The Molecular Characterisation Of Pregnancy-Associated Plasma Protein-A (PAPP-A)

By

Steven Evans

A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy.

Research Conducted At Sheffield Hallam University.

April 1996

CONTENTS

List of Tables

List of Figures

List of Plates

List Of Plate Insets

(Note insets were numbered from figures in which they were placed)

Dedication

This Thesis is dedicated to the memory of my father, Mr T. E. Evans.

Acknowledgements

I would like to extend my deepest gratitude to my supervisor Maria Blair for all the help, encouragement and financial support during this project, also for an inordinate amount of tolerance and patience during the production of the bound thesis.

I would also like to thank everyone within the Universities in Sheffield that were involved with this project, especially those within the School of Science. For their continued understanding, usually when a piece of equipment miraculously disappeared for that vital experiment. Thank you all. Special thanks to those that made me eventually draw a line under things, especially to Di, Anne, Barry and those that at Langhill.

Lastly thanks to my family for their continuing encouragement as friends were starting to think that Connie and Alex were a one-parent family.

I wish to acknowledge the financial support of the Medical Research Council and The School of Science, Sheffield Hallam University.

List Of Abbreviations Used in the Body of This Thesis

α	Alpha.
α_2M	Alpha-2-macroglobulin.
α-CN	4-Chloro-1-Napthol.
A	Adenine.
$A_{xxx\ nm}$	Absorption at xxx nm.
ACS	American Chemical Society.
AD	Alzheimer's disease.
AFP	Alpha-Fetoprotein.
Arg	Arginine.
APP-KD	Amyloid β-protein precursor-Kunitz domain.
APS	Ammonium Persulphate.
ATZ	Anilinothiazoline
β	Beta.
BCA	Bicinchoninic Acid.
Bp	Base pairs.
BPB	Bromophenol Blue.
BSA	Bovine serum albumin.
C	Cytosine.
Ca^{2+}	Calcium
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid.
CBB	Coomassie Brilliant Blue®.
cDNA	complementary DNA.
CL	Cornelia-de-Lange.
CNBr	Cyanogen Bromide.
Con-A	Concanavalin - A.
Cong.	Congenital.
CPM	Counts per minute.
CV	Coefficient of variation.
CVS	Chorionic villus sample.
d.	Density.
DEAE-Trisacryl	Diethyl aminoethyl(Trisacryl).
DMAB	p-dimethyl aminobenzaldehyde.
DMF	Dimethyl formamide.
DMSO	Dimethyl sulphoxide.
DNA	Deoxyribonucleic acid.
dNTP's	Deoxyribonucleoside Triphosphates
DS	Down syndrome.
DTT	Dithiothreitol.
EDTA	Ethylenediamine tetra-acetic acid.
EEO	Electro-endosmosis.
EGF	Epidermal growth factor
EGF-BP	Epidermal growth factor binding protein
ELISA	Enzyme Linked Immunosorbent Assay.
EtBr	Ethidium Bromide.
EtOH	Ethanol.
$F(ab')_2$	Divalent antigen binding region.

FISH	Fluorescence *in-situ* hybridisation.
FPLC	Fast performance liquid chromatography™.
Fig.	Figure.
G	Guanine.
g.	Gramme.
GalNAc	N-Acetylgalactosamine
gl.	Glacial.
GlcNAc	N-Acetylglucosamine.
GPR	General Purpose Reagent Grade.
gr.	Grade.
hCG	human Chorionic Gonadotrophin.
HCl	Hydrochloric acid.
HGE	Human Granulocyte Elastase.
HOAc	Acetic acid.
hPL	Human placental lactogen.
HPLC	High performance liquid chromatography.
HRP	Horseradish Peroxidase.
HSA	Human serum albumin.
IPTG	β-D-isopropyl-thiogalactopyranoside.
IU	International units.
K	10^3.
kDa	10^3 daltons.
λ	Lambda.
LB	Luria Broth.
LCA	Lentil lectin (*Lens culinaris*) agglutinin
LP4	Size of plastic tube (Denley).
M	Molar.
mA	10^{-3} Amps.
mAb	Monoclonal antibody.
Mat.	Maternal.
mCi	10^{-3} Curie.
MEGA-10	Decanoyl-N-Methyl Glucamide
MeOH	Methanol.
mg	10^{-3} gramme.
ml	10^{-3} litre.
mm	10^{-3} Metre.
mM	10^{-3} Molar.
Mol. Biol. Gr.	Molecular Biology Grade chemical.
MP	Membrane associated placental protein.
mRNA	messenger RNA.
MW	Molecular weight.
NaCl	Sodium Chloride.
NAG	N-Acetylglucosamine.
NaOAc	Sodium acetate.
nd.	Not determined.
NDS	Napthalene-1-5-disulphonic acid-disodium salt.
NeuAc	Sialic acid (N-Acetyl-neuraminic acid).
nmole	10^{-9} Mole.

NSB	Non-Specific background.
NT	Nuchal translucency.
^{o}C	Centigrade.
2-ME	2-mercapto-ethanol.
*X	Stock *times concentrate(where *= No.)
[ ]	Concentration.
PAPP-A	Pregnancy-associated Plasma Protein-A.
PEG	Polyethylene Glycol(Average MW 6000).
PET	Pre-eclamptic toxaemia.
pfu	Plaque forming unit.
pg	10^{-12} gramme.
PHA	Phytohaemagglutinin.
pI	Iso-electric point.
pmole	10^{-12} Mole.
PMSF	Phenyl methyl sulphonyl fluoride.
PP	Placental protein.
PBS	Phosphate Buffered Saline.
PCR	Polymerase Chain Reaction.
PITC	Phenyl isothiocyanate
proMBP	Proform of eosinophil major basic protein.
PTC	Phenylthiocarbamoyl
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
PVP-360	Polyvinylpyrolidone-360.
PZP	Pregnancy zone protein.
RCF	Relative Centrifugal force.
R_f	Relative mobility
RIA	Radioimmunoassay.
RID	Radial Immunodiffusion.
RIE	Rocket Immunoelectrophoresis.
RMM	Relative molecular Mass.
RP-HPLC	Reverse Phase-HPLC
rpm	Revolutions per minute.
RT	Ambient Room Temperature (Approx. 21^{o}C).
SDS	Sodium dodecyl sulphate.
ss.	Single stranded
Sp. Ac.	Specific activity.
SP1	Schwangerschaft spezifische β glycoprotein-1.
T	Thymidine.
TAE	Tris-Acetate-EDTA buffer.
TAME	Nα-p-Tosyl-L-Arginine Methyl Ester.
TBE	Tris-Borate-EDTA buffer.
TBS	Tris Buffered saline.
TCA	Trichloroacetic acid.
TEMED	N,N,N',N'-tetramethyl ethylene diamine.
TFA	Trifluoroacetic Acid.
TMB	3,3',5,5'- Tetramethylbenzidine.
TMC	Threatened Miscarriage.

TPCK	N-Tosyl-L-Phenylalanine Chloro-Methyl Ketone.
TLCK	Nα-p-Tosyl--L-Lysine Chloro-methyl Ketone.
TRIS(TRIZMA)	Tris(hydroxymethyl)aminomethane.
Triton® X-100	Octyl polyethylene ether.
Tween®-20	Polyoxyethylenesorbitan Monolaurate.
μg	10^{-6} g.
μl	10^{-6} l.
μM	10^{-6} M.
uE3	Unconjugated Oestriol.
v/v	Volume : volume ratio.
V_o	Void volume. .
V_t	Total volume.
V_c	Column volume.
V8	Alternative name for Endoproteinase Glu-C.
w/v	Weight : volume ratio.
X-gal	5-Bromo-4-Chloro-3-Indolyl-β-galactopyranoside.
XIE	Crossed Immunoelectrophoresis

Coomassie Brilliant Blue® is a registered trademark of ICI.

Tween® is a registered trademark of ICI.

Triton® is a registered trademark of Rohm & Haas, USA.

Other Abbreviations are as found and understood by general texts as illustrated by the scientific literature and texts such as that devoted to the usage of Abbreviations (Baron, **1994**).

Other activities undertaken as part of the PhD programme

Post-graduate courses, attendance at conferences, active participation in research seminars and teaching that was undertaken as part of the programme of research that was required as a partial fulfilment of the requirements for the degree of PhD.

Attendance at biochemical society meetings and a British Society for Immunology meeting.

Regular participation in research seminars at:

- The school of science.
- Jessop hospital for Women.
- The Institute of Virology, The Royal Hallamshire hospital.

The presentation of a research seminar on the molecular characterisation of PAPP-A to the school of science. Regular participation in the postgraduate PhD rolling training programme.

Teaching experience at a variety of levels with:

- Lectures on the MSc pathological sciences course and postgraduate DNA technology course.
- Supervision of Practical Classes for the HND, BSc and MSc.
- Part-supervision of projects on HND, BSc and MSc.

Actively involved in the development of material that was used for course material on the HND and BSc courses run in the Biomedical Sciences Division.

Abstract

PAPP-A is a large glycoprotein with α_2 - electrophoretic mobility that is produced by the placenta during pregnancy. In this thesis a biochemical and molecular characterisation of PAPP-A was performed.

The polyclonal antiserum (DAKO) directed against PAPP-A has been shown to also interact with proteins other than PAPP-A. These non-specific interactions were abolished by performing Western blotting immunodetection at a high salt concentration (0.6M NaCl). At this salt concentration a single band of 195 kDa was immunodetected and this corresponded to the monomeric PAPP-A molecule. It was also discovered that a subset of paratopes in this antiserum reacted, under the described high salt concentration conditions, with the glycan component of PAPP-A.

A placental cDNA library was screened using this antibody for the PAPP-A cDNA but this did not yield a clone for PAPP-A. A possible explanation is that the interaction with this antibody requires carbohydrate components to be present on the PAPP-A molecule. It is known that proteins expressed in bacterial systems are not post-translationally modified. Therefore another approach to the isolation of the PAPP-A cDNA clone was adopted, but this required some primary amino acid sequence of this protein that was unavailable at the time. To generate this information, PAPP-A was purified using its previously unpublished affinity for L-arginine in combination with the already described procedures of ammonium sulphate precipitation, ion exchange and gel filtration. Final purification of PAPP-A was achieved by SDS-PAGE electrophoresis. The isolated monomeric PAPP-A gave a unique single N-terminal amino acid sequence: **N-EARGATEEPS.**

The N terminal sequence combined with the sequence obtained from limited proteolytic digestion of PAPP-A were used to design oligonucleotide primers specific for PAPP-A. These primers were used in a PCR reaction that produced 500 and >1200 bp fragments using the cDNA library as DNA template; thus demonstrating that PAPP-A is synthesised in the placenta.

PAPP-A was shown to have O and N-linked carbohydrate chains. Enzymatic deglycosylation demonstrated that the N-linked chains were 8% (w/w) of the molecule. The O-linked groups were extensively modified with the presence of oligomers of N-acetyl-glucosamine. It was also shown that it was these groups the PAPP-A antibodies bind to at high salt concentration.

A physical interaction of PAPP-A with endoproteinase Arg-C (EGF-BP) was observed. It was seen that they form a 1:1 (PAPP-A: endoproteinase) sub-unit complex that was stable in SDS. A further investigation revealed that PAPP-A interacted with the endoproteinase Arg-C and this resulted in a 30% inhibition of the esterolytic activity of this enzyme.

CHAPTER ONE

Chapter One

Pregnancy and Pregnancy Proteins

1.1 Introduction

The changes that are imposed on the maternal human system by pregnancy have led to an interest in the molecules that are synthesised during pregnancy. The placenta is a highly invasive organ that is analogous to a locally invasive tumour. It is hoped that an understanding of the control mechanisms and molecules produced at the trophoblastic interface will lead to a better understanding of how the conceptus in the majority of cases survives to term.

1.2 The Establishment of the Human Foeto-Maternal Interface

After fertilisation of the human female oocyte by the male spermatozoan a series of mitotic cleavage divisions occur and two distinctive groups of blastomeres emerge, surrounded by the zona pellucida. One of these groups form the inner cell mass which include the embryogenic cells, while the other consists of a mass of flattened polyhedral cells that compose the blastocystic trophoblast. At this stage the repeated divisions produce a characteristic mulberry-shaped mass of cells (a morula). By the fifth day following ovulation and fertilisation the zona pellucida is shed. The uterine wall now consists of two main layers, the myometrium (a thick layer of muscles) and the endometrium (an inner mucous membrane, Figure 1.1a). Implantation of the morula now occurs with the trophoblastic cells adhering to the endometrium. On adhering they exert histiolytic activity on the endometrial epithelium with invasion and destruction of a part of the maternal vascular epithelia. The endometrium then undergoes a series of

changes that results in the decidualisation of the secretory endometrium. These cells acquire a characteristic polygonal shape and this decidual layer thickens and becomes more heavily vascularised (Figure 1.1b).

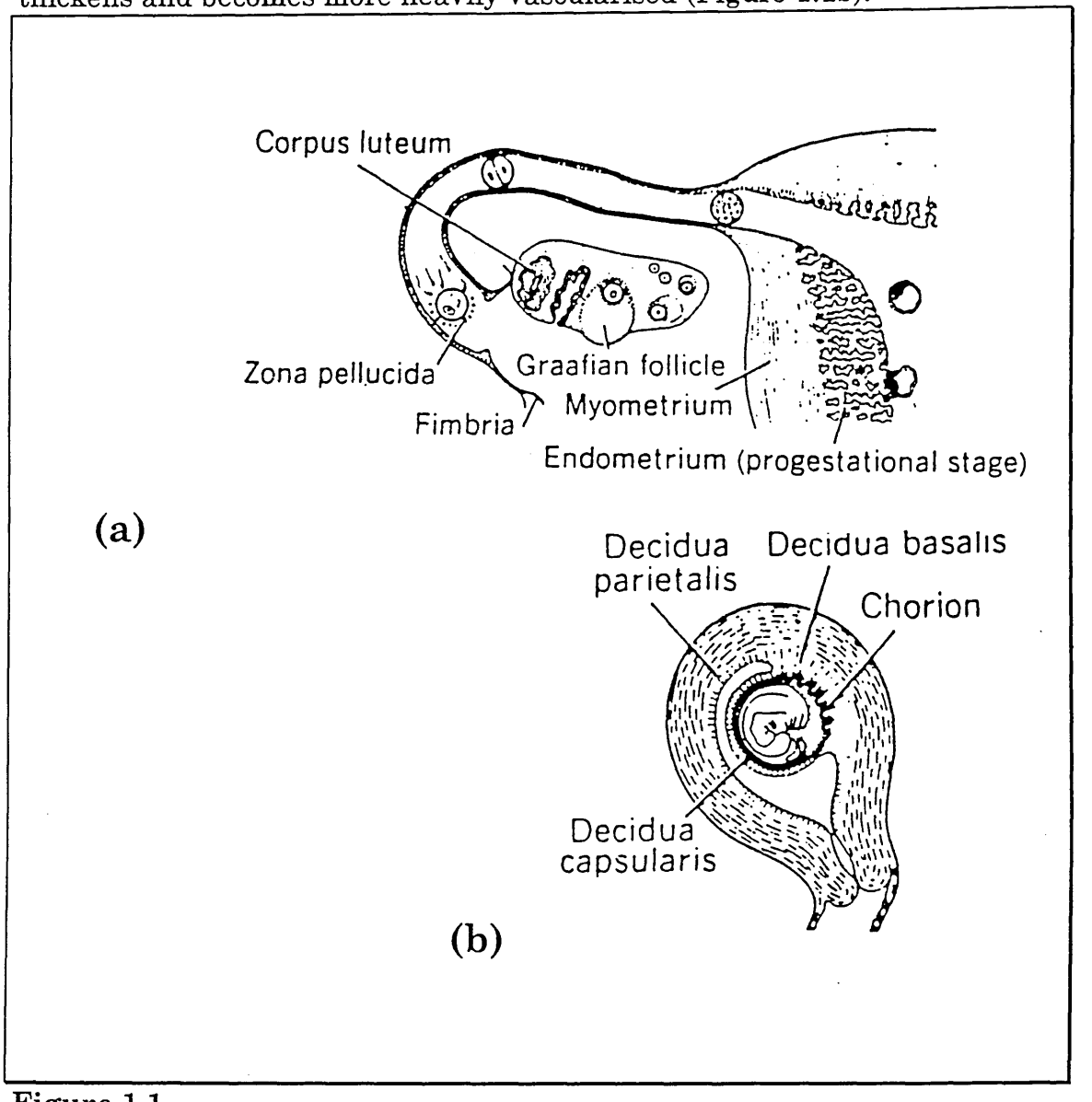


Figure 1.1
Schematic illustration of events leading to implantation (**a**) and the development of the Chorion and Decidua (**b**).
(Adapted from Langman, **1975**)

During this invasive process the trophoblastic cells divide with great rapidity and the progeny fuse with each other to form a thin polarized membrane (the syncytio-trophoblast). This places the chorionic (trophoblastic and foetal vascular tissue) attachment to the uterus in direct contact with the maternal blood stream and so this is a haemochorial type of placentation (Bjorkman,**1985**).

During the described invasive process large lacunar spaces are formed and after enlargement become blood filled intervillous spaces that initially contain a labyrinthine chorionic structure that develops into a secondary villous structure (Williams and Warick, **1980**). The chorion is vascularized by the allantoic blood vessels and so is termed chorioallantoic. The chorionic attachment enlarges in a discoidal pattern to cover approximately 25% of the endometrium at about the third month of pregnancy. At this stage this attachment is known as the placenta; which is deciduate as maternal tissue and placental membranes are shed as part of the afterbirth at term (Figures 1.2a and 1.2b).

