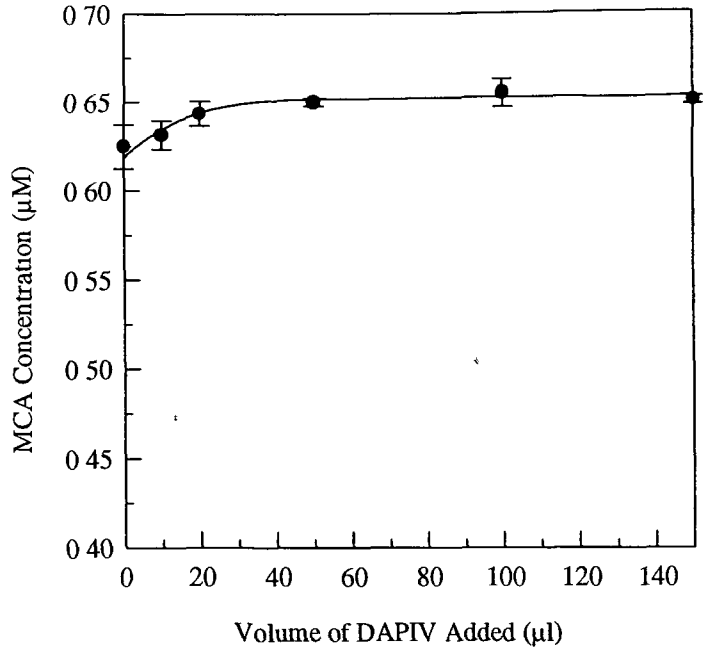
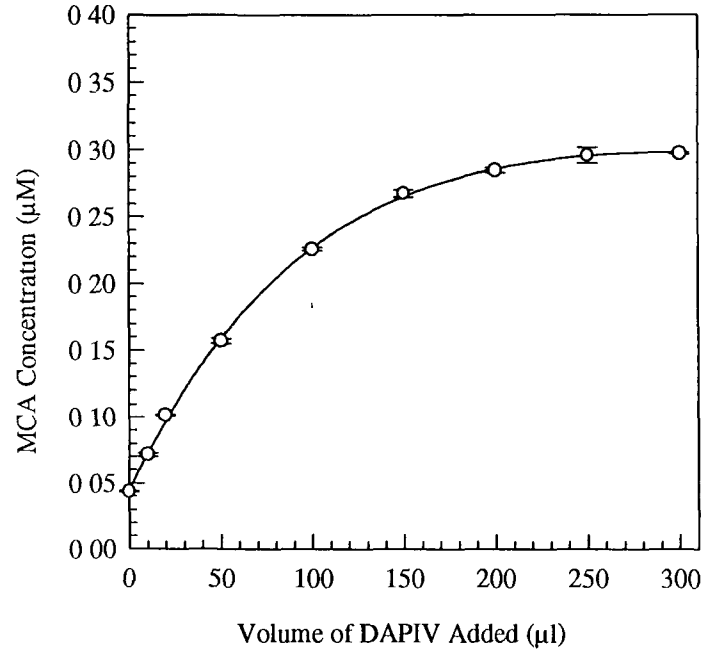


Figure 3 7.3 2



Figures 3.7.3.1 and 3.7 3.2 The Effect of DAPIV on MCA Release. Following the reaction of crude homogenate (•—•) or purified PAPII (o—o) with pGlu-His-Pro-MCA, a range of volumes of DAPIV was added as outlined in section 2 9 3 After incubation at 37°C for 1 hour liberated MCA was determined as outlined in section 2 2 1 1 Error bars represent the SEM of triplicate readings

Figure 3.7.4.1

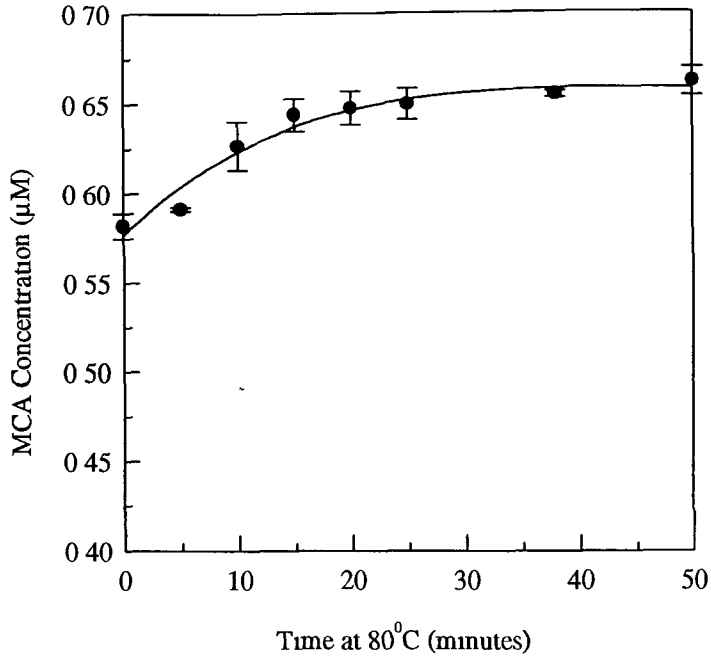
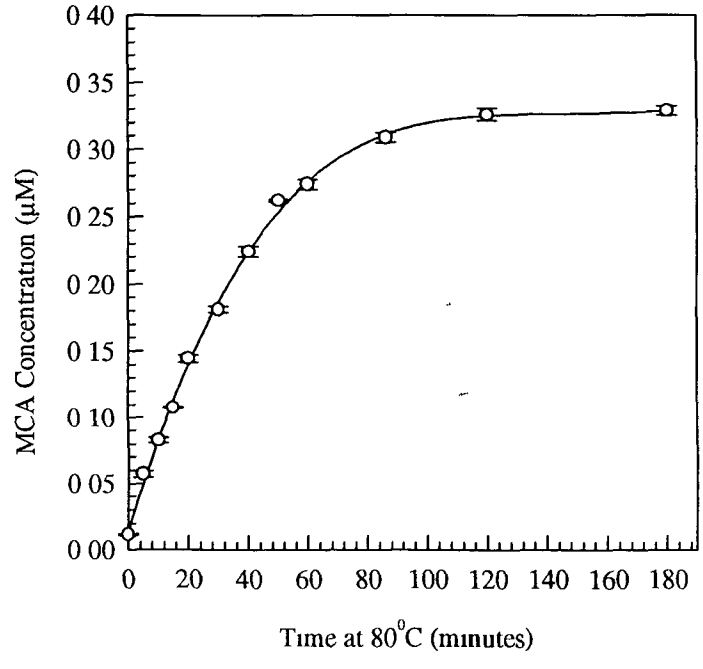


Figure 3.7.4.2



Figures 3.7.4.1 and 3.7.4.2 The Effect of Time at 80°C on MCA Release. Following the reaction of crude homogenate (•—•) or purified PAPII (o—o) with pGlu-His-Pro-MCA, samples were incubated for a range of times at 80°C as outlined in section 2 9 4 After cooling in an ice water-bath, liberated MCA was determined as outlined in section 2 2 1.1. Error bars represent the SEM of triplicate readings

Figure 3.7.5.1

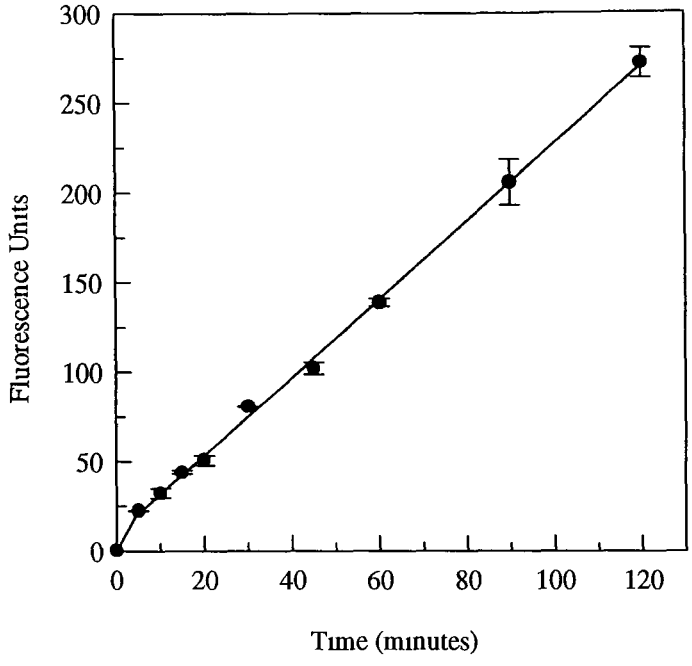
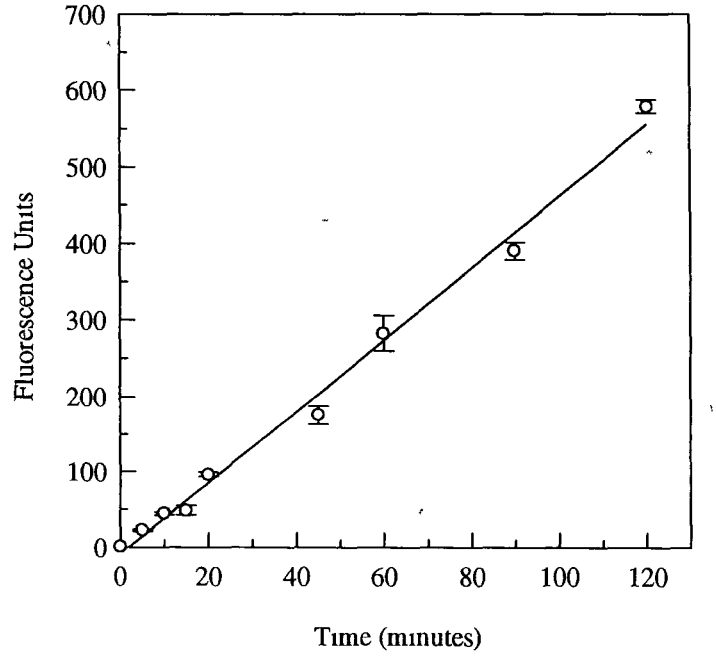


Figure 3.7.5.2



Figures 3.7.5.1 and 3.7.5.2 Linearity of PAPII Activity with respect to Time Crude homogenate (•—•) or purified PAPII (o—o) was assayed over a range of times at 37°C as outlined in section 2 9 5 Liberated MCA was determined as outlined in section 2 2 1 1 Error bars represent the SEM of triplicate readings

3.7.6 Linearity of the Non-Enzymatic Cyclisation PAPII Assay with respect to Enzyme Concentration

The linearity of the non-enzymatic cyclisation PAPII assay with respect to enzyme concentration was investigated as outlined in section 2 9 6 Figure 3 7 6 1 illustrates the relationship between PAPII activity and concentration of purified enzyme containing no BSA, using the non-enzymatic cyclisation assay described in section 2 2 1 2 The relationship between PAPII activity and concentration of purified enzyme containing 1% w/v BSA, using the non-enzymatic cyclisation assay with 30 minutes incubation at 80°C (see section 2 2 1 2) and 2 hours incubation at 80°C (see section 2 9 7) is illustrated in figures 3 7 6 2 and 3 7 6 3 respectively A non-linear relationship between enzyme activity and enzyme concentration was observed in the absence of BSA In the presence of 1% w/v BSA a linear relationship was observed when either 30 minute or 2 hour incubations at 80°C were employed

Figure 3.7.6.1

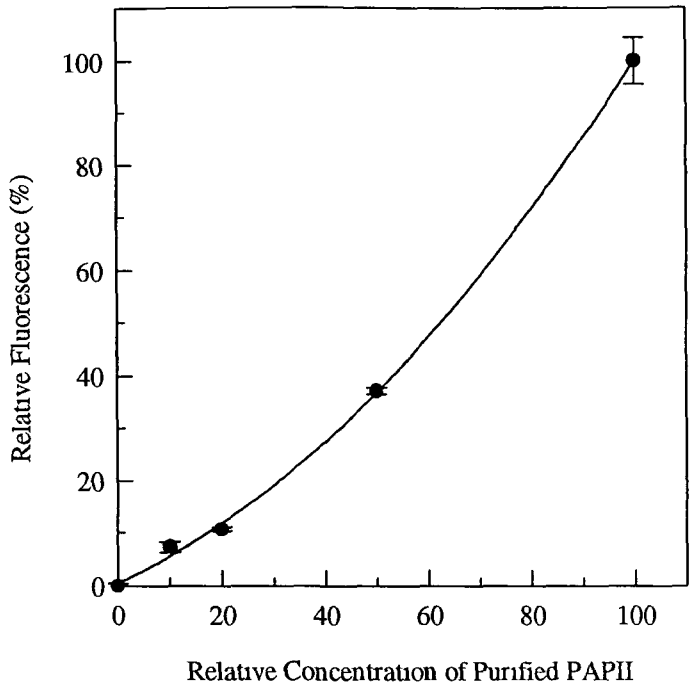
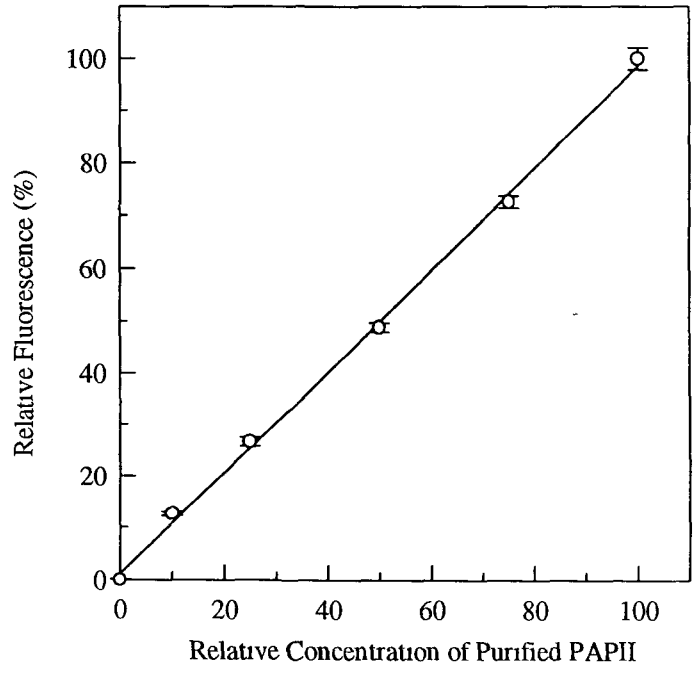


Figure 3.7 6.2



Figures 3.7.6.1 and 3.7.6.2 Linearity of the Non-Enzymatic Cyclisation PAPII Assay with respect to Enzyme Concentration. Plots of PAPII activity versus enzyme concentration A range of dilutions of post-Sephacryl S200 PAPII containing no BSA (•—•) or 1% w/v BSA (o—o), were assayed using the non-enzymatic cyclisation assay, employing a 30 minute incubation at 80°C, described in section 2 2 1 2 Error bars represent the SEM of triplicate readings

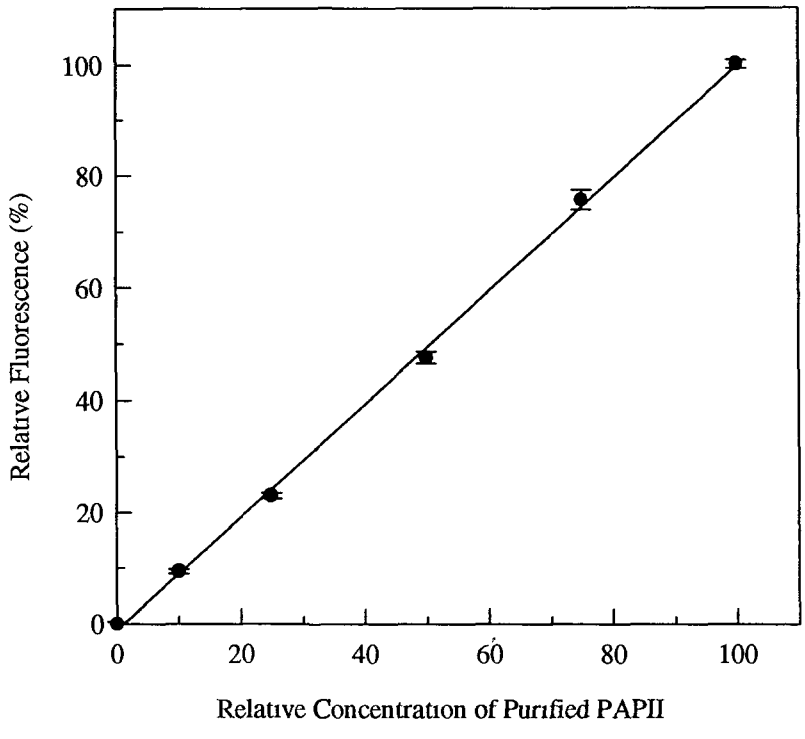


Figure 3.7.6.3 Linearity of the Non-Enzymatic Cyclisation PAPII Assay with respect to Enzyme Concentration. Plot of PAPII activity versus enzyme concentration A range of dilutions of post-Sephacryl S200 PAPII containing 1% w/v BSA was assayed using the non-enzymatic cyclisation assay, employing a 2 hour incubation at 80°C, as described in section 2 9 7 Error bars represent the SEM of triplicate readings

3.8 Characterisation of PAPII

3.8.1 Relative Molecular Mass Determination via Gel-Filtration Chromatography

A 230ml Sephacryl S-200 HR gel-filtration column was calibrated as outlined in section 2.10.1. The void volume (V_O) of the column (i.e. the elution volume of blue dextran) was determined to be 104ml as outlined in section 2.10.1.1. The elution volumes (V_e) of a range of molecular mass standards were determined as described in section 2.10.1.2. A plot of Log_{10} of molecular mass versus V_e/V_O yielded the calibration curve shown in figure 3.8.1. The elution volume of PAPII activity was determined to be 138ml. Based on the equation of the calibration curve,

$$Log_{10} MW = 6.095 - 2.837 (V_e/V_O)$$

the molecular mass of PAPII was estimated to be 214,051 Da.

3.8.2 Stability of Purified PAPII Under Various Storage Conditions

Post-Sephacryl S-200 PAPII, in the presence of (i) 1% w/v BSA, (ii) 20%v/v glycerol or (iii) in the absence of stabilising agents, was stored at 4°C, -20°C and -80°C as outlined in section 2.10.2. Over a three week period, aliquots of enzyme were removed and assayed for residual PAPII activity as outlined in section 2.9.7. Enzyme stored at 4°C, in the presence of BSA was not assayed beyond the fourth day due to microbial contamination of the sample. Plots of residual PAPII activity versus storage time are presented in figure 3.8.2.

When stored at 4°C, total loss of activity was observed after 14 days in the sample containing no stabilising agents. After 21 days, 7% of the initial activity remained in the sample containing glycerol. 88% of the initial activity remained in the sample containing BSA after 4 days (last day assayed).

When stored at -20°C, total loss of activity was observed in the sample containing no stabilising agents after 7 days. After 21 days, 21% of the initial activity remained in the sample containing glycerol and 81% of the initial activity remained in the sample containing BSA.

When stored at -80°C, total loss of activity was observed in the sample containing no stabilising agents after 24 hours (earliest time assayed). After 21 days, 47% and 93% of the initial activity remained in the samples containing glycerol and BSA respectively.

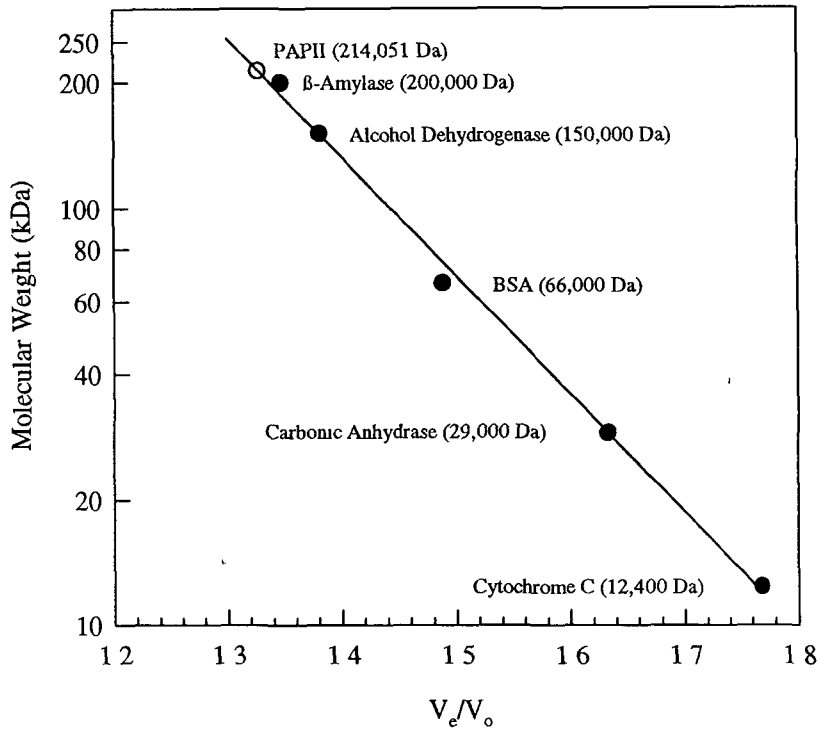


Figure 3.8.1 Sephacryl S-200 Molecular Mass Calibration Curve. Plot of Log_{10} molecular weight versus V_e/V_o where V_e is the elution volume of molecular mass standards (•—•) or PAPII (o) and V_o is the void volume of the column (104ml), determined by the elution volume of blue dextran Based on linear regression analysis of the calibration curve, as outlined in section 2 10 1, the molecular mass of PAPII was estimated to be 214,051 Da

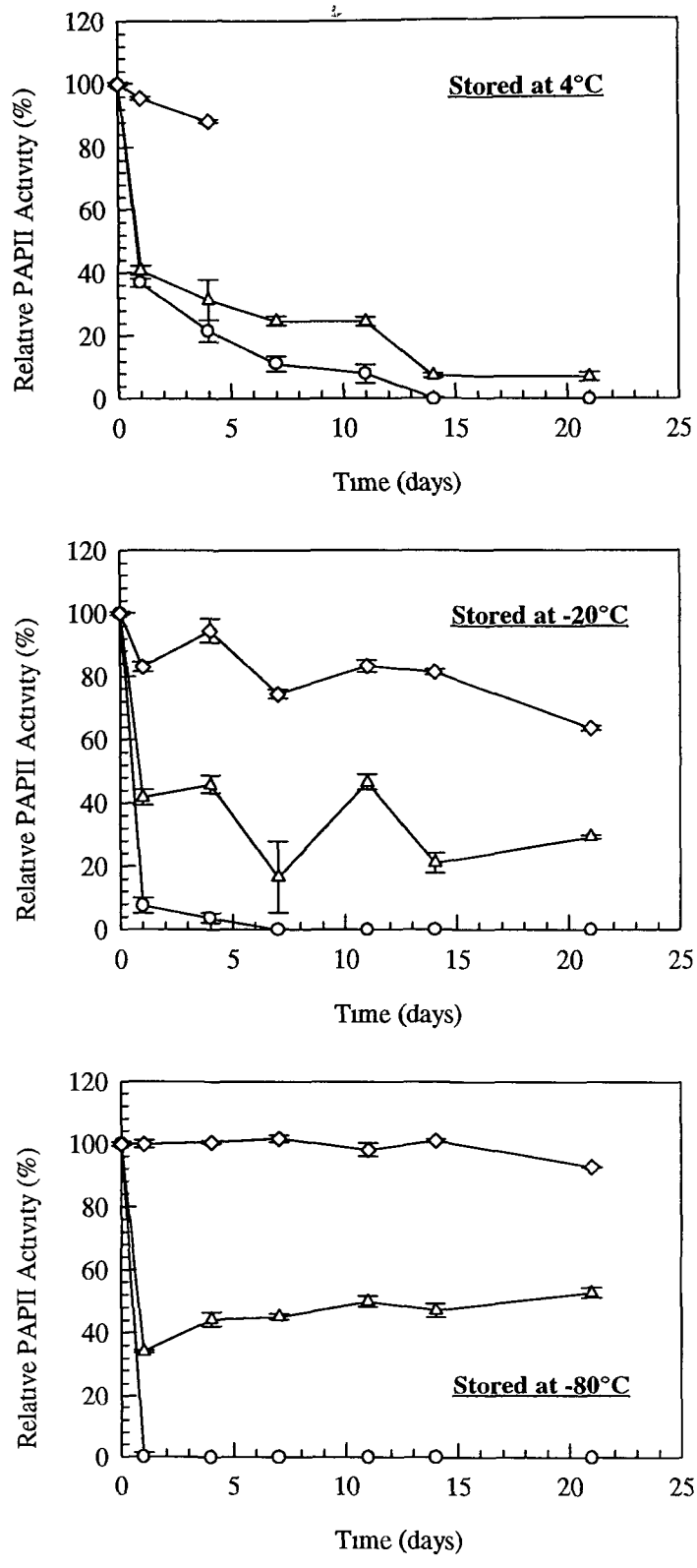


Figure 3.8.2 Stability of Purified PAPII. Plots of relative PAPII activity versus time stored at 4°C, -20°C and -80°C Post-Sephacryl S-200 PAPII was stored in the presence of 1% w/v BSA (◇—◇), 20% v/v Glycerol (Δ—Δ) or without any additions (○—○) as outlined in section 2 10 2 Error bars represent the SEM of triplicate readings