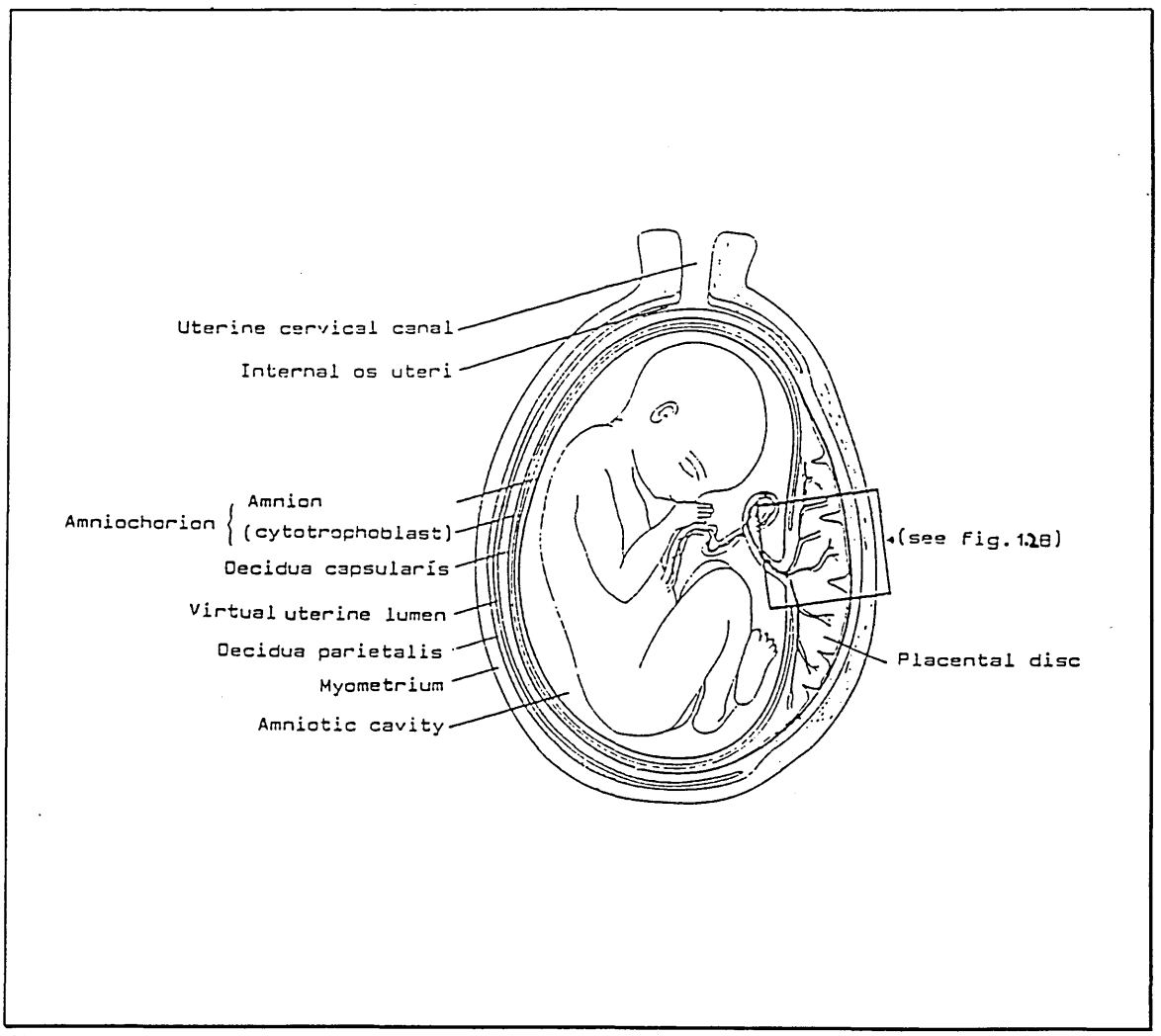


Figure 1.2a
Schematic illustration of the foetal placental intrauterine complex
(Adapted from Panigel et al, **1985**)

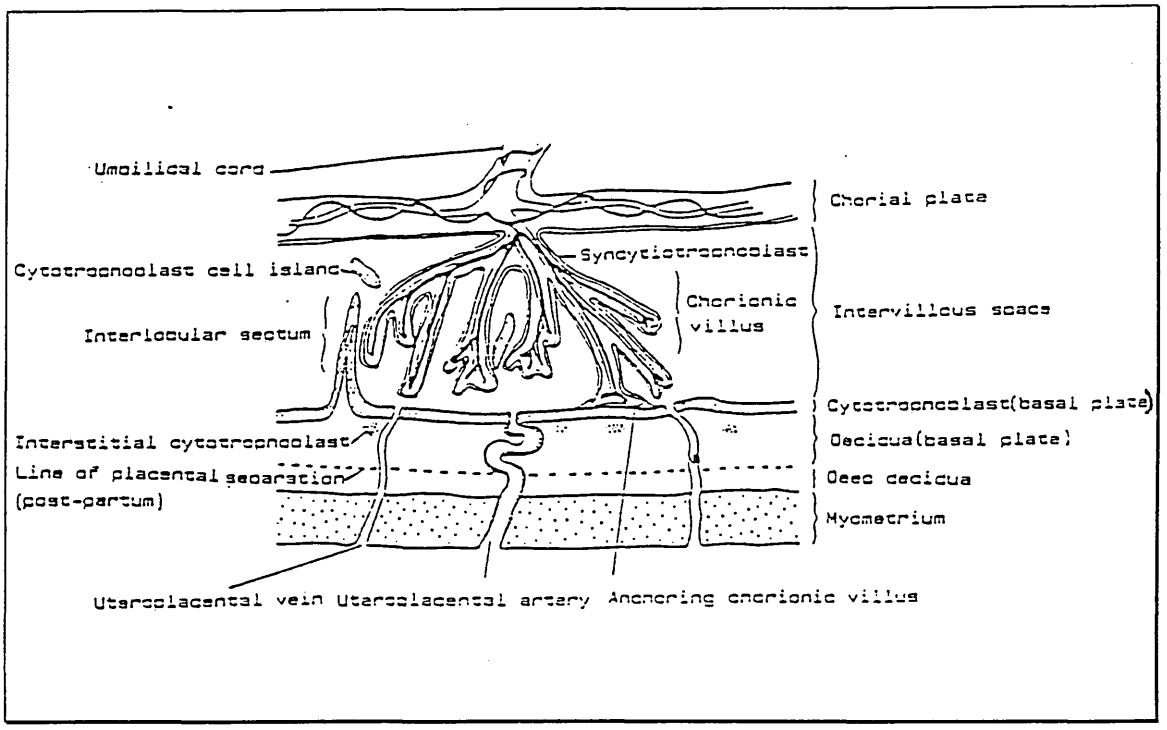


Figure 1.2b
Schematic enlargement of inset seen in Figure 1.2a

If you consider Mossman's (**1987**) definition of the placenta as an " apposition of foetal and parental tissues for the purpose of physiological exchange. " then it is well suited to this task from a structural point of view as its surface area is equivalent to the calculated absorptive area of the entire adult gastro-intestinal tract.

The placenta also acts as an anchoring device for the foetus, providing it with buoyancy and allowing for freedom of movement and growth in an aquatic environment. Allowing for these described features the placental unit must provide for effective synthesis, storage, transport and breakdown of compounds that are vital for foetal growth. These physiological processes have been reviewed by Bjorkman (**1985**) and it is not proposed to further cover them here.

The foetus also contains material acquired from the paternal genotype, which to the mothers immune system must make the placenta look somewhat allogeneic, so it comes as a surprise that the semi-allogeneic

gestation survives and proceeds to term. This apparent contradiction is further deepened with the demonstration by Voisin et al, **(1985)** that:

- The immunological capacity of the mother to defend against microbial infections is not significantly impaired.
- The maternal immune system recognises the foreign paternal antigens.
- The haemochorial placentation bathes the paternal antigens present on the trophoblast in blood and hence one of the major channels for transport of cells involved in immune surveillance and response.

Therefore it is a surprise that the immune system does not react as expected to the presence of this semi-allogeneic allograft. It is also likely that any communication between the foetal and maternal systems will be endocrine in nature (Bell, **1988**). So a more detailed understanding of the trophoblast and related tissues is needed, as they are likely to play a key role in this physiological and immunological exchange that occurs between the foetal and maternal systems.

1.3 Molecules Produced In Response to the Foetal Maternal Intra-Uterine Complex

The placenta, foetus and endometrial tissues produce a wide variety of biologically active proteins, which have historically been split into two groups (Bohn and Winkler, **1988**).

- Group-A: Molecules that were identified on the basis of their activity.
- Group-B: Molecules that were identified using an immunochemical approach.

The first group-A molecule was discovered by Aschheim and Zondek in 1927 who found a hormone with gonadotrophic activity and named it human gonado-tropin (later renamed human chorionic gonadotropin, hCG). Some of the molecules identified in this group are identical or analogues to functional proteins that have been found in other tissues, whilst others are

biologically active substances that are necessary for the maintenance of pregnancy.

Studies of the group B proteins were initiated by MacLaren et al, (**1959**) and their cited work on the protein composition of blood in human pregnancy demonstrated the presence of additional antigenic determinants, when compared to sera from non-pregnant or male donors. This demonstration led to the suggestion that a new group of pregnancy-specific proteins exist during pregnancy. Using the immunochemical approach Tatarinov and Masyukevich (cited from **1970)** and Bohn (cited from **1971)** isolated pregnancy-specific proteins in the early '70s. Lin et al, (**1974a, 1974b**) produced antisera to human pregnancy plasma that had been adsorbed with plasma from males. Using this antisera they showed the presence of four distinct immunoprecipitin lines. They were named alphabetically from the immunoprecipitin pattern seen as pregnancy-associated plasma protein's A, B, C and D. Since then numerous placental proteins have been isolated, with Bohn et al, (**1988**) isolating more than 50 different antigens over the past 20 years. The initial work utilised whole term placental tissue that was composed of foetal and maternal tissue. This led to the identification of a number of maternal proteins initially defined as **P**'lacental **P**'roteins (PP), e.g. PP2 has subsequently shown to be ferritin (Bohn and Winkler, **1988** cited from Bohn's initial work, **1973**). Subsequent work by other investigators has used a more closely defined tissue source and has led to the characterisation of :-

- Foetal antigens (Fay et al, **1989**, Price et al, **1995**).
- Soluble tissue proteins (PP's). and
- Membrane associated placental proteins (MP's).

The immunochemical approach has led to many proteins being identified,

some of the components have been seen in more than one tissue compartment and have been shown to be immunochemically identical, e.g. MP2-C shown to be identical to PP21 (Bohn and Winkler, **1988**).

An overall class of proteins defined as pregnancy-associated has emerged, several of these proteins have been found outside of pregnancy e.g. in the seminal plasma and follicular fluid. To keep these proteins as a class it was suggested that a more appropriate term be Reproductive Proteins (Chard, **1985**). One of the proteins whose levels are modulated by pregnancy is termed pregnancy-associated plasma protein-A (PAPP-A) and is considered further.

1.4 Pregnancy-Associated Plasma Protein-A (PAPP-A)

1.4.1 The Isolation and Purification of PAPP-A

Lin et al (**1974a**) were the first group to purify PAPP-A and raise a polyclonal antiserum against it. Folkersen et al (**1979**) subsequently isolated a protein they called pregnancy specific protein-4, which was shown using immunological methods to be identical to PAPP-A. Subsequently Sutcliffe et al (**1979**), Bischof (**1979a**), McIntyre et al (**1981**), Davey et al (**1982**) and Oxvig et al, (**1994**) isolated PAPP-A by a variety of chromatographic techniques (Table 1.1). The described purification procedures were applied to purify PAPP-A from maternal blood samples, although PAPP-A has also been purified from placental homogenates (Lin and Halbert, **1976** and Davey et al, **1983**). In all purification schemes it was found to be difficult to obtain a homogeneous preparation, without resorting to negative (or positive) immuno-affinity chromatography or utilising PAPP-A's interaction with heparin (Sinosich et al, **1981**). Techniques that utilise positive affinity chromatography, i.e. selective binding of PAPP-A to an antibody directed against it require subsequent elution with chaotropic agents (Sutcliffe et al, **1979**).

All schemes for the purification of PAPP-A except that of Oxvig et al, (**1994**) were published prior to the practical work of this thesis. A guide to the yield and purity of PAPP-A prepared by the various schemes outlined in Table 1.1 is shown in Table 1.2. An assessment of the purity can also be made by looking at specificity of antisera produced by the various investigators, in which they used this material as immunogen for the production of antisera directed against PAPP-A (Table 1.5).

1.4.2 Physico-Chemical Characteristics of PAPP-A

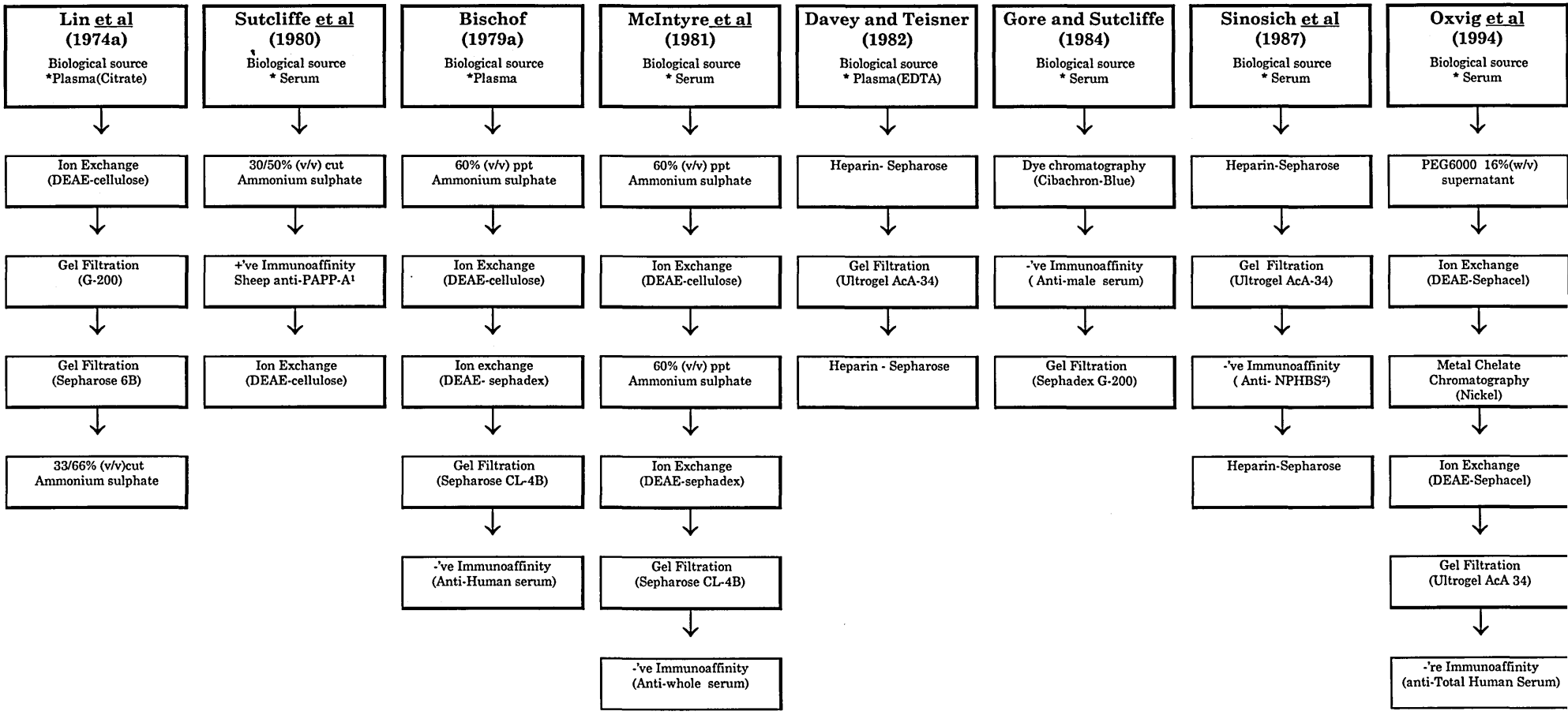
PAPP-A is a glycoprotein containing 19.2-19.4% (Sutcliffe et al, **1980.** Sinosich et al, **1990**) carbohydrates with the major sugar residue being glucose (48%). The carbohydrate content has been analysed using lectins which have revealed the presence of sialic acid, α-D-glucose, α-D-mannose, N-Acetyl-glucosamine and N-Acetyl-galactosamine (Sinosich, **1988a**). A summary of the various findings is given in Table 1.3

1.4.2.1 Molecular Organisation

Prior to the work by Oxvig et al, (**1993**) it was proposed that PAPP-A is a homo-tetramer with a Mr of 710 - 820 kDa, (Lin et al, **1974b.** Bischof, **1979a.** Sinosich et al, **1987**) which consists of two dimers held together by van der Waal's forces, each dimer being composed of identical subunits of Mr 200 - 236 kDa covalently linked by disulphide bridges (Bischof, **1979b.** Sinosich et al, **1990**). This proposed structure was also shown not to contain any thiol-esters. The carbohydrate moiety has also been suggested to play a role in the maintenance of PAPP-A's structure (Sinosich, **1990**). Recently Oxvig et al, (**1994**) have suggested that PAPP-A is a heterotetramer composed of two sub-units of PAPP-A with two sub-units of proMBP with a calculated M_r of 474 kDa. The exact tertiary form of PAPP-A present in the maternal serum is unknown, it has not been established whether all of the

Table 1.1

PAPP-A purification schemes that have been adopted by various research groups.



[Legend: *The biological source of PAPP-A was from late 3rd trimester plasma/serum or from placental homogenates.
[1](Sutcliffe et al, **1979**). [2]Anti-NPHBS: An antisera raised against "Normal plasma heparin binding proteins. "Sinosich et al, **1987**).]

Table 1.2 The yield and purity of PAPP-A obtained by various investigators.

Investigator(s)	Source **Protein Concentration** **Volume used:(mg/ml)** ***[1]%PAPP-A***	**Final purification factor**	**Yield (Percentage of start material)**	**Purity (*Subsequent contaminants found*)**
Lin et al, (1974a).	Term plasma **400 mls:(ns: mg/ml)** ***%ns.***	**X 115 Fold.**	**ns.**	**Stated not pure but enriched. (*Monospecific antisera produced by negative immunoadsorption)***
[2]Bischof (1979a).	Pooled Late 3rd Trimester Plasma **1100 mls (126 mg/ml)** ***%ns.***	**X 294 Fold.**	**19.6%**	**Stated not pure but enriched. *(Antisera produced contained specifities for at least 6 other proteins)***
Sutcliffe et al, (1980).	Term serum pool **900 mls (64 mg/ml)** ***0.16%***	**X 505 Fold.**	**16%**	**Estimated to be 95% pure, with major contaminant at approx. 85 KDa.**
Sinosich et al, (1987).	Term serum **100 mls (ns.)** ***0.07%***	**X 1483 Fold.**	**22%**	**Estimated pure by authors *(no other specificities detected)***

[Legend: The Yield and Purity quoted were based upon the individual investigators findings and were very much related to the sensitivity of the assay system used to assess these factors (Chapter 4). **ns:** not-stated. [1]%PAPP-A was given as %(PAPP-A Vs other blood proteins). [2]Bischof **(1979b)** did produce another scheme with a %Purity at start of 0.07% and a final %Purity of >98% (X1400 fold purification factor) but only a 2.7 % final yield.]

PAPP-A is in a complex with proMBP or if it also exists as a homotetramer as described by previous investigators.

Table 1.3 Glycan components of PAPP-A as found by other investigators

PAPP-A purified and measured by:	**Carbohydrate residues found on purified material:**
Bischof (**1979b**)	No Galactosamine but contains 3.1% Glucosamine.
Sutcliffe et al, (**1980**)	Presence of sialic acid as determined by treatment with neuramindase, N-linked groups present as determined by incubation with the glycosidic enzyme, Endo-H. % Total carbohydrate: 19.2% (w/w)
Sinosich et al,(**1990**)	3.8% Glucuronic acid 9.4% Glucose 3.1% Fucose 2.3% Mannose 0.8% Galactose. % Total carbohydrate: 19.4% (w/w)
Oxvig et al, (**1994**)	PAPP-A monomer PAPP-A/proMBP nd.....Glucuronic acid ..... 0.92% 0.42% ..... Fucose ..... 0.41% 3.39% ..... Mannose ..... 3.05% 0.03% ..... Glucose ..... 0.03% 1.87% ..... Galactose ..... 3.11% 4.52% .....N-Acetylglucosamine .. 6.34% 0% ..... N-Acetylgalactosamine.. 0.23% 3.09% .N-Acetylneuraminic acid..3.27% 13.4% Total Carbohydrate (w/w) 17.4%

[Legend: The Carbohydrate composition of PAPP-A, pro-MBP and PAPP-A/pro-MBP complex were as determined by Oxvig et al, (**1994**) compared with that obtained for PAPP-A (PAPP-A/proMBP complex) by Sinosich et al, (**1990**). The 13.4% of glycan present on PAPP-A monomer being equivalent to 26.5kDa carbohydrate and the PAPP-A polypeptide chain,172 kDa as calculated from the cDNA sequence, Kristensen et al, **1994**)]

1.4.2.2 Electrophoretic Mobility

PAPP-A has α_2 electrophoretic mobility (Lin et al, **1974a**. Bischof, **1979b**) but its isoelectric point (pI) seems to depend on the body compartment from which it was isolated (Table 1.4).

Table 1.4 The pI of PAPP-A isolated by various investigators

Source of PAPP-A measured	pI
Serum	4.4[1] 4.5[2]
EDTA plasma	4.35[2]
Citrated plasma	4.3 - 4.5[3]
Placental extract	4.75[2]

[**Note:** Investigators were, [1]Halbert and Lin (**1979**), [2]Sinosich (**1988a**), [3]Davey and Teisner (**1982**) and [4]Sinosich et al,(**1983**)]

1.4.2.3 Other Characteristics

It has also been shown that PAPP-A is a metalloprotein containing zinc (Sinosich et al,**1983**) and that it interacts with heparin (Sinosich et al,**1981**). Detection of PAPP-A by immunological means is not affected by repeated freeze/thaw cycles or exposure to a pH between 4 - 10 at 4°C for 2hrs, but it is destroyed at pH 2 and 12 (Lin et al,**1974b**). Part of the PAPP-A molecule is cleaved during incubations with chondroitinase suggesting that it is a proteoglycan (Sinosich,**1990**).