3.8.3 The Effect of Assay Temperature on PAPII Activity

Purified PAPII, containing 1% w/v BSA was assayed for 15 minutes at a range of temperatures as outlined in section 2 10 3 Figure 3 8 3 1 illustrates the effect of assay temperature on enzyme activity Maximal enzyme activity is observed at 45°C Incubation at 60°C resulted in no observed enzyme activity

Purified PAPII, containing 1% w/v BSA was assayed over a range of times at 45°C as outlined in section 2 10 3 The linear relationship between enzyme activity and time is illustrated in figure 3 8 3 2

3.8.4 The Effect of pH on PAPII Activity

The effects of pH on PAPII activity was investigated as outlined in section 2 10 4 The pH activity profile, described in section 2 10 4 1, is presented in figure 3 8 4 1 The enzyme exhibits a pH optimum of 6 8 to 7 6 Within the optimum pH range, maximal activity is observed in the citric acid / potassium phosphate buffering system

The pH inactivation curve, described in section 2 10 4 2, is presented in figure 3 8 4 2 Following preincubation for 15 minutes at 37°C, at a range of pH values, the enzyme assay was performed within the optimal pH range of 6 8 to 7 6 At pH values above 9 2 and below 4 0 the enzyme is inactivated

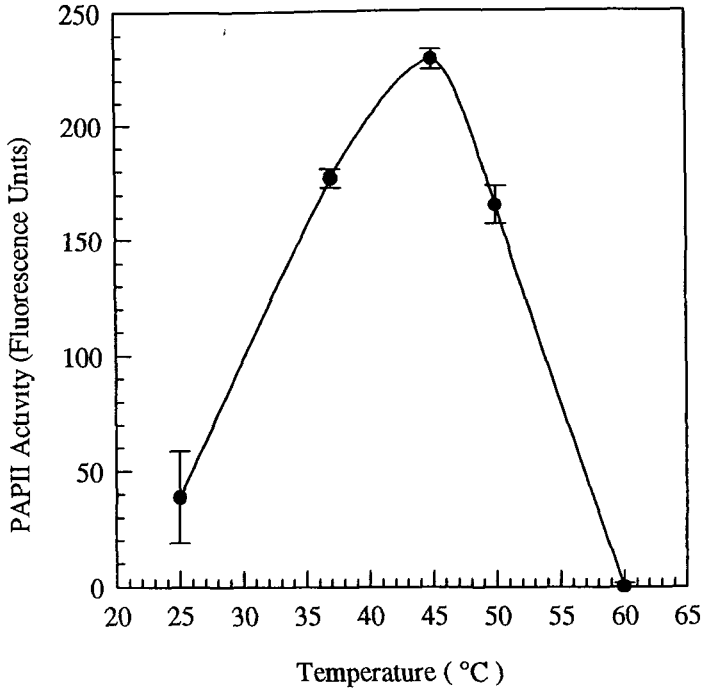


Figure 3.8.3.1 The Effect of Assay Temperature on PAPII Activity. Plot of PAPII activity versus assay temperature Following a 2 minute preincubation of substrate and sample, purified PAPII containing 1% w/v BSA was assayed for 15 minutes at a range of temperatures as outlined in section 2 10 3 Error bars represent the SEM of triplicate readings

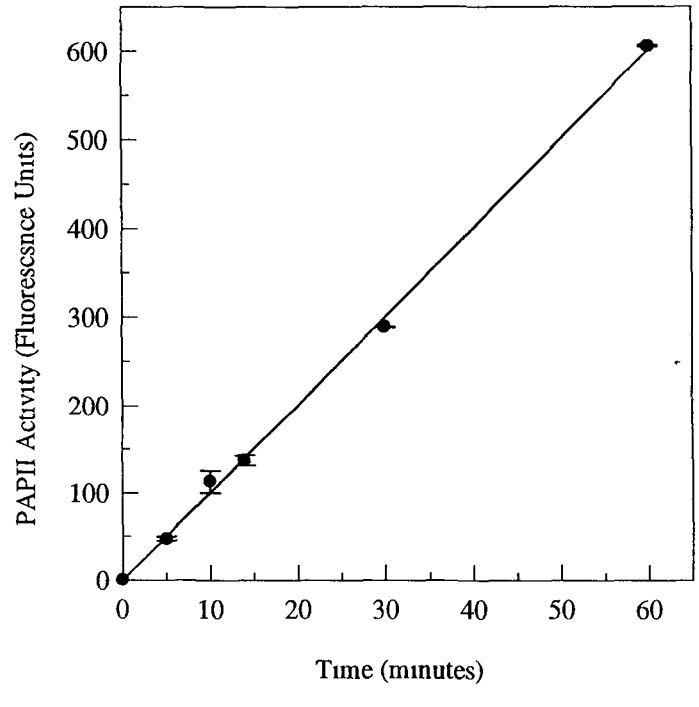


Figure 3.8.3.2 Linearity of PAPII Activity with respect to Time at 45°C. Plot of PAPII activity versus time at 45°C Following a 2 minute preincubation of substrate and sample, purified PAPII containing 1% w/v BSA was assayed for a range of times at 45°C, as outlined in section 2 10 3 Error bars represent the SEM of triplicate readings

Figure 3.8.4.1

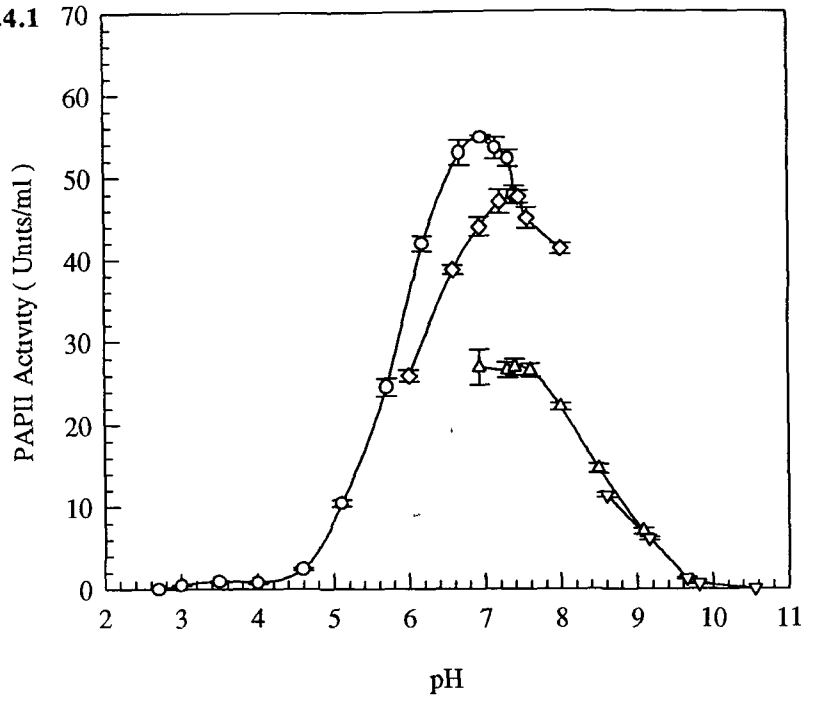
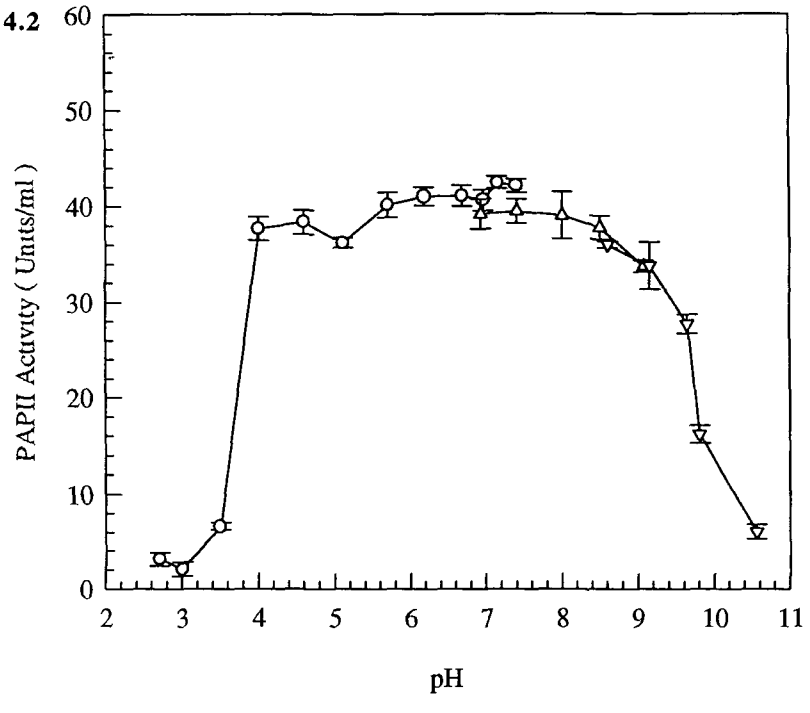


Figure 3.8 4.2



Figures 3.8.4.1 and 3.8.4.2 The Effect of pH on PAPII Activity. Plots of PAPII activity versus pH Figure 3 8 4 1 illustrates pH activity profile of PAPII as outlined in section 2 10 4 1 Purified enzyme, containing 1% w/v BSA, was preincubated for 15 minutes at 37°C in a range of buffers at a range of pH values and was assayed with substrate prepared in the same buffer at the same pH The pH inactivation curve of the enzyme, prepared as described in section 2 10 4 2, is illustrated in figure 3 8 4 2 Following a 15 minute preincubation at 37°C in a range of buffers at a range of pH values, purified PAPII containing 1% w/v BSA was assayed using substrate prepared in potassium phosphate buffer within the pH range 6 8 to 7 6 The buffering systems used are citric acid / di-basic potassium phosphate (o—o), potassium phosphate (◇—◇), tris / HCl (Δ—Δ) and glycine / NaOH (▽—▽) Error bars represent the SEM of triplicate readings

3.8.5 The Effect of Functional Reagents on PAPII Activity

The effect of various functional reagents on purified PAPII activity was tested as outlined in section 2 10 5 Table 3 8 5 presents the residual enzyme activity, as a percentage of that in an untreated sample, following 15 minutes preincubation at 37°C with functional reagents, at the concentrations indicated Total inhibition of enzyme activity was observed following treatment with 10mM 1,10-phenanthroline Residual PAPII activities of 33 7% and 3 2% resulted from incubation with AEBSF (Pefabloc SC) at concentrations of 1mM and 10mM respectively Significant inhibition of greater than 20% was observed following treatment with EGTA (10mM), 8-hydroxyquinoline (1mM and 10mM), 1,7-phenanthroline (10mM), 4,7-phenanthroline (10mM),*N*-Ethylmaleimide (10mM) and dithiothreitol (10mM)

3.8.6 The Effect of Metal-Complexing Agents on PAPII Activity with respect to Time

The effect of various metal-complexing agents and the non-chelating 1,7- and 4,7- phenanthrolines on purified PAPII activity was tested, with respect to time, as outlined in section 2 10 6 Figures 3 8 6 1 and 3 6 8 2 illustrate the effect of these compounds on enzyme activity when incubated on ice with purified PAPII, containing 1% w/v BSA, over a 24 hour period The residual activities after 24 hours are presented in table 3 8 5 The inhibition by 1,7- or 4,7-phenanthroline is not time dependent Inhibition with imidazole increases over the initial 3 hour period but does not increase further, upon incubation beyond this time 1,10-phenanthroline, 8-hydroxyquinoline, EDTA, CDTA, and EGTA inhibit the enzyme in a time dependent manner over the 24 hour period

Compound Class.	Compound Name.	Residual PAPII Activity as a Percentage of that in an Untreated Sample ± SEM		
			Concentration (mM) †	
			1.0	10
Cysteine Protease Inhibitors	Iodoacetamide		93.2 ± 0.3	89.8 ± 0.8
	Iodoacetate		91.5 ± 2.7	89.1 ± 2.1
	N-Ethylmaleimide		97.4 ± 0.8	78.3 ± 0.7$^{\surd}$
	Dithiobisnitrobenzoic acid		89.8 ± 0.5	-
Cysteine Protease Activators	Dithiothreitol		88.9 ± 1.3	52.1 ± 1.1$^{\surd}$
	2-Mercaptoethanol		95.0 ± 1.4	89.5 ± 1.6
	Thioglycolic acid		91.4 ± 2.8	94.2 ± 3.1
Serine Protease Inhibitors	AEBSF (Pefabloc SC)		33.7 ± 0.3$^{\surd}$	3.2 ± 0.6$^{\surd}$
	Phenylmethanesulfonylfluoride		97.9 ± 3.2	-
Metallopeptidase Inhibitors	Imidazole		99.3 ± 2.6 [86.3 ± 1.1]*$^{\surd}$	96.2 ± 1.0
	CDTA		96.2 ± 2.8 [46.4 ± 1.7]*$^{\surd}$	89.8 ± 0.9$^{\surd}$
	EDTA		97.7 ± 1.1 [52.8 ± 0.8]*$^{\surd}$	95.9 ± 2.2
	EGTA		92.5 ± 1.3 [46.1 ± 0.6]*$^{\surd}$	70.7 ± 1.4$^{\surd}$
	8-Hydroxyquinoline		85.9 ± 1.0$^{\surd}$ [68.6 ± 2.6]*$^{\surd}$	32.2 ± 2.5$^{\surd}$
	1,10-Phenanthroline		90.6 ± 3.5 [59.1 ± 1.1]*$^{\surd}$	0$^{\surd}$
	1,7-Phenanthroline		86.3 ± 3.6 [83.3 ± 2.3]*	33.1 ± 2.1$^{\surd}$
	4,7-Phenanthroline		82.9 ± 3.4 [83.4 ± 1.7]*$^{\surd}$	44.1 ± 2.5$^{\surd}$
Other Substances	2-Pyrrolidone (50mM)†	92.9 ± 2.8	-	-
	Bacitracin (1mg/ml)†	91.9 ± 1.1	-	-
	Puromycin		83.1 ± 1.1$^{\surd}$	-
	Benzamidine		90.7 ± 3.2	94.4 ± 1.0

Table 3.8.5 The Effect of Functional Reagents on PAPII Activity The effect of functional reagents on PAPII activity was determined as outlined in section 2.10.5. Purified enzyme containing 1% w/v BSA was preincubated for 15 minutes at 37°C with the test compound at the concentration indicated (†). Residual PAPII activity was determined as outlined in section 2.9.7.

* The number in square brackets refers to the residual activity following incubation for 24 hours on ice.

$^{\surd}$ Indicates $P < 0.05$, when statistical analysis of the results was performed using the paired Student's t-Test.

Figure 3.8.6.1

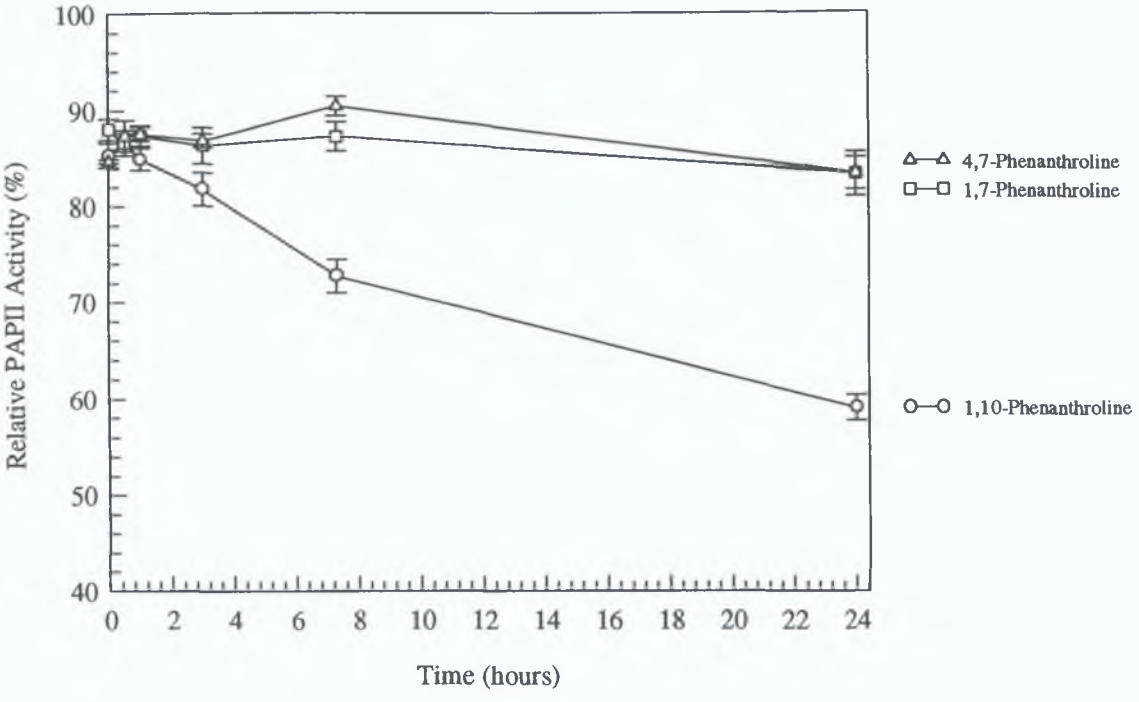
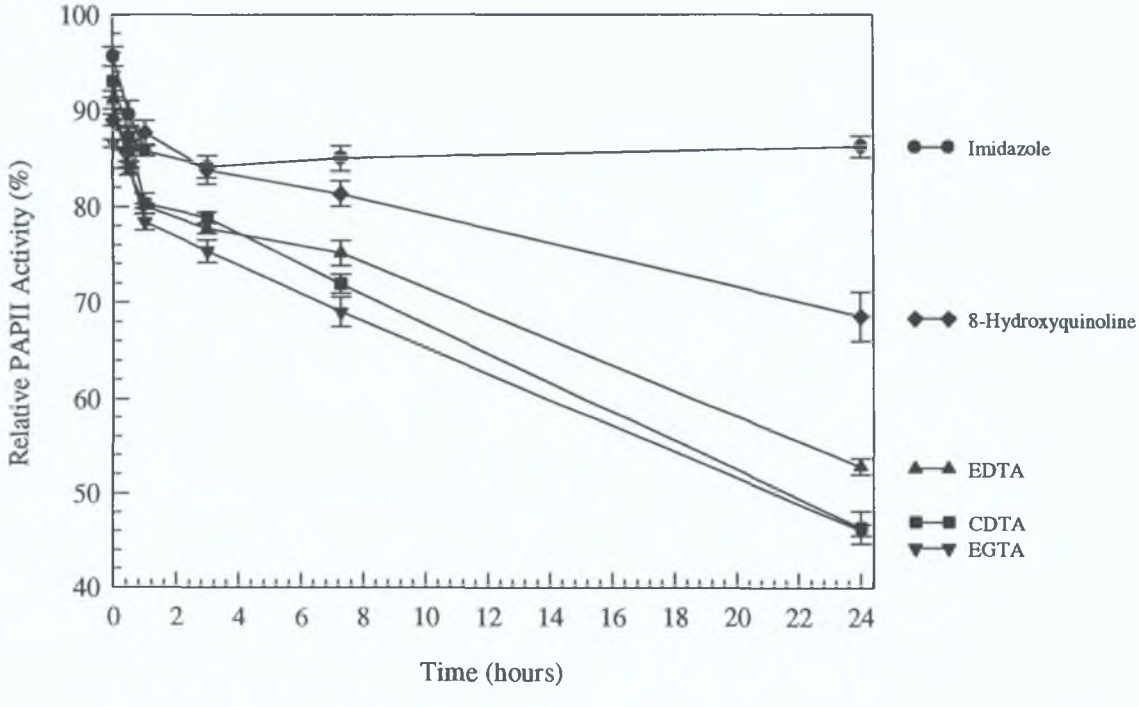


Figure 3.8.6.2



Figures 3.8.6.1 and 3.6.8.2 The Effect of Metal-Complexing Agents on PAPII Activity with respect to Time. The effect of metal-chelators, and the non-chelating 1,7- and 4,7-phenanthrolines, on PAPII activity with respect to time was investigated as outlined in section 2.10.6. Purified PAPII containing 1% w/v BSA was incubated over a 24 hour period on ice with each of the test compounds at a concentration of 1mM. Residual enzyme activity was determined as outlined in section 2.9.7. Error bars represent the SEM of triplicate readings.

3.8.7 The Effect of Metal Ions on PAPII Activity

The effect of various metal ions on purified PAPII activity was tested as outlined in section 2 10 7 Table 3 8 7 presents the residual enzyme activity, as a percentage of that in an untreated sample, following 15 minutes preincubation at 37°C with metal salts, at the concentrations indicated 50% inhibition or greater was observed following exposure to $CdSO_4$ (0 1mM and 1mM), $HgSO_4$ (1mM) and $ZnSO_4$ (1mM) Significant inhibition was also observed with $CuSO_4$ (0 1mM and 1mM) and $MgSO_4$ (0 1mM) An enhancement of enzyme activity to 161% and 559% of the initial activity was observed following exposure to $CoSO_4$ at 0 1mM and 1mM respectively

Metal Salt	Residual PAPII Activity as a Percentage of that in an Untreated Sample ± SEM	
	Concentration (mM) †	
	0.1	1.0
$CaSO_4$	91 1 ± 2 5	96 0 ± 2 2
$CdSO_4$	50 0 ± 1 6√	1 6 ± 0 5√
$CoSO_4$	161 3 ± 3 1√	559 1 ± 0 8√
$CuSO_4$	96 7 ± 0 9√	79 3 ± 0 5√
$FeCl_3$	96 8 ± 2 5	98 7 ± 1 9
$HgSO_4$	91 4 ± 1 8	24 1 ± 1 8√
$MgSO_4$	93 3 ± 0 5√	91 3 ± 1 4√
$MnSO_4$	112 0 ± 14	94 9 ± 3 0
$NiSO_4$	105 1 ± 2 4	125 0 ± 5 7
$ZnSO_4$	99 3 ± 1 2	39 1 ± 11 6√

Table 3.8.7 The Effect of Metal Ions on PAPII Activity. The effect of metal ions on PAPII activity was determined as outlined in section 2 10 7 Purified enzyme containing 1% w/v BSA was preincubated for 15 minutes at 37°C with the metal salt at the concentration indicated (†) Residual PAPII activity was determined as outlined in section 2 9 7

√ Indicates $P < 0\ 05$, when statistical analysis of the results was performed using the paired Student's t-Test

3.8.8 Reactivation of EDTA-Inactivated PAPII by Metal Ions

Purified PAPII containing 1% w/v BSA was incubated for 7 hours at 25°C with 10mM EDTA as outlined in section 2 10 8 1 Following this treatment, 16% of the enzyme activity remained when compared to the control sample Sephadex-G25 gel filtration, as outlined in section 2 10 8 2, resulted in an increase in volume from 1ml to 1 8ml for the control sample and 3ml to 5 4ml for the EDTA-treated sample Following EDTA removal, the EDTA treated sample displayed 32% of the activity found in the control sample

A range of metal salts were added to the post-gel filtration EDTA treated sample at final concentrations of 0 1mM and 1mM, as described in section 2 10 8 3 Figure 3 8 8 illustrates the PAPII activity, determined as outlined in section 2 9 7, following 15 minutes preincubation at 37°C with the metal salts Reactivation to 67% was observed following incubation with 1mM $CaSO_4$ Treatment with 0 1mM $MnSO_4$ and $ZnSO_4$ resulted in reactivation to 56% and 108% respectively No significant change in activity was observed following treatment with these metals at the higher concentration of 1mM Reactivation to 587% and 185% was observed following treatment with 1mM $CoSO_4$ and $NiSO_4$ respectively

3.8.9 The Effect of DMSO on PAPII Activity

The effect of DMSO on PAPII activity was investigated as outlined in section 2 10 9 Figure 3 8 9 illustrates the effect of increasing concentrations of DMSO in the substrate, on the purified enzyme The activity expressed using substrate containing 2% v/v DMSO, is 5% higher than that using substrate containing no DMSO 4% v/v and 6% v/v DMSO in the substrate has no significant effect on enzyme activity A decrease of 10% and 21% of enzyme activity is observed when the substrate contains 8% v/v and 10% v/v DMSO respectively

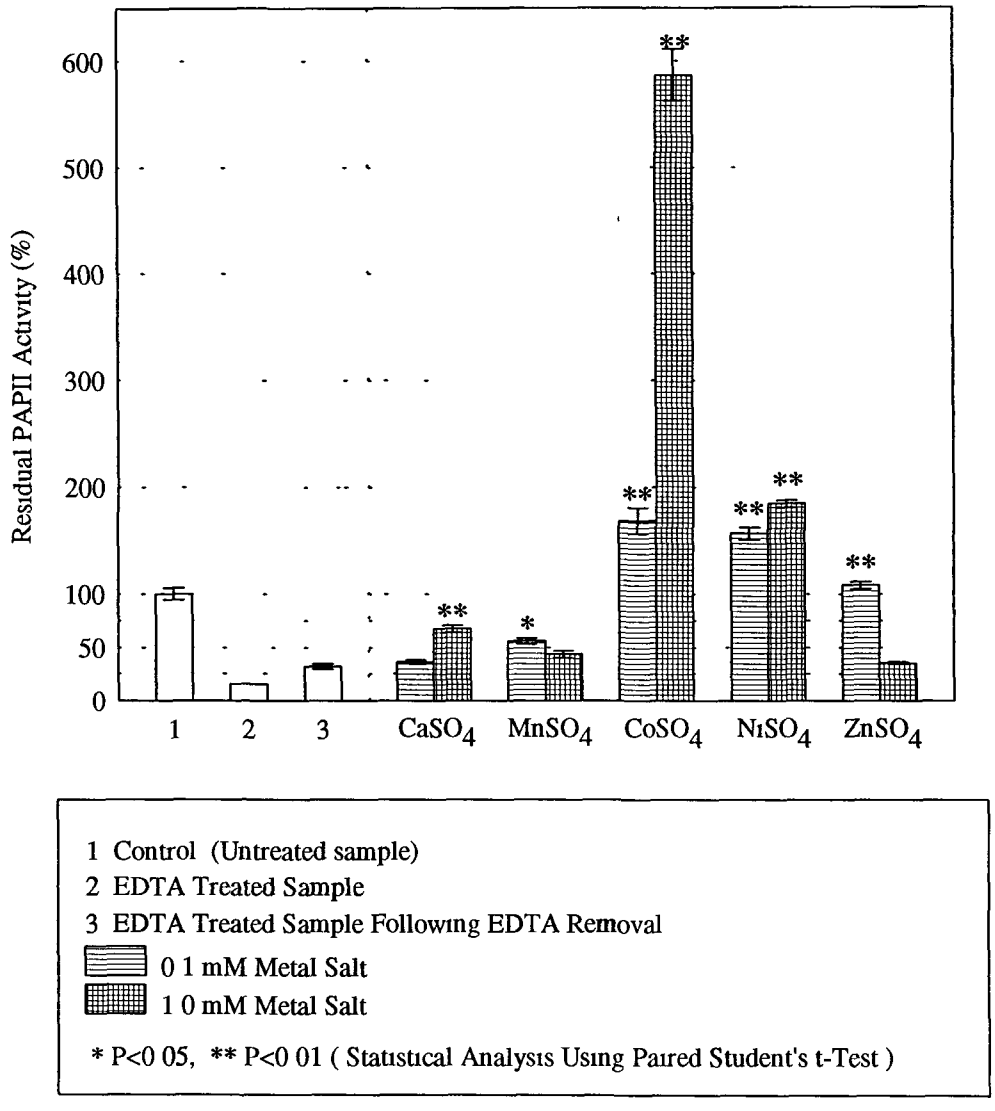


Figure 3.8.8 Reactivation of EDTA-Inactivated PAPII by Metal Ions Purified PAPII containing 1% w/v BSA was incubated for 7 hours at 25°C with 10mM EDTA as outlined in section 2 10 8 1 EDTA was removed by Sephadex G-25 gel filtration as described in section 2 10 8 2 A range of metal salts were added to the post-gel filtration EDTA treated sample as outlined in section 2 10 8 3 Following 15 minutes preincubation at 37°C with the metal salts, at the concentrations indicated, the residual PAPII activity was determined as outlined in section 2 9 7 Error bars represent the SEM of triplicate readings

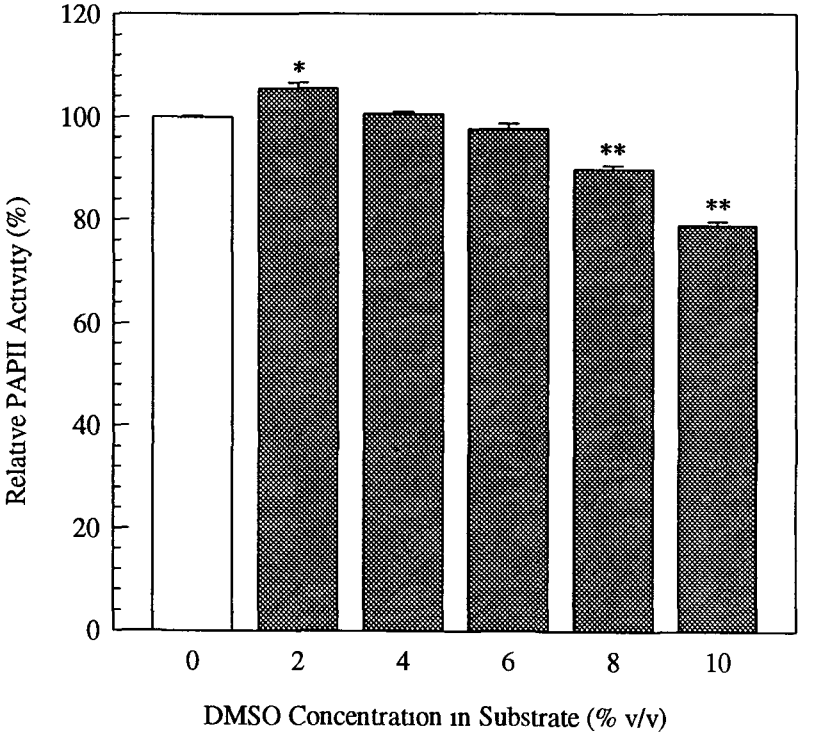


Figure 3.8.9 The Effect of DMSO on PAPII Activity Plot of relative PAPII activity, as a percentage of that expressed using substrate containing no DMSO, versus DMSO concentration Purified PAPII containing 1% w/v BSA was assayed using 0 1mM pGlu-His-Pro-MCA containing various concentrations of DMSO as outlined in section 2 10 9 Error bars represent the SEM of triplicate readings

3.8.10 Substrate Specificity of PAPII

3.8.10.1 The Activity of PAPII Against a Range of Quenched Fluorimetric Substrates

The ability of PAPII to hydrolyse a range of quenched fluorimetric substrates was tested as outlined in section 2 10 10 1 Table 3 8 10 1 illustrates that none of the substrates were cleaved by the purified enzyme with the exception of the PAPI substrate pGlu-MCA

Substrate	Whether Hydrolysed
Z-Gly-Pro-MCA	No
Gly-Pro-MCA	No
Pro-MCA	No
Ala-MCA	No
pGlu-MCA	Yes

Table 3.8.10.1 Cleavage of Quenched Fluorimetric Substrates by PAPII 100µl of purified PAPII was incubated with 400µl of each substrate (at a concentration of 0 1mM), for 1 hour at 37°C The reaction was terminated by the addition of 1ml of 1 5M acetic acid Liberated MCA was determined as outlined in section 2 2 1 1 All assays were performed in triplicate with suitable negative controls as described in section 2 10 10 1

3.8.10.2 The activity of PAPII Against pGlu-MCA, pGlu-ßNA and pGlu-pNA

The activity of purified PAPII against the PAPI substrates pGlu-MCA, pGlu-ßNA and pGlu-pNA was investigated as outlined in section 2 10 10 2 No activity was observed towards the substrate pGlu-pNA Figures 3 8 10 1 and 3 8 10 2 show the effect of incubating PAPII (+ 2-pyrrolidone), over 1 hour at 37°C, with pGlu-MCA and pGlu-ßNA respectively Also shown on these plots is the breakdown of the substrates in the absence of enzyme when 20mM potassium phosphate buffer at pH 7 5 is included in place of PAPII Both plots illustrate that the substrates are hydrolysed by the enzyme and that the reactions proceed in a linear fashion over 1 hour

Figure 3.8.10.1

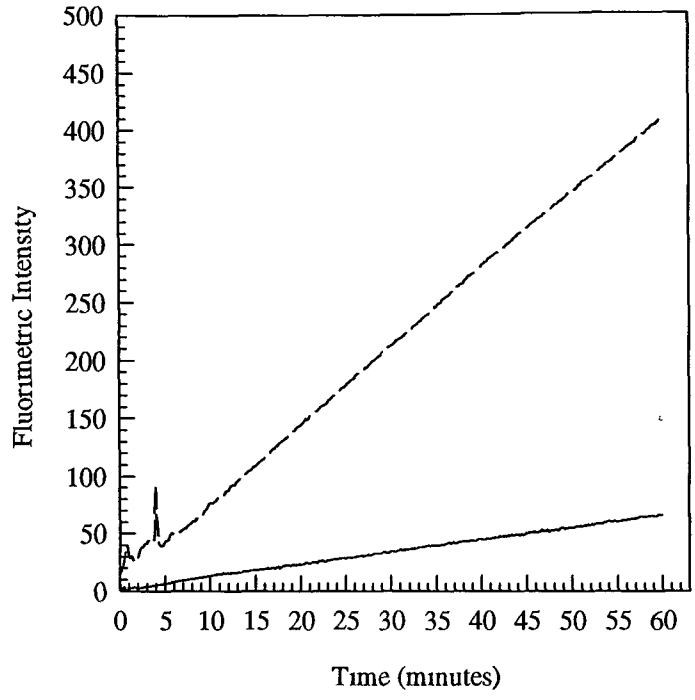
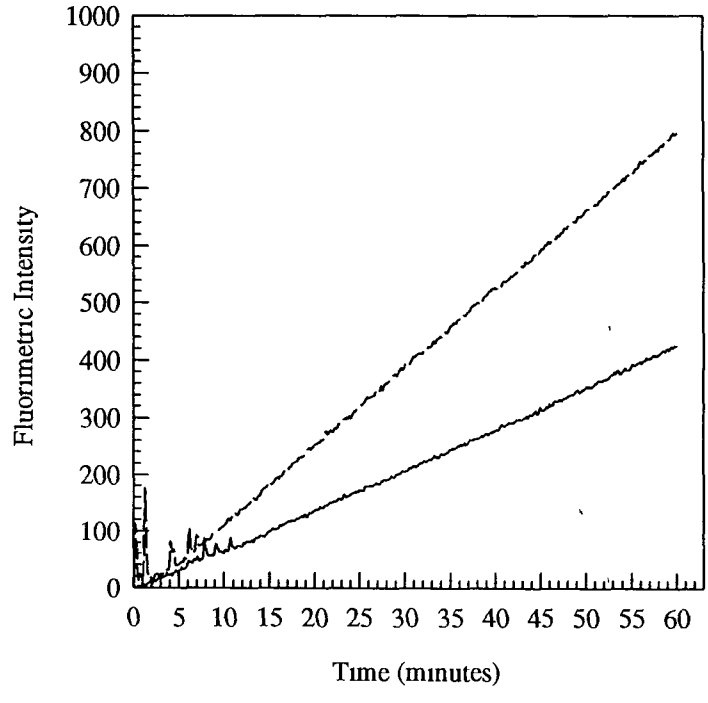


Figure 3.8.10.2



Figures 3.8.10.1 and 3.8.10.2 Cleavage of pGlu-ßNA and pGlu-MCA by PAPII. 60µl of 1 3M 2-pyrrolidone and 240µl of purified PAPII containing 1% w/v BSA (- - -) or 240µl of 20mM potassium phosphate buffer at pH 7 5 (—), was incubated for 1 hour at 37°C with 1 2ml of 0 2mM pGlu-ßNA (Figure 3 8 10 1) or pGlu-MCA (Figure 3 8 10 2) Liberated ßNA or MCA was monitored continuously over 1 hour as outlined in section 2 10 10 2 Both substances were monitored fluorimetrically with excitation and emission wavelengths of 370nm and 440nm respectively for MCA and 340nm and 410nm respectively for ßNA Excitation and emission slit widths of 10nm and 20nm respectively were used throughout

3.8.10.3 Investigation of the Substrate Specificity of PAPII by HPLC

The ability of purified PAPII to hydrolyse a range of synthetic pyroglutamic acid containing substrates was investigated using HPLC, as outlined in section 2 10 10 3 The HPLC chromatograms illustrating the cleavage of acid TRH, pGlu-Phe-Pro-NH_2, pGlu-His-Pro-Gly-OH and pGlu-His-Pro-Gly-NH_2 are shown in figures 3 8 10 3 to 3 8 10 6 The cleavage of TRH is illustrated on the HPLC chromatograms shown in figure 3 8 10 7 In each case, cleavage of the peptide is determined by a reduction of, or total elimination of the substrate peak and the formation of a pyroglutamic acid peak In the case of acid TRH cleavage, His-Pro-OH and a small amount of cyclo-His-Pro were identified as the other cleavage products (figure 3 8 10 3) Cyclo-His-Pro was identified as a minor cleavage product following pGlu-His-Pro-Gly-OH and pGlu-His-Pro-Gly-NH_2 hydrolysis (figures 3 8 10 5 and 3 8 10 6) His-Pro-NH_2 and cyclo-His-Pro were identified as the other cleavage products following TRH degradation (figure 3 8 10 8) Other cleavage products could not be conclusively identified due to the fact that standard fragments were not available Table 3 8 10 2 lists the peptides which were tested and whether or not they were cleaved by the enzyme

3.8.10.4 Monitoring the Cleavage of Substrates over Time by PAPII, Using HPLC

Standard concentrations of pyroglutamic acid, TRH, acid TRH, pGlu-His-Pro-Gly-NH_2 and pGlu-His-Gly-OH were prepared as outlined in section 2 10 10 4 Following application to, and elution from the HPLC column under the conditions outlined in section 2 10 10 3, plots of peak area versus peptide concentration were constructed The standard curves thus obtained are presented in figure 3 8 10 8

The peptides listed above were incubated with purified PAPII at 37°C for a range of times as outlined in section 2 10 10 4 Following HPLC analysis, the concentrations of pyroglutamic acid and peptide in the incubation mixture were determined using the standard curves Plots of peptide and pyroglutamic acid concentration, versus incubation time are presented in figures 3 8 10 9 to 3 8 10 12 Total exhaustion of substrate coupled with an increase of the concentration of pyroglutamic acid, to a level equivalent to the initial substrate concentration, was observed after incubation for 6 hours in the cases of TRH and acid TRH and after incubation for 10 hours in the case of pGlu-His-Pro-Gly-NH_2 After 20 hours incubation, approximately 30% of the pGlu-His-Gly-OH was hydrolysed (figure 3 8 10 12)

The cleavage of TRH over time is illustrated by the HPLC chromatograms shown in figure 3 8 10 8 After 6 hours all of the TRH has been hydrolysed resulting in the formation of pyroglutamic acid, His-Pro-NH_2 and cyclo His-Pro Following 20 hours incubation, the pyroglutamic acid peak remains the same size, the His-Pro-NH_2 peak has disappeared and the cyclo His-Pro peak has increased in size A similar pattern of degradation was observed for pGlu-His-Pro-Gly-NH_2 After 10 hours, all of the substrate was hydrolysed resulting in the formation of pyroglutamic acid and two other metabolites, (i) a major peak with a retention time of 10 5 minutes and (ii) a small amount of cyclo-His-Pro Following a further 10 hours incubation, the major peak is reduced in size while the cyclo-His-Pro peak is increased in size

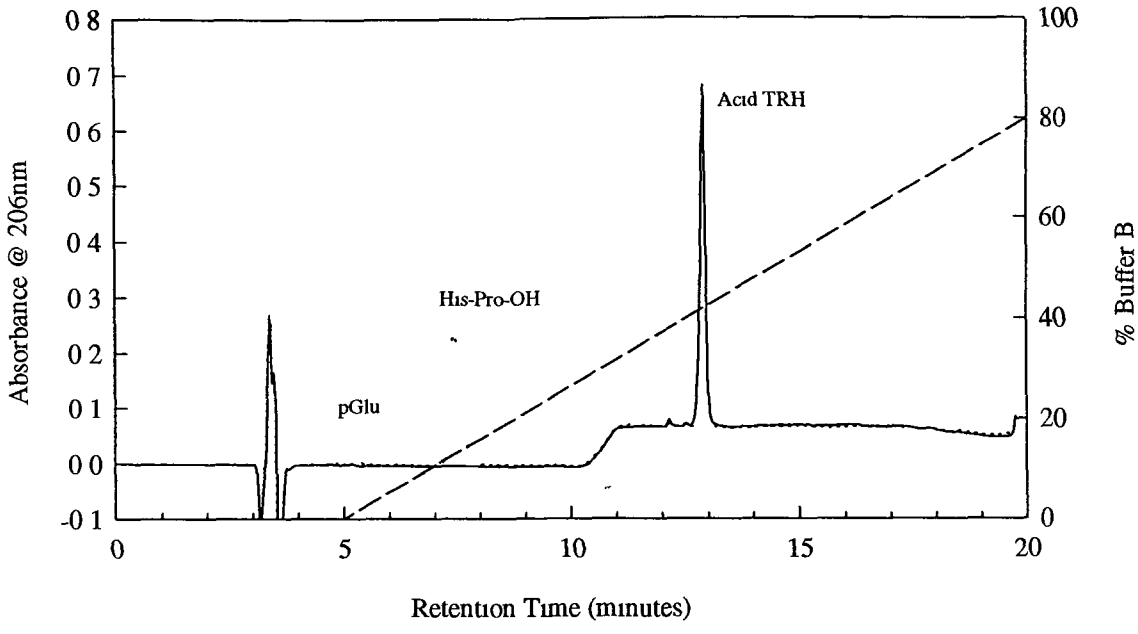


Figure 3.8.10.3 Cleavage of Acid TRH by PAPII. Plot of absorbance at 206nm versus retention time Substrate specificity studies using reverse phase HPLC were carried out as outlined in section 2 10 10 3 Plots present data obtained from the negative control (——) and following the incubation, for 18 hours at 37°C, of acid TRH (0 35mM) with purified PAPII ( ) A linear gradient from 0 to 80% buffer B is illustrated (- - -)

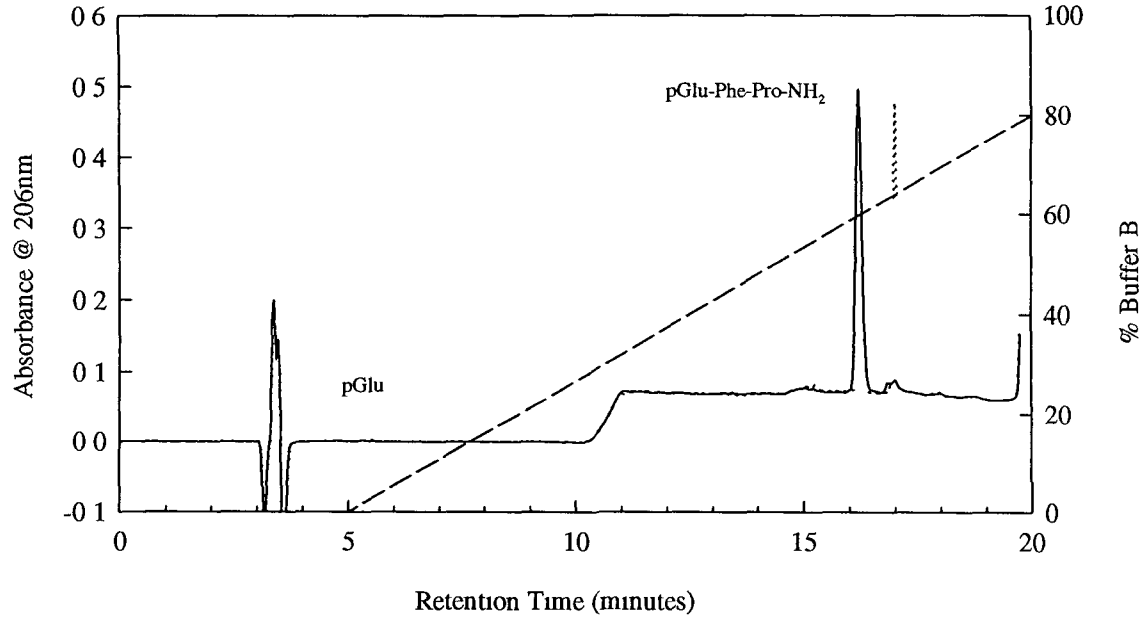


Figure 3.8.10.4 Cleavage of pGlu-Phe-Pro-NH_2 by PAPII. Plot of absorbance at 206nm versus retention time Substrate specificity studies using reverse phase HPLC were carried out as outlined in section 2 10 10 3 Plots present data obtained from the negative control (——) and following the incubation, for 18 hours at 37°C, of pGlu-Phe-Pro-NH_2 (0 375mM) with purified PAPII ( ) A linear gradient from 0 to 80% buffer B is illustrated (- - -)

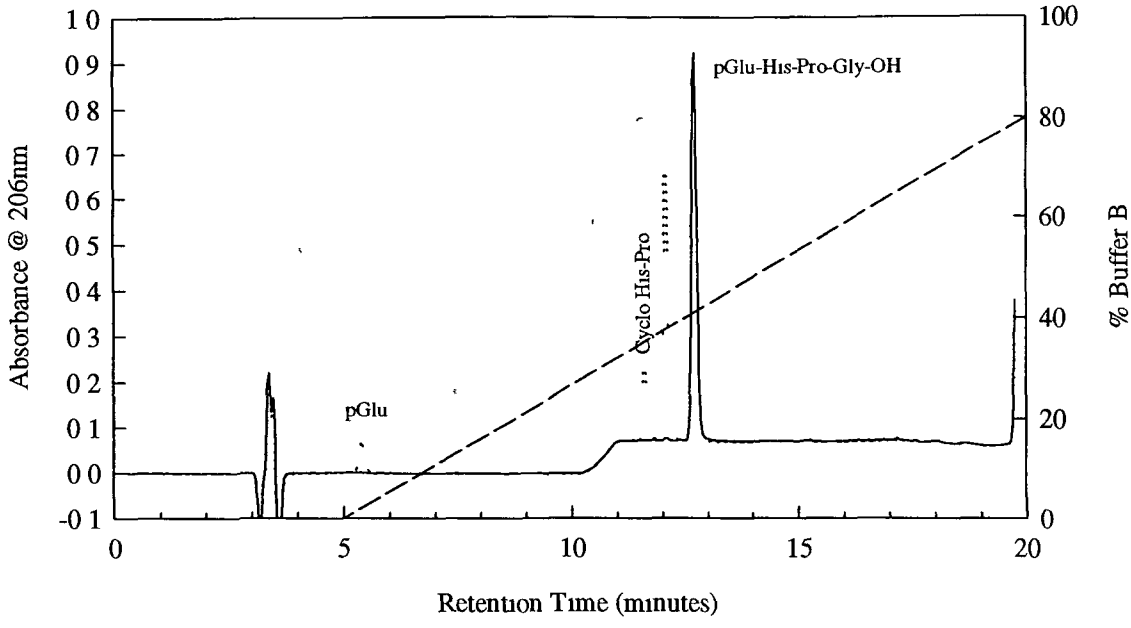


Figure 3.8.10.5 Cleavage of pGlu-His-Pro-Gly-OH by PAPII. Plot of absorbance at 206nm versus retention time Substrate specificity studies using reverse phase HPLC were carried out as outlined in section 2 10 10 3 Plots present data obtained from the negative control (——) and following the incubation, for 18 hours at 37°C, of pGlu-His-Pro-Gly-OH (0 5mM) with purified PAPII ( ) A linear gradient from 0 to 80% buffer B is illustrated (- - -)