1.4.3 PAPP-A Localisation and Control of Its Synthesis

Work published after the completion of the practical aspects of this thesis by Silahtaroglu et al, (**1993**) has shown by using a cDNA probe (pPA-1, Kristensen et al, **1994**) and fluorescence *in-situ* hybridisation (FISH) that the PAPP-A gene is located on Chromosome 9 at position, p33.1.

1.4.3.1 The Trophoblast

Lin and Halbert (**1976**) demonstrated by immuno-fluoresence that PAPP--A is present in the placenta. Wahlstrom et al, (**1981**) confirmed this, but found that it was almost entirely restricted to the apical border of the syncytiotrophoblast. McIntyre et al, (**1981**) also showed it to be localised in the syncytiotrophoblast but found that not all tissue sections stained strongly for PAPP-A. However just because it is present in the syncytiotrophoblast does not necessarily mean that this is its site of synthesis, e.g. Isaka and Bischof (**1986**) demonstrated that PAPP-A binds to placental subfractions of the trophoblast with a similar binding affinity to that of insulin for its GnRH receptor.

Barnea et al, (**1986b**) examined long term cultures of primary cells from the trophoblast to see if they were capable of producing PAPP-A. They demonstrated that PAPP-A was produced and this process was inhibited by incubating these cells with inhibitors of protein synthesis. Bersinger et al, (**1988**) also showed that PAPP-A was produced by a perfused placenta and that its synthesis was energy dependant.

Recently the identification of PAPP-A cDNA (Kristensen et al, **1994**) has allowed the production of PAPP-A RNA probes that have confirmed that the site of production of PAPP-A mRNA are the placental X-cells and syncytiotrophoblast (Bonno et al, **1994b**).

Bischof et al,(**1986a**) also demonstrated that trophoblastic production of PAPP-A could be inhibited by RU486 (a progestogen) and that the inhibition could be overcome by addition of progesterone, with the suggestion that PAPP-A is a progesterone dependant protein. Sorensen et al. (**1995**) found that differing rates of production by the placenta were seen between the proteins: PAPP-A, SP1, hPL and hCG with the suggestion that a more complicated control mechanism exists for synthesis of PAPP-A and hCG. Work on animal models by Pepe et al,(**1994**) demonstrated using a Baboon animal model that by performing a foetectomy (i.e. removal of the foetus, not the placenta) in mid-gestation resulted in normal delivery of the placenta at term. They also observed continued secretion of PAPP-A but found that maternal PAPP-A serum levels did not continue to rise when compared to a

normal control group of baboon pregnancies. This suggests that PAPP-A is regulated by factors that regulate placental growth, contradictory findings to that seen by Bischof et al, (**1986a**) were observed in that progesterone was not seen to affect the maternal serum levels of PAPP-A. The difference in control mechanisms will have to await further molecular studies that can now be undertaken as the cDNA for PAPP-A has been produced.

1.4.4 Extra-Trophoblastic sites of production for PAPP-A

1.4.4.1 The Endometrium

Duberg et al, (**1982**) have found that the endometrium contains 3-4 times more PAPP-A than the trophoblast, with the levels of PAPP-A tending to follow histological staging, they were lowest during the inactive phase and highest in the secretory phase. PAPP-A levels in the endometrium also correlated with the level of steroids during the phases. Duenas et al, (**1988**) have localised PAPP-A in endometrial (glandular and stromal) tissues during its proliferative and secretory phases. Bischof and Tseng (**1986**) have also shown that like the primary trophoblast cells, a primary cell culture of human endometrial cells is capable of producing PAPP-A and that it is also progesterone dependent. This endometrial link for an extra-trophoblastic site of production is also strengthened by the finding of Ikarashi and Takeuchi (cited from **1987**) that by analysis of PAPP-A and Tissue polypeptide antigen levels it is possible to screen for endometrial cancer. However in light of the findings by Tornehave et al (**1987**) it is likely that the PAPP-A seen in the endometrium is an artefact of the immunovisualisation technique that was used. This has subsequently been confirmed at a molecular level by Bonno et al, (**1994b**).

1.4.4.2 Other Sites

Schindler et al, (**1986**) have visualised PAPP-A in the male genital tract amongst cells in the Leydig population, the epithelium of the Rete testis, the

head of the epididymis and the seminal vesicles. Duenas et al, (**1988**) have also found PAPP-A in the stroma of the ovary, cervix and ductal epithelium of the breast. It should however be pointed out that PAPP-A localised by the immunoperoxidase technique is very dependant upon the specificity of the primary antibody and the technique used for immunovisualisation (Tornehave et al, **1986**).

Tornehave et al, (**1987**) have already demonstrated that the PAPP-A antisera available are not monospecific. Kuhajda et al, (**1989**) for example identified a cDNA clone for a haptoglobulin related protein using the commercially available antisera. It has also been shown that PAPP-A specific staining of tissues from Stage I breast cancer could be abolished by pre-incubating the primary antibody with haptoglobulin. It is therefore clear that care is needed in interpreting those studies that have localised PAPP-A using an immunovisualisation technique.

Recently Chemnitz et al, (**1986**) have also shown that the antisera developed by Bischof and used in many localisation studies is not monospecific for PAPP-A but contains specificities for at least 6 other antigens. In their study they compared four different polyclonal antisera for PAPP-A and showed that if Bischof's antisera was adsorbed with human serum and foetal/connective tissue then they could abolish the detection of PAPP-A on the decidualised endometrium. They found that PAPP-A was only found in the cytoplasm of the syncytiotrophoblast, suggesting that PAPP-A is localised in the trophoblast and that other immuno-localisations in other tissues may be due to using a polyspecific antisera. Westergaard et al, (**1988**) have also confirmed these findings, but have also noted that by setting up a competitive system between PAPP-A added and the tissue for the antisera it is possible to demonstrate that PAPP-A is associated with the apical rim of the syncytiotrophoblast, confirmation at a molecular level using anti-sense probes confirmed that PAPP-A mRNA was produced by syncytio-trophoblast cells (Bonno et al, **1994b**). PAPP-A mRNA was however

demonstrated in the testes and follicular cells (Kristensen et al, **1994**). The use of animal models (Pepe et al, **1994**) also allows an *in - vivo* model to be used to look at the affect of hormones on regulating the placental production of PAPP-A.

1.4.5 Assay Systems For The Measurement of PAPP-A

The immunoassays which have been used by various investigators are listed in Table 1.5. Investigators term pregnancy pools are compared to an International World Health Organisation term serum reference pool (W.H.O. 78/610) which consists of a pool of late pregnancy serum allowed the comparison of PAPP-A levels that have been found by different investigators and revealed very disparate levels for PAPP-A with over a 400% difference seen in the concentrations determined ((3) and (4a), Table 1.5).

Various antisera have been raised against PAPP-A (Table 1.6) but as observed by Chemnitz et al (**1986**) not all of the antisera produced had epitopes directed solely against PAPP-A. This variability in the quality of one of the primary reagents will ultimately have an affect on assays for PAPP-A that utilise an immunochemical measurement system e.g. the immunoassays and the subsequent levels found for PAPP-A using assays will also be affected by the quality of the primary reagents that have been used.

The Investigators represented in Table 1.6 reported that their antisera were specific for PAPP-A. The differences described in specificity were probably due to the methods chosen to evaluate the antisera produced. For example, Bischof et al (**1979c**) reported his antisera to be specific for PAPP--A by immunodiffusion, whereas this antisera was subsequently shown to have specifities directed against other serum proteins when tested using Line immunoelectrophoresis, a more sensitive method of analysis.

A variety of non-specific effects on the described assay systems for PAPP-A (Table 1.5) have been observed, see Table 1.7. The ELISA described suffered from non-specific matrix effects with markedly increased (by 50%) concentrations of PAPP-A found in haemolysed plasma when compared to matched non-haemolysed controls, suggesting that a red cell component can affect the levels of PAPP-A found when using this ELISA system.

Table 1.5 PAPP-A immunoassays used by other investigators.

Method	[a]Sensitivity	[b]Term cf Ref.
Crossed Immunoelectrophoresis[1]	Mid-2nd trimester	156
Rocket Immunoelectrophoresis[2]	2nd trimester	123
Radiorocket line electrophoresis[3]	30 µg/l.	65
Radioimmunoassay		
a) - (Bischof et al,**1981b**)	33 µg/l.	258
b) - (Sinosich et al,**1982b**)	3 µg/l.	
c) - (Anthony et al,**1983**)	20 µg/l.	
d) - (Pinto-Furtado et al,**1984**)	5 µg/l.	110
Radioimmunometric4	5 µg/l.	110
Enzymeimmunoassay[5]	2-3rd trimester	90

[**Legend:** [a] The sensitivity quoted were the own investigators measure of the minimum levels that the listed assay was capable of measuring, if no figure was quoted then it was given as an estimate of the minimum level able to be measured during pregnancy. [b] Term cf Ref: This referred to the investigators own late third trimester serum pool compared to the W.H.O. standard.]
(**[1]**Lin et al, **1976b**. **[2]**Bischof et al, **1979c**. **[3]**Folkersen et al, **1981.[4]**Mowles et al, **1986**. **[5]**Pledger and Bellfield, **1983.**)

Table 1.6 Antisera to PAPP-A produced by various investigators

Investigator (designation)	[a]Type	Antisera raised in:	Immunadsorbed with:	[b]Specificity
Lin et al [1] (Miami)	P	Rabbits	HNPPP	
Bischof et al [2] (Aberdeen)	P	Rabbits	HNPPPO	[c] at least 6. others
Sutcliffe et al [3] (Glasgow)	P	Sheep	HMS	
Folkersen et al[4] (Odense)	P	Goats	HNPPP+FCS	[d]Specific
DAKO[5] (Commercial)	P	Rabbits	HNPPP	[e,f] Other specificities
Sinosich et al[6] (Sydney)	P	Rabbits	MHS	[d] Specific
Mowles et al[7] (London)	M	Mice	-	-

[LEGEND: [a]Type: **(P)** Polyclonal. **(M)** Monoclonal. [b]Specificity: Cross - reactivity, also see text. [c] :PZP, α_2-M, β-lipoprotein, AT-III, SP1 plus 1 undefined specificity. [d]:Sinosich **(1988a)**. [e] : Kuhajda et al **(1989)** antibody shares epitopes with haptoglobulin related protein. [f] : also binds SP1 (Sinosich, **1988c**). **HNPPP**: Human Non-Pregnant Plasma Proteins. **HNPPPO**: Human Non-Pregnant Plasma Proteins from women on Oestrogen therapy. **MHS**: Male Human Serum. **FCS**: Foetal Connective Tissue.]
([1]Lin et al, **(1974a)**, [2]Bischof et al, **(1979c)**, [3]Sutcliffe et al, **(1980)**, Folkersen et al, **(1979)**, [5]DAKOPATTS (Appendix 1), [6] Sinosich et al, **(1987)** and [7] Mowles et al, **(1986)).**

Two RIA procedures (Table 1.5) gave markedly different results depending on the type of anti-coagulant that was used to collect the blood samples. The difference in PAPP-A levels that were found when different anti-coagulants were used was most marked in this type of assay when serum was compared against EDTA plasma (The RIA described by Sinosich

et al, (**1982b**) noted a 100% increase in levels). The RIA described by Pinto-Furtado et al, (**1984**) did find a statistically significant difference in the levels found in serum compared to EDTA plasma but this RIA procedure had a far less pronounced difference (20% increase) in the levels that were seen. The exact nature and cause of these observed differences is unknown.

Table 1.7 The affect of anti-coagulants on the measured levels of PAPP-A

Type of Assay	Type of Anticoagulant Used					Effect of Haemolysis
	[d]ACD	Citrate	Oxalate	Heparin	EDTA	-
RIE[1]	-	nc	-	[a]Inc.	-	-
RIE[2]	Dec.	-	-	Inc.	Inc.	-
RIA[3]	Dec.	nc	nc	nc	Inc.	-
RIA[4]	-	-	-	[a]Inc.	[b]Inc.	-
ELISA[5]	-	-	-	-	-	[c]Inc.

[Legend: Levels compared against its serum value in that assay. (-): Not determined. **(nc)**: No significant change in levels observed. **(Inc.)**: Increase in levels observed in relation to that seen in serum. **(Dec.)**: Decrease in levels observed in relation to that seen in serum. [a](Action reversed by protamine sulphate). [b](Increase neutralised if chelation affect abolished). [c](Higher values observed in haemolysed samples from males). [d] (Acid Citrate Dextrose). **]** ([1]Westergaard et al **(1983a)**, [2]Toop and Klopper **(1983)**,[3]Pinto-Furtado et al **(1984)**, [4]Sinosich et al **(1985)** and [5]Pledger and Bellfield **(1983)**).

It has been demonstrated that it is the nature of the PAPP-A molecule used for the RIA standard and tracer in these assays that was responsible for these observed differences and not due to the antisera that was used (Bischof and Meisser, **1988**); these investigators re-evaluated their RIA in terms of tracer, PAPP-A standard and antibody used and concluded that the PAPP-A isolated from late pregnancy EDTA plasma had a component that was recognised by an antiserum against human serum proteins. They also noted that irrespective of the source of antiserum used (Table 1.6) that coagulation also changed the level of PAPP-A detected, therefore some form of immunological heterogeneity has been observed for PAPP-A. It has been recently shown (Oxvig et al, **1993**) that PAPP-A is linked by disulphide bonds to pro-MBP. It is unknown however whether all the PAPP-A found in

the maternal serum of pregnant women is complexed with pro-MBP or what effect this complex has on the different assay systems. Sinosich et al, (**1991**) have also demonstrated that PAPP-A levels when measured by crossed immuno-electrophoresis are reduced when Human Granulocyte Elastase (HGE) was included in an intermediate gel, thus suggesting an effect on the measured level of PAPP-A when PAPP-A was interacting with HGE. It is not known whether a similar effect is seen for the suggested PAPP-A/proMBP complex.

PAPP-A is a metallo-protein (Sinosich et al, **1983**) and was demonstrated to bind Zinc. This has been confirmed at a structural level as the PAPP-A cDNA (Kristensen et al, **1994**) contains a Zinc binding region. Therefore it is possible that the effect observed by Sinosich et al, (**1985**) that changes in the PAPP-A levels could be removed, if the chelating affect of the EDTA was neutralised immediately by the addition of an excess of metal ions. It is interesting to speculate that as this reversible change in PAPP-A levels may be due to structural changes in the conformation of PAPP-A that are effected by the chelation of Zinc with EDTA which is reversed when this affect was abolished. Similar changes in the PAPP-A levels seen when heparin was present were reversed if the heparin antagonist, protamine sulphate was used. This indicates that the tertiary structure of PAPP-A affects the PAPP-A levels measured in the blood using the described immunoassays

1.4.6 PAPP-A Levels Found In The Maternal Blood During Pregnancy

The affects of the PAPP-A immunoassays described in this Chapter may explain the different biochemical profiles that were obtained for PAPP-A when different investigators have followed the levels of PAPP-A found in the maternal blood during pregnancy (Figures 1.3 and 1.4).

Bischof and Meisser (**1988**) have also observed that depending upon whether the PAPP-A used to prepare the tracer in an RIA was from EDTA

plasma or serum, that markedly different results were obtained that were independant of the PAPP-A antisera used. The results affected, effectively changed whether or not PAPP-A was found in non-pregnant individuals and may explain why some investigators find PAPP-A in the blood of males/non-pregnant females (Bischof and Megevand, 1986. Bueler and Bersinger, 1989), whilst others could not (Anthony et al, 1983. Pinto-Furtado et al, 1984).

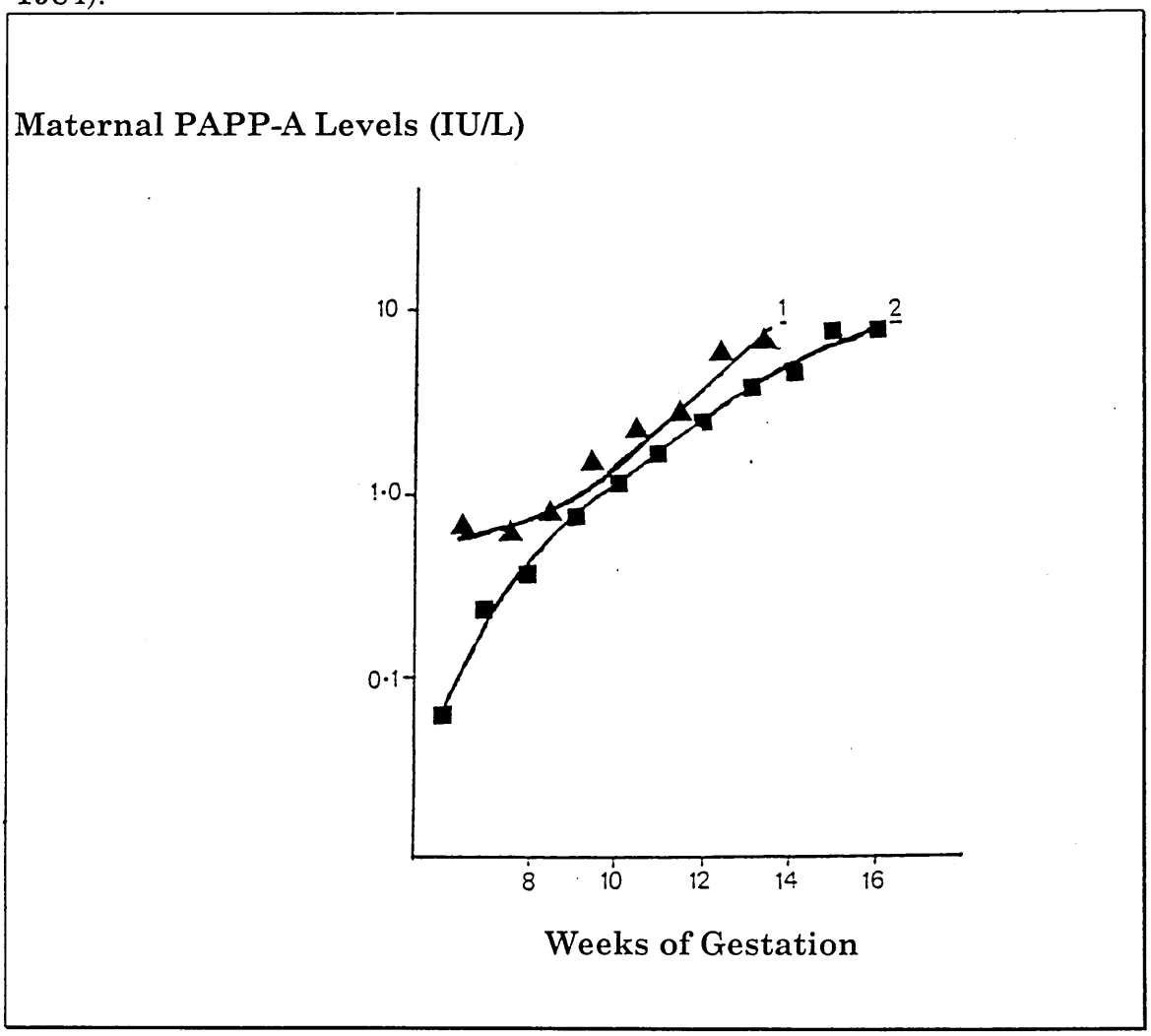


Figure 1.3

The levels of PAPP-A found in maternal blood during the first trimester of pregnancy.

[**Legend:** [1]: Levels as found using a RIA described and adapted from Bischof et al,(1981a) with measurement of EDTA plasma samples. [2]: Levels as found using a RIA described and adapted from Pinto Furtado et al,(1984) with measurement of serum samples. **Note:** Assumed that the International Reference Standard 78/610, contained 100 IU/L.]