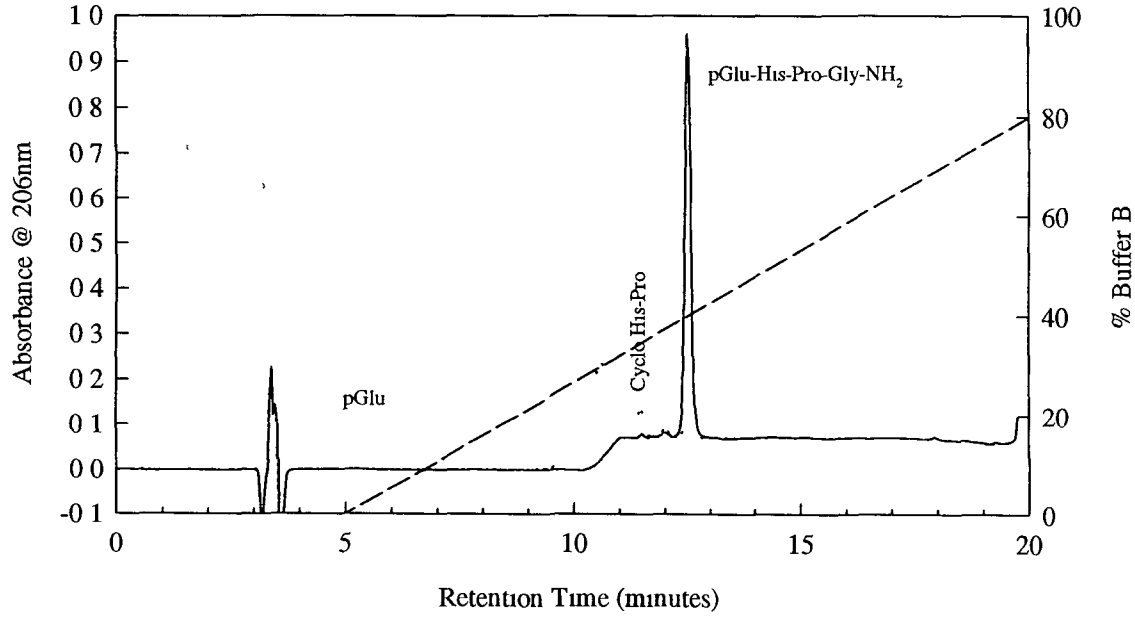


Figure 3 8.10.6 Cleavage of pGlu-His-Pro-Gly-NH_2 by PAPII. Plot of absorbance at 206nm versus retention time Substrate specificity studies using reverse phase HPLC were carried out as outlined in section 2 10 10 3 Plots present data obtained from the negative control (——) and following the incubation, for 18 hours at 37°C, of pGlu-His-Pro-Gly-NH_2 (0 5mM) with purified PAPII ( ) A linear gradient from 0 to 80% buffer B is illustrated (- - -)

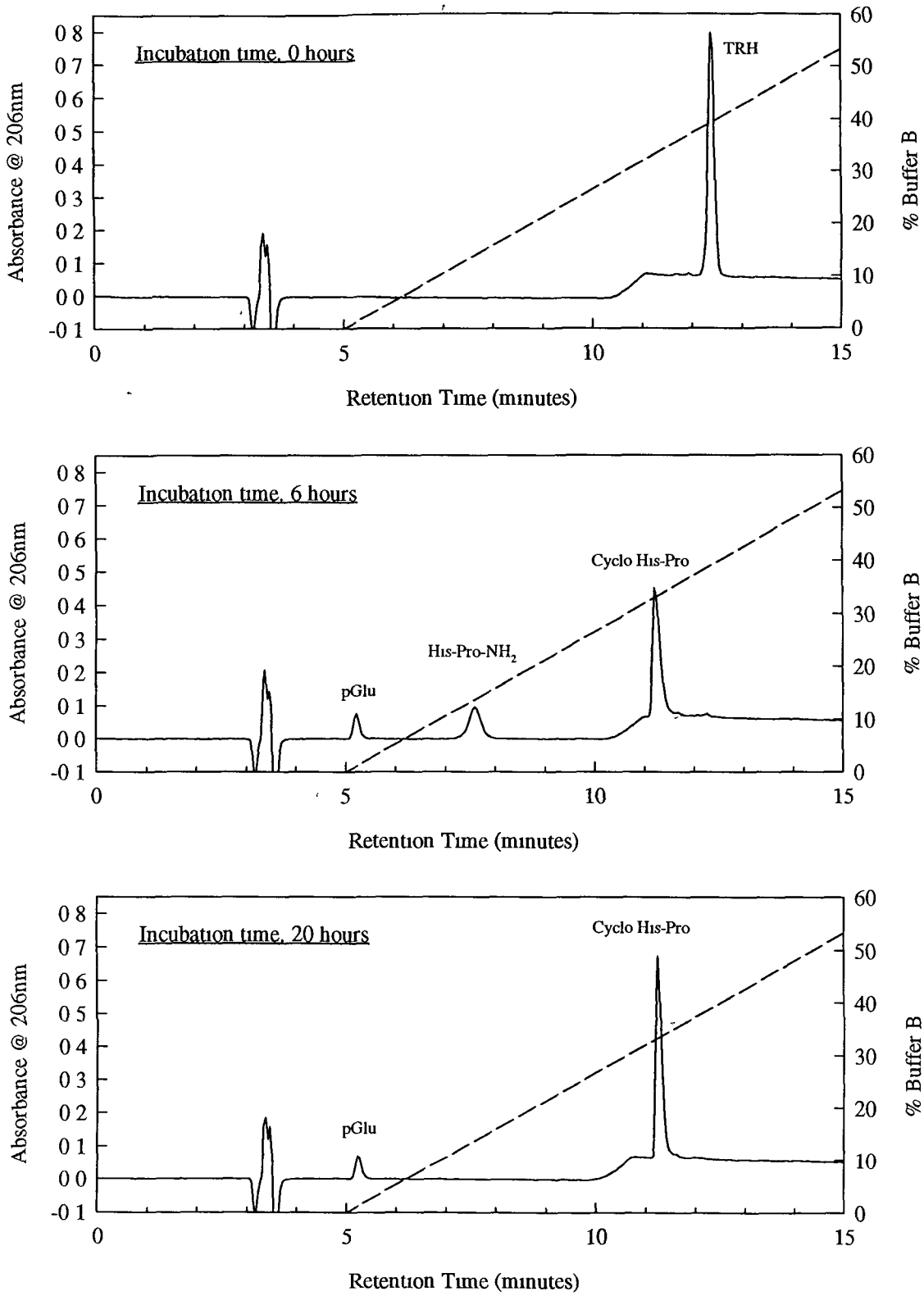


Figure 3.8.10.7 Cleavage of TRH by PAPII. Plots of absorbance at 206nm versus retention time The cleavage of TRH by PAPII was monitored over a 20 hour period using reverse phase HPLC, as outlined in section 2 10 10 4 Plots present data obtained following the incubation of TRH (0 5mM) with purified PAPII for 0, 6 and 20 hours at 37°C- A linear gradient from 0 to 80% buffer B is illustrated (- - -)

Peptide	Peptide Concentration (mM)†	Whether Hydrolysed
pGlu-Ala-OH	0 5	No
pGlu-His-OH	0 5	No
pGlu-Gly-OH	0 5	No
pGlu-Phe-OH	0 5	No
pGlu-Val-OH	0 5	No
pGlu-Pro-NH_2	0 5	No
pGlu-His-Gly-OH	0 5	**Yes**
pGlu-His-Gly-NH_2	0 5	**Yes**
pGlu-His-Pro-OH (Acid TRH)	0 35	**Yes**
pGlu-His-Pro-NH_2 (TRH)	0 5	**Yes**
pGlu-Phe-Pro-NH_2	0 375	**Yes**
pGlu-His-Pro-Gly-OH	0 5	**Yes**
pGlu-His-Pro-Gly-NH_2	0 5	**Yes**
pGlu-Glu-Pro-NH_2	0 5	No
LHRH 1-5	0 5	No
LHRH 1-6	0 5	No
LHRH 1-7	0 5	No
LHRH	0 5	No
Eledoisin	0 175	No
Neurotensin	0 175	No
pGlu-MCA	0 375	**Yes**

Table 3.8.10.2 Investigation of the substrate Specificity of PAPII Purified PAPII containing 1% w/v BSA was incubated at 37°C for 18 hours with a range of peptides Following analysis of the incubation mixture by reverse-phase HPLC as outlined in section 2 10 10 3, cleavage of the peptide was determined by a reduction (or elimination) of the peptide peak coupled with the production of pyroglutamic acid (†) The concentration of the peptides under assay conditions is indicated

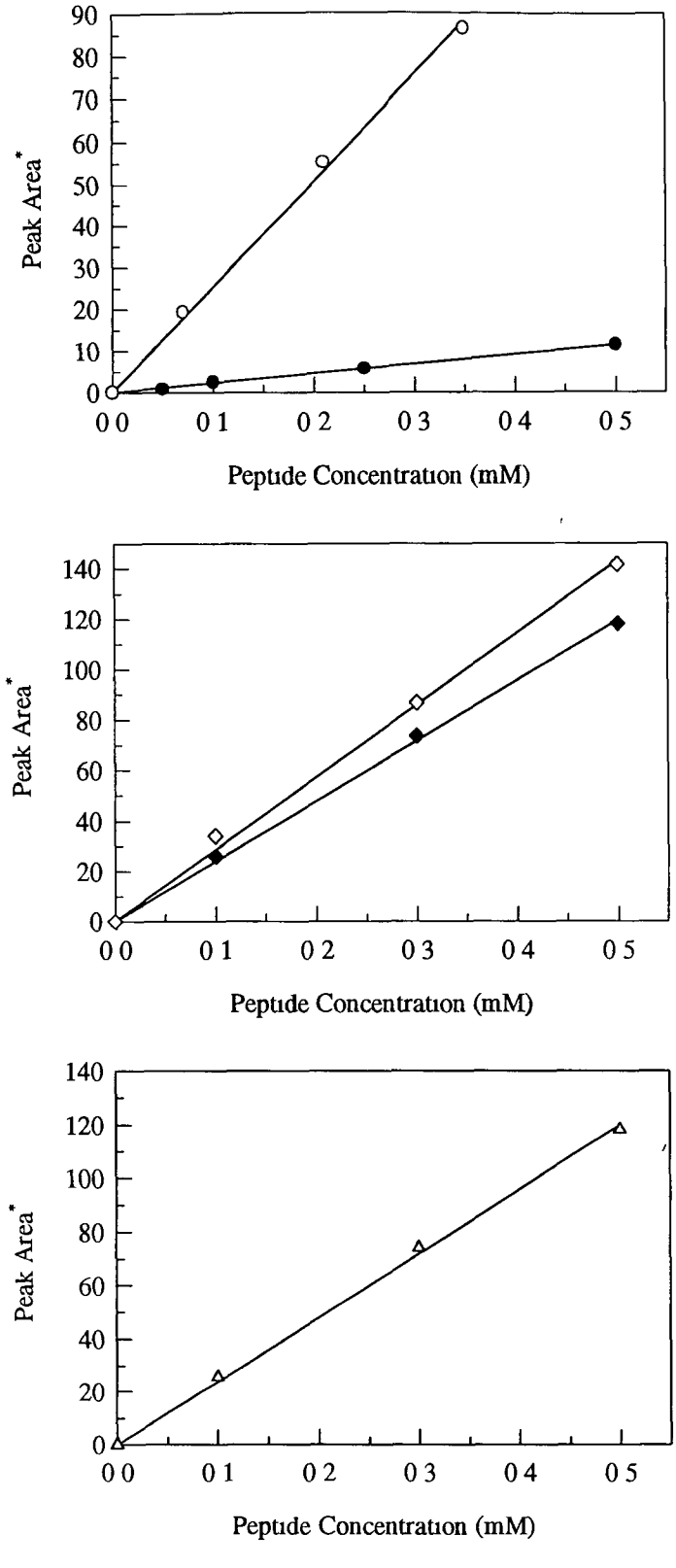


Figure 3.8.10.8 Peptide Standard Curves. Plots of peak area (*Absorbance Units) versus peptide concentration Standard concentrations of peptides were prepared and run on the HPLC system as outlined in section 2 10 10 4 Plots present data obtained from acid TRH (o—o), pyroglutamic acid (•—•), pGlu-His-Pro-Gly-NH_2 (◇—◇), TRH (◆—◆) and pGlu-His-Gly-OH (Δ—Δ)

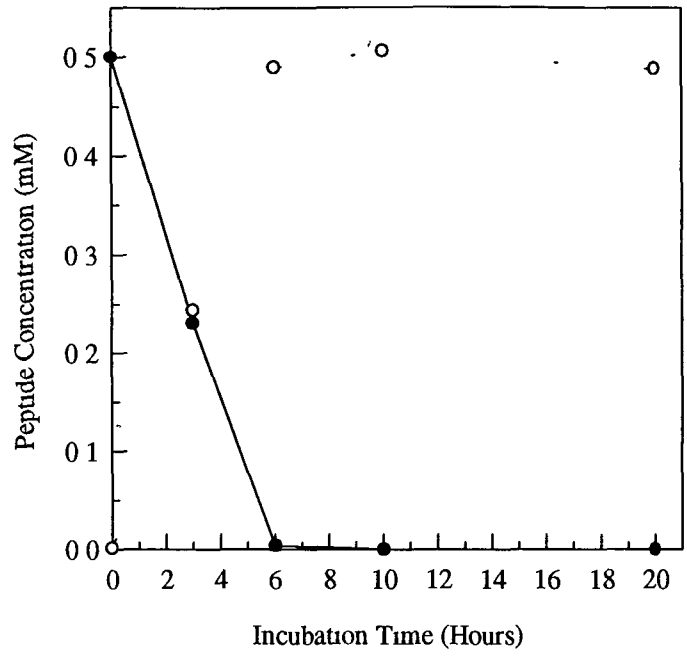


Figure 3.8.10.9 Cleavage of TRH by PAPII over Time. Plots of peptide concentration versus incubation time at 37°C The cleavage of TRH by PAPII was monitored over a 20 hour period using reverse phase HPLC, as outlined in section 2 10 10 4 Plots present the decrease in TRH concentration (•—•) and the increase pyroglutamic acid concentration (o o) over time

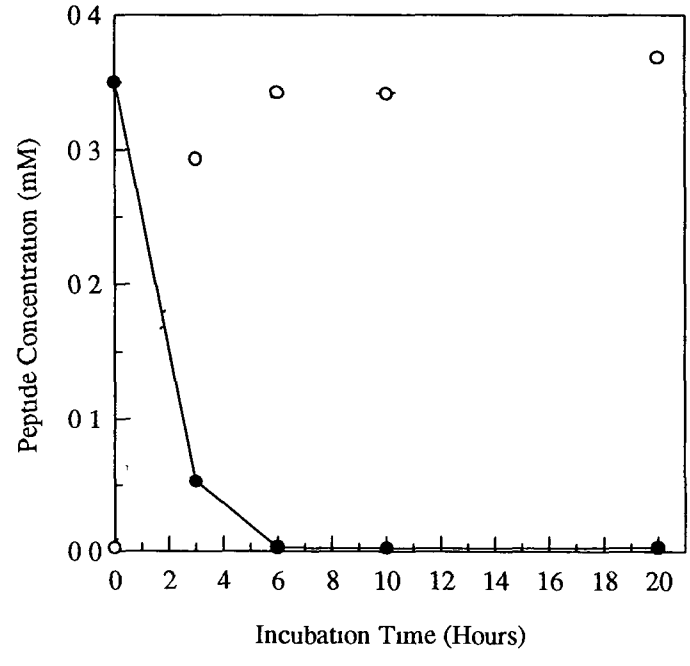


Figure 3 8.10.10 Cleavage of Acid TRH by PAPII over Time. Plots of peptide concentration versus incubation time at 37°C The cleavage of acid TRH by PAPII was monitored over a 20 hour period using reverse phase HPLC, as outlined in section 2 10 10 4 Plots present the decrease in acid TRH concentration (•—•) and the increase pyroglutamic acid concentration (o o) over time

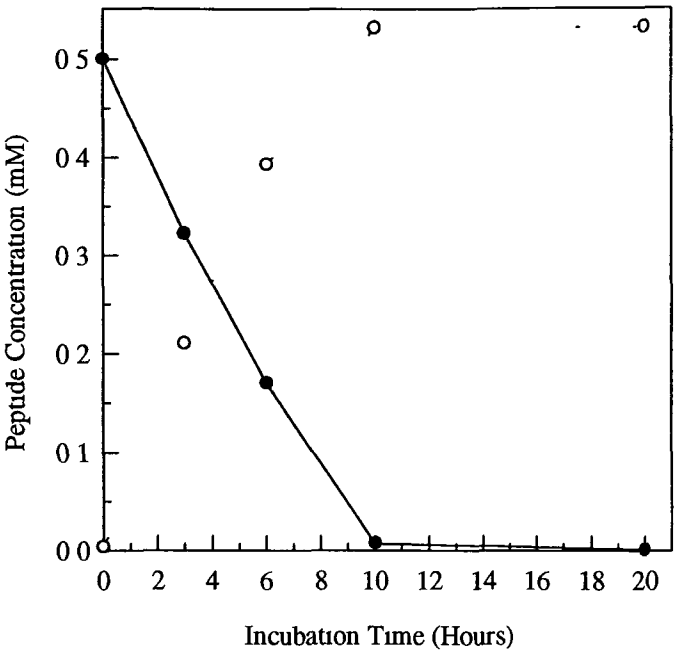


Figure 3.8.10.11 Cleavage of pGlu-His-Pro-Gly-NH_2 by PAPII over Time. Plots of peptide concentration versus incubation time at 37°C The cleavage of pGlu-His-Pro-Gly-NH_2 by PAPII was monitored over a 20 hour period using reverse phase HPLC, as outlined in section 2 10 10 4 Plots present the decrease in pGlu-His-Pro-Gly-NH_2 concentration (•—•) and the increase pyroglutamic acid concentration (o o) over time

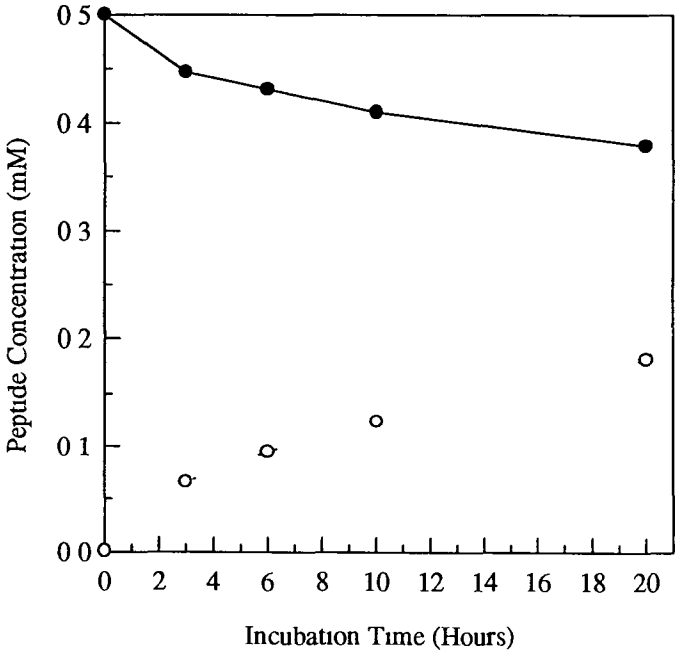


Figure 3.8.10.12 Cleavage of pGlu-His-Gly-OH by PAPII over Time Plots of peptide concentration versus incubation time at 37°C The cleavage of pGlu-His-Gly-OH by PAPII was monitored over a 20 hour period using reverse phase HPLC, as outlined in section 2 10 10 4 Plots present the decrease in pGlu-His-Gly-OH concentration (•—•) and the increase pyroglutamic acid concentration (o o) over time

3.8.11 Kinetic Studies

3.8.11.1 Determination of K_m of PAPII for pGlu-MCA and pGlu-His-Pro-MCA

A K_m value was determined for the reaction of purified PAPII with pGlu-His-Pro-MCA and pGlu-MCA as outlined in section 2 10 11 1 and 2 10 11 2 respectively The data obtained is presented as Michaelis-Menten, Eadie-Hofstee, Lineweaver-Burk and Hanes-Woolf plots, in figures 3 8 11 1 to 3 8 11 4 for pGlu-His-Pro-MCA and in figures 3 8 11 5 to 3 8 11 8 for pGlu-MCA The K_m and V_{max} values for the substrates were determined from these plots and also from Direct Linear Plot analysis (plot not presented) The values obtained are presented in table 3 8 11 1 K_m values of 419 8μM and 2 53μM and V_{max} values of 2,815 units/mg and 36,144 units/mg, were determined for pGlu-MCA and pGlu-His-Pro-MCA respectively, using the Direct Linear Plot analysis

Kinetic Analysis	pGlu-His-Pro-MCA		pGlu-MCA	
	K_m (μM)	V_{max} (Units/mg)†	K_m (μM)	V_{max} (Units/mg)†
Michaelis-Menten	2 46	36,584	351 4	2,582
Eadie-Hofstee	2 63	36,954	412 2	2,801
Lineweaver-Burk	2 76	37,573	448 8	2,964
Hanes-Woolf	1 67	35,358	385 4	2,677
Direct Linear Plot	2 53	36,144	419 8	2,815

Table 3.8.11.1 K_m and V_{max} Values for the Reaction of Purified PAPII with the Substrates pGlu-His-Pro-MCA and pGlu-MCA.

† V_{max} values are presented in Units/nig, defined as that which liberates 1 picomole of MCA per minute at 37°C, per mg of purified protein (not including the BSA added to the purified enzyme)

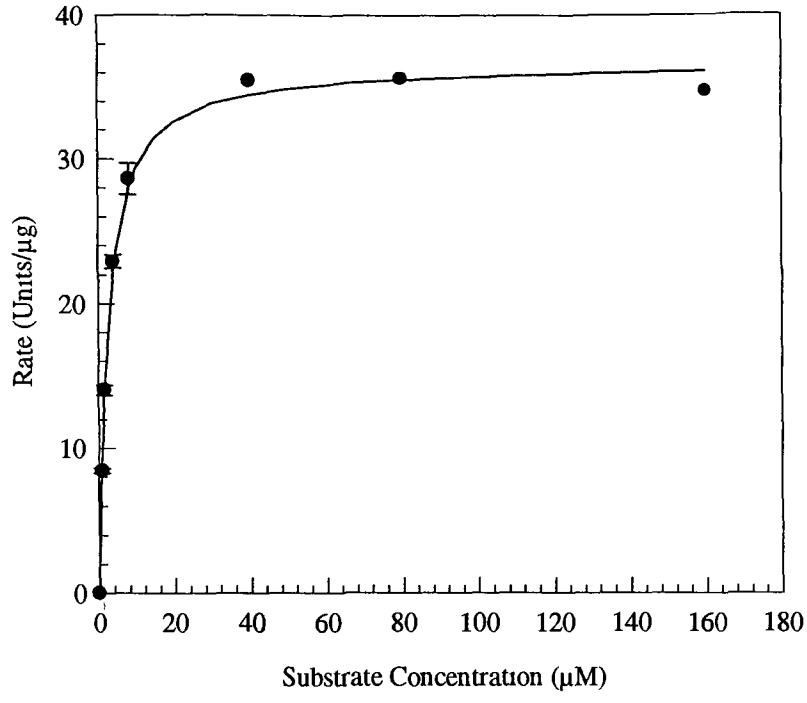


Figure 3.8.11.1 Michaelis-Menten Plot for pGlu-His-Pro-MCA.
Data was obtained as outlined in section 2 10 11 1 K_m and V_{max} values for the substrate were determined to be 2 46µM and 36,584 units/mg respectively Error bars represent the SD of triplicate readings

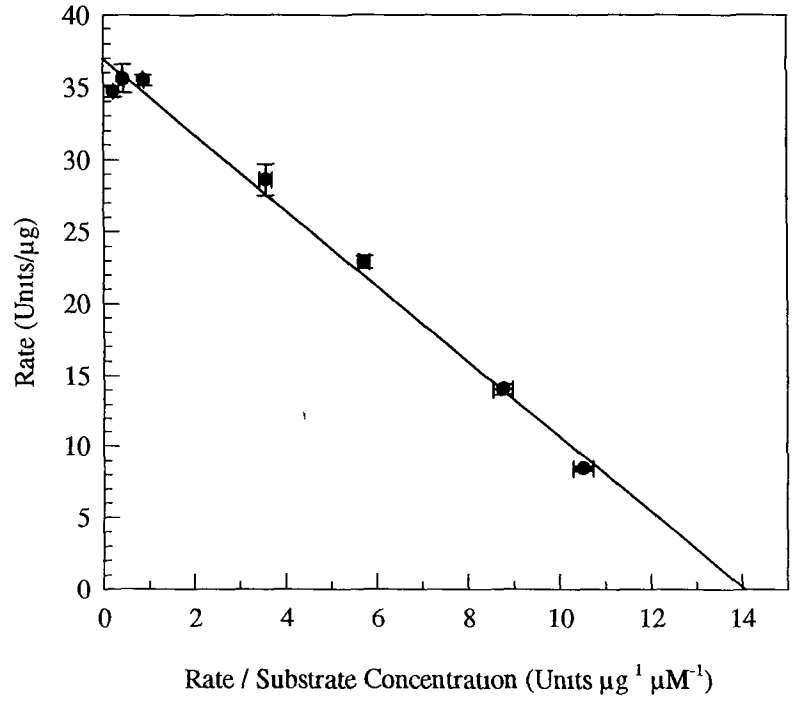


Figure 3.8.11.2 Eadie-Hofstee Plot for pGlu-His-Pro-MCA.
Data was obtained as outlined in section 2 10 11 1 K_m and V_{max} values for the substrate were determined to be 2 63µM and 36,954 units/mg respectively Error bars represent the SD of triplicate readings