The differences in the measured level of PAPP-A are dependant upon assay type, state of the blood (e.g. serum or plasma) and are maintained throughout pregnancy (Figure 1.4). It is however generally believed that PAPP-A can be detected in the blood from the fourth week of gestation and that it continues to rise until parturition. The problems outlined make the interpretation of many of the clinical studies performed not only dependent upon clinical differences between patients but also to artefactual differences in the measurement of PAPP-A and may explain why such conflicting clinical data has accumulated (Section 1.4.7).

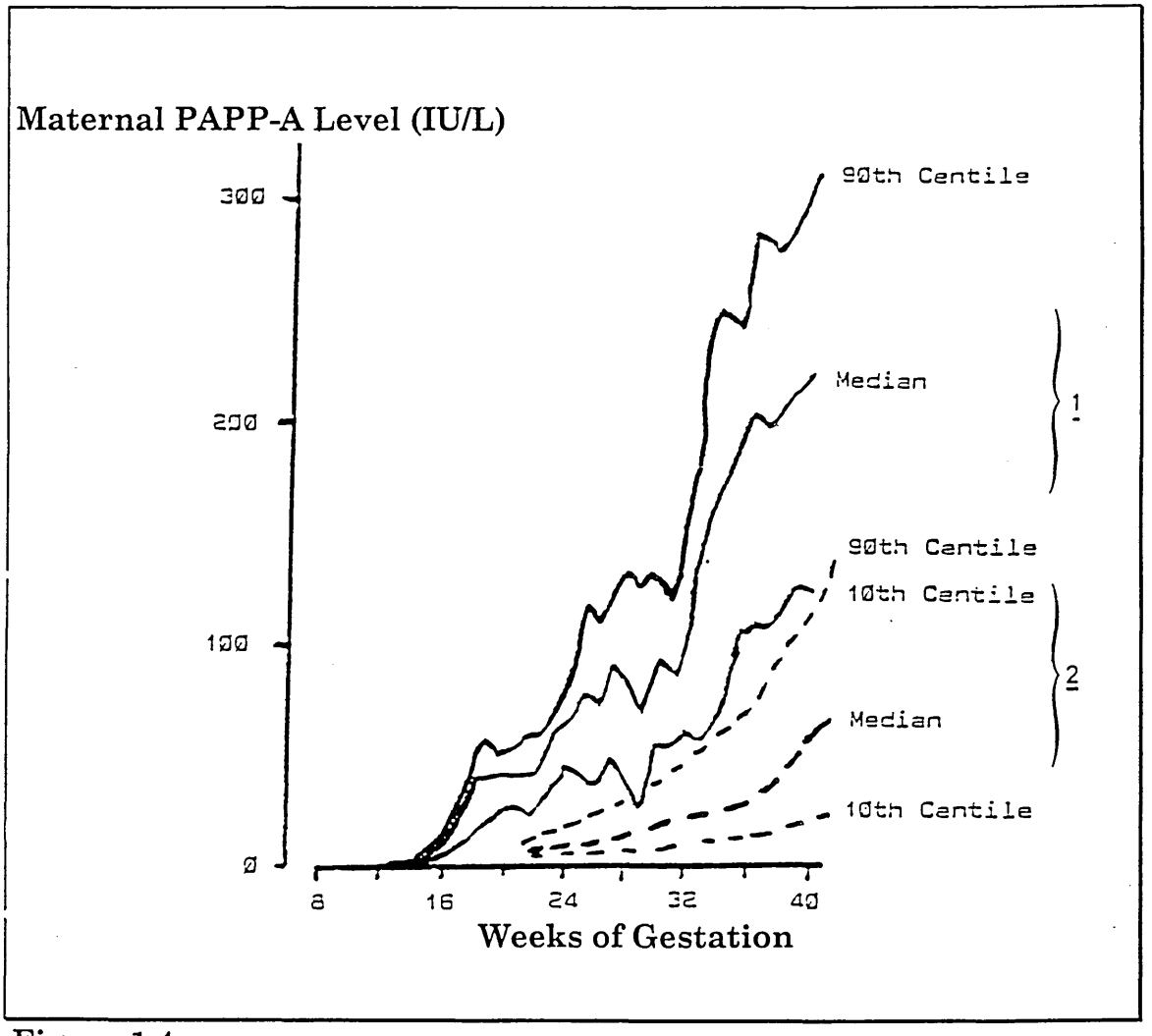


Figure 1.4

Schematic illustration of the maternal levels of PAPP-A found in the blood during pregnancy.

[Legend: [1]**:** Adapted from Barnea et al, **(1986a).** [2]**:** Adapted from Westergaard and Teisner, **(1983b). Note:** Assumed that the International Reference Standard 78/610 contained 100 IU/L.]

1.4.7 The Diagnostic Usefulness Of PAPP-A Measurements

Many studies have been performed to see if circulating PAPP-A levels can be used as a biochemical parameter in obstetric diagnosis. The basic criterion for a biochemical parameter of foetal/placental function is whether that parameter can be used to distinguish normal from pathological pregnancies. PAPP-A is found in relatively high concentrations in the maternal serum, therefore it can be measured by simple immunochemical methods. It also shows very little diurnal variation and so fulfils some of the secondary criteria for an obstetric biochemical parameter (Chard, **1976**). Quantification of maternal PAPP-A levels have been performed to see if it would be of use in the prediction and management of certain abnormalities of pregnancy:

- Threatened miscarriage.
- Ectopic pregnancy.
- Pre-eclamptic toxaemia.
- Congenital disorders.

1.4.7.1 Threatened Miscarriage/Abortion

" Abortion, spontaneous abortion and miscarriage..... refer to the unexpected loss of a pregnancy prior to the period of fetal viability. " (as quoted from Winter et al,1988). Threatened Miscarriage (TMC) being the appearance of preliminary signs of being about to abort the foetus/embryo but stopping before this outcome is reached. It is estimated that TMC occurs in up to 25% of all pregnancies (Stabile et al, **1988**) and this is a figure that has remained unchanged for decades. TMC can be diagnosed by direct vaginal examination if the cervix is dilated, however a diagnosis of TMC is not possible by physical examination where the cervix is closed. Initially it was hoped that bleeding could be used as an indicator of TMC, but as many

as 20% of patients have vaginal bleeding. It has been shown that a higher percentage of this group of patients who present with vaginal bleeding do subsequently abort when compared to those patients who do not present with vaginal bleeding. This diagnostic problem has partially been solved with the advent of Ultrasound, now it is possible to reliably determine the presence of a live foetus after six weeks gestation and therefore alter the clinical management of women with TMC.

Stabile et al, (**1988**) have showed that ultrasound is of use in the management of TMC post 7 weeks gestation, with the prognosis that if foetal life is confirmed during the first trimester of pregnancy then 95 - 98% of these women will progress to the normal outcome of pregnancy.

Of the possible biochemical indicators that can be used it has been found that if PAPP-A levels are depressed when compared to normal pregnancy, it can then be used as a predictor of miscarriage (Bischof et al, **1986a**. Stabile et al, **1988**. and Bischof et al, **1989**). Westergaard et al,(**1985**) has subsequently qualified this in that PAPP-A can be used as a biochemical indicator if foetal life is evident by ultrasound. If the heart action was normal and the level of PAPP-A were normal, then the chance of a normal outcome of pregnancy was in excess of 98%. It was also found (Westergaard et al, **1983b**) that of the biochemical parameters (hCG, SP1, hPL and PAPP-A), only PAPP-A levels were depressed if foetal heart action was present and a subsequent abortion occurred. Moussa et al, (**1988**) have also shown that PAPP-A levels were consistently lower in those patients who spontaneously abort when compared against another group in which abortion was elected for by the patient. However the usefulness of PAPP-A to predict subsequent abortions has not been found to be superior to other biochemical parameters by all investigators. Bersinger et al, (**1987**) did find that PAPP-A levels were depressed in TMC but that it did not improve upon the predictability conferred by a hCG measurement.

1.4.7.2 Ectopic Pregnancy

Implantation of the blastocyst normally occurs in the endometrium of the uterine body, but it may also occur in an extrauterine or ectopic site. Implantation occurring near the internal os uteri (Figure 1.2a) gives rise to a condition known as placenta praevia which can cause severe ante-partum haemorrhage. This condition has been found to occur in 0.5 % of pregnancies. Modern diagnostic methods and rapid surgical intervention have led to a fall in maternal mortality caused by ectopic pregnancy. However better treatment of the other causes of maternal mortality has led to Ectopic pregnancy being an even greater contributor to maternal mortality in the western world (Westergaard et al, **1988**). The risk of ectopic pregnancy has also been compounded by a study that has shown a link between those patients undergoing tubal surgery and IVF-ET having a higher incidence of ectopic pregnancies (Bischof et al, **1989**). These two factors have led to a renewed interest in methods that can be used to diagnose ectopic pregnancy. The diagnostic procedure followed should show that the patient is pregnant and whether the foetal-placental tissue is located outside its normal position in the uterus.

Bischof et al, (**1989**) have reported that PAPP-A levels are depressed in ectopic pregnancy with the finding that depressed levels in the maternal blood occur from day 30 onwards. They found it to be an excellent prognostic parameter for ectopic or intrauterine abortion with a sensitivity of 87.5% and a predictive value of 100%. They concluded that PAPP-A can be used to monitor post-implantation embryo viability following IVF-ET. Sinosich et al, (**1985**) have also found that in conjunction with a positive pregnancy test and clinical history, that severely depressed or undetectable serum PAPP-A was indicative of suspected ectopic pregnancy.

A larger study of 207 women with suspected ectopic pregnancy (Westergaard et al, **1988**) also bears out the use of PAPP-A to detect ectopic pregnancies, but they also suggested that it was not the ideal test prior to

8 weeks. Sauer et al, (**1989**) also came to the conclusion that a single PAPP-A measurement was of little value in determining normal from abnormal pregnancies prior to 8 weeks gestation. The need for early diagnosis of ectopic pregnancy seem therefore to exclude PAPP-A as a biochemical parameter for it diagnosis, however Tornehave et al, (**1987**) have also shown that the syncytiotrophoblast tissue staining for PAPP-A matches its maternal serum levels. Ectopic tissues studied had PAPP-A values all below the 10^{th} centile, with PAPP-A levels being implicated in this pathological condition by measurements at both local (tissue) and at the periphery (blood). Its use therefore as a diagnostic tool for the diagnosis of ectopic pregnancy has not yet been ruled out.

1.4.7.3 Pre-eclamptic toxaemia

Pre-eclamptic toxaemia (PET) occurs in 3 - 5% of pregnancies, the cause has not yet been elucidated. It is characterised by a raised blood pressure, proteinuria and oedema. Eclampsia is a severe degree of PET and if untreated leads to the death of the patient.

PET is one of the earliest recognised disorders being observed over 100 years ago and is present in about 7% of pregnancies, three quarters develop hypertensive pregnancies during the last trimester of pregnancy, it is responsible for over half of all perinatal maternal deaths seen in the USA (Blazer et al, **1991**). Surgical intervention in the form of caesarean section has led to the mortality rate being reduced to 1% in some clinics. Smith et al, (**1979**) observed that maternal levels of PAPP-A rose in those patients that suffered from PET and that this rise often preceded any overt signs of this condition, the significance of this still has to be determined.

1.4.7. 4 Cornelia-de-Lange Syndrome

This is a rare syndrome with an estimated occurrence of 1: >10,000 live births. It is known as the Cornelia de Lange (CL) syndrome with the first published account by this Paediatrician in 1933, the condition was also described by Dr Brachmann in 1915 (Opitz, **1985**). It is a serious syndrome producing severe mental retardation in the infant and has characteristic facial and limb dysmorphic features; with small stature, hypoplastic middle phalanx of the index fingers and gastroesophageal reflux. It is not always a fatal syndrome and current diagnosis relies upon recognising the dysmorphic features. Early recognition is essential as dietary changes combined with surgical treatment for the gastroesophageal reflux has been seen to reduce morbidity and mortality in children with this syndrome (Sataloff et al, **1990** and Rosenbach et al, **1992**). The lack of foetal markers makes antepartum diagnosis difficult but it has been suggested (Bruner and Hsia, **1990**) that a diagnosis is possible by comparing the triad of:

- Abnormally low maternal serum AFP.
- Symmetrical Intrauterine growth retardation.
- Ultrasound examinations for foetal abnormalities in the second trimester.

It has also been suggested that hormonal derangement may play a role in the manifestations of this syndrome (Sataloff et al, **1990**). CL has a relatively poor prognosis as relatively few patients survive into adulthood. Some familial transmission has been seen, however the majority of CL cases are sporadic. An autosomal dominant mode of inheritance has been suggested, but no clear chromosomal region has been identified. The only candidate regions that have been seen were a balanced 3;17 chromosomal translocation and ring 3 and 9 chromosomes. No consistent chromosomal abnormality has been observed in CL cases. PAPP-A levels have been suggested as another biochemical indicator as they have been demonstrated to be undetectable in the maternal serum or trophoblastic tissues of patients with this rare syndrome (Westergaard et al, **1983c**). This combined with the observation that the locus for PAPP - A is on Chromosome 9

(Silahtaroglu et al, **1993**) and one case of CL has been associated with a ring 9 chromosomal abnormality (Opitz, **1985**) suggests that PAPP-A may be implicated in the aetiology of this syndrome but further studies are needed to define it's role.

1.4.7.5 Down Syndrome

John Down in 1866 described a series of patients with what is now defined as Down Syndrome (DS). The cause of this phenotype was found to be trisomy of chromosome 21 in 1959. Approximately 1 in 600 - 800 live births are born with DS (Hernandez and Fisher, **1996**). A range of clinical features seen with this syndrome are described in Table 1.8.

Table 1.8 Clinical Features of DS

[1]Clinical Features	Frequency of Features Seen in DS Patients
Mental Retardation	99[2,3] %
Neonatal Hypotonia	100[3] %
Dysmorphology	- 90[2] % Flattened facial features. - 50[2] % Speckled Iris.
AD Histopathology	100[3] % by the age of 35 years (30% have AD-dementia by age of 40.)
Cong. Heart Defects	40 - 60[2,3] %
Growth Retardation	31[4] % below 10th centile at term.
Acute Leukaemia	1[3] %

[Legend: AD: Alzheimer Disease. Cong.: Congenital. [1]Clinical Features: These are not a comprehensive list but represent a selected range of features; A comprehensive listing of DS features can be found in: [2]: Buyse, **1990**. [3]: Hernandez and Fisher, **1996**. [4]:Cuckle and Wald, **1992.]**

It is the most common form of aneuploidy seen in Man; the observation that a maternal serum marker (alpha-fetoprotein, AFP) is reduced in DS pregnancies (Cuckle et al, **1987**) led to the possibility of screening for this aneuploidy. Wald (**1988**) suggested the use of the biochemical maternal serum triple test assaying for AFP, Total hCG and Unconjugated free in Oestriol between 15 and 22 weeks of pregnancy and this is now established many countries.

1.4.7.5a Maternal Serum Screening for DS in the 2nd Trimester

A multi-centre evaluation of maternal serum screening methods (Ward, **1994**) demonstrated that the measurement of unconjugated Oestriol adds little to the risk assessment (probably because it includes a maternally derived component, Cuckle and Wald, **1992**). A superior assessment of risk is obtained by measuring levels of free β-Chain of hCG and AFP (Table 1.9).

Table 1.9 Detection Rates for DS in the 2nd Trimester when analysing different maternal serum analytes.

Analyte measured:	% [1]Detection rate:	% [2]False positives:
[3]AFP + Free βhCG	63.8	5.9
[3]AFP + Total hCG	55.3	6.5
[3]AFP + ThCG + UE3	55.1	7.1

[Legend: [1]: Given as proportion of affected pregnancies with positive results. [2]: Given as proportion of unaffected pregnancies with positive results. [3]: Figures given were derived from Ward, **1994**.]

PAPP-A levels have been shown not to be significantly different in Down syndrome pregnancies when compared to normal unaffected pregnancies in the second trimester (Knight et al, **1993**), It is thus not a useful maternal serum marker in the second trimester. PAPP-A measurements in the first trimester of pregnancy are significantly lower in trisomic foetuses than in normal controls (Wald et al, **1992**). This may therefore form the basis of an effective first trimester screening program.

1.4.7.5b Maternal Serum Screening for DS in the 1st Trimester

It has been demonstrated that maternal serum PAPP-A levels are reduced in chromosomally abnormal foetuses (Wald et al, **1992**). Bersinger et al, (**1994**) and Brambati et al, (**1994**) have also raised the possibility of maternal serum screening in the first trimester with the indication that a combination of age and PAPP-A levels offer a higher aneuploidy detection rate when compared to βhCG and maternal age alone.

The conclusion of multi-centre trial assessing first trimester screening for DS against a panel of seven maternal serum markers concluded that maternal serum screening for DS is now a practical proposition in the first trimester of pregnancy. The most discriminatory markers appeared to be a combination of β-hCG and PAPP-A (Wald et al, **1996,** Table 1.10). Approximately 50 % of DS pregnancies abort between the first trimester and term. It is likely that the maternal biochemistry at the time of CVS may reflect the process of miscarriage, Stabile et al, (**1988**) have shown that the maternal levels of PAPP-A in pregnancies that go on to miscarry are reduced. Thus screening for DS during the first trimester will also detect pregnancies that would not have proceeded to term.

Table 1.10 Comparison of maternal serum DS screening during first and second trimester of pregnancy

	First Trimester (8 - 14 wks)		Second Trimester (15 - 22 wks)	
[1]Analytes measured:	% [2]DR	% [3]FP	% [2]DR	% [3]FP
[4]PAPP-A,β-hCG+ mat. age	62	5	54	5
[5]Triple Test	**nd**	**nd**	59	5
[6]Quadruple Test	**nd**	**nd**	72[7]: (65[7])	5
[8]Nuchal translucency (NT)	86	5	**nd**	**nd**
NT + Serum Markers[8]	91	5	**nd**	**nd**

[Legend: [1]: Maternal serum markers used for DS screening. [2]: Detection rate as defined in Table 1.9. [3]: False positives as defined in Table 1.9. [4]: Figures derived from Wald et al, **1996.** [5]: Triple test as defined by Wald (**1988**) and listed above. [6]: Quadruple test as defined by Wald et al, **1995.** [7]: Percentage detected if gestational age determined by ultrasound dating (Percentage detected if gestational age based on LMP dates). [8]: Data taken from Wald et al, **1995. Note:** Mat.: Maternal **nd.:** Not determined/or reported.**]**

It has been proposed by Wald et al, (**1995**) that by combining ultrasound examination of nuchal translucency during the first trimester with a biochemical screening program will increase the detection rate to >80% and reduce the false positive rate to < 5%, but this will have to await a combined control trial.

1.4.7.6 The use of Biochemical Tests To Assess Foetal Well-being

It has been suggested that biochemical tests indicating foetal well-being have fallen into disfavour (Klopper, **1987**). This has mainly been because they have a large normal range, thus making it difficult to separate normal from pathological conditions in pregnancy. The situation is further compounded in that most of the new placental proteins have no ascribed function. This class of proteins may have a role in antenatal monitoring, PAPP-A can now be used as a biochemical indicator for first trimester screening of Down syndrome (section 1.4.7.5).

1.4.8 *In-Vitro* Biological Roles Attributed To PAPP-A

During the period since PAPP-A's isolation several biochemical effects have been attributed to PAPP-A (Table 1.11).

1.4.8.1a Immunosuppression

Martin-du-Pan et al, (**1983**) did not demonstrate that PAPP-A inhibited PHA induced lymphoblastogenesis as they used seminal plasma and correlated levels of PAPP-A with the degree of immunosuppression seen. Bischof et al, (**1983**) used PAPP-A that was contaminated with other serum proteins that were immunosuppressive. Thus the contradictory findings that PAPP-A does not inhibit PHA induced lymphoblastogenesis by McIntyre et al, (**1981**) and Sinosich et al, (**1984**) confirm that it is unlikely that PAPP-A is involved in immune suppression.

1.4.8.1b/c Inhibition of Complement(b) and Fibrinolytic(c) systems

PAPP-A was shown to inhibit complement fixation and thrombin induced coagulation (Bischof, **1979a.** Meisser et al, **1985**) whereas others could not show this (Gore et al, **1984**, Sinosich et al, **1990**). Meisser et al, (**1988a, 1988b**) demonstrated that the initial activity attributed to PAPP-A was an artefact and both the reported inhibitory actions were likely to be due to the presence of heparin that is known to inhibit both the complement and

Table 1.11 *In - Vitro* Biological Roles Attributed To PAPP-A

Biological Effect Attributed to PAPP-A	Effect Noted By:	Effect Refuted By:
1) Inhibition of:		
a) Immune System		
- PHA induced lymphoblastogenesis	Martin-du-Pan et al, (1983)[E] Bischof et al, (1983)[P]	McIntyre et al, (1981), Sinosich (1990)
b) Complement	Bischof (1979a)[P]	Gore et al, (1984), Meisser et al, (1988a)
- Classical pathway		Sinosich (1990)
- Alternate pathway		Sinosich (1990)
c) Fibrinolytic System		
- Inhibits thrombin induced coagulation of fibrinogen	Meisser et al, (1985)[P-CH]	Meisser et al (1988a)
d) Protease's		
- Plasmin	Bischof (1979a)[P]	Gore et al, (1984), Sinosich (1990), Oxvig et al, (1994)
- Granulocyte elastase (non-competitive inhibition)	Sinosich et al, (1982)[P*]	Bischof et al, (1990), Oxvig et al, (1994)[P*]
2) Sperm Motility	Bolton et al, (1986)	
3) Carrier Protein		
- Zinc	Sinosich et al, (1983)	

[**Legend:** [E]: Effect described likely to be due to experimental design, see section 1.4.8.1a. [P]: Effect described likely to be due to the use PAPP-A contaminated with other serum proteins, see section 1.4.8.1b/c. [P-CH]: Effect likely to be due to heparin contaminating purified PAPP-A material, see section 1.4.8.1b/c.[P*]:Contradictory results probably due to differences in PAPP-A material prepared by research groups, see section 1.4.8.1d.]

fibrinolytic systems. The initial material and effect noted by Bischof in 1979 used PAPP-A that was not contaminated with heparin. The initial inhibition demonstrated was probably due to the purity of PAPP-A used as this material has been subsequently shown to be contaminated with other serum proteins. PAPP-A has not been shown to convincingly inhibit either of these two systems.

1.4.8.1d Proteolytic Inhibition

Bischof (**1979a**) demonstrated inhibition of plasmin but as already described the material used was contaminated with other serum proteins and others (Gore et al, (**1984**), Sinosich (**1990**)) did not demonstrate an inhibition of plasmin. It is thus unlikely that PAPP-A inhibits either of these two proteases.

It has been suggested by Sinosich et al,(**1982a, 1991**) that PAPP-A is a specific inhibitor of human granulocyte elastase (HGE). Other investigators have suggested that this inhibition is due to a heparin contaminant (Bischof et al, **1990**) that is responsible for the inhibition of HGE. Oxvig et al, (**1994**) have found that the described inhibition is non-specific in nature (Table 1.12). The inhibition of HGE by PAPP-A is considered below.

Bischof et al, (**1990**) found that HGE was inhibited by heparin sepharose column washings in absence of PAPP-A under hypotonic (>0.15 M NaCl) conditions, with suggestion that the reported inhibition of HGE by PAPP-A was due to a leeched heparin contaminating component. In contrast Sinosich and Zakher (**1991**) found PAPP-A's inhibition of HGE was labile, inactive PAPP-A did not to inhibit HGE at isotonic or hypertonic NaCl concentrations. In contrast heparin that was found to inhibit HGE under isotonic conditions but not at hypertonic (>0.15M NaCl) conditions. PAPP-A inhibition could be shown to specifically inhibit HGE under hypertonic conditions where heparin was shown to have no inhibitory affect.

Contradictory findings were observed by Oxvig et al, (**1994**) who suggested that the inhibition is likely to be due to electrostatic interactions between the glycosaminoglycan on proMBP linked to PAPP-A in their

purified PAPP-A and a set of Arg-residues located near the active site of HGE.

Table 1.12 Type of HGE Inhibition observed with PAPP-A

Investigator (s)	Type of Inhibition	[1]Inhibition (<0.15M NaCl)	[2]Inhibition (>0.15M NaCl)
Sinosich et al, (**1982a**)	Non-Competitive (Specific)	**nd**	**nd**
Sinosich et al, (**1990**)	Non-Competitive (Specific)	**nd**	**nd**
Sinosich/Zakher (**1991**)	**nd** (Specific)	++	++
Oxvig et al, (**1994**)	Competitive, (Non-specific)[3]	++	-

[Legend: nd: not determined (or reported). **++:** Inhibition positive. **-:** Inhibition negative. [1]: Reaction performed under hypotonic NaCl conditions. [2]: Reaction performed under hypertonic NaCl conditions. [3]: Cathepsin-G competitively inhibited.]

A possible explanation of the contradictory results obtained by Sinosich et al, (**1982a, 1990, 1991**) and Oxvig et al, (**1994**) for the inhibition of HGE by PAPP-A could be due to differences in composition of PAPP-A purified by these two groups. It has been demonstrated that proMBP binds to heparin and is purified by affinity chromatography to this matrix (Popken-Harris et al, **1994**). Sinosich et al, (**1987**) use a heparin affinity matrix and negative immunoaffinity against non-pregnancy heparin binding proteins. Thus it is possible that this antibody removes the PAPP-A/proMBP complexes that have been described by Oxvig et al, (**1994**) to yield PAPP-A free of proMBP. The discrepancy in type and specificity of inhibition of HGE by PAPP-A found by these two groups is at present unresolved.

1.4.8.2 Sperm Motility

It has been seen that the levels of PAPP-A found in seminal plasma correlate with sperm motility (Bolton et al, **1986**), with increased PAPP-A levels found in samples in which the sperm had a motility >60%. It is not known whether this represents an effect on sperm motility, or is due to the

presence of increased accessory gland secretions that have been noted to have an affect on sperm motility. The significance of this observation is at present unknown.

1.4.8.3 Carrier Protein

Sinosich et al, (1983) noted that PAPP-A interacted and chelated, Zinc. They also demonstrated that PAPP-A has an absorption maximum at 214 nm that is characteristic of an interaction for a zinc carrier protein. An analysis of PAPP-A cDNA (Kristensen et al, 1994) reveals the presence of a putative Zinc binding site that is characteristic of metalloproteinases. Thus PAPP-A binds Zinc, the function of which is at present unknown.

1.4.8.4 *In-Vivo* Biological function of PAPP-A

That such a diverse set of ascribed functions has been attributed to PAPP-A *In-Vitro* that have been confirmed and refuted by different investigators is probably a reflection on the purity of the PAPP-A material that was used. The biological function of PAPP-A *in-vivo* has probably yet to be elucidated.

1.5 Aims of The Study

The aim of this study was to further characterise the PAPP-A molecule; to this end a molecular approach was undertaken. This necessitated an assessment of the antibodies available for PAPP-A and involved the isolation of the PAPP-A protein in a homogeneous form that enabled primary sequence information to be generated for PAPP-A. This information allowed the production of PAPP-A specific oligonucleotide probes that were used to screen a placental cDNA library. The isolation of a pure PAPP-A monomer allowed a further characterisation of the PAPP-A monomeric sub-unit.

CHAPTER TWO

Chapter Two

Materials And Methods

2.1 Materials and General Equipment

A detailed list of materials, equipment and suppliers used in this study can be found in Appendix 1. Unless otherwise indicated all other chemicals were of BDH 'Analar' grade or its equivalent. The preparation of stock reagents and buffers is described in Appendix 2. The distilled water referred to as dH_2O in the text was glass-distilled, de-ionised and filtered (0.2 μm) nanopure grade (> 16.7 Megaohms).

Aseptic techniques were performed as described in Sambrook et al **(1989)**. General sterilisation of plasticware and solutions (unless otherwise indicated) was achieved by autoclaving at 15 lbs. pressure for 15 minutes. Filter sterilisation where required was through 0.22μm syringe filters (Sarstedt).

Blood samples were obtained in collaboration with Dr T.C. Lee, Jessop's hospital, Sheffield. Maternal blood was obtained from women at term with their informed consent. All blood samples were collected as either EDTA or Citrated Plasma unless otherwise stated (section 2.7.2, Chapter 2).

An International Reference serum 78/610 was used initially as a standard for PAPP-A. It was assumed that this contained 110 μg/ml (Pinto-Furtado et al,**1984**) of PAPP-A. This was a kind gift of the International Agency for Research on Cancer, 150 Cours Albert-Thomas, Lyon, France.

Monoclonal antibodies to PAPP-A were produced and kindly provided by Ms E.A. Mowles, Department of Biology and Biochemistry, University of East London.

Heparin-Sepharose (pre-1984) was kindly donated by Mr R. Stoker, Department of Biology and Biochemistry. University of East London.

Partially purified PAPP-A used initially for radioiodination was kindly supplied by Miss K. Price, The Royal London Hospital, Whitechapel.

Allesheria terrestris culture supernatant was a kind gift of Dr E. Kvesitadze, Republic of Georgia.

2.2. Services Used During This Study:

2.2.1 Custom Oligonucleotide Synthesis

These were produced in - house using PAC phosphoramidites on a Pharmacia LKB Gene Assembler Plus by Mr P. Loxley. De-protection and end-processing was as described in Appendix 2.

2.2.2 Protein Sequencing

- Dr K. Lilley, Department of Biochemistry, University of Leicester.
- Mr J. Gilroy, Department of Biological Sciences, University of Durham.
- Dr A. Moir. Department of Molecular Biology and Biotechnology, University of Sheffield.

2.2.3 Amino Acid Analysis

- Dr A. Moir. Department of Molecular Biology and Biotechnology, University of Sheffield.

2.3 General Methods

2.3.1 The Determination of Protein Concentration

Protein concentration can be estimated by spectrophotometric methods either directly by absorption of UV light at 280 nm or following the addition of chemicals that interact with proteins leading to the formation of a coloured complex with a maximum of absorption within the visible range. The amount of protein in an unknown sample can then be determined by comparing its absorbance with that of the standard. Two methods were used in this study, one based on the colour change when CBB dye binds to a protein (Bradford, **1976**). The other utilised protein interaction with BCA, as originally described by Smith et al, (**1985**).

2.3.1.1 Bradford Protein Assay

When the Dye CBB G-250 binds to basic and aromatic amino acids (Compton and Jones, **1985**) there is a shift in the absorption maxima of the dye from the red form to the blue form that can then be used as a measure of the amount of protein present. The microassay procedure as described in the Bio-Rad Protein Estimation kit was used, using BSA as the standard protein. Briefly, 800 µl of samples/standards diluted in PBS were mixed with 200 µl of Bio-Rad dye concentrate by vortexing. The samples were left for 10 minutes and then the absorbance was read at 595 nm.

The main problem with this assay was due to the dependence of dye binding on the number of basic and aromatic amino acids, leading to differences in binding due to the protein primary structure. It was also seen to suffer from interference by buffer components that interact with the protein or dye components. However the assay components were very stable over time and it was used mainly to determine protein levels when loading electrophoresis gels.

2.3.1.2 BCA protein assay

Proteins are known to reduce alkaline Cu(II) to Cu(I) (Lowry, **1951**). Bicinchoninic acid has been shown to be a specific chromogenic reagent for Cu(I), on interaction it forms a purple complex whose resulting absorbance is directly proportional to the protein concentration. The BCA assay and its composition is essentially that of Smith et al (**1985**). A modified BCA assay was used using a microtitre plate format. Briefly 20 µl of sample (or standard) was placed in a microtitre plate well, 20 µl of 0.1 M NaOH and 200 µl of BCA reagent was then added to each well. The microtitre plate was mixed at RT for 60 minutes using a Denley well-mix . It was found that a modification of an alkali addition to this micro-assay did not result in a change in levels measured in the protein standard (BSA) used , Figure 2.1. It did however result in less variation in protein levels that were found and measured during the purification of PAPP-A. The measured levels were closer to the levels observed using other methods of protein estimation such as the absorbance at 280 nm and integration of elution profiles. This

modified BCA microtitre plate assay was used to monitor protein levels found during the purification of PAPP-A.

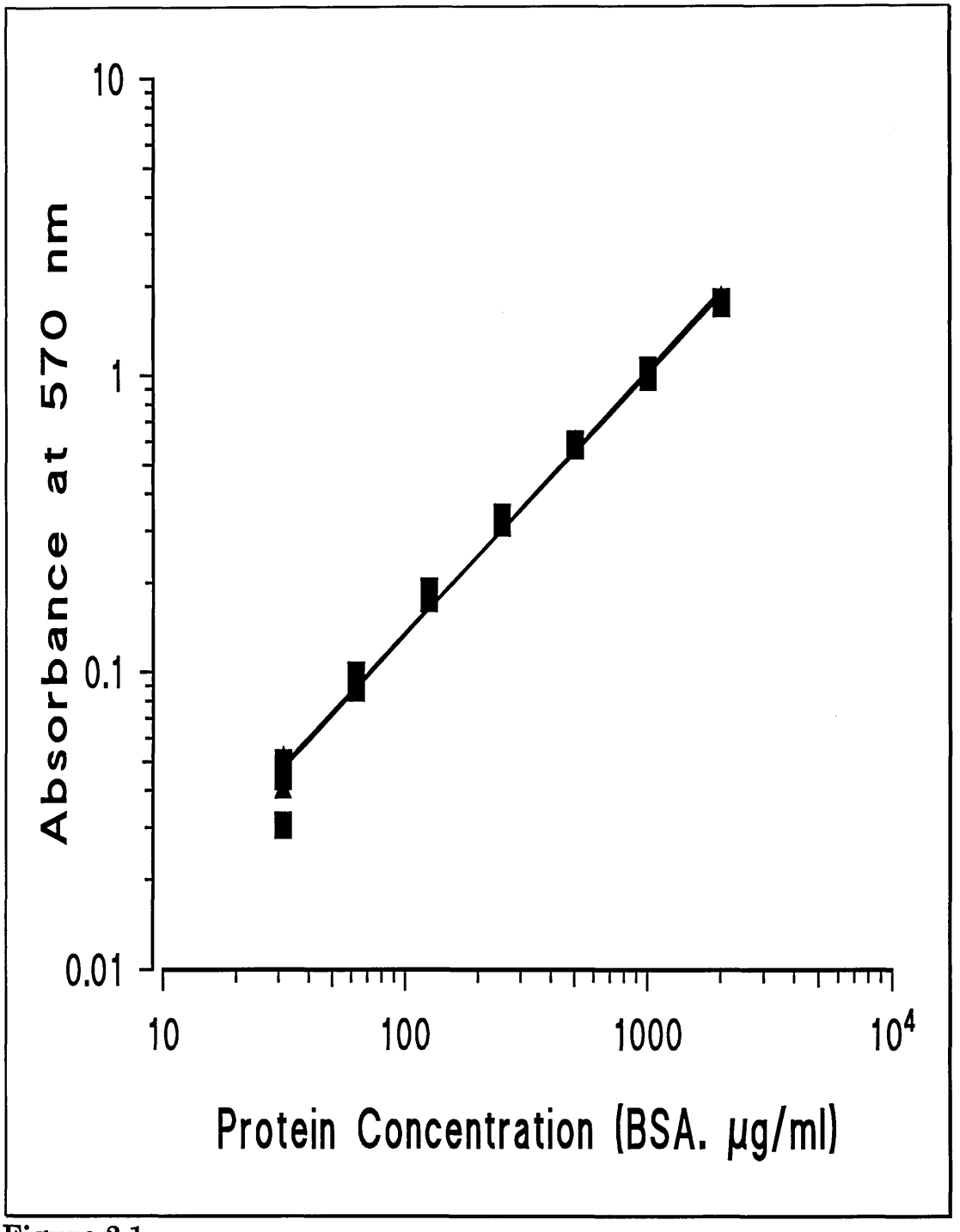


Figure 2.1

The BCA protein micro-assay (+/- alkali addition) with BSA used as the protein standard.

[Legend: All points were in triplicate. (■); indicates standard plus alkali addition, $r^2 = 0.992$. (▲); indicates PBS in place of alkali addition, $r^2 = 0.996$**].**

2.3.2 Electrophoretic Methods

Electrophoresis is a method whereby charged molecules migrate in response to the application of an electric field. Proteins are amphoteric, so in a solution whose pH is above the proteins iso-electric point then the protein will have a net negative charge. Nucleic acids are negatively charged a broad range of pH, so under these conditions macromolecules will migrate towards the anode.

2.3.2.1 Horizontal Agarose Slab Gel Electrophoresis of DNA

Aaij and Borst (**1972**) demonstrated that the electrophoretic mobility of DNA molecules in agarose and polyacrylamide gels is inversely proportional to the log of their molecular weight. By varying the agarose concentration and buffer composition it is possible to separate DNA in the size range 20bp - 10Kbp. Submerged horizontal electrophoresis was performed as described in Sambrook et al, (**1989**). In brief an X% (w/v) agarose gel (low EEO) was prepared in Tris-Acetate buffer, on cooling ethidium bromide was added to a final concentration of 1 μg/ml. This was then poured into a horizontal gel former (Biometra) and a comb positioned. The gel was allowed to set for a minimum of 30 minutes and was subsequently submerged in Tris-Acetate buffer. The submerged gel allows quick dissipation of any heat generated during electrophoresis, so reducing any band distortion. Samples were pre-treated by adding 0.2:1 (v/v) of sample buffer:sample (Appendix 2) and loaded into gel slots. Electrophoresis was performed at 120 V (constant voltage) until the BPB front was approximately 2/3rds the length of the gel and visualisation was by exposure of the gel to UV - light.

2.3.2.2 SDS-PAGE

A modified Laemmli (**1970**) SDS PAGE system was used, which has the following essential features:

- A modification of that originally described by Ornstein (**1964**) who outlined the theory and its practical application, as described by Davis (**1964**) for a discontinuous system that has a non-restrictive large pore stacking gel that is layered on top of a resolving gel. Each of these gels was

made with a different buffer, the tank buffers was also of a different composition from that present in the gel. This ensured that the protein loaded migrates at an intermediate rate that was determined by the mobility of the buffer ion in the stacking gel (leading ion) and the mobility of the buffer ion in the upper tank (trailing ion). When electrophoresis starts then the ions and proteins start to migrate into the stacking gel. The proteins then concentrate into a very thin zone, the stack between the leading and trailing ions. This continues until they reach the resolving gel and results in a loading of a sharp band onto the resolving gel.

- The majority of proteins have been shown to bind 1.4 g of SDS per 1g. of protein (Reynolds and Tanford,**1970**), this ensures that proteins in effect become negatively charged rods that have equal charge densities over their entire length. If electrophoresis occurs through a matrix with a constant pore size, separation will occur as function of size as molecular sieving will take place. This slows down the migration rate of a larger protein when compared to its smaller counterpart, thus resulting in an inverse linear relationship when plotting the log M_r of the protein Vs its relative mobility.

A highly uniform gel structure with a consistent pore size is produced by polymerising acrylamide under controlled chemical conditions using an initiator which produces oxygen free radicals (e.g. ammonium persulphate) from a base catalyst (e.g. TEMED). Oxygen acts as an inhibitor of polymerisation so the top of the resolving gel is covered with water saturated n-Butanol to limit this effect and reduce any meniscus effects at the top surface of the resolving gel.

2.3.2.2a The Laemmli (1970) SDS-PAGE System

This system with the following modifications was used:

- Samples were diluted 1:1 (v/v) in sample buffer (Appendix 2) and then boiled for 2 minutes, centrifuged briefly and loaded onto the gel. Prior to sample loading the stacking gel slots were washed with running buffer to remove any unpolymerised acrylamide.