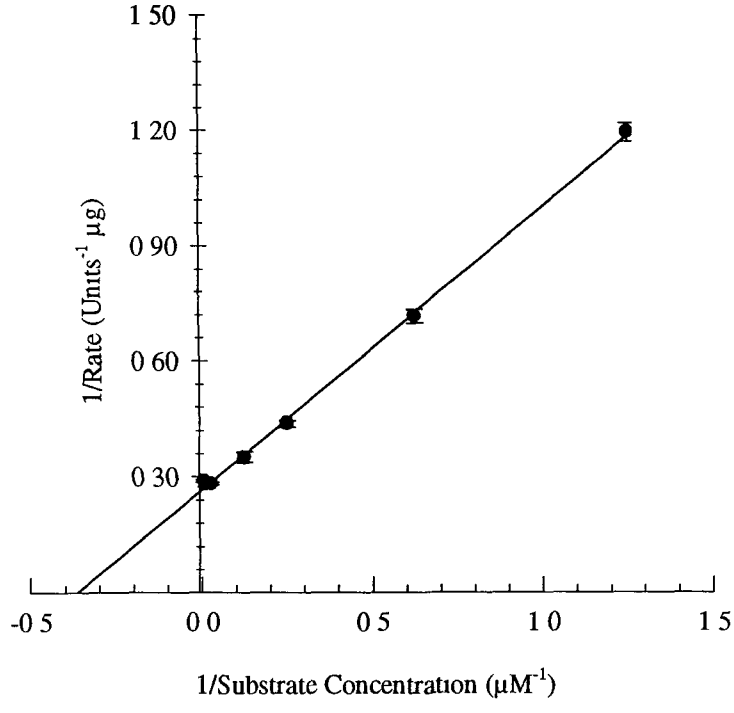


Figure 3.8.11.3 Lineweaver-Burk Plot for pGlu-His-Pro-MCA.
Data was obtained as outlined in section 2 10 11 1 K_m and V_{max} values for the substrate were determined to be 2 76µM and 37,573 units/mg respectively Error bars represent the SD of triplicate readings

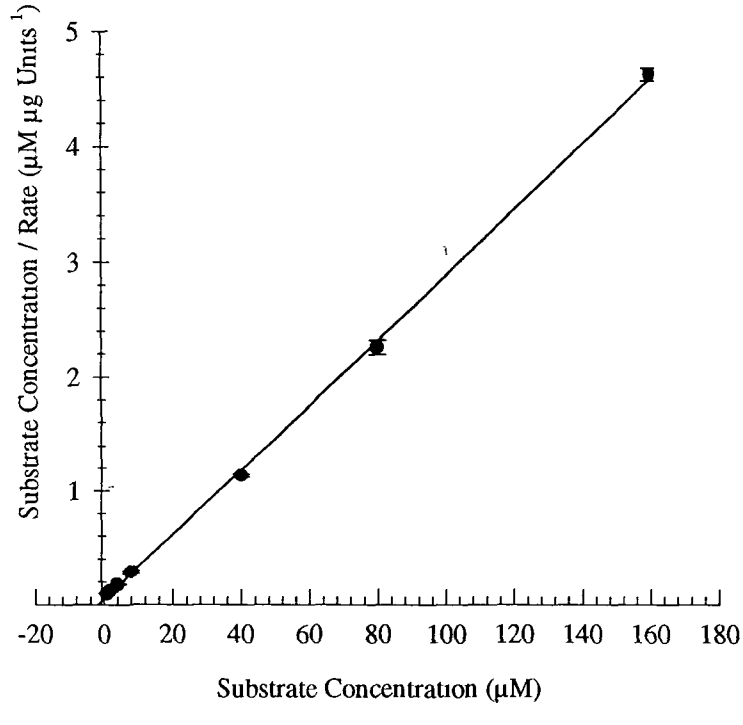


Figure 3.8.11.4 Hanes-Woolf Plot for pGlu-His-Pro-MCA.
Data was obtained as outlined in section 2 10 11 1 K_m and V_{max} values for the substrate were determined to be 1 67µM and 35,358 units/mg respectively Error bars represent the SD of triplicate readings

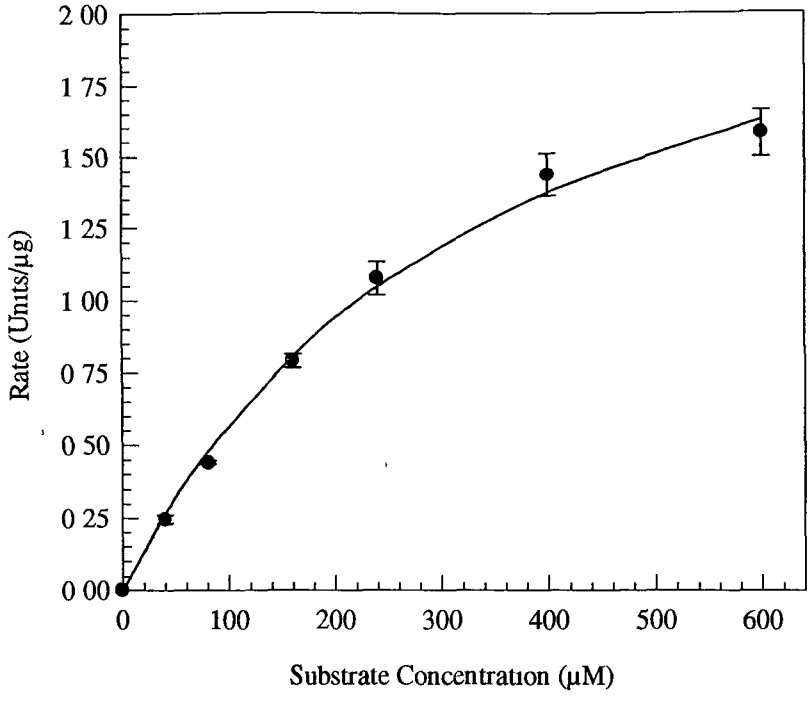


Figure 3.8.11.5 Michaelis-Menten Plot for pGlu-MCA.
Data was obtained as outlined in section 2 10 11 2 K_m and V_{max} values for the substrate were determined to be 351 4µM and 2,582 units/mg respectively Error bars represent the SD of triplicate readings

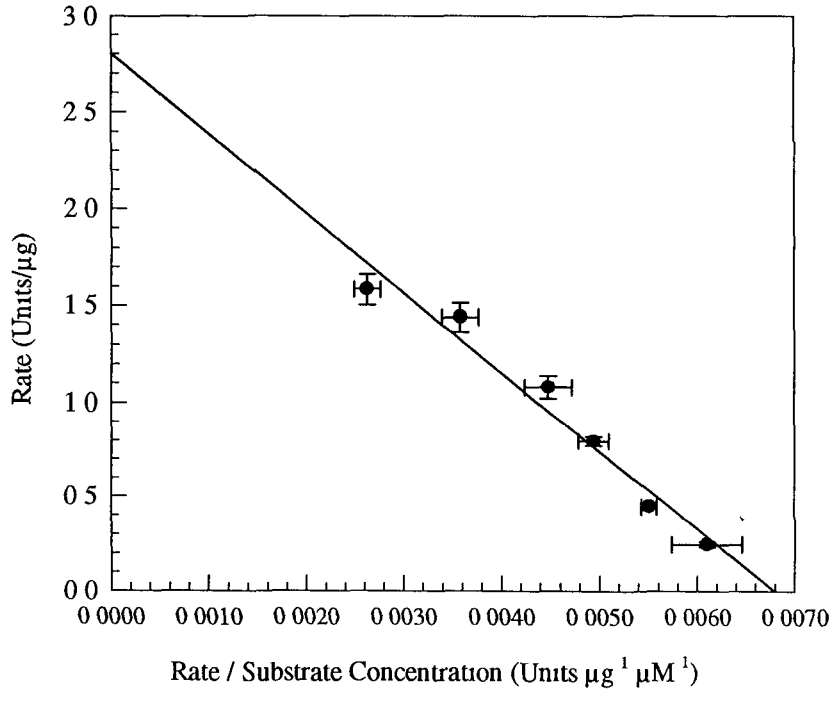


Figure 3.8.11.6 Eadie-Hofstee Plot for pGlu-MCA.
Data was obtained as outlined in section 2 10 11 2 K_m and V_{max} values for the substrate were determined to be 412 2µM and 2,801 units/mg respectively Error bars represent the SD of triplicate readings

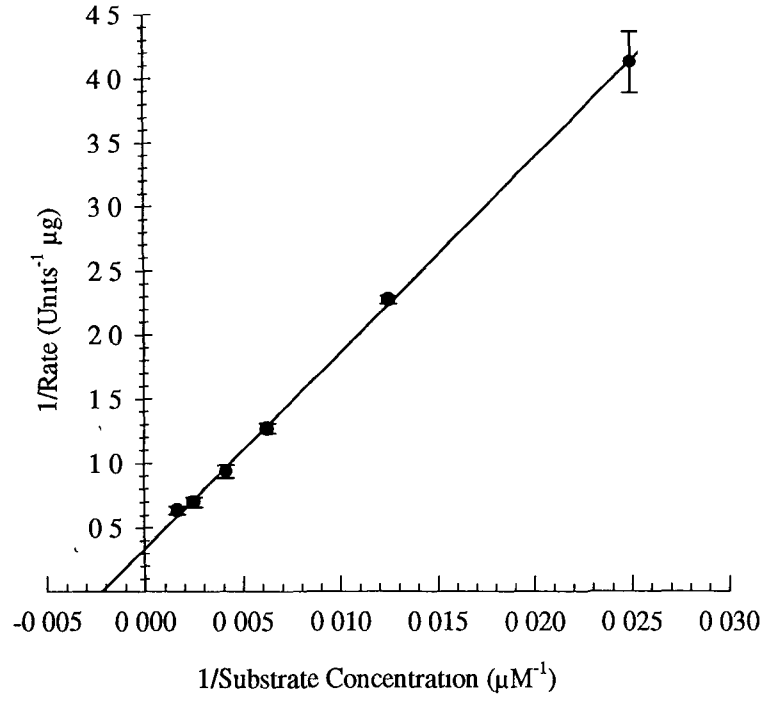


Figure 3.8.11.7 Lineweaver-Burk Plot for pGlu-MCA
Data was obtained as outlined in section 2 10 11 2 K_m and V_{max} values for the substrate were determined to be 448 8µM and 2,964 units/mg respectively Error bars represent the SD of triplicate readings

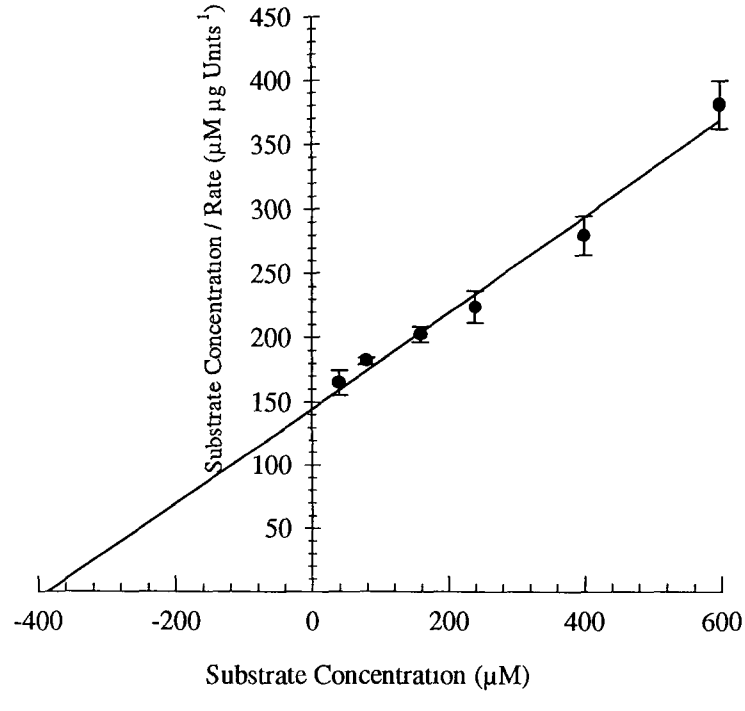


Figure 3.8.11.8 Hanes-Woolf Plot for pGlu-MCA.
Data was obtained as outlined in section 2 10 11 2 K_m and V_{max} values for the substrate were determined to be 385 4µM and 2,677 units/mg respectively Error bars represent the SD of triplicate readings

3.8.11.2 Determination of K_i values for Pyroglutamyl Peptides on PAPII Activity

The inhibitor constant (K_i) for a range of pyroglutamyl peptides on purified PAPII activity was determined as outlined in section 2 10 11 3 The data obtained was analysed using the Michaelis-Menten, Eadie-Hofstee, Lineweaver-Burk, Hanes-Woolf and Direct Linear Plots models The nature of inhibition observed, when pyroglutamyl peptides were introduced into reactions between purified enzyme and pGlu-His-Pro-MCA, was determined using the Lineweaver-Burk plot Figures 3 8 11 9 to 3 8 11 12 demonstrate the competitive nature of the inhibition observed with all of the peptides tested The K_i values for the peptides are presented in table 3 8 11 2

Peptide	Concentration (mM)	Type of Inhibition	K_i (μM)				
			M-M	E-H	L-B	H-W	DLP
None*	-	-	(2 3)	(2 5)	(2 7)	(1 1)	(2 4)
pGlu-His-Pro-OH (Acid TRH)	0 5	C	233 7	254 6	283 7	101 1	236 7
pGlu-His-Pro-NH_2 (TRH)	0 5	C	43 9	45 1	57 0	18 9	40 3
pGlu-Phe-Pro-NH_2	0 1	C	67 8	76 7	87 7	31 6	73 1
pGlu-His-Gly-OH	0 5	C	2,347	1,506	1,043	908	1,671
pGlu-His-Gly-NH_2	0 5	C	602 1	606 8	634 0	296 2	582 2
pGlu-His-Pro-Gly-OH	0 5	C	58 1	60 0	54 4	25 8	58 8
pGlu-His-Pro-Gly-NH_2	0 325	C	32 3	32 3	37 4	14 2	29 6
LHRH 1-5	0 175	C	10 1	11 3	10 3	4 7	10 8
LHRH 1-6	0 175	C	19 1	21 5	31 0	8 6	19 4
LHRH 1-7	0 175	C	61 3	61 5	69 1	28 1	60 3
LHRH	0 175	C	9 1	7 5	7 6	3 2	8 1

Table 3.8.11.2 K_i Values for a Range of Pyroglutamyl Peptides in the Reaction of Purified PAPII with pGlu-His-Pro-MCA. K_i values for a range of pyroglutamyl peptides were determined as outlined in section 2 10 11 3 Data was analysed using Michaelis-Menten (M-M), Eadie-Hofstee (E-H), Lineweaver-Burk (L-B), Hanes-Woolf (H-W) and the Direct Linear Plot (DLP) models

* The figures in brackets represent the K_m values obtained for the substrate pGlu-His-Pro-MCA in the absence of peptide / inhibitor

C Indicates competitive inhibition

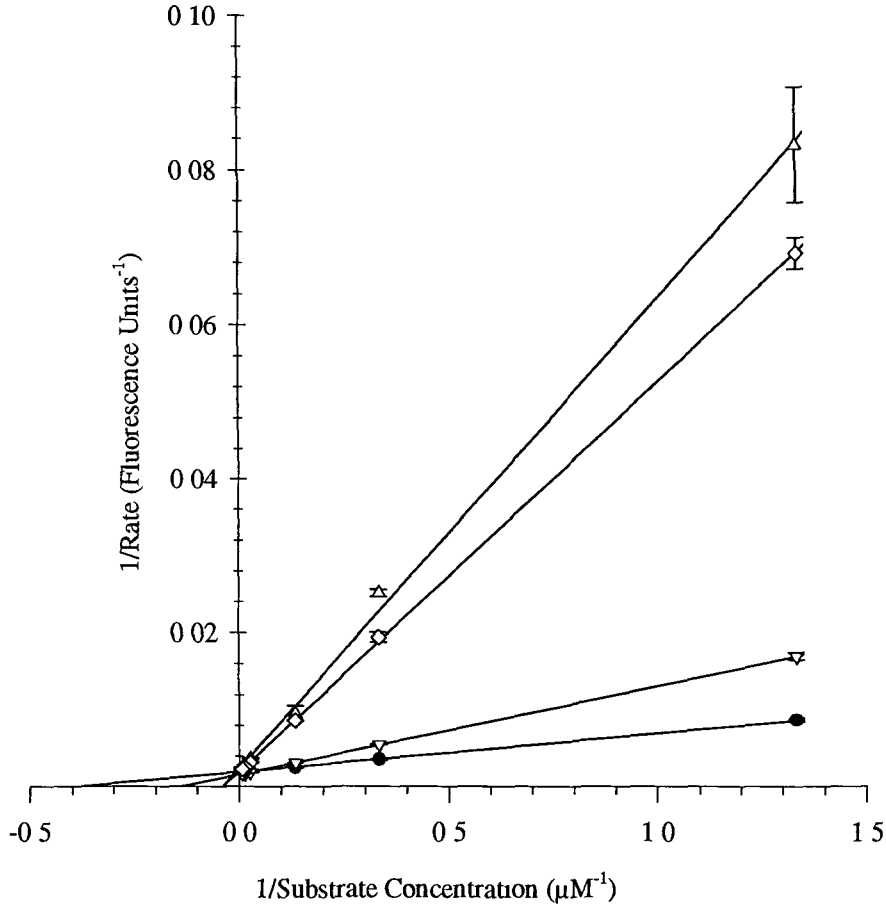


Figure 3.8.11.9 Lineweaver-Burk Plot for Pyroglutamyl Peptides
The effect of pyroglutamyl peptides on the reaction of purified PAPII with pGlu-His-Pro-MCA, was investigated as outlined in section 2 10 11 3 Lineweaver-Burk plots are presented for assays incorporating TRH (Δ—Δ), pGlu-His-Pro-Gly-NH_2 (◇—◇), acid TRH (▽—▽) or buffer (•—•) Competitive inhibition is indicated by the intersection of the lines on the Y-axis K_i values are presented in 3 8 11 2 Error bars represent the SD of triplicate readings

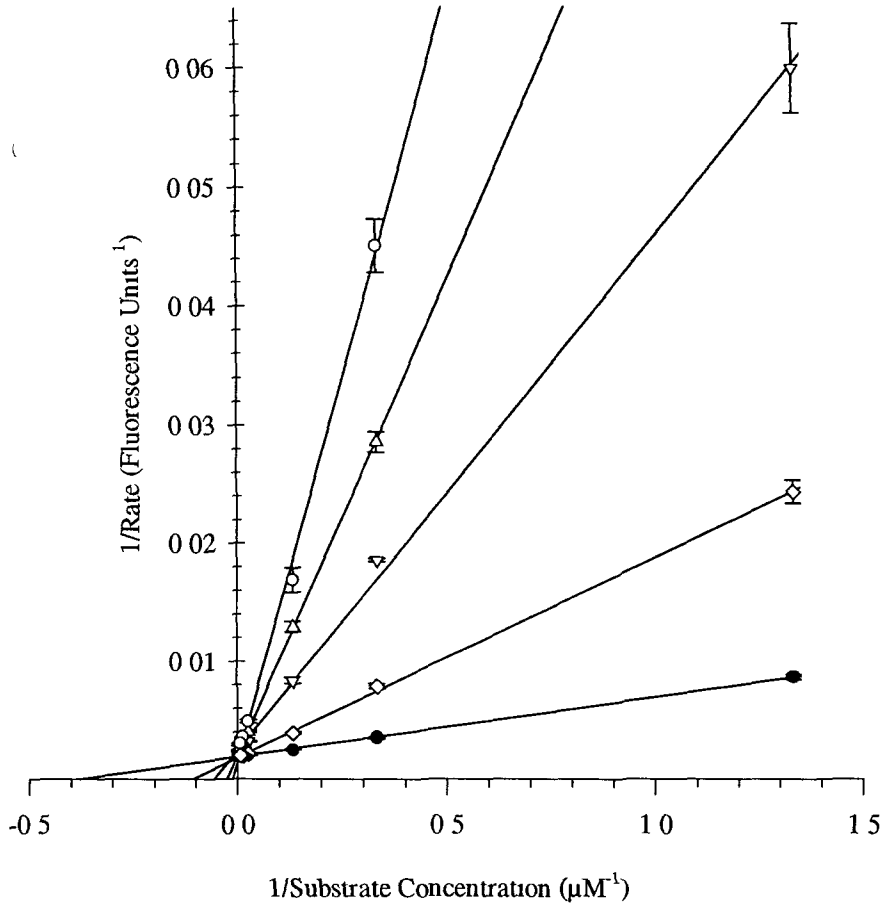


Figure 3.8.11.10 Lineweaver-Burk Plot for Pyroglutamyl Peptides.
The effect of pyroglutamyl peptides on the reaction of purified PAPII with pGlu-His-Pro-MCA, was investigated as outlined in section 2 10 11 3 Lineweaver-Burk plots are presented for assays incorporating LHRH (o—o), LHRH 1-5 (Δ—Δ), LHRH 1-6 (∇—∇), LHRH 1-7 (◇—◇) or buffer (•—•) Competitive inhibition is indicated by the intersection of the lines on the Y-axis K_I values are presented in 3 8 11 2 Error bars represent the SD of triplicate readings

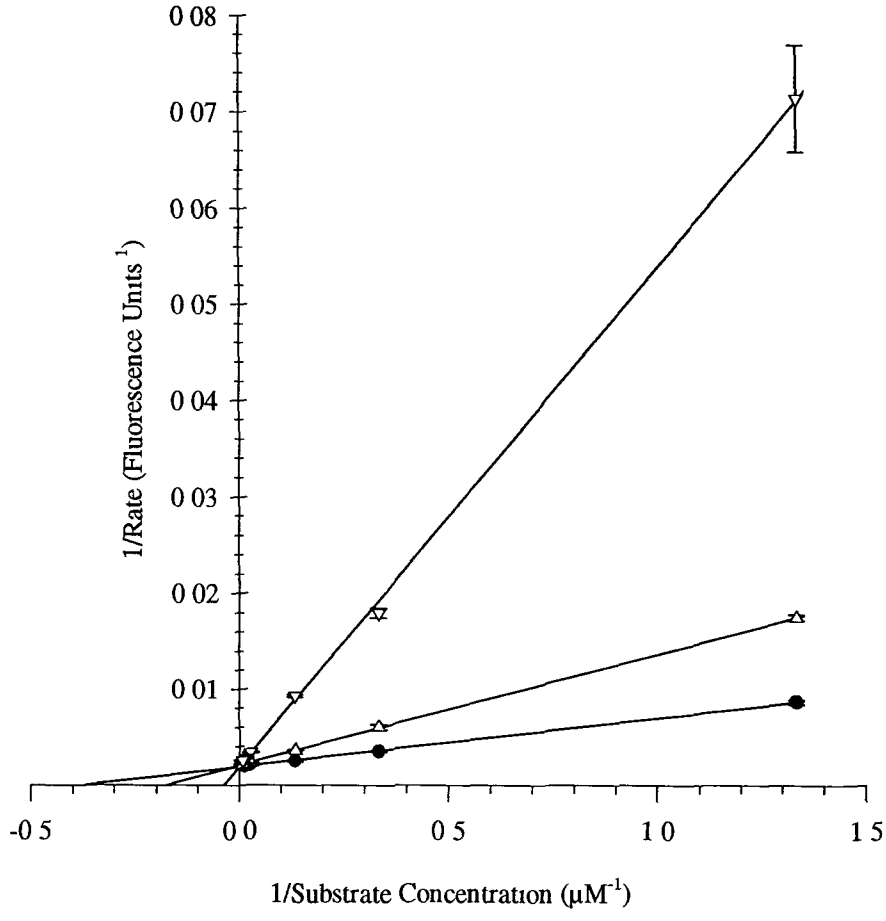


Figure 3.8.11.11 Lineweaver-Burk Plot for Pyroglutamyl Peptides.
The effect of pyroglutamyl peptides on the reaction of purified PAPII with pGlu-His-Pro-MCA, was investigated as outlined in section 2 10 11 3 Lineweaver-Burk plots are presented for assays incorporating pGlu-His-Pro-Gly-OH (∇—∇), pGlu-Phe-Pro-NH_2 (Δ—Δ) or buffer (•—•) Competitive inhibition is indicated by the intersection of the lines on the Y-axis K_1 values are presented in 3 8 11 2 Error bars represent the SD of triplicate readings