- A 5% (w/v) polyacrylamide (30%T: 2.7% C) separating gel was cast using Tris-HCl at pH 8.6, with the stacking gel at pH 6.8. Both gels contained a final concentration of SDS of 0.1% (Table 2.1).
- The initiator and catalyst of polymerisation were ammonium persulphate and TEMED respectively.
- The tank stock running buffer (Appendix 2) was diluted tenfold with distilled water to give a 2.5 mM Tris/190 mM glycine buffer at pH 8.6 containing 0.1% SDS .
- Electrophoresis was at a constant voltage of 50 Volts overnight.

Table 2.1 Composition of Laemmli SDS-polyacrylamide gels.

Components	Stacking Gel	Resolving Gel
Volume(**ml**)	10	40
30 %Acrylamide/Bis(**ml**)	1.5	6.6
10 % SDS(**ml**)	0.1	0.4
1 M Tris-HCl(**ml**)	(**pH 6.8**) 1.25	(**pH 8.6**) 15
10 % APS(**ml**)	0.1	0.4
dH_2O(**ml**)	7	17.4
TEMED(**ml**)	0.01	0.04
	Volume sufficient for one 14 x 20 cm gel	

2.3.2.2b Modifications to SDS-PAGE System if sample was to be N-terminally sequenced.

- The resolving gel was cast and left to age overnight prior to use.
- The stacking gel was cast at least 3 hours prior to use.
- The upper tank buffer included 0.2 mM sodium thioglycolate, which was pre-run for 1/2 hour prior to the samples being loaded.

2.3.2.2c SDS Linear Gradient Gel Electrophoresis.

The SDS-PAGE system described above was used for monitoring the purification procedure, however a separation system was needed which yielded sharper bands over a wider size range than that obtained with the described SDS-PAGE system. A linear gradient of increasing acrylamide

concentration (%T) produces a gel that introduces the element of size selection on the basis of a decreasing pore size, thus also size fractionating proteins that were loaded. A gradient mixer system as described by Hames (**1991**) was used to cast these gels but as they were going to be used to separate proteolytic digests of PAPP-A. A modified buffer system as originally described by Schagger and Von-Jagow (**1989**) was used that enabled a greater size range of proteins to be separated. The inclusion of a glycerol gradient also aided in the preparation of a reproducible gradient system. The gel composition was as given in Tables 2.2 and 2.3.

Table 2.2 The standard linear gradient SDS-PAGE gel system

Components of resolving gel	**Acrylamide Concentration**	
	5%T.	**20%T.**
Volume(**ml**)	17.5	17.5
30 % Acrylamide/Bis (**ml**)	2.9	11.7
10 % SDS (µl)	175	175
3 M Tris-HCl (pH 8.8, **ml**)	2.2	2.2
Glycerol (**ml**)	(1%) 0.175	(10%) 1.75
dH_20 (**ml**)	12	1.6
20 % APS (µl)	31	31
TEMED (µl)	6	6
Components of stacking gel		
Volume(**ml**)	10	
30 % Acrylamide/Bis (**ml**)	1.5	
10 % SDS (µl)	100	
1 M Tris-HCl (pH 6.8, **ml**)	1.25	
dH_20 (**ml**)	7.1	
20 % APS (µl)	50	
TEMED (µl)	10	
	Volume sufficient for one 14 x 20 cm gel	

Table 2.3 The modified linear gradient SDS-PAGE gel system

Modified gradient system using Schagger and Von-Jagow Buffers		
Components of resolving gel	**Acrylamide Concentration**	
	5%	**20%**
Volume(**ml**)	17.5	17.5
30 % Acrylamide/Bis (**ml**)	2.9	11.7
Peptide gel buffer (Appendix 2, **ml**)	3.5	3.5
Glycerol (**ml**)	(1%) 0.175	(10%) 1.75
dH_20 (**ml**)	10.8	0.45
10 % SDS (µl)	70	70
20 % APS (µl)	31	31
TEMED (µl)	6	6
Components of stacking gel		
Volume(**ml**)	10	
30 % Acrylamide/Bis (**ml**)	1.35	
10 % SDS (µl)	25	
Peptide gel buffer (Appendix 2, **ml**)	2.5	
dH_20 (**ml**)	5.9	
20 % APS (µl)	50	
TEMED (µl)	10	
	Volume sufficient for one 14 x 20 cm gel	

The tank running buffer for the standard gradient system was as described in section 2.3.2.2a. The modified system had different anodic and cathodic running buffers (Appendix 2) but were briefly a 0.1 M Tris/Tricine SDS cathodic buffer and 0.2 M Tris-HCl (pH 8.9) as the anodic buffer. As was seen from Figure 2.2 the modified gel system used in this thesis:

- Extended the size range of proteins that can be separated.
- Yielded a wider linear range for the of proteins that were separated.

It was thus a suitable system to separate limited proteolytic digestions of PAPP-A. (Chapter 6).

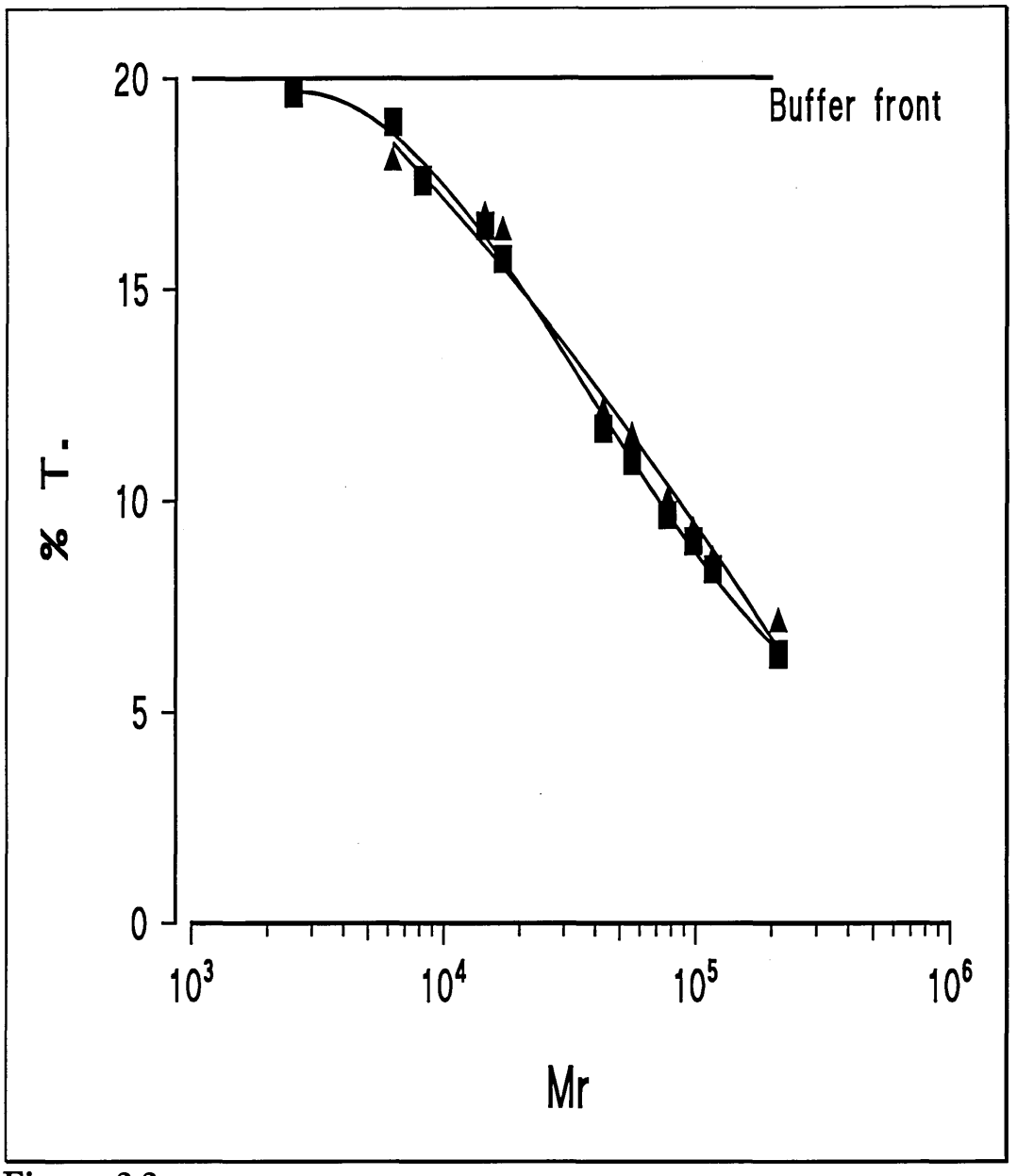


Figure 2.2

Linear Gradient SDS-PAGE

(The standard gradient system Vs modified system developed in this thesis)

[Legend: %T: This was calculated as a function of expected linear gradient with known start (5%) and finishing points (20%) of the gradient. **M_r:** Size from use of molecular weight marker standards. Molecular weight standards were the high, medium and peptide markers from BDH (Appendix 1); These were taken as Myosin (205 kDa), β-Galactosidase (116.3 kDa), Phosphorylase-b (97.4 kDa), Ovotransferrin (77 kDa), Albumin (66.3 kDa), Glutamate dehydrogenase (55.5 kDa), Ovalbumin (42.7 kDa) Carbonic anhydrase (30 kDa), Myoglobin, (Equine 17.2 kDa), Myoglobin (horse-heart, 16.95 kDa), Myoglobin peptide fragment I (14.4 kDa), Cytochrome-c (12.3 kDa), Myoglobin peptide fragment II (8.16 kDa), Myoglobin peptide fragment III (6.2 kDa) and Myoglobin peptide fragment IV (2.5 kDa). Standards were run in duplicate. (▲) samples/standards were run in standard gradient system. (■) Samples/standards were run in modified system described in this thesis, section 2.3.2.2c.]

2.3.2.2d Protein Detection: CBB Staining of SDS-PAGE Gels

General protein staining of SDS-PAGE gels was performed as described by Wilson and Yuan (**1989**), In brief, the gel was incubated in 0.04% pre-warmed (42^{0}C) G-250 reagent (Appendix 2) for 60 minutes at 42^{0}C. The gel was then destained in 5 % acetic acid for about 30 minutes and stored in sealed plastic bags.

2.3.2.2e Protein Detection: Silver Staining of SDS-PAGE Gels

SDS-PAGE gels were silver stained using a method first described by Merril et al, (**1981**). A Bio-Rad silver staining kit was used. Briefly, the gels were fixed in Fixer 1 for 30 minutes. They were then fixed in Fixer 2 for 2 X 15 minutes. The gel was then placed in an oxidiser (yellow) for 5 minutes and then washed in multiple changes of distilled water until all visible traces of yellow within the gel have disappeared. The gel was then placed for 20 minutes in silver reagent, briefly washed with distilled water and then the gel was placed in pre-warmed developer (37^{0}C). The developer was replenished every five minutes and development stopped by incubating the gel in 5% acetic acid for at least 5 minutes. The gels were stored in sealed plastic bags. This silver stain procedure was very sensitive to any impurities present in distilled water, so all preparation of reagents and manipulations were carried out in clean distilled water washed glass apparatus.

2.3.3 Measurement of Chitinase Activity

Chitinase activity was assessed by the measurement of the N-acetyl amino sugars that were released from chitin. It was performed essentially as described by Thomas (**1981**). Crude chitin was also purified as described by Thomas (**1981**) using a method that was described in Appendix 2. An aliquot (0.5 ml) of a stock suspension of purified chitin was incubated at 30^{0}C with 0.1 ml of chitinase standard (or sample). The reaction was terminated by removing an aliquot (0.1 ml) and adding it to 0.1 ml of 0.8 M potassium tetraborate, pH 9.2, this was boiled for 3 minutes and the N-acetyl-glucosamine (NAG) content was measured (section 2.3.4).

2.3.4 Measurement of N-Acetyl-glucosamine (NAG)

The NAG content was determined using the method cited from Reissig et al (**1955**) with modifications as described by Thomas (**1981**). The stopped reaction from section 2.3.3 after cooling to RT was incubated with 1 ml of DMAB reagent (Appendix 2) at 37^{0}C for 20 minutes. The absorbance at 585 nm was read and compared to a set of NAG standards that were prepared in PBS (Figure 2.3).

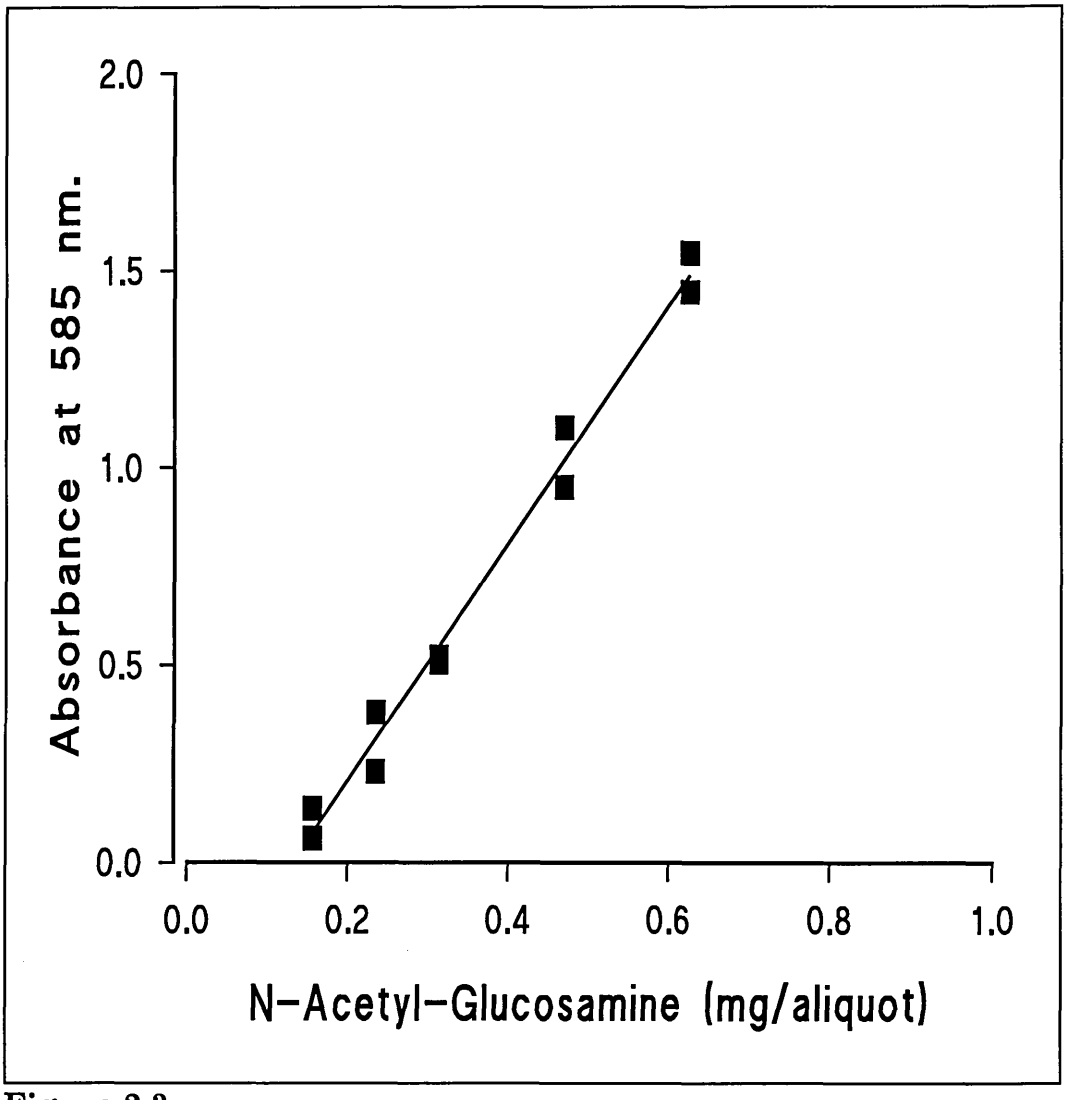


Figure 2.3

Standard calibration curve for N-acetyl-glucosamine using DMAB reagent

[Legend: The calculated absorbance was plotted as standard value minus the value at 0.12 mg/aliquot of NAG. Standards and samples were run in duplicate and as outlined in sections 2.3.3 and 2.3.4.]

2.3.5 Assessment of General Proteolytic Activity

Proteolytic activity can be assessed by a variety of methods. The advantages and disadvantages of each method have been described by Sarath et al, (**1989**). The assay developed here had the advantage that it was quick and required no special equipment. It utilises the protease's ability to digest a standard protein (BSA) and then the discrimination between the digested material and native protein by TCA precipitation. The products of digestion that were present in the supernatant were determined using the protein assays described in this Chapter (sections 2.3.1.1 and 2.3.1.2).

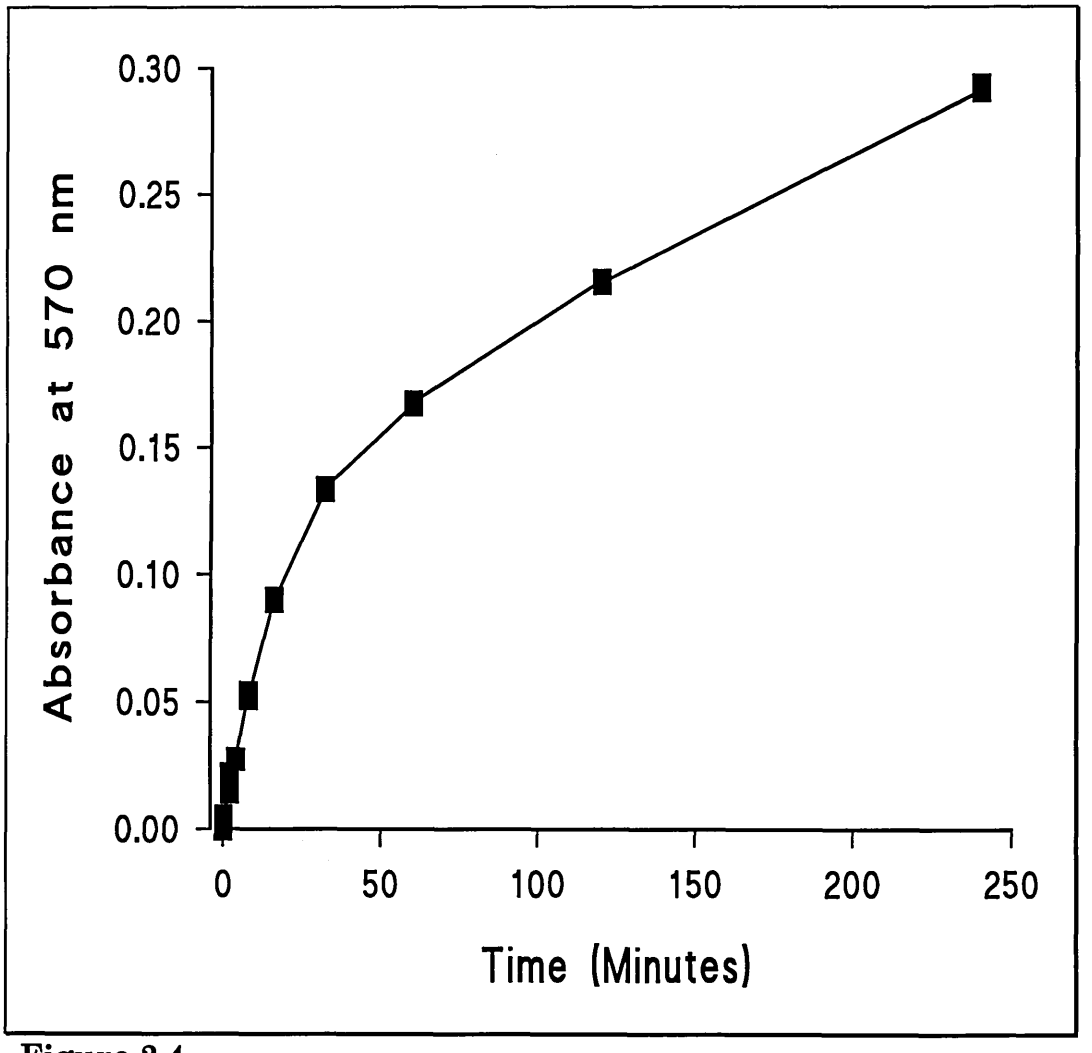


Figure 2.4

The general protease assay with detection using the BCA protein assay

[Legend: (■) 25 Units (Appendix 1) of standard protease/aliquot. (The standard protease used was chymotrypsin, Appendix 2), protein measurement was as described in section 2.3.1.2).]