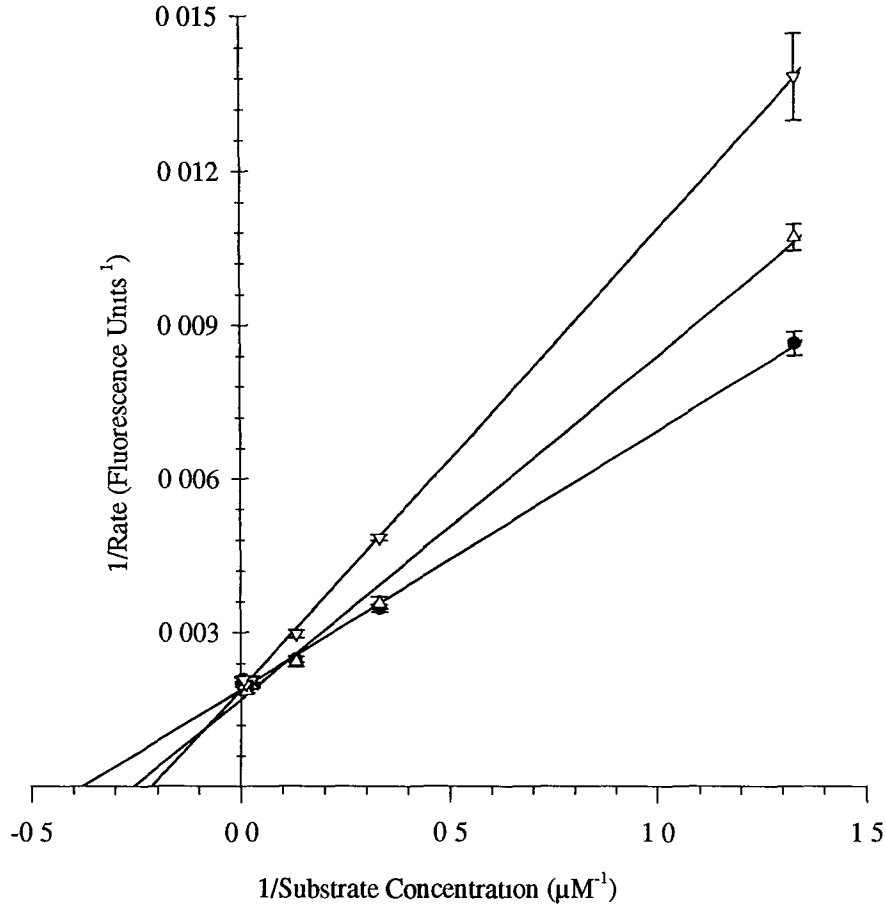


Figure 3.8.11.12 Lineweaver-Burk Plot for Pyroglutamyl Peptides.
The effect of pyroglutamyl peptides on the reaction of purified PAPII with pGlu-His-Pro-MCA, was investigated as outlined in section 2 10 11 3 Lineweaver-Burk plots are presented for assays incorporating pGlu-His-Gly-NH_2 (∇—∇), pGlu-His-Gly-OH (Δ—Δ) or buffer (•—•) Competitive inhibition is indicated by the intersection of the lines on the Y-axis K_I values are presented in 3 8 11 2 Error bars represent the SD of triplicate readings

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 samples of the standard curves obtained The inclusion of biological samples such as bovine serum or crude homogenate of bovine brain in the standards, results in a decrease in the slope of the curve, as illustrated in figures 3 1 1 and 3 1 2 This decrease is due primarily 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 the emitted radiation (440nm for MCA) before it leaves the cell Other terms used to describe this effect include *self-absorption, screening, 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 removal 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 the inner filter effect (Lloyd, 1981)

Considering the purification of DAPIV from serum illustrates an example of how failure to consider the inner filter effect, could lead to misinterpretation of results If a standard curve incorporating serum (figure 3 1 1) had not been prepared the total DAPIV activity in serum would have been calculated to be 86,128 units, 26% lower than the actual value of 116,389 units (table 3 3 1) Similarly, if a standard curve incorporating the partially purified sample had not been prepared the total DAPIV activity in this sample would have been calculated to be 69,078 units, 5 7% lower than the actual value of 73,253 units (table 3 3 1) As a result of this, an erroneous yield of 80 2% would have been determined instead of the actual yield of 62 9%

Preparation of a standard curve, consisting of 10 MCA concentrations, each in triplicate and incorporating enzyme, requires 3ml of enzyme sample Although the presence of a biological sample causes a decrease in the slope, the standard curve remains linear over the MCA concentration range (figures 3 1 1 and 3 1 2) Therefore, in order to minimise the amount of sample required, *mini-standard curves* were prepared

which incorporated only 2 standard 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 1% w/v BSA in 20mM potassium phosphate buffer at pH 7 5 could be used instead of purified PAPII containing 1% 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 Buffers have a significant effect on fluorescence, with tris / HCl at pH 7 5 causing a 13% decrease in slope when compared to a corresponding standard curve prepared in 20mM potassium phosphate buffer at pH 7 5 Decreasing the pH of the buffer used to prepare the standard curve causes a corresponding decrease in slope (see section 2 10 4) Functional reagents also contribute to the inner filter effect A decrease in the slope of 46% was observed when sample containing 10mM 8-hydroxyquinoline (0 67mM in the assay mixture) was incorporated into the standard curve

The final factor to be discussed in relation to fluorescence spectrometry 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, 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 of sensitivity, due to the optical fibres contained in the microplate reader accessory, microplate enzyme assays were used only for non-quantitative determinations The exaggerated inner filter effect observed with microplate assays is illustrated in the elution profile of DAPIV from SP-Sepharose chromatography (figure 3 3 1) The DAPIV run-through peak appears to be split into two peaks, due to drop in activity in fractions 4-8 This apparent drop in activity is in-fact due to the increased inner filter effect in these fractions as a result of their high protein concentration and/or colour

4.2 Partial Purification of Dipeptidyl Aminopeptidase Type IV (DAPIV) From Bovine Serum

DAPIV is required for the coupled enzyme assays for PAPII described in sections 2 2 1 1 and 2 9 3 Due to the fact that the enzyme is not commercially available it was necessary to purify DAPIV in the laboratory Bovine serum was chosen as the source of the enzyme since it is readily available in large quantities and is rich in DAPIV activity

As illustrated in figure 3 3 1, the majority of DAPIV activity does not bind to the SP-Sepharose column under these conditions, but merely runs through with the bulk of the protein The bulk of the Z-Gly-Pro-MCA degrading activity, however, is bound to the column and is eluted with 500mM NaCl It should be noted that two distinct Z-Gly-Pro-MCA degrading activities are represented here That which co-elutes with DAPIV can be totally inhibited by Z-Pro-Prolinal and on this basis is identified as prolyl endopeptidase The second Z-Gly-Pro-MCA degrading activity, which binds to the column, is totally insensitive to Z-Pro-Prolinal and appears to be a distinct proline specific peptidase These activities will not be discussed here since they have been studied in detail by other workers in our laboratory

Phenyl Sepharose hydrophobic interaction chromatography (figure 3 3 2) successfully removes the remaining Z-Gly-Pro-MCA degrading activity (PE) from the greater part of the DAPIV activity which, once again, runs through the column Despite the co-elution of the major DAPIV peak with the major protein peak from both columns, thus reducing the purification factors, it was decided that removal of contaminating Z-Gly-Pro-MCA degrading activity was the most important consideration An overall purification factor of only 4 9 fold was achieved using this procedure, however, a relatively high yield of 62 9% was obtained

Due to the fact that this partially purified DAPIV activity was shown to be free from aminopeptidase activity and enzymes capable of pGlu-His-Pro-MCA hydrolysis, PAP and PE, it was decided that this material was suitable for use in the coupled enzyme assays for PAPII

4.3 Determination of Optimal Conditions for the Solubilisation of PAPII from the Particulate Fraction of Bovine Brain

PAPII is an ectoenzyme, i e an integral plasma membrane enzyme with an extracellularly localised active site (Charli *et al*, 1988) Analysis of the amino acid sequence, deduced from cDNA encoding PAPII, reveals a transmembrane-spanning domain, consisting of 22 amino acids, near the amino terminus of the enzyme (Schauder *et al*, 1994) Integral membrane proteins may be classified into four groups depending on the proportion of their structure that is in contact with the hydrophobic part of the lipid bilayer (see figure 4 3) (Findlay, 1990) Class I proteins traverse the bilayer several times and have the bulk of their total structures within the hydrophobic phase Class II proteins are bound to the bilayer via a phosphotidyl inositol-carbohydrate moiety attached to the C-terminus of the polypeptide chain Class III proteins are anchored in the bilayer by a single transmembrane segment Class IV proteins are associated with the bilayer via fatty acyl and / or diacylglycerol moieties covalently attached to the N-terminus of the polypeptide chain The latter three categories contain most or all of their mass in the aqueous phase

The first step in the purification of a particular membrane protein involves the isolation of a membrane fraction enriched in the protein The next step involves the solubilisation of the protein This may be achieved usmg detergents or enzymes Detergents are amphiphilic molecules (i e they contain spatially distinct hydrophobic and hydrophilic regions) which are soluble in aqueous conditions on account of their hydrophilic regions If sufficient amounts of a suitable detergent is present , the membrane *solubilises*

into its lipid and protein components The former generally form mixed micelles with the amphiphile, while the latter exist surrounded by a monomolecular layer of the detergent which intercedes between the hydrophobic surface of the protein and the bulk aqueous medium The problems of obtaining amphiphilic proteins in aqueous solution using detergents can be circumvented in the case of proteins belonging to classes II, III and IV Proteins of this type can often be liberated from the membranes by the use of proteases or phospholipases to release the soluble hydrophilic fragment, bearing biological activity, from its protein or lipid membrane anchor, which remains behind in the bilayer

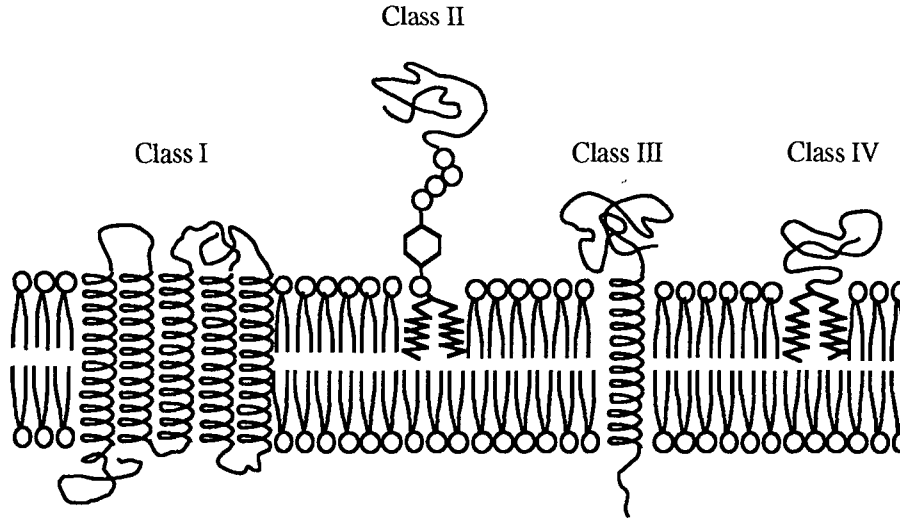


Figure 4.3 *Structural Types of Integral Membrane Proteins*

Following homogenisation of bovine brain, the particulate fraction was washed with a high ionic strength buffer (1M NaCl) to remove loosely bound peripheral proteins Subsequent washing with low ionic strength (distilled water), osmotically shocked the membranes to remove occluded protein Greater than 90% of enzyme activity was recovered following each of these steps, resulting in an overall recovery of 78 6% with only 53% of the total protein remaining These findings are in agreement with other reports which have demonstrate conclusively that PAPII is a class III ectoenzyme which, within the CNS, is located on the synaptosomal membranes (O'Connor and O'Cuinn, 1984, Horsthemke *et al*, 1984, Friedman and Wilk, 1986, Charli *et al*, 1988, Wilk and Wilk, 1989a, Bauer, 1994, Schauder *et al*, 1994, and O'Leary and O'Connor, 1995a)

It was attempted to solubilise PAPII from the washed membranes using a range of detergents and proteases The enzyme was not solubilised when membranes were incubated for 1 hour with up to 100µg/ml phospholipase C, indicating that the enzyme is not anchored by a phosphatidylinositol-glycan residue (see figure 4 3, class II protein) This is in agreement with the findings of Bauer (1994)

The detergents tested were shown to solubilise the enzyme with variable efficiency (see figure 3 4 1) At a concentration of 1%, Triton X-100 and CHAPS released 100% and 90% of enzyme activity respectively

However, it should be noted that at detergent concentrations above 0 4%, the supernatant obtained following centrifugation was not totally clear This may indicate membrane disruption rather than true solubilisation at high detergent concentrations

The enzyme was successfully solubilised using proteases (figures 3 4 2 and 3 4 3), with trypsin being the most- and chymotrypsin the least efficient Maximal solubilisation was obtained following incubation of the membranes with 5µg/ml trypsin for 1 hour at 25°C Solubilisation of the enzyme with proteases suggests that the enzyme belongs to class III integral membrane proteins as presented in figure 4 3 This agrees with the structure proposed by Schauder *et al* (1994)

The final choice of an enzyme solubilisation regime was based on two further factors, (i) the PAPII specific activity in the supernatant and (ii) the stability of the solubilised enzyme Figure 3 4 4 shows a comparison of the specific activities in supernatants obtained from various solubilisation regimes Solubilisation with trypsin results in the highest enzyme activity, lowest protein concentration and therefore, the highest specific activity The values obtained for papain are not significantly different from those for trypsin However, in the case of the three detergents tested, significantly lower enzyme activity, higher protein concentration and therefore lower specific activity is observed Sodium deoxycholate, being the worst of the detergents yields a specific activity 75% lower than that of the trypsin solubilised supernatant.

The stability of the enzyme, at 4°C and -20°C, following solubilisation by various regimes, is illustrated in figures 3 4 5 and 3 4 6 At both temperatures, the trypsin solubilised enzyme was the most stable with no loss of activity following storage at 4°C for 14 days and only 23% loss of activity after 14 days at -20°C PAPII solubilised with Triton X-100 and sodium deoxycholate was extremely unstable with 95% and 65% of activity respectively being abolished after 24 hours at -20°C In both cases, almost 50% of activity was destroyed after 24 hours at 4°C Although the enzyme solubilised by papain and CHAPS was relatively stable at 4°C, there was a marked decrease in their stability at -20°C, with greater than 50% of activity being destroyed after 3 days The instability of PAPII solubilised by detergents has been reported previously by O'Connor and O'Cuinn (1984), Wilk and Wilk (1989a) and Bauer (1994) Although non-ionic detergents, in general, have a less destructive effect on biological activity (Findlay, 1990), this was not found to be so in this case The non-ionic detergent, Triton X-100, had an equally detrimental effect on PAPII activity as the anionic detergent sodium deoxycholate and was considerably more destructive than the zwitterionic detergent CHAPS The instability of the solubilised enzyme may be due to proteolytic attack since membrane disruption is often accompanied by protease activation (Findlay, 1990) However, this is unlikely to be the case since the enzyme is less stable when frozen at -20°C where proteolytic action would not occur Although the role of phospholipids in stabilising membrane proteins is not fully understood, complete removal of lipids from biological membranes inactivates most membrane proteins, even in the presence of detergents (Jones *et al*, 1987) This problem may be overcome by the addition of 'protective' lipids to the solubilised protein and to all buffers during the purification procedure Of the detergents tested, CHAPS had the least detrimental effect on PAPII

stability This may be due to the fact that this steroid derivative has the ability to substitute for cholesterol, a lipid which may stabilise the structures of certain membrane structures (Jones *et al*, 1987), thus indicating that delipidation is the reason for the poor stability following solubilisation with detergents

On the basis of the high specific activity and adequate stability, solubilisation with trypsin was accepted as the method of choice Proteolytic enzymes have been used previously by other researchers for the solubilisation of PAPII O'Connor and O'Cuinn (1984) used papain, while trypsin has been employed by Wilk and Wilk (1989a), (4 3µg of trypsin per mg of membrane protein for 2 hours at 37°C) and Bauer (1994), (0 05µg of trypsin per mg of membrane protein for 15 minutes at 4°C) In this study, 5µg/ml trypsin (0 46µg of trypsin per mg of membrane protein) for 1 hour at 25°C was used Under these conditions 92% of enzyme activity and only 15% of total membrane protein was solubilised, thus producing a purification factor of 6 28 fold

4.4 Purification of PAPII from the Membrane Fraction of Bovine Brain

PAPII was further purified from the trypsin solubilised supernatant using a range of conventional chromatographic techniques which exploit the physiochemical properties of the enzyme The entire purification procedure was carried out at 4°C and the pH was maintained at 7 5 in order to minimise the loss of activity Adsorption processes were used early in the procedure due to their high capacity while gel-filtration, which has a much lower capacity, was used as the final step The sequence in which the chromatographic steps were arranged served to minimise the number of intermediate conditioning stages such as buffer / ionic strength changes (by dialysis) and concentration

4.4.1 Q-Sepharose High Performance Anion Exchange Chromatography

Following the application of the trypsin solubilised supernatant to a Q-Sepharose column, the bulk of the contaminating protein (86%) was not retained by the anion exchange resin while all of the PAPII activity was bound (see figure 3 5 1) Isocratic elution with 100mM NaCl resulted in excellent recovery of enzyme activity (95 4%) and a 7-fold purification factor This purification step also serves as a concentration step since it reduces the sample volume from the 78 5ml loaded, to only 22ml in the post Q-Sepharose pool, thus making the product stream easier to handle

4.4.2 Chelating-Sepharose Fast Flow Immobilised Metal Ion Chromatography

Metal chelate chromatography is a useful technique for the separation of high molecular weight and conformationally unstable biopolymers Protein sorption occurs by the coordination of surface histidine, cysteine and tryptophan residues to transition metal ions such as Cu^{2+} or Zn^{2+} (Roe, 1989) Initial attempts to purify PAPII using a chelating sepharose column charged with Zn^{2+} were only partially successful since a large portion of the activity (approximately 40%) failed to bind to the resin and merely ran through the column This run-through was not due to column overloading since increasing the size of the column or decreasing the volume of sample applied failed to resolve the problem Moreover, raising the pH of the sample and of the equilibration buffer to pH 8 0, which would be expected to promote

stronger adsorption (Roe, 1989), also failed to reduce the run-through of enzyme activity The inclusion of a small column of uncharged chelating-Sepharose, connected in series after the main column which was saturated with Zn^{2+}, successfully eliminated the run-through of activity This configuration essentially produced a 19ml column of which only the top 74% was charged with metal ions Metal chelate chromatography is not usually recommended for the purification of metalloproteins as they tend to scavenge the metal ions from the column (Pharmacia, 1993) It is likely that the second uncharged column prevents the leakage of metal ions which have been scavenged from the upper portion of the column by PAPII

As described in section 2 7 3, 150mM NaCl is included in the sample and the running buffers in order to minimise non-specific electrostatic interactions Under these running conditions PAPII activity is successfully resolved from the bulk of the contaminating protein (see figure 3 5 2) As with the ion-exchange step, a good recovery of enzyme activity (86 3%) was achieved resulting in a 5 7-fold purification factor Dialysis of the post chelating-Sepharose pool served two important functions, (i) removal of imidazole which inhibits PAPII activity in a time-dependent manner (see section 3 8 6) and (ii) preparation of the sample for calcium phosphate-cellulose chromatography

4.4.3 Calcium Phosphate-Cellulose Chromatography

Calcium phosphate resin was prepared as outlined in section 2 7 4 The addition of Na_2HPO_4 to $CaCl_2$ results in the formation of a precipitate which consists mainly of brushite (CaHPO4 $2H_2O$) At pH values above 7 0, brushite is unstable and may be transformed into hydroxylapatite ($Ca_5(PO_4)_3OH$) with the liberation of phosphoric acid This may even happen during chromatography and thus lead to complications and inconsistent chromatographic properties Treatment of brushite with ammonia causes a transformation to a more stable product which probably consists largely of hydroxylapatite (Tiselius *et al*, 1956) The material produced by this procedure is quite fine grained and must be mixed with a filter-aid such as cellulose prior to column packing in order to improve the flow characteristics of the column

The exact mechanism of protein adsorption onto calcium phosphate / hydroxylapatite is not fully understood, however, it is thought to involve both Ca^{2+} and PO_4^{3-} groups on the resin surface Since these charged groups are closely arranged on the resin it is likely that dipole-dipole interactions exist between adsorbent and protein (Roe, 1989) A drawback to the use of calcium phosphate prepared in the laboratory over commercially available hydroxylapatite is the potential for batch to batch or even laboratory to laboratory variations and therefore inconsistent chromatographic properties However, it should be noted that commercial hydroxylapatite may be inferior to fresh laboratory prepared material (Roe, 1989)

Calcium phosphate-cellulose chromatography of the post chelating-Sepharose pool, under the conditions outlined in section 2 7 5 successfully resolved PAPII activity from almost 85% of contaminating protein (see figure 3 5 3) However, a relatively low recovery of enzyme activity (61 3%) gives rise to a modest purification factor of 3 88-fold

4.4.4 Sephacryl S-200 Gel-Filtration Chromatography

Sephacryl S-200 gel-filtration chromatography was used as a final *clean-up* step in the purification procedure An un-exceptional purification factor of 2 11-fold was achieved using this technique This is due, in part, to a relatively low recovery of enzyme activity (66 4%)

An overall purification factor of 3,047-fold with a 24 2% recovery was obtained for the purification of the enzyme from bovine whole brain (see table 3 5 1) These figures compare favourably with those obtained by other investigators for the purification of PAPII O'Connor and O'Cuinn (1985) (3,558-fold purification, 17% recovery from guinea pig whole brain), Wilk and Wilk (1989a) (5,266-fold purification, 12 5% recovery from washed membranes of rabbit brain), O'Leary and O'Connor (1995a) (600-fold purification, 17% recovery from washed membranes of bovine brain) However, the purification factor is considerably lower than that obtained by Bauer (1994) (201,272-fold purification, 20% recovery) for the purification of PAPII from the washed membranes of porcine brain

4.5 Assessment of the Effectiveness of the PAPII Purification Procedure

4.5.1 Polyacrylamide Gel Electrophoresis

Multiple bands were visualised by silver staining following SDS PAGE of post Sephacryl S-200 PAPII (see figure 3 6 1) The presence of multiple bands suggests that the enzyme was not purified to homogeneity It is possible that at least some of the bands occurred as a result of fragmentation of the enzyme Bauer (1994) demonstrated a similar banding pattern following 200,000-fold purification of trypsin solubilised PAPII from porcine brain The author demonstrated that membrane solubilisation using milder conditions and subsequent purification of the enzyme resulted in a single band This proved that the enzyme had been cleaved by trypsin at several peptide bonds during solubilisation but the overall structure of the enzyme was maintained by non-covalent protein-protein interactions Treatment of washed membranes from bovine brain using milder conditions (5µg/ml for 1 hour on ice) resulted in only 20% of PAPII activity being solubilised Following enzyme purification as outlined in section 2 7 and 250-fold concentration of the purified sample, only very faint bands were visualised by silver staining following SDS PAGE There was however no reduction in the number of bands observed following electrophoresis of this sample compared to that produced by harsher trypsin treatment

Despite the failure to conclusively demonstrate that the presence of multiple bands is due to PAPII fragmentation, there is some evidence to suggest that this may be the case From the Sephacryl S-200 elution profile illustrated in figure 3 5 4, it can be seen that the PAPII pool contains only protein which eluted between 130ml and 150ml From the Sephacryl S-200 calibration curve shown in figure 3 8 1, it can be determined that proteins with an elution volume greater than 150ml have a relative molecular mass of less than 100,000 Da It is therefore reasonable to assume that, following SDS PAGE, protein bands which have molecular weights less than 100,000 Da are fragments or subunits of larger proteins More than half of the bands observed following electrophoresis of the post Sephacryl S-200 pool have such a molecular weight

4.5.2 Determination of the Enzymatic Purity of the PAPII Preparation

The enzymatic purity of the PAPII preparation obtained following Sephacryl S-200 chromatography was investigated as outlined in section 2 10 10 1 Table 3 8 10 1 illustrates that this preparation is free from contamination by PE and DAPIV, enzymes capable of primary and secondary hydrolysis of TRH and the fluorimetric substrate pGlu-His-Pro-MCA No aminopeptidase activity was detected using the substrates Ala-MCA and Pro-MCA Aminopeptidases, if present, may have led to inaccurate results when investigating the substrate specificity of PAPII It should be noted that contaminating aminopeptidase activity was detected in some BSA preparations (Sigma - A7906) Since BSA was used to stabilise the purified enzyme (see sections 3 8 2 and 3 7 6) it was important to ensure that protease free BSA (Sigma - A3294) was used for this purpose

4.6 Development of pGlu-His-Pro-MCA Based PAPII Assays

Assays based on the hydrolysis of radiolabelled TRH have been used by many researchers for the determination of PAPII activity Although sensitive and accurate for the quantitation of enzyme activity, these assays are also labour-intensive and expensive (see section 1 2 2 4) In 1986, Friedman and Wilk developed a colourimetric, coupled enzyme assay for the detection of PAPII activity, based on the cleavage of the substrate pGlu-His-Pro-2NA in the presence of excess DAPIV A modification of this assay was described by O'Leary and O'Connor (1995a), who used the quenched fluorimetric substrate pGlu-His-Pro-MCA These coupled enzyme assays are less expensive and labour-intensive than the radiolabelled assays and have the added advantage that the use of radioactivity is avoided

4.6.1 Inhibition of PE and PAPI

In order for assays based on the cleavage of TRH or the TRH substrate analogue, pGlu-His-Pro-MCA, to be specific for PAPII, it is important to ensure that other enzymes capable of its hydrolysis (PE and PAPI) are inhibited under assay conditions Figures 3 7 1 1 and 3 7 1 2 illustrate the total inhibition of PE by the specific inhibitors Z-Pro-Pro-Prolinal and fmoc-Pro-Pro-Nitrile respectively Figure 3 7 2 illustrates the inhibition of PAPI by 2-pyrrolidone The inhibition of PAPI is particularly important when assaying crude samples which contain high levels of the cytosolic enzyme Washed membrane preparations and further purified samples are less likely to contain PAPI activity, however, bacterial contamination of samples, thereby resulting in the presence of the bacterial PAP has been observed (results not shown)

4.6.2 Development of Coupled Enzyme Assays for PAPII

Two variations of the pGlu-His-Pro-MCA based coupled enzyme assay were used in the course of this work In the first, described in section 2 2 1 1, DAPIV is included in the assay mixture at the same time as the PAPII containing sample This procedure is similar to that described by Friedman and Wilk (1986) and assumes that as His-Pro-MCA is being produced by the action of PAPII on the substrate, DAPIV

simultaneously catalyses the liberation of MCA from this metabolite, thus allowing PAPII activity to be quantified fluorimetrically This assay represents a fast and simple method for the detection of enzyme activity The microplate assay described in section 2 2 1 1, which used this methodology, proved to be especially useful for the detection of PAPII activity in the large number of fractions generated during the development of the purification procedure

The second variation of the coupled enzyme assay is described in section 2 9 3 In order to ensure that MCA is liberated from all of the His-Pro-MCA produced by the action of PAPII, the two enzyme reactions were performed separately Following the termination of the PAPII reaction by heating the reaction mixture to 80°C, DAPIV was added to catalyse the liberation of MCA from His-Pro-MCA This reaction was subsequently terminated by the addition of acetic acid This assay procedure is a modification of that described by O'Leary and O'Connor (1995a)

Figures 3 7 3 1 and 3 7 3 2 illustrate the effect of increasing the amount of DAPIV added following the reaction of crude homogenate or purified PAPII with pGlu-His-Pro-MCA The addition of 25µl of DAPIV is sufficient to catalyse the liberation of all MCA following the reaction of crude homogenate with the substrate The DAPIV reaction increases the concentration of MCA by only 4% The liberation of such a high concentration of MCA without the addition of DAPIV can be explained by the fact that crude homogenate of brain contains relatively high levels of endogenous DAPIV and DAPII activity (see section 1 3 8)

Following the reaction of purified PAPII with pGlu-His-Pro-MCA, 250µl of DAPIV must be added to catalyse the complete hydrolysis of His-Pro-MCA Since the purified PAPII preparation contains no DAPIV activity (see section 4 5 2), it is likely that the low concentration of MCA observed when no exogenous DAPIV is added, occurs as a result of non-enzymatic cyclisation of His-Pro-MCA to cyclo His-Pro and free MCA during the two hour incubation

4.6.3 Development of a Non-Enzymatic Cyclisation Assay for PAPII

Although the coupled enzyme assays described have distinct advantages over radiolabelled assays, there are nonetheless some disadvantages As mentioned previously, since DAPIV is not commercially available it must therefore be purified in the laboratory Moreover, the use of a coupled enzyme assay may cause complications in some of the PAPII characterisation studies For instance, in the investigation of the effect of pH, functional reagents or metals on enzyme activity or in the determination of K_i values of peptides, it may be difficult to determine whether an observed result is due to the effect on PAPII, DAPIV or both For these reasons in was decided to develop a fluorimetric assay based on the non-enzymatic cyclisation of His-Pro-MCA to cyclo His-Pro and free MCA

The non-enzymatic cyclisation of His-Pro-NH_2 to cyclo His-Pro has been shown to occur in aqueous solution at pH 2-10 at 37°C (Moss and Bundgaard, 1990) Following the termination of the PAPII

reaction by the addition of acetic acid, the cyclisation of His-Pro-MCA was promoted by heating the incubation mixture at 80°C The effect of incubation time at 80°C on MCA release, following the reaction of crude homogenate or purified PAPII with pGlu-His-Pro-MCA, is illustrated in figures 3 7 4 1 and 3 7 4 2 Following the reaction of crude homogenate with the substrate, only a short incubation of 15-20 minutes at 80°C is required to promote the complete cyclisation of His-Pro-MCA An increase in the MCA concentration of only 10% was observed as a result of this incubation, indicating that 90% of His-Pro-MCA was already hydrolysed by endogenous DAP activity in this sample

An incubation of 2 hours at 80°C was required for the complete cyclisation of His-Pro-MCA produced by the reaction of purified PAPII with pGlu-His-Pro-MCA In the absence of endogenous DAP activity in this purified sample, the extremely low concentration of MCA observed when the reaction mixture was not incubated at 80°C can be accounted for by a slow rate of cyclisation of His-Pro-MCA during the initial reaction at 37°C

The possibility of shortening the incubation time necessary for the complete cyclisation of His-Pro-MCA was considered In view of the fact that the cyclisation of His-Pro-NH_2 has been shown to occur more rapidly at pH 6-7 (Moss and Bundgaard, 1990), the possibility of terminating the PAPII reaction by means other than the addition of acetic acid, which reduces the pH of the reaction mixture to approximately 2 2, was considered The PAPII reaction could be terminated by transferring the reaction mixture directly to an 80°C water-bath where the cyclisation reaction could proceed at pH 7 5 However, this option was not pursued further since it was decided that the addition of acetic negated the possibility of inter-sample pH variations affecting the rate of cyclisation Slight pH variations within the pH range 6-8 have profound effects on the rate of cyclisation of His-Pro-NH_2 while slight pH variations within the pH range 2-3 have little or no effect on the rate of cyclisation (Moss and Bundgaard, 1990) Moreover, it was shown that the incubation time at 80°C could be shortened from 2 hours to 30 minutes with no loss of assay-linearity with respect to enzyme concentration (section 3 7 6)

4.6.3.1 Linearity of the Non-Enzymatic Cyclisation Assay with respect to Time and Enzyme Concentration

The progress curves of most enzyme reactions are of the form shown in figure 4 6 3, in which the velocity decreases with time Various factors may contribute to this decrease For instance, (i) the products of the reaction may inhibit the enzyme, (ii) the degree of substrate saturation of the enzyme may decrease due to a fall in substrate concentration as the reaction proceeds, (iii) the reverse reaction may become more important as the product concentration increases or (iv) the enzyme may undergo some inactivation at the temperature or pH of the reaction Enzyme activity is therefore usually quantified by measuring the initial velocity of the reaction It is only at the initial point that the conditions are known and the factors just mentioned have not yet had time to operate (Dixon and Webb, 1979a) The initial velocity of the reaction can be obtained simply by drawing a tangent, through the origin, to the early, linear part of the progress curve as illustrated in figure 4 6 3 Due to the nature of the assays described for

the detection of PAPII activity, both of which require a second step after the initial reaction of the enzyme with the substrate, continuous monitoring of activity is not possible These methods therefore represent discontinuous assays in which the amount of product formed by the enzyme is measured at one discrete point on the reaction curve With such assay systems it is important to ensure that the measurement is taken from the linear part of the progress curve thereby representing the initial velocity of the reaction

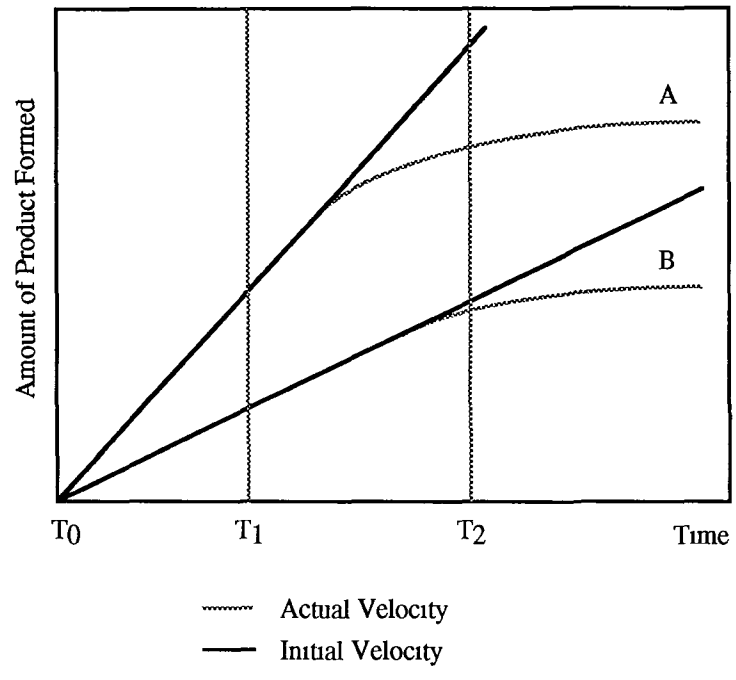


Figure 4.6.3 *Schematic of progress curves with two different amounts of enzyme (A and B)*

The progress curves, A and B, illustrated in figure 4 6 3 might represent experiments at two different pHs or with two different substrates or with two different amounts of enzyme Assuming, for simplicity, that the curves represent two different amounts of enzyme Single point measurement of product formed at time T_1 gives a true indication of the initial activity of both samples since the amount of product formed is proportional to the amount of enzyme present The amount of product formed at time T_2, however, is far from being proportional to the amount of enzyme present in either sample The apparent velocities, obtained by dividing these values by the time (T_2), give quite misleading results Not only would the absolute values for the activity of each sample be incorrect, but the apparent activity of each sample would also be disproportionate

Progress curves of PAPII activity against the substrate pGlu-His-Pro-MCA are illustrated in figures 3 7 5 1 and 3 7 5 2 These curves illustrate that whether crude homogenate or purified PAPII is used as the enzyme source, the reaction proceeds in a linear fashion over a 2 hour period It is therefore safe to assume that the assays described in sections 2 2 1, 2 9 3 and 2 9 7 give a true measurement the initial activity of PAPII since an incubation time of 1 hour is employed

In order to ensure that the non-enzymatic cyclisation assay is quantitative for the measurement of PAPII activity, standard curves of enzyme concentration versus MCA released, were constructed Figure 3 7 6 1 illustrates a non-linear relationship between MCA released and the concentration of post Sephacryl S-200 PAPII An upward curvature such as this is usually indicative of the presence of an activator Dilution of the enzyme sample reduces the concentration of the activator below that at which optimal enzyme activity is expressed (Dixon and Webb, 1979b) In this case protein can be considered to be the enzyme activator or perhaps more correctly, the absence of protein-protein interactions in the dilute sample causes enzyme inactivation The presence of 1% w/v BSA in the enzyme results in a linear relationship between product formed and enzyme concentration as illustrated in figures 3 7 6 2 and 3 7 6 3

In view of the facts presented here the non-enzymatic cyclisation assays described in section 2 9 7 were deemed to be suitable for the quantitative determination of PAPII activity A 2 hour incubation period at 80°C was employed for studies which required *absolute* values of PAPII activity such as construction of the purification table or kinetic studies for the determination of V_{max} values Where *relative* values of enzyme activity were required, the shorter assay in which the reaction mixture is incubated for 30 minutes at 80°C, was employed

4.6.4 Development of a Continuous PAPII Assay

Although a number of investigators have reported that the PAPI substrates pGlu-ßNA, pGlu-pNA and pGlu-MCA are not cleaved by PAPII (O'Connor and O'Cuinn, 1985, Wilk and Wilk, 1989a), it has recently been demonstrated that the purified enzyme is capable of pGlu-ßNA hydrolysis (Czekay and Bauer, 1993, Bauer, 1994) The results presented in section 3 8 10 2 clearly illustrate that the purified bovine brain enzyme is capable of cleaving pGlu-ßNA and pGlu-MCA, even in the presence of the PAPI inhibitor 2-pyrrolidone Hydrolysis of the colourimetric substrate pGlu-pNA was not observed It is possible that the failure to detect hydrolysis of this substrate can be attributed to the lower sensitivity of the spectrophotometric method employed for the detection of pNA compared to the fluorimetric methods of ßNA and MCA detection

The ability of the enzyme to hydrolyse these substrates offers a simple method to continuously monitor PAPII activity It should be noted that these assays can only be used to detect enzyme activity in relatively pure samples which contain high levels of PAPII activity, and are much less sensitive than the aforementioned pGlu-His-Pro-MCA based assays This fact can be explained by the kinetic characteristics of the enzyme for the substrates pGlu-His-Pro-MCA and pGlu-MCA, presented in section 3 8 11 1 and summarised in table 3 8 11 1 For reasons which will be discussed in section 4 7 9, the K_m and V_{max} values referred to here will be those derived from the Direct Linear Plot The affinity of the enzyme for pGlu-MCA is 165-times lower than for pGlu-His-Pro-MCA (K_m values of 420µM and 2 5µM respectively) Furthermore, the V_{max} value for pGlu-MCA (2,815 U/mg protein) is almost 13-times lover than that of pGlu-His-Pro-MCA (36,144 U/mg protein) These two factors combined, indicate that pGlu-MCA is an extremely poor substrate for the enzyme

4.7 Characterisation of PAPII

4.7.1 Stability of PAPII under Various Storage Conditions

Purified bovine brain PAPII appears to be less stable than PAPII purified from other sources Bauer (1994) reported that, irrespective of protein concentration, the purified porcine brain enzyme remained fully active for more than 2 months at 4°C In the absence of stabilising agents the bovine brain enzyme is extremely labile with more than 50% of activity being abolished after only 24 hours at 4°C (see figure 3 8 2) Freezing the enzyme at -20°C destroys 90% of activity while freezing at -80°C results in total loss of activity A similar effect was observed by Bauer (1994) who reported that at protein concentrations below 1mg/ml the porcine brain enzyme was sensitive to freezing and thawing

The addition of 20% v/v glycerol affords only moderate protection to the enzyme upon freezing and little or no stabilising effect when stored at 4°C This finding contrasts with those of O'Connor and O'Cuinn (1985) who reported the purified guinea pig brain enzyme to be extremely stable at -20°C or 15°C in the presence of 30% glycerol Bauer (1994) demonstrated that in the presence of 25% glycerol, sucrose or trehalose, more than 90% of activity could be recovered after freezing and thawing the porcine brain enzyme at -80°C

The inclusion of 1% w/v BSA in the purified enzyme preparation greatly improves the stability of the bovine brain PAPII at each of the storage temperatures tested After 3 weeks at -80°C the BSA containing sample retained 93% of the initial activity A similar effect of BSA on the stability of the enzyme upon freezing and thawing was reported by Bauer (1994) The improved stability of the purified enzyme in the presence of BSA lends credence to the suggestion that protein-protein interactions are important for the stability / activity of PAPII (see section 4 6 3 1)

4.7.2 Relative Molecular Mass Determination

The relative molecular mass of PAPII was determined via gel-filtration chromatography as outlined in section 2 10 1 The results of this study are presented in section 3 8 1 A relative molecular mass of approximately 214,000 Da was determined for the trypsin solubilised enzyme This value is slightly lower than estimates of 230,000 - 240,000 Da reported for previously characterised PAPIIs (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994, O'Leary and O'Connor, 1995a) There are a number of possible explanations for this discrepancy Following solubilisation of the enzyme with trypsin, the molecular mass presented here represents that of a truncated enzyme since the transmembrane spanning domain and intracellular domain have been cleaved from the extracellular part of the enzyme This is an attractive hypothesis when the value obtained here is compared to a value of 240,000 Da obtained by O'Leary and O'Connor (1995a) for the intact, Triton X-100 solubilised enzyme from bovine brain However relative molecular weights of approximately 230,000 Da have been reported by other investigators for protease solubilised PAPII (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994)

It is possible that the lower molecular mass obtained in this study is a reflection on the inaccuracies of the method used Close examination of the Sephacryl S-200 calibration curve illustrated in figure 3 8 1 and the calibration equation presented in section 3 8 1, reveals that a 1ml difference in the elution volume of a protein results in a difference of approximately 13,000 Da in the estimation of its molecular mass In view of this relatively large potential for error, the relative molecular mass of the enzyme presented here does not differ considerably from previous estimates

As mentioned previously in section 4 5 1, SDS PAGE analysis of the purified PAPII preparation resulted in the visualisation of multiple bands, thereby making it impossible to determine the subunit structure of the enzyme It is clear however, that the enzyme does not exist as a monomer since the highest molecular weight band visualised was at 141,000 Da (see section 3 6) Suen and Wilk (1990) reported a 48,000 Da subunit structure of PAPII from Y-79 human retinoblastoma cells Bovine brain PAPII does not appear to contain such a subunit since the lowest molecular weight band visualised was at 55,000 Da Bauer (1994) demonstrated that the porcine brain enzyme exists as a dimer consisting of two identical subunits of 116,000 Da The possibility that bovine brain PAPII exists as a dimer is supported by the visualisation of a band with a molecular weight of approximately 114,000 Da following SDS PAGE of the purified enzyme

4.7.3 The Effect of Assay Temperature on PAPII Activity

The effect of assay temperature on PAPII activity was investigated as outlined in section 2 10 3 Samples were preincubated for 2 minutes at each temperature prior to assay in order to ensure that the observed activity genuinely reflected the activity of the enzyme at the temperature under investigation Failure to preincubate the samples would result in the observed activity representing the activity as the sample reached thermal equilibrium and the activity at the temperature under investigation The assay time was shortened to 15 minutes in order to ensure that the measurement of enzyme activity represented the initial activity of the sample (see figure 4 6 3) The results of this study are presented in section 3 8 3 Optimal activity was observed at 45°C The activity at 45°C was approximately 30% higher than at 37°C At 55°C the enzyme is partially inactivated, while 2 minutes preincubation at 60°C results in total inactivation of the enzyme This temperature profile is similar to that observed for serum PAP (Bauer and Nowak, 1979)

In addition to displaying optimal activity at 45°C, the enzyme was shown to be stable at this temperature for at least 1 hour This is illustrated by the progress curve in figure 3 8 3 2 which demonstrates a linear relationship between enzyme activity and reaction time at 45°C

4.7.4 The Effect of pH on PAPII Activity

The effect of pH on PAPII activity was investigated as outlined in section 2 10 4 The results of this study are presented in section 3 8 4 A narrow pH optimum of 6 8-7 6 was observed for the enzyme as

illustrated in figure 3 8 4 1 A similar pH optimum of 7 0-7 5 was reported for the rabbit brain enzyme by Wilk and Wilk (1989a,b) Within this pH range, highest activity was observed using the citric acid/potassium phosphate buffering system, with only slightly lower activity being observed in potassium phosphate buffer At pH 7 0, the activity of the enzyme in tris/HCl was only 50% of that in citric acid/potassium phosphate buffer This low activity may reflect a genuine preference of the enzyme for citric acid/potassium phosphate buffer over tris/HCl or it may be caused by the effect of the buffers on the rate of His-Pro-MCA cyclisation during the assay The latter possibility is supported by the observation that His-Pro-NH_2 cyclisation proceeds at a faster rate in the presence of phosphate buffer than in the presence of tris, acetate, or borate buffers (Moss and Bundgaard, 1990) Further investigation is necessary to determine the reason for the difference in the levels of activity, however, it is clear that in each of the buffering systems highest enzyme activity is observed in the pH range 6 8-7 6

The pH inactivation curve of PAPII is presented in figure 3 8 4 2 This curve differs from the pH activity profile in that the former is prepared by preincubating the enzyme at a wide range of pH values and performing the assay at the optimum pH while the latter is prepared by preincubating and assaying the enzyme at a range of pH values From the pH inactivation curve it can be seen that the enzyme is almost totally inactivated below pH 4 0 Enzyme inactivation also occurs above pH 9 2 and exposure to pH 10 5 results in almost total inactivation of the enzyme

By comparing the two curves it can be deduced that the observed pH activity profile is due to kinetically important ionisations rather than the effect of pH on enzyme structure Although pK_as are dependent on temperature, ionic strength and microenvironment of the ionisable group, the pH activity profile indicates the presence of a critical group which must be in the basic form for activity ($pK_a \approx$ 6 0), possibly due to imidazole groups (histidine) and a group which must be in the acidic form for activity ($pK_a \approx$ 8 5), possibly due to hydroxyl groups (tyrosine or serine) (Dunn, 1989) The presence of histidine and tyrosine residues as catalytically important elements of PAPII has been clearly demonstrated by O'Connor and O'Cuinn (1987) and Czekay and Bauer (1993)

4.7.5 The Effect of Functional Reagents on PAPII Activity

The effect of functional reagents on PAPII activity was investigated as outlined in section 2 10 5 The pH of all stock solutions was adjusted to pH 7 5 in order to ensure that any observed effects were not simply due to pH effects on the enzyme The results of this study are presented in section 3 8 5

The cysteine protease inhibitors had a slightly inhibitory effect on enzyme activity The cysteine protease activators were also found to inhibit PAPII activity A significant inhibition of 48% was observed with 10mM DTT The mild inhibition of the enzyme by cysteine protease inhibitors and the inhibition by DTT is consistent with previously characterised PAPIIs (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994, O'Leary and O'Connor, 1995a), and clearly demonstrates that this enzyme is not a member of the family of cysteine proteases

The effect of two serine protease inhibitors on PAPII activity yielded conflicting results In agreement with previous reports (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994, O'Leary and O'Connor, 1995a), PMSF was shown to have no inhibitory effect on enzyme activity AEBSF however, was found to be a potent inhibitor of the enzyme resulting in 63% and 97% inhibition at 1mM and 10mM respectively Further work is necessary to determine the mechanism of inhibition with this reagent.