Briefly; 1 ml of a 1% BSA solution in PBS (Appendix 2) was pre-incubated at 37°C. Aliquots (50 - 100 µl) of sample (or standard protease, as detailed in legend of Figure 2.4) were added and incubated at 37°C. At various times, a 100 µl aliquot was removed and placed in 900 µl of TCA reagent (Appendix 2), which was heated to 90°C for 3 minutes. This was then centrifuged and an aliquot of the supernatant removed and assayed for the protein concentration that was present (Figure 2.4) as outlined in section 2.3.1.2.

2.3.6 Measurement of Tosyl-arginine methyl ester (TAME)

The measurement of this ester is based upon the quantitative colorimetric reaction that was described by Castellano and Sodetz (**1969**). TAME reacts with hydroxylamine to yield hydroxamic acid, the concentration of this acid can be measured by the conversion of ferric chloride to a ferric ion-hydroxamic acid complex that can be determined using a colorimetric measurement of this complex at 525 nm.

Briefly a 50 µl aliquot containing the TAME substrate (Appendix 2) was mixed with 40 µl of Reagent A (Appendix 2) and incubated for 30 minutes at RT. Hydroxamic acid formation was complete after 30 minutes and was measured by the addition of 80 µl of a TCA solution (Appendix 2) and 800 µl of a ferric chloride solution (Appendix 2), this was mixed and the absorbance at 525 nm was determined for each sample.

2.4 Miscellaneous Membrane Associated Techniques

2.4.1 Semi-Dry Electroblotting (Western blotting)

Proteins present in an SDS-PAGE resolving gel can be transferred onto a membrane by electroblotting. The choice of a blotting membrane was dictated by the purpose of the blot. Nitrocellulose (Schleicher and Schuell), was used if the blot was to be probed with antibodies or lectins. Immobilon - P (Millipore) or a modified PVDF (Fluorotrans, Pall) were used if the blot was to be stained for proteins or used for N-terminal protein sequencing.

A semi-dry method of protein transfer as outlined by Kyhse-Andersen (1984) was used. Blotting membranes were allowed to equilibrate for at least 10 minutes in transfer buffer prior to electroblotting. If PVDF membranes were used they were pre-wetted in 100% methanol and then equilibrated in transfer buffer. The gel was equilibrated in transfer buffer on an electroblotter (Biometra) as a sandwich shown in Figure 2.5. The blotter was cooled by the passage of cold water through the base of the electroblotter. Electroblotting was carried out at 5 mA/cm^2 for 10 minutes followed by 10 mA/cm^2 for 20 minutes.

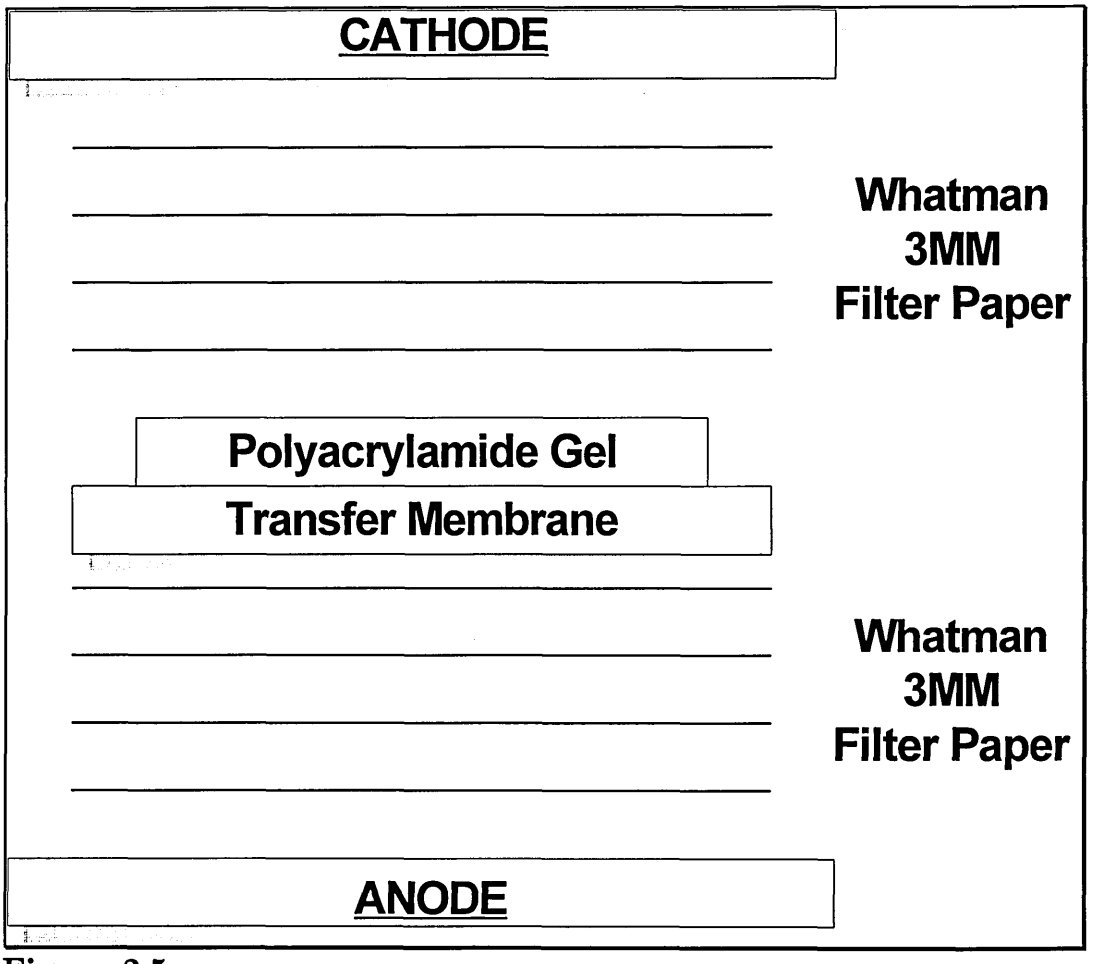


Figure 2.5

Schematic illustrating semi-dry electroblotting sandwich.

2.4.2 Detection of Protein on PVDF Blots

After blotting the membrane was rinsed briefly in distilled water, followed by 100% methanol and then stained with CBB-Stain (Appendix 2). The protein bands were visible in about 1 minute. The blot was then

destained with frequent changes of destain (Appendix 2), rinsed with distilled water and left to air dry.

2.4.3 Probing Western Blots

The efficient transfer of proteins from a gel to a membrane allows the blot to be probed by a variety of molecules. The technique of probing the blot for specific antigens using antibodies was first described by Towbin et al **(1979)**. The bound antibody can then be detected by a variety of detection systems. In this study an avidin/biotin-enzyme system was used for all Western blots. The blocking step was overnight incubation at 4^0C in 5% (w/v) Marvel™ in TBS buffer. All subsequent steps were performed at RT with shaking. The blot was washed 3 times in TBS-T (Appendix 2) for 12 minutes and then incubated for 3 hours in TBS-TM containing a dilution of the primary antibody (dilutions of the primary antibody are indicated in the relevant Figure legend). The blots were washed as indicated above and then incubated with a 1:100 dilution of biotinylated secondary antibody (Appendix 2) in TBS-TM for 1 hr at RT. After washing to remove the secondary antibody the blot was incubated with a 1:100 dilution of Steptavidin-HRP (Appendix 2) in TB-MT for 1 hr at RT. The blot was then washed for 3 x 6 minutes in TB-T and 3 x 6 minutes in TB (Appendix 2). Detection was obtained by mixing colour reagent-A with B (Appendix 2) just prior to use and applying to the blot. The reaction was stopped by washing in distilled water and the blot was dried between paper towels.

2.4.4 Lectin Probing

Proteins present on the blots were probed with lectins to determine the glycan component as described by Kijimoto-Ochiai et al, **(1985)**, the following lectins were used:

- *Bandeiraea simplicifolia* lectin - II (BSL-II).
- *Datura stramonium* lectin (DSL).
- *Erythina cristagalli* lectin (ECL).
- *Artocarpus integrifolia* (Jacalin).
- *Lycopersicon esculentum* lectin (LEL).

- *Solanum tuberosum* lectin (STL).
- *Vicia villosa* lectin (VVA).

These were provided in a kit from VECTOR laboratories (Appendix 1). When lectins were used as a probe then the blot was treated as described in section 2.4.3 except that it was blocked with TBS-G (Appendix 2) and the primary antibody was replaced by a biotinylated lectin in TBS-GT (Appendix 2). Briefly, a 1:100 dilution of each lectin in TB-GT (Appendix 2). was used and the blots were incubated for 1 hour at RT, (washing was as described in section 2.4.3). They were then treated with a 1:100 dilution of Streptavidin-HRP in TB-GT and then as described in section 2.4.3.

2.4.5 Periodate Chemical Treatment of Nitrocellulose Blots.

The antigenicity of the glycan component of PAPP-A was studied using the chemical cleavage method that by periodate oxidation that was originally described by Woodward et al, (**1985**). It was used to further examine the glycan components of PAPP-A antigenic determinants that were known to bind to a sub-group of paratopes present in the polyclonal PAPP-A antisera (Chapter 3). The blot was incubated in TBS (Appendix 2) or sodium periodate at 2 - 10 mM in Buffer A (Appendix 2) for 1 hour at RT. It was then incubated with 50 mM sodium borohydride in PBS for 1/2 hour at RT and washed 5 times in PBS-T (Appendix 2). The blot was then probed with DAKO anti-PAPP-A antibodies (as described in section 2.4.3).

2.4.6 Enzyme Digest Treatment of Proteins Electroblotted Onto Nitrocellulose.

The blots were prepared as described in section 2.4.1. After blocking and washing the membrane twice with TBS-T, they were incubated for 2 hours at RT in TBS-M with/without 0.5 units of chitinase (see Plate 3.4 legend, Chapter 3) that had been pre-incubated with an aliquot of a protease inhibitor cocktail 1 hour prior to use (Appendix 2) and then with another aliquot of this cocktail just before use. Following washing, the immunodetection with polyclonal anti-PAPP-A antibody was performed as described in section 2.4.3. The effect that the protease inhibitor cocktail had

on the glycosidic activity and the level of contaminating proteolytic activity of the chitinase preparation was measured using the methods described in sections 2.3.3 and 2.3.5.

2.4.7 Enzymatic Deglycosylation and Subsequent Detection

Selective removing of the glycan components by glycosidic enzymes is one tool that can be used to examine their structure. A review of glycosidic enzymes that have been used for this purpose can be found in Maley et al, (**1989**). In this study the N-linked glycan groups were removed with recombinant N-Glycanase™ (PNGase F: EC 3.5.1.52, Appendix 1) which has a wider specificity than Endo-H. It was also free of contaminating proteases and it is known to be unaffected by presence of sulphated residues or substituted N-linked residues that have been shown to inhibit Endo-H. O-linked groups were selectively removed by treatment of the protein with O-Glycanase™ (Appendix 1). The inclusion of detergents is required as it has been demonstrated (Hasselbeck and Hosel, **1988**) that they not only prevent denaturation of the glycosidic enzymes used to digest the glycan part of a molecule but also aid in the removing of these groups. Non-ionic detergents such as Triton X-100 and Nonidet P-40 are efficient at protecting these enzymes but they need to be removed prior to fractionation of the de-glycosylated protein by SDS-PAGE, therefore a glucoside (MEGA-10) was used as it had the same protective effect but did not have to be removed prior to electrophoresis.

The enzymatic treatment were performed as described by Patterson and Bell (**1990**) except that an enriched PAPP-A fraction and the control proteins, HSA (Grade V, Appendix 1), Fetuin (Type IV, Appendix 1) and Ovalbumin (Appendix 1) were used as the protein substrates. Prior to deglycosylation an aliquot of the proteins were boiled for 2 minutes with an equal volume of sample buffer (Appendix 2) and equilibrated to pH 7.2 with phosphate buffer containing 0.5 % MEGA-10 (Appendix 1). This was then boiled for 2 minutes and after cooling, deglycosylated as described below:

- N-Glycanase treatment: 1 Unit of N-glycanase/10 μg of protein substrate was incubated in 20 mM phosphate buffer, pH 7.2 in capped Eppendorf tubes overnight at 37^{0}C.
- Neuraminidase (*Clostridium perfringens.*, appendix 1): Proteins were equilibrated in 25 mM phosphate/citrate buffer, pH 5.8 and incubated with 1 mUnit of enzyme/μg of protein substrate for 2 hours at 37^{0}C in capped Eppendorf tubes.
- O-Glycanase (Endo-alpha-N-Acetyl-D-galactosaminidase, Appendix 1) treatment: Any substitution on the core disaccharide results in inhibition of this enzyme, therefore prior to incubation with this enzyme, proteins were de-sialated by treatment with neuraminidase (as above). After de-sialation they were incubated with 1 mUnit of O-Glycanase/10μg of protein substrate for 6 hours at 37^{0}C in capped Eppendorf tubes.

All enzyme reactions were stopped by boiling for 2 minutes and the samples were stored frozen. The fractionation of the deglycosylated proteins was as described in the legends on Plates 7.2 and 7.3 (Chapter 7).

2.5 Materials and Methods Section for cDNA Screening

A Clik Clontech human cDNA library (mRNA source: 34 week Placenta with a cDNA insert size range of 0.8 - 3.6 kb: Appendix 1) was used. Buffers, reagents and media are described in detail in Appendix 2.

2.5.1 Screening A Placental cDNA Library

2.5.1.1 Storage and Preparation of Competent Bacterial Cells for Phage Infection

E. coli (strain: Y1090) was stored freeze dried in vials and used to prepare slopes for inoculation into TB medium (Appendix 2). 10 ml containing 100 μg/ml of ampicillin in sterile universals. The cultures were shaken overnight at 37^{0}C.

2.5.1.2 Preparation of Agar Plates Used for Screening

Autoclaved agar (Appendix 2) was poured into sterile Petri dishes (Northumbria Biologicals) and allowed to set. On cooling the plates were inverted and allowed to dry before use. The poured plates were stored at room temperature.

2.5.1.3 Infection of *E.coli* (strain: Y1090) With Bacteriophage (λgt11)

Two different Petri dish sizes (90 and 140 mm diameter) were used and their size determined the volume of infected bacterial culture and top agarose that was used to accommodate phage plaques. A dilution of library in lambda phage diluent (Appendix 2) was used for infection that would give an estimated number of plaques per plate as illustrated in Table 2.4.

Table 2.4 The relationship of number of plaques to Petri dish diameter.

Petri dish diameter:	90 mm.	140 mm.
Volume of culture used:	200 µl	600 µl
Volume of top agarose used:	3.3 mls.	9.9 mls.
Estimated number of plaques[1]:	8,000	20,000

[Legend: [1]: The cDNA library was diluted to give the estimated number of pfu's for the given size of Petri dish as described in section 2.5.1.]

The diluted library was incubated with bacterial culture in a water bath at 37°C for 20 minutes to allow infection to take place. Top agarose (Appendix 2) which had been equilibrated to 50°C was then added and mixed by gentle inversion. It was then swirled onto plates and allowed to set for 10 minutes.

2.5.1.4 Titration of The Library.

The library was diluted with lambda phage diluent in sterile capped polypropylene tubes to a 10^{-7} dilution. Then 10, 20 and 30 µl of the diluted library was used to infect *E. coli* as described in Section 2.5.1.3. Inoculated plates were inverted and incubated for 3 hours at 42°C, followed by 3 hours at 37°C. The number of plaques detected per plate is illustrated in Table 3.1 (Chapter 3).

2.5.1.5 The Ratio of Recombinant/Vector Phage in The Library.

A 10 µl aliquot of 10^{-6} dilution of library was added to the top agarose as described in section 2.5.1.3, except that the top agarose also contained 9 mM iso-propyl-β-D-galactoside (IPTG) and 10 µM X-gal. The plates were incubated at 37°C overnight (Plate 3.2, Chapter 3).

2.5.1.6 Plaque Formation.

Plates were prepared as in section 2.5.1.3. and incubated at 42°C for 3 hours, followed by 1 hour at 37°C. They were then overlaid with a notched/numbered 0.45 µm nitro-cellulose filter membrane that had been impregnated (Appendix 2) with IPTG. The plates were further incubated for 3 hours at 42°C. The membranes were always handled wearing latex gloves and forceps were used to carefully remove the membrane overlay from the plate so as to avoid disturbing the top agarose lawn and limit any tearing of the membrane. The membrane orientation was marked on the plate and inverted plates were stored at 4°C.

2.5.1.7 Blocking of Nitrocellulose Overlays and Immunodetection.

Filters prepared as described in section 2.5.1.6 were placed back to back in a plastic bag and 3 (9) mls of TBS-TB added for a 90 (140) mm filter. Air bubbles were removed and the bag was sealed and left overnight at 4°C. The filters were then washed three times for 10 minutes each in TBS-T and placed in a plastic bag with a 1:300 dilution of DAKO anti-PAPP-A in TBS-T for 3 hours at room temperature. Following washing (as above) the filters were transferred into a plastic bag with a 1:100 dilution of biotinylated anti-rabbit IgG (SIGMA Extra-3 kit, Appendix 1) in TBS-T for 1 hour at room temperature with rocking. They were then washed (as above) and sealed in plastic bags with a 1:100 dilution of avidin-horseradish peroxidase conjugate (SIGMA Extra-3 kit, Appendix 1) in TBS-T and incubated for 1 hour at room temperature with rocking. The filters were washed in TBS (3 times, 10 minutes each wash). Components (A and B, Appendix 2) of a chromogenic substrate were prepared just before use and approximately 5 (15) ml were applied to each 90 (140) mm filter. The colour was allowed to develop for

approximately 20 minutes. The filters were blotted dry and examined for coloured areas.

2.5.1.8 Isolation of Putative Recombinant Phage Plaques.

Filters containing coloured areas (a positive immunoblot) were aligned with the Petri dish using the notch and filter outline on the dish as a guide. The agar/agarose containing the plaque that corresponded to the coloured area was removed using a sterile Pasteur pipette tip. This agar plug was placed into a sterile capped polypropylene tube and vortexed with 50 µl of chloroform to lyse the bacterial cells. 1 ml of lambda phage diluent was then added and it was incubated for 1 hour at 37°C. The lysate was diluted 10^3 fold and 50 µl of this was used to infect *E. coli* cells for re-screening as described in section 2.5.1.3.

2.6 Materials and Methods Section for The Quantitative Measurement of PAPP-A

2.6.1 Rocket Immunoelectrophoresis

RIE was performed as originally described by Bischof et al, (**1979**) but with the following modifications. Litex HSA agarose at a concentration of 0.75% (w/v) in Tris-barbital buffer containing a 1:40 dilution of anti- PAPP-A antibody (DAKO) and 2% (w/v) PEG 6000 were cast onto coated glass plates (Appendix 2) and allowed to set for 1 hour at RT. Wells were cut out and 5 (10) µl of sample/standards were loaded (as shown on the individual plate captions). The RIE was performed in a Pharmacia LKB electrophoresis system at 10 Volts/cm (constant voltage) as measured by a voltage probe across the plate. The electrophoresis system was cooled to 10°C and run for a minimum of 14 hours. Visualisation of the rockets required each plate to be pre-treated (Appendix 2) prior to staining with Coomassie Brilliant Blue.