Since 2-pyrrolidone was used in the PAPII assays, it is important to note that, at a concentration of 50mM, this compound did not significantly inhibit the enzyme The microbial protease inhibitors bacitracin and benzamidine produced no significant effect on enzyme activity while, in agreement with the findings of O'Connor and O'Cuinn (1984), puromycin was found to be inhibitory

4.7.6 The Effect of Metal-Complexing Agents on PAPII Activity

PAPII has recently been identified as a zinc metallopeptidase (Czekay and Bauer, 1993, Schauder *et al*, 1994) Initial studies on the inhibition of the purified enzyme by metal chelators were carried out as outlined in section 2 10 5 The results of this study are presented in section 3 8 5 At a concentration of 1mM, only 8-hydroxyquinoline had a significant inhibitory effect of 14% on the enzyme At a concentration of 10mM, no inhibition by imidazole or EDTA was observed and only modest inhibition of 10% and 29% was observed with CDTA and EGTA respectively Strong inhibition of 68% was observed with 8-hydroxyquinoline at a concentration of 10mM Exposure to the chelator 1,10-phenanthroline at this concentration resulted in total inhibition of the enzyme However, the non-chelating compounds 1,7-phenanthroline and 4,7-phenanthroline, also resulted in strong inhibition of the enzyme

The degree of enzyme inhibition by metal chelators is considerably lower than that reported previously for PAPII (O'Connor and O'Cuinn, 1984, Wilk and Wilk, 1989a, Bauer, 1994, Czekay and Bauer, 1994, O'Leary and O'Connor, 1995a) It should be noted that the results obtained in this study relate specifically to the method employed thereby making direct comparison with previous studies difficult For instance, a pH of 7 5 was maintained throughout these experiments A lower pH would favour metal removal owing to competition between H^+ ions and metal ions for the enzyme ligands, therefore stronger inhibition by metal chelators would be observed (Auld, 1995) It is also possible that the presence of 1%w/v BSA in the enzyme preparation affords some protection to the enzyme from these compounds

To further investigate the effect of metal chelators and the non-chelating phenanthrolines, their effect on enzyme activity was monitored over time as outlined in section 2 10 6 The results of this study are presented in section 3 8 6 It can be seen from figures 3 8 6 1 and 3 8 6 2 that inhibition of the enzyme by the chelators occurs in a time-dependent manner while inhibition by the non-chelating 1,7-phenanthroline and 4,7-phenanthroline is independent of time A time-dependent inhibition of

metallopeptidases by chelators is not unprecedented and has been previously reported for many enzymes, including PAPII (Czekay and Bauer, 1993) These authors also reported the inhibition of the enzyme by the non-chelating phenanthrolines to be independent of time They therefore suggested that this inhibition was due to non-specific hydrophobic interactions of the enzyme with the aromatic structures of these compounds It is interesting that in the initial study which employed a 15 minute preincubation of enzyme with the test compound, 8-hydroxyquinoline and 1,10-phenanthroline were found to be the most inhibitory chelators (see table 3 8 5) However, following 24 hours preincubation, the chelators CDTA, EDTA and EGTA resulted in the strongest inhibition of PAPII activity (see figures 3 8 6 1 and 3 8 6 2) The initial, rapid inhibition of the enzyme by 8-hydroxyquinoline and 1,10-phenanthroline may occur as a result of the aforementioned non-specific hydrophobic interactions of the enzyme with the aromatic structures of these compounds, with the subsequent time-dependent inhibition resulting from their metal-complexing abilities It is also possible that the initial inhibition occurs as a result of the formation of a ternary enzyme / metal / chelator complex which is followed by dissociation of the chelator-bound metal in a time dependent manner This mode of inhibition by 1,10-phenanthroline has been observed for other enzymes including astacin (Stocker *et al*, 1988) In the absence of chelators, the half life of metal release from enzymes can vary from seconds to years, for example angiotensin converting enzyme $t_{1/2}$ = 36 seconds, carbonic anhydrase $t_{1/2}$ = 3 years (Auld, 1995) This fact explains the time-dependent inhibition of metallopeptidases by chelators

While EDTA is a universal complexing agent which chelates most divalent ions, EGTA is reported to be specific for Ca^{2+} 1,10-phenanthroline has a much higher affinity for Zn^{2+} than for Ca^{2+} and inhibition of an enzyme by this compound is usually indicative of a zinc metallopeptidase (Salvesen and Nagase, 1989) Since PAPII is inhibited by all of these chelators, further studies were necessary to confirm the identity of the metal at its active site

4.7.7 The Effect of Metal Ions on PAPII Activity

The effect of metal ions on PAPII activity was investigated as outlined in section 2 10 7 This study was performed at pH 7 0 since many of the metal salts were found to be insoluble at higher pH values No single buffer was found to be suitable for the dissolution of all of the metal salts, therefore tris and potassium phosphate buffers were used as described in table 2 10 7 Since all attempts to dissolve $FeSO_4$ at pH 7 0 were unsuccessful, $FeCl_3$ was used in its place Sulphate salts of all other metals were used so as to ensure that observed effects were due to metal ions rather than their counterions The results of this study are presented in section 3 8 7 The enzyme was not affected by Ca^{2+}, Fe^{3+} or Mn^{2+}, however, significant inhibition was observed following exposure to Zn^{2+}, Hg^{2+} and Cd^{2+} Inhibition by Zn^{2+} and other transition metals has been reported for most metallopeptidases (Auld, 1995), including PAPII (Wilk and Wilk, 1989a, Bauer, 1994, Czekay and Bauer, 1994)

Exposure of the enzyme to Ni^{2+} caused a slight increase in PAPII activity while exposure to 1mM Co^{2+} caused an increase in activity to more than 5-times that in the control Cobalt has been shown to be the

one metal that substitutes for zinc in carboxypeptidases, endopeptidases and aminopeptidases In some cases, cobalt has been shown to cause up to a 10-fold enhancement of the activity of zinc metallopeptidases (Auld, 1995) In 1993, Czekay and Bauer reported that the addition of 15μM $CoSO_4$ to purified PAPII resulted in a slow increase in the reaction rate to 170% of the control In a subsequent report, Bauer (1994) showed the enzyme to be inhibited by 71% following 15 minutes preincubation with 1mM $CoSO_4$ This inhibition with 1mM $CoSO_4$ contrasts sharply with the large increase in activity observed under similar conditions in this study It is possible that the presence of 1% w/v BSA in this enzyme preparation necessitates a higher concentration of the metal (mM rather than μM range) to enhance enzyme activity

The effect of metal ions on EDTA-treated PAPII was investigated as outlined in section 2 10 8 Figure 3 8 8 illustrates that the enzyme was only partially reactivated in the presence of Ca^{2+} or Mn^{2+} while full enzymatic activity could be restored by the addition of 1mM $ZnSO_4$ As expected from previous studies on the native enzyme, Co^{2+} or Ni^{2+} were shown to be capable of restoring activity to levels greater than the control value These results correlate well with previous PAPII reactivation studies by Czekay and Bauer (1993) with the exception that these authors showed Zn^{2+} to be more efficient in the reactivation of the enzyme than Ni^{2+} This inconsistency may be explained by the differences in the methodologies used As outlined in sections 2 10 8, following the inactivation of the enzyme with EDTA, the chelator was removed by gel filtration Addition of metal salts to the EDTA free enzyme ensures that only the ability of the metal ion to reactivate the enzyme is reflected in the resulting activity In their study, Czekay and Bauer inhibited the enzyme in the presence of EDTA and subsequently added an equimolar amount of the metal under investigation Under these conditions, the observed effect on enzyme activity reflects both the affinity of EDTA for the metal and the ability of the metal to reactivate the enzyme Since the binding strength of EDTA for Ni^{2+} has been shown to be stronger than for Zn^{2+} (Auld, 1995), the concentration of Ni^{2+} available to the enzyme would be lower that of Zn^{2+} It is possible that the relative abilities of these metals to reactivate the enzyme is not truly represented

4.7.8 The Effect of DMSO on PAPII Activity

DMSO is necessary for the dissolution of the substrate pGlu-MCA (see sections 2 2 2 and 2 10 10 1) but is not necessary for the dissolution of pGlu-His-Pro-MCA (see section 2 2 1) Before direct comparisons could be made between the kinetic characteristics of PAPII for these two substrates, it was necessary to determine the effect of DMSO on PAPII activity as outlined in section 2 10 9 The results of this study are presented in section 3 8 9 The presence of 2% v/v DMSO in the substrate causes the enzyme activity to increase to 105% of the control While the activity is not significantly affected by 4% or 6% v/v DMSO, increasing the concentration to 8% and 10% v/v causes the activity to decrease by 10% and 21% respectively In view of the fact that PAPII activity is not affected by the presence of 4%v/v DMSO, direct comparisons can be made between the K_m and V_{max} values of the substrates pGlu-MCA and pGlu-His-Pro-MCA, as presented in section 3 8 11 1 and discussed in section 4 6 4

The notion that nature intended enzymes to be catalytically active in water and that organic solvents serve only to destroy their catalytic power is incorrect (Dordick, 1989) Many enzymes, such as PAPII, function in naturally hydrophobic environments, usually in the presence of, or immobilised in a lipid membrane While water is an absolute requirement for the catalytic function of enzymes, it is unlikely that the bulk water concentration of 55M in aqueous solutions is necessary It is more likely that just a monolayer of water around the enzyme is necessary and therefore, it is not surprising that enzymes are catalytically active in organic solvent systems (Klibanov, 1986) The enhancement of PAPII activity in a low concentration of DMSO followed by its inactivation at higher concentrations represents a commonly reported effect of organic solvents on enzyme activity Some examples of the effects of organic solvents on enzyme activity include, increased trypsin activity in 10% v/v acetomtrile (Batra and Gupta, 1994) and a 2-fold increase in the esterase activity of α-thrombin in the presence of 20% v/v DMSO (Neidleman, 1990)

4.7.9 Substrate Specificity of PAPII

The ability of PAPII to hydrolyse a range of pyroglutamyl peptides was monitored by reverse phase HPLC as outlined in sections 2 10 10 3 and 2 10 10 4 The standard curves presented in figures 3 8 10 3 to 3 8 10 7, illustrate the quantitative nature of the system for the detection of peptides and the metabolite pyroglutamic acid The HPLC profiles presented in figures 3 8 10 3 to 3 8 10 7, illustrate the cleavage of pyroglutamyl peptides by the enzyme, while figures 3 8 10 9 to 3 8 10 12 illustrate the cleavage of some of these peptides over time These figures clearly show hydrolysis of the peptides, indicated by a reduction in peak area, coupled with simultaneous increase in pGlu concentration The results of this study are summarised in table 3 8 10 2

In agreement with previous studies on PAPII (O'Connor and O'Cuinn, 1985, Wilk and Wilk, 1989a, Elmore *et al*, 1990, O'Leary and O'Connor, 1995a), the enzyme was shown to be specific for only TRH and closely related peptides In addition to the peptides which these investigators showed to be hydrolysed, the enzyme was also shown to cleave pGlu-His-Gly-OH (anorexogenic peptide / colon mitosis inhibitor), pGlu-His-Gly-NH_2 and pGlu-His-Pro-Gly-NH_2

A number of TRH-like peptides have recently been identified in mammalian semen (Cockle, 1995) pGlu-Glu-Pro-NH_2 (fertilisation promoting peptide) originates in the prostate and is involved in sperm maturation (Linden *et al*, 1996) This peptide has also been found to be present in the CNS and anterior pituitary where it may function as a paracrine or autocrine modulator of pituitary function (Cockle, 1995, Rondeel *et al*, 1995) In agreement with a recent report that PE is the enzyme responsible for the degradation of this tripeptide (Siviter and Cockle, 1995), no cleavage by PAPII was observed pGlu-Phe-Pro-NH_2 originates in the testes and is also secreted into the semen Although its function has not yet been elucidated, it is thought to have a role distinct from that of pGlu-Glu-Pro-NH_2 (Linden *et al*, 1996) In contrast with the findings of Lanzara *et al* (1989) and Elmore *et al* (1990), PAPII was shown to be capable of hydrolysing pGlu-Phe-Pro-NH_2 Since PAPII has been shown to be present in rabbit testes

(Vargas *et al*, 1992a) and is regulated by sex hormones (see section 1 4 2 3), it is possible that the enzyme plays a part in the regulation of this tripeptide *in vivo* Previous studies on the substrate specificity of PAPII have suggested an absolute requirement for the N-terminal pGlu-His sequence (Elmore *et al*, 1990) The ability of the enzyme to hydrolyse pGlu-Phe-Pro-NH_2 demonstrates that the enzyme tolerates substitution of the penultimate His residue

Figure 3 8 10 7 illustrates the cleavage of TRH by PAPII over time From these plots the non-enzymatic cyclisation of His-Pro-NH_2 to cyclo His-Pro, after cleavage of the pGlu-His bond by PAPII, is clearly demonstrated Figures 3 8 10 3, 3 8 10 5 and 3 8 10 6 demonstrate that cyclo His-Pro is formed following the metabolism of acid TRH, pGlu-His-Pro-Gly-OH and pGlu-His-Pro-Gly-NH_2 It is clear, however, that the rate of cyclisation in each of these cases is slower than that of His-Pro-NH_2, since cyclo His-Pro represents only a minor metabolite after 18 hours incubation at 37°C The TRH precursor pGlu-His-Pro-Gly-OH has recently been shown to interact directly with TRH-Rs (Yamada *et al*, 1995) It is possible that PAPII plays a part in the regulation of this tripeptide *in vivo*, and in doing so it may contribute to *in vivo* levels of cyclo His-Pro The conversion of His-Pro-Gly-OH to cyclo His-Pro, following the *in vitro* action of PAPI on pGlu-His-Pro-Gly-OH, has previously been demonstrated by Miyashita *et al* (1993)

In view of the fact that the enzyme has been shown to hydrolyse pGlu-ßNA and pGlu-MCA, albeit at a low rate and with low affinity (see sections 3 8 10 2, 3 8 11 1 and 4 6 4), it would be expected that some pGlu-Xaa dipeptides, such as pGlu-His or pGlu-Phe, might be cleaved However none of the dipeptides tested were hydrolysed by the enzyme It is possible that limited hydrolysis of these dipeptides does occur, but a more sensitive method such as capillary electrophoresis is necessary for its detection However, even if such hydrolysis was to be observed, it is unlikely to be of physiological importance

The kinetic characteristics of purified PAPII towards a range of pyroglutamyl peptides were determined as outlined in section 2 10 11 The K_m, K_i and V_{max} values for these peptides were determined using a range of kinetic models based on the Michaelis-Menten equation Henderson (1992) has reviewed the suitability of these models for the determination of K_m, K_i and V_{max} values While very useful for display purposes, because of their linearity and familiarity, the reciprocal plots (Eadie-Hofstee and Hanes-Woolf plots) and especially the double reciprocal plot (Lineweaver-Burk plot), are statistically unsound for the determination of K_m and V_{max} The author has suggested that one reason for the continued popularity of the Lineweaver-Burk plot, is that it conceals a poor fit between the data and a straight line Using a computer to calculate the least-squares fit of the data points to a *rate* versus *substrate concentration* hyperbola (Michaehs-Menten curve) provides statistically sound estimates of K_m and V_{max} Although not suitable for display purposes, the most statistically sound method for the determination of these values is the Direct Linear Plot (Eisenthal and Cornish-Bowden, 1974) This method uses a non-parametric criterion of best-fit, in which the median value of a series of estimates of K_m and V_{max} is taken (Henderson, 1992) In view of these facts, K_i and K_m values referred to hereafter are those determined from the Direct Linear Plot The Lineweaver-Burk plots illustrated in figures 3 8 11 9 to

3 8 11 12 were used to determine the mode of inhibition of the peptides All of the peptides tested were found to act as competitive inhibitors of the enzyme as summarised in table 3 8 11 2

In agreement with previous reports (O'Connor and O'Cuinn, 1985, Elmore *et al*, 1990, O'Leary and O'Connor, 1995a), a K_I of 40μM was obtained for the reaction of the enzyme with TRH The K_m value determined for the TRH analogue, pGlu-His-Pro-MCA, (2 4-2 5μM), is considerably lower than for TRH itself, indicating that the enzyme has a preference for a large, hydrophobic group such as MCA, at the carboxy-terminus of the substrate This finding contrasts sharply with the findings of O'Leary and O'Connor (1995a), who reported a K_m of 100μM for this substrate The reason for this discrepancy is not clear, however, it is possible that the presence of 0 3% v/v Triton X-100 in the enzyme preparation described by these investigators is responsible for this, and other, differences in the biochemical characteristics displayed by the enzyme It is also possible that the intact, Triton X-100 solubilised enzyme behaves differently to the truncated, trypsin solubilised enzyme

In agreement with the findings of Elmore *et al* (1990), the peptides pGlu-His-Pro-OH, pGlu-His-Gly-OH and pGlu-His-Pro-Gly-OH each displayed higher K_I values than the corresponding amides, suggesting that the enzyme prefers an amide group at the carboxy-terminus of peptide substrates It is interesting to note that the enzyme has a higher affinity for pGlu-His-Pro-Gly-NH_2 (K_I = 30μM), than for TRH (K_I = 40μM) However, the rate of TRH hydrolysis by the enzyme is faster than that of pGlu-His-Pro-Gly-NH_2 as illustrated by the HPLC time-courses presented in figures 3 8 10 9 and 3 8 10 11

The cleavage of pGlu-His-Trp (LHRH 1-3), with a K_I of 130-170μM, has previously been demonstrated by O'Connor and O'Cuinn (1985), Elmore *et al* (1990), and O'Leary and O'Connor (1995a) As expected, extending this sequence to LHRH 1-5, 1-6, 1-7 or to the complete decapeptide LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), abolishes the ability of the enzyme to remove the N-terminal pGlu The K_I values obtained for these peptides display an interesting trend The enzyme displays a marked increase in affinity for LHRH 1-5 (K_I of 11μM) over LHRH 1-3 Extending the sequence further to LHRH 1-6 and 1-7 causes an increase in the K_Is to 19μM and 60μM respectively This indicates that elongation of the peptide causes the affinity of the enzyme to be reduced Based on this trend, the K_I of the enzyme for the complete decapeptide LHRH would be expected to be even higher than 60μM However, this was not found to be the case In fact, LHRH, with a K_I of only 8μM, was found to be the most potent peptide inhibitor tested It is possible that this higher than expected affinity is due, at least in part, to the previously mentioned preference of the enzyme for peptide substrates with an amidated carboxy-terminus This result compares well with the findings of O'Connor and O'Cuinn (1985) who reported LHRH to be a potent competitive inhibitor of PAPII (K_I = 20μM) However, it contrasts sharply with the findings of O'Leary and O'Connor (1995a), who reported the enzyme to be non-competitively inhibited by LHRH with an extremely high K_I of 820μM The reason for this discrepancy is not clear

In view of the facts presented herein, it is clear that the substrate specificity of PAPII is not as narrow as previously believed It is nonetheless clear that only TRH and closely related peptides are hydrolysed by

the enzyme The physiological significance, if any, of the enzymes ability to hydrolyse peptides other than TRH remains to be elucidated

4.8 Summary

PAPII was solubilised from the membrane fraction of bovine brain by treatment with trypsin The enzyme thus released, was found to be considerably more stable than that obtained by solubilisation using a range of detergents The trypsin solubilised enzyme was further purified by Q-Sepharose anion-exchange, immobilised Zn-affinity, calcium phosphate cellulose and Sephacryl S200 gel filtration chromatography, resulting in a 3,047-fold purification with a 24% recovery of enzyme activity

A number of fluorimetric assays for the detection of PAPII were developed, two of which are based on the TRH substrate analogue pGlu-His-Pro-MCA The first of these is a coupled enzyme assay Following the removal of the N-terminal pGlu by PAPII, liberation of MCA from the metabolite His-Pro-MCA is catalysed by the addition of, partially purified, bovine serum DAPIV The second assay is based on the non-enzymatic cyclisation of His-Pro-MCA to cyclo His-Pro and free MCA This cyclisation is catalysed by incubation of the reaction mixture for up to 2 hours at 80°C A third assay, based on the ability of the enzyme to hydrolyse the substrate pGlu-MCA, facilitated the continuous monitoring of PAPII activity in purified samples containing relatively high levels of activity These fluorimetric assays offer expeditious alternatives to the existing radiolabelled TRH assays, for the detection of PAPII activity

A relative molecular mass of 214,000 Da was determined for the enzyme, by gel filtration chromatography However, the subunit structure could not be determined due to the visualisation of multiple bands following SDS PAGE

The purified enzyme was found to be relatively labile However, the presence of 1% v/v BSA was shown to greatly improve its stability The enzyme exhibits optimal activity in the pH range 6 8-7 6 and was found to be stable over a wide pH range of 4 0-9 0 Optimal enzyme activity was observed at 45°C

The enzyme was identified as a metallopeptidase on the basis of its inhibition, in a time dependent manner, by metal-complexing agents and its subsequent reactivation in the presence of metal ions, including Zn^{2+}, Ni^{2+} and Co^{2+} Enzyme inhibition by cysteine protease activators and inhibitors and by the serine protease inhibitor AEBSF, was also observed

TRH and a number of closely related peptides, including pGlu-His-Gly-OH, pGlu-His-Gly-NH_2, pGlu-His-Pro-Gly-NH_2 and pGlu-Phe-Pro-NH_2, were hydrolysed by the purified enzyme LHRH and LHRH 1-5, although not substrates for the enzyme, were shown to be potent, competitive inhibitors of the enzyme, with K_i values of 8μM and 11μM respectively

In conclusion, the characteristics of the PAPII activity purified from bovine brain are similar to those reported for previously characterised PAPIIs It can therefore be assumed that this enzyme represents a "classical" pyroglutamyl aminopeptidase type-II (EC 3 4 19 6)

5. Bibliography

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Appendices

A.1. Kinetic Analysis

Data obtained from kinetic investigations was analysed using the following models based on the Michaelis-Menten equation In these equations, V = initial velocity, V_{max} = maximal velocity, [S] = substrate concentration, [I] = inhibitor concentration, K_m = Michaelis constant and K_i = inhibitor dissociation constant

A.1.1 Michaelis Menten Analysis

The rate equation for the Michaelis-Menten model of enzyme catalysed reactions is as follows

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

A plot of V versus [S] yields a rectangular hyperbola At $V = V_{max}/2$, $[S] = K_m$

A.1.2 Lineweaver-Burk Analysis

The Michaelis-Menten equation can be rearranged to yield the following equation

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

A double reciprocal plot of 1/V versus 1/[S] yields a straight line The Y-Axis intercept represents $1/V_{max}$ and the X-Axis intercept represents $-1/K_m$

A.1.3 Eadie-Hofstee Analysis

The Michaelis-Menten equation can be rearranged to yield the following equation

$$V = V_{max} - \frac{V K_m}{[S]}$$

A reciprocal plot of V versus V/[S] yields a straight line The Y-Axis intercept represents V_{max} and the slope of the line represents $-K_m$

A 1.4 Hanes-Woolf Analysis

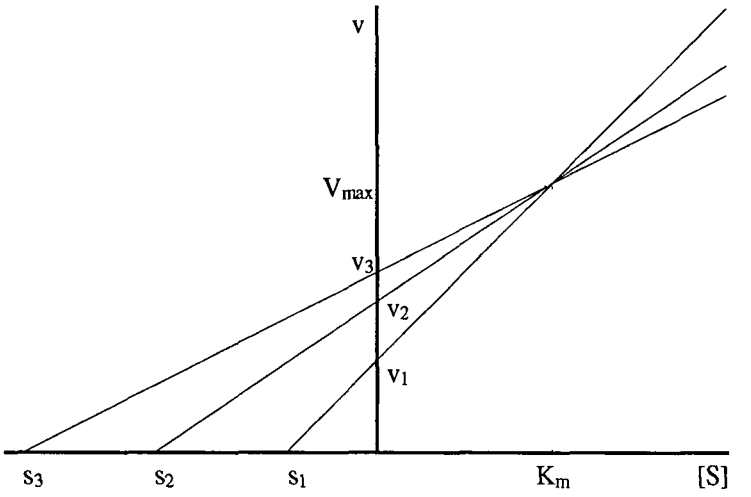
The Michaelis-Menten equation can be rearranged to yield the following equation

$$\frac{[S]}{V} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}}$$

A reciprocal plot of [S]/V versus [S] yields a straight line The X-Axis intercept represents $-K_m$ and the slope of the line represents $1/V_{max}$

A.1.5 Direct Linear Plot

This method is based on plotting substrate concentration and initial velocity values as lines in in parameter space rather than as points in observation space Where as in the previously mentioned models, substrate concentration and initial velocity values were used to place a point in observation space at particular X, Y coordinates, in this model a line is constructed by joining a point at a distance of -[S] from the origin on the X-axis to a point at a distance V from the origin on the Y-axis and extending that line into positive X and Y space Plotting multiple lines using multiple substrate concentration and initial velocity combinations will lead ideally to a unique intersection point, whose X and Y coordinates represent K_m and V_{max} respectively



In practise, many intersection points are obtained and the K_m and V_{max} parameters are represented by the median values Beacuse the median value of the list of possible determinations is used, the Direct Linear Plot is less sensitive to the effects of outliers, making it statistically better than the previouly described models A simple BASIC program was created to take data obtained from kinetic investigations and calculate the intesection point of lines created as described, and to determine the median values

A.1.5 Determination of K_I Values

In the presence of a *competitive inhibitor* the Michaelis Menten equation is replaced by following equation

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_I}\right)\left(\frac{1}{[S]}\right)$$

On the basis of this equation, the K_I value can be determined from the following equation in which K_m is determined in the absence of inhibitor and K_{app} is the apparent K_m in the presence of the inhibitor at a concentration [I] are determined using the kinetic models described previously

$$K_I = \frac{K_m\,[I]}{K_{app} - K_m}$$

in which K_m is determined in the absence of inhibitor and K_{app} is the apparent K_m in the presence of the inhibitor at a concentration [I] are determined using the kinetic models described previously

A.2. Statistical Analysis

Most results are presented as the mean of three individual determinations, (a, b and c), ± the standard deviation (SD) or ± the standard error of the mean (SEM) These values are calculated as follows

$$\text{Mean} = \frac{a + b + c}{3}$$

$$\text{Variance} = \frac{a^2 + b^2 + c^2}{3} - \text{Mean}^2$$

$$\text{SD} = \sqrt{\text{Variance}}$$

$$\text{SEM} = \frac{\text{SD}}{\sqrt{3}}$$