2.6.2 Radial Immunodiffusion

SRID was performed as originally described by Mancini et al (**1964**), except for the following modifications. Litex HSA agarose at a concentration of 0.75% agarose (Appendix 2) was prepared containing a 1:75 dilution of anti-PAPP-A antibody (DAKO) and poured into sealable plastic SRID plates (Appendix 1). The agarose was allowed to set and then wells were cut out and the plates allowed to equilibrate for at least 24 hours at 4^0C before use. The SRID Plates were stored at 4^0C and allowed to equilibrate at room temperature before use. Samples/standards were diluted in assay diluent (Appendix 2) containing 5 µl of male (Citrate) plasma, (the salt concentration present in the loaded sample/agarose was as described in the appropriate Figure legend). The plates were sealed and left at room temperature for a minimum of 24 hours. Visualisation and quantification of the precipitin rings were the same as that for RIE, except a longer wash pre-treatment prior to CBB staining was required (Appendix 2). Quantification was achieved by comparison of unknown sample with a standard reference serum (WHO 78/610) . A typical calibration curve is as illustrated in Figure 2.6.

2.6.3 Sandwich ELISA for PAPP-A

2.6.3.1 Preparation of PAPP-A F(ab')$_2$ Fragments

A pepsin digest of the DAKO anti-PAPP-A was performed as described by Amaral et al,(**1966**). Briefly a calculated amount of IgG (7 mgs in 0.5 ml) was mixed with 0.5 ml of 0.2 M sodium acetate buffer at pH 4.5 containing 50 µg (175 U) of pepsin. This was left to incubate overnight at 37^0C. The digestion was stopped by raising the pH to 8.6 with 3 M Tris. This stock digest was diluted to a final concentration of 2.5 mg/ml and stored in aliquots at -70^0C.

2.6.3.2 ELISA Coating and Blocking of Solid Phase

A 100 µl aliquot of antibody (see Figure legend in text for type and concentration) in ELISA coating buffer was applied to the wells of a Falcon 3915 microtitre plates. This was sealed with Clingfilm and left overnight at 4°C n a humid chamber. The plates were then washed with PBS and

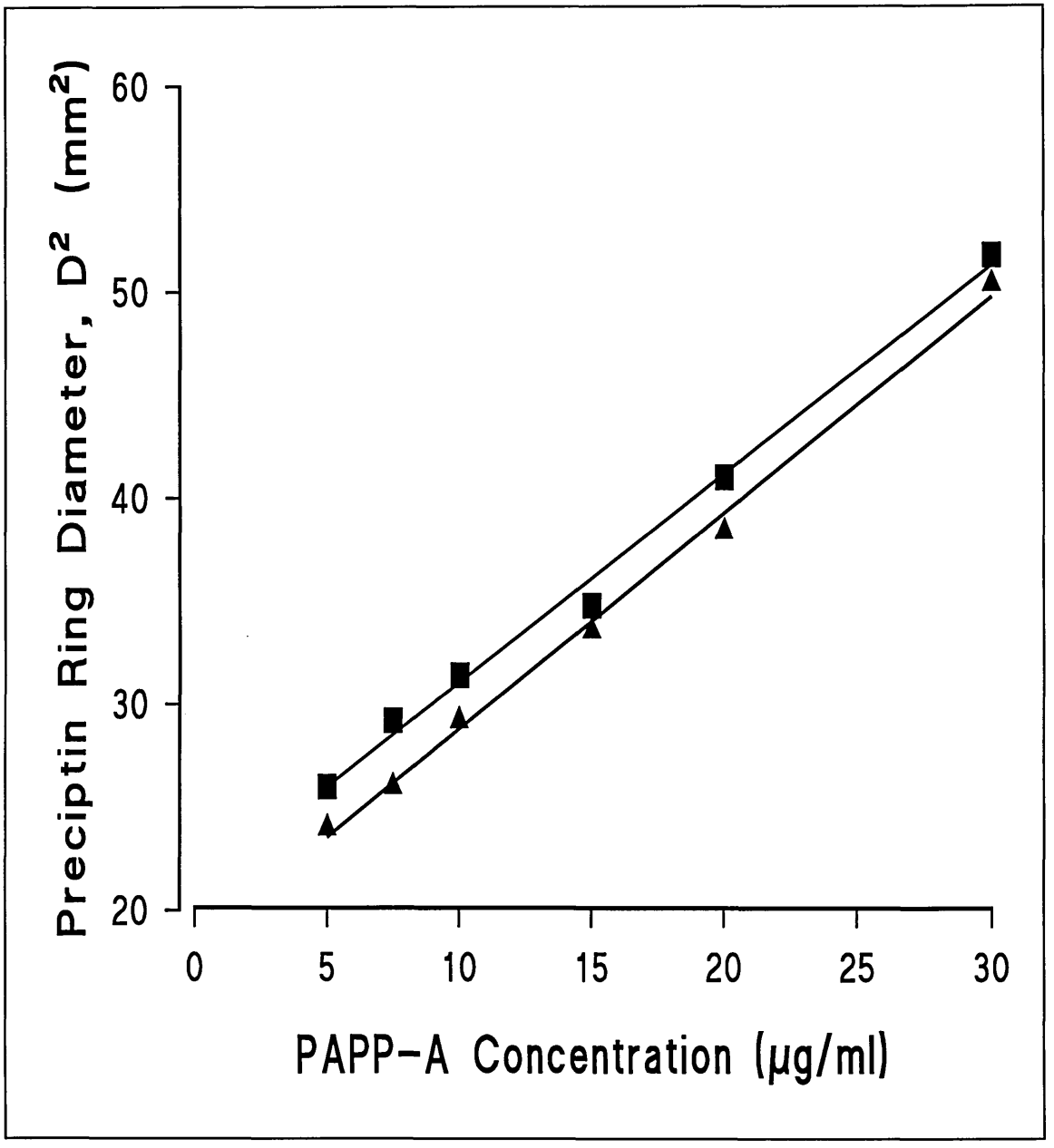


Figure 2.6
Calibration curve for PAPP-A SRID

[Legend: (■: Sample was loaded in assay diluent containing male (citrate) plasma, plate was made in assay diluent. $r^2 = 0.994$). (▲: Sample was loaded in assay diluent with a final concentration of 1M salt and containing male (citrate) plasma, plate was made in assay diluent. $r^2 = 0.996$). The assay was performed as outlined in section 2.6.3.3.].

subsequently 300 µl of ELISA blocking buffer was dispensed into all wells. The plates were covered with Clingfilm and left for 2 hours at RT. The plates were then washed 3 times (as before) but with ELISA wash buffer.

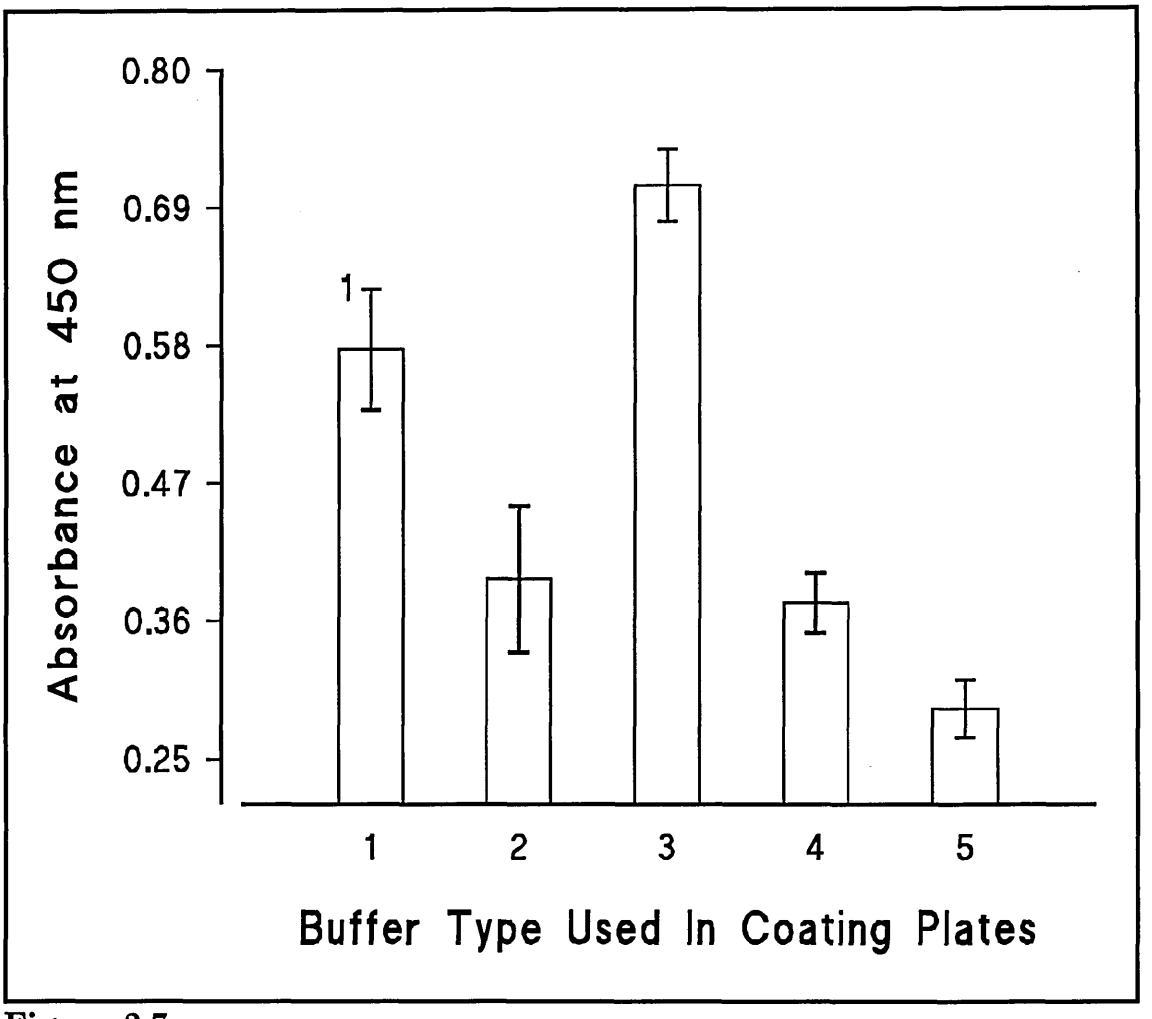


Figure 2.7
Determination of optimum conditions for coating PAPP-A F(ab')$_2$ fragments onto Falcon 3915 plates.
[Legend: 1: 0.25 M Tris-HCl (pH 8.8). 2: 0.01 M Tris-HCl (pH 8.8). 3: 0.25 M Carbonate Buffer (pH 9.6). 4: 0.25 M Acetate/Citrate Buffer (pH 5.6). 5: 0.01 M Acetate/Citrate Buffer (pH 5.6). [1](Results were expressed as mean +/- standard deviation (n=9)) **Note:** F(ab')$_2$ fragments were detected by performing the ELISA as described in section 2.6.3.3 with all wells being incubated with assay diluent containing a 1:5 dilution of a late pregnancy plasma pool as described in section 2.6.4. Anti-PAPP-A was used at a 1:2.5 K dilution in assay diluent and detected with a 1:1K dilution of protein-A-HRP conjugate in assay diluent.]

The most efficient buffer for coating the PAPP-A F(ab')$_2$ fragment to Falcon microtitre plates was found to be 0.25 M Carbonate buffer (pH 9.6). The optimum concentration of the fragment for this type of plate was then determined (Figure 2.8).

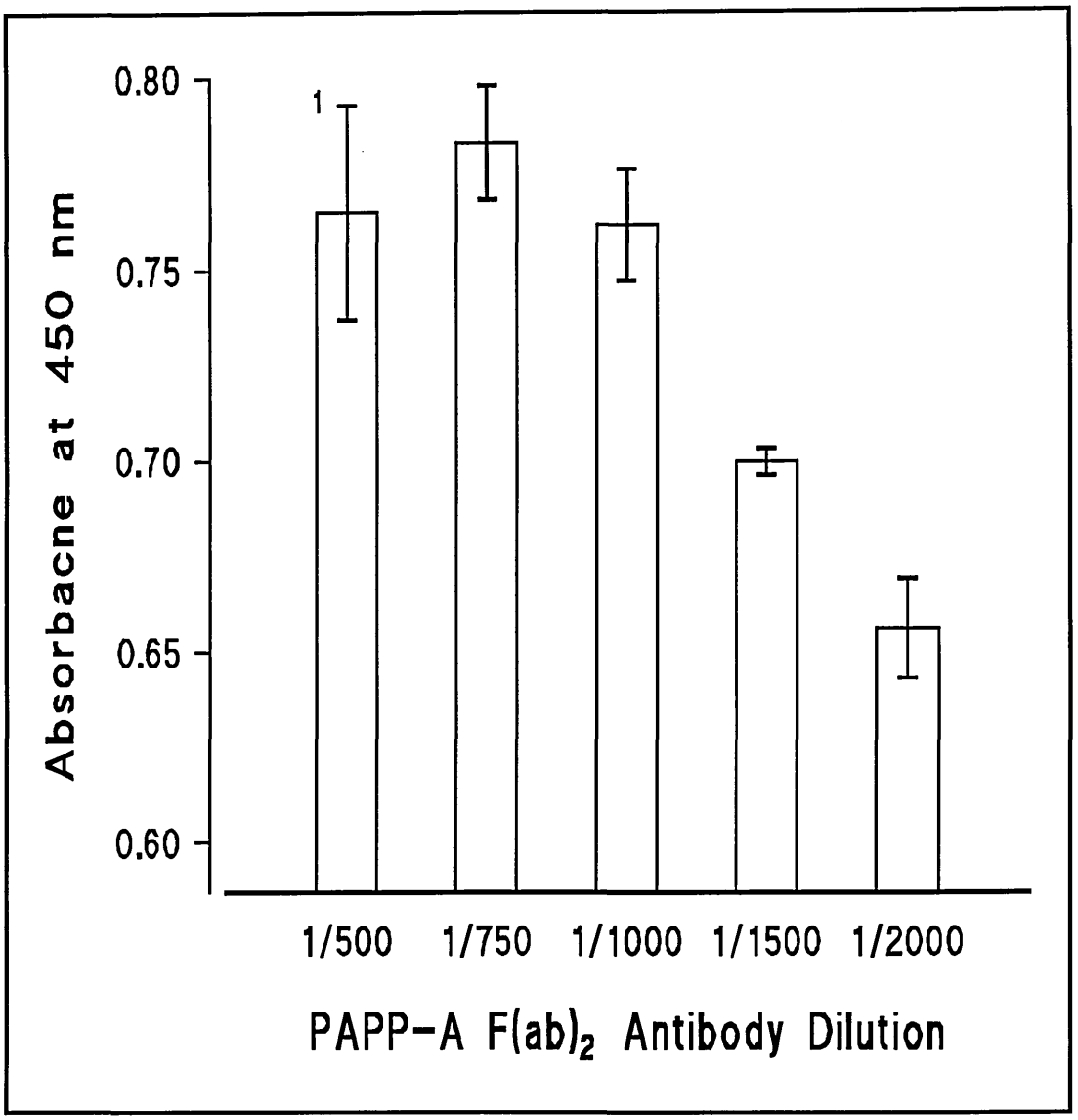


Figure 2.8

Determination of optimum concentration of $F(ab')_2$ for coating Falcon 3915 plates.

[Legend: [1](Results were expressed as Mean +/- standard deviation (n=9)) **Note:** The $F(ab')_2$ fragments were detected as described in Figure legend 2.7]

The optimum dilution of anti-PAPP-A $F(ab')_2$ fragment to coat this type of plate was found to be a 1:750 dilution (Figure 2.8). A 100µl aliquot at this dilution was subsequently used to coat the wells of microtitre plates by incubation overnight at 4°C as described in section 2.6.3.2. These plates were then wrapped in Clingfilm and stored at 4°C in PBS until required.

2.6.3.3 The ELISA Assay Procedure

The samples/standards were diluted in ELISA diluent (Appendix 2) and then 100 µl aliquots were placed into the microtitre plate wells and incubated for 1 hour at RT. The plates were washed three times in ELISA wash buffer (Appendix 2) and then incubated with 150 µl of second stage antibody (see Figure legends 4.3 and 4.4 in Chapter 4 for the type and concentration of antibody that was used) in ELISA diluent for 1 hour at RT. The plates were washed (as above) and then incubated with a Protein-A HRP-conjugate (See Figure legends 4.3 and 4.4 in Chapter 4 for the concentration of conjugate) in ELISA diluent for 1 hour at RT. They were then washed (as above) and then were given a final wash, three times in PBS and blotted dry.

2.6.3.4 Colorimetric Detection and Reading of Results

200 µl of ELISA colour reagent (Appendix 2) was added to all wells and incubated at room temperature for approximately 15 minutes at RT. The colour development was stopped by adding 75 µl of ELISA stop reagent (Appendix 2) and the plates were read at 450 nm.

2.6.4 The PAPP-A Radioimmunoassay

A number of radioimmunoassay procedures have been described for PAPP-A (Bischof et al, **(1981b)**, Sinosich et al, **(1982)** and Anthony et al, **(1983)**), however the RIA procedure chosen in this study was one developed originally by Pinto-Furtado et al, **(1984)** and it was used with some modifications.

An iodination grade enriched PAPP-A preparation that had been derived from maternal serum was kindly supplied by Miss K. Price, this preparation was used in the original iodinations. This material was present at a concentration of 400 µg/ml with each aliquot containing 4 µg and it was stored at -70^{0}C until use. Initial calibration was against the reference serum (WHO 78/610), but later a plasma (Citrate and EDTA) pool from the late 3rd trimester of pregnancy was calibrated against this and stored in

aliquots of 100 µl at -70°C. Each set of standards was used only once and a second set of working standards for use in the assay was prepared by dilution in assay diluent buffer with a concentration range of 4 - 4000 µg/l. The antisera used was polyclonal anti-PAPP-A (DAKO, Appendix 1).

2.6.4.1 The Chloramine-T Method For Iodination of PAPP-A

The method used to iodinate PAPP-A was as described (Pinto-Furtado et al, **1984**) except that 5 µg of PAPP-A was used for a 0.5 mCi iodination with 5 µg of chloramine-T . The labelled protein was separated from the other products of iodination by de-salting on a disposable G-25 column (Sephadex-G25 fine grade, Appendix 1). Before separating the products of iodination this column was pre-blocked by running an aliquot of a 30% BSA solution made in assay diluent (Appendix 2). The PAPP-A tracer that was produced had a mean specific activity of 29 µCi/µg.

2.6.4.2 Affinity Purification of Tracer For Use in The PAPP-A RIA.

The process of iodination can lead to damage of the material that has been labelled (Bolton, **1981**). Pinto-Furtado et al, (**1984**) demonstrated that a further affinity purification of the PAPP-A tracer prior to it's usage in an RIA increased the percentage of tracer that bound to the PAPP-A antibody. A disposable heparin-affigel (Bio-Rad, Appendix 1) column was used to affinity isolate the PAPP-A tracer, the tracer was eluted from the gel with assay diluent containing 0.8 M NaCl (Figure 2.9). The affinity purified tracers binding to the PAPP-A antibody was significantly improved by this affinity purification. A maximal binding of only 50% was seen at excess antibody concentrations, this was probably due to the sample that was used for iodination containing proteins other than PAPP-A that were iodinated but did not bind the PAPP-A antibody. The presence of other proteins in the sample used for iodination was confirmed using N-terminal sequencing (Chapter 6). This method of iodination and the affinity purification of the PAPP-A stock tracer just prior to it's use, produced a tracer that was used in a PAPP-A RIA (Chapter 4, section 4.3.3).

2.6.4.3 Antibody Titration Curves

Serial two fold dilutions of anti-PAPP-A antisera (DAKO) were prepared from 1:4 K - 1:512 K in the assay diluent buffer. 100 µl aliquots of the appropriate dilution was added to plastic tubes (LP4, Appendix 1). 100 µl of PAPP-A tracer (2 ng/ml) and 100 µl of assay diluent was added to all tubes.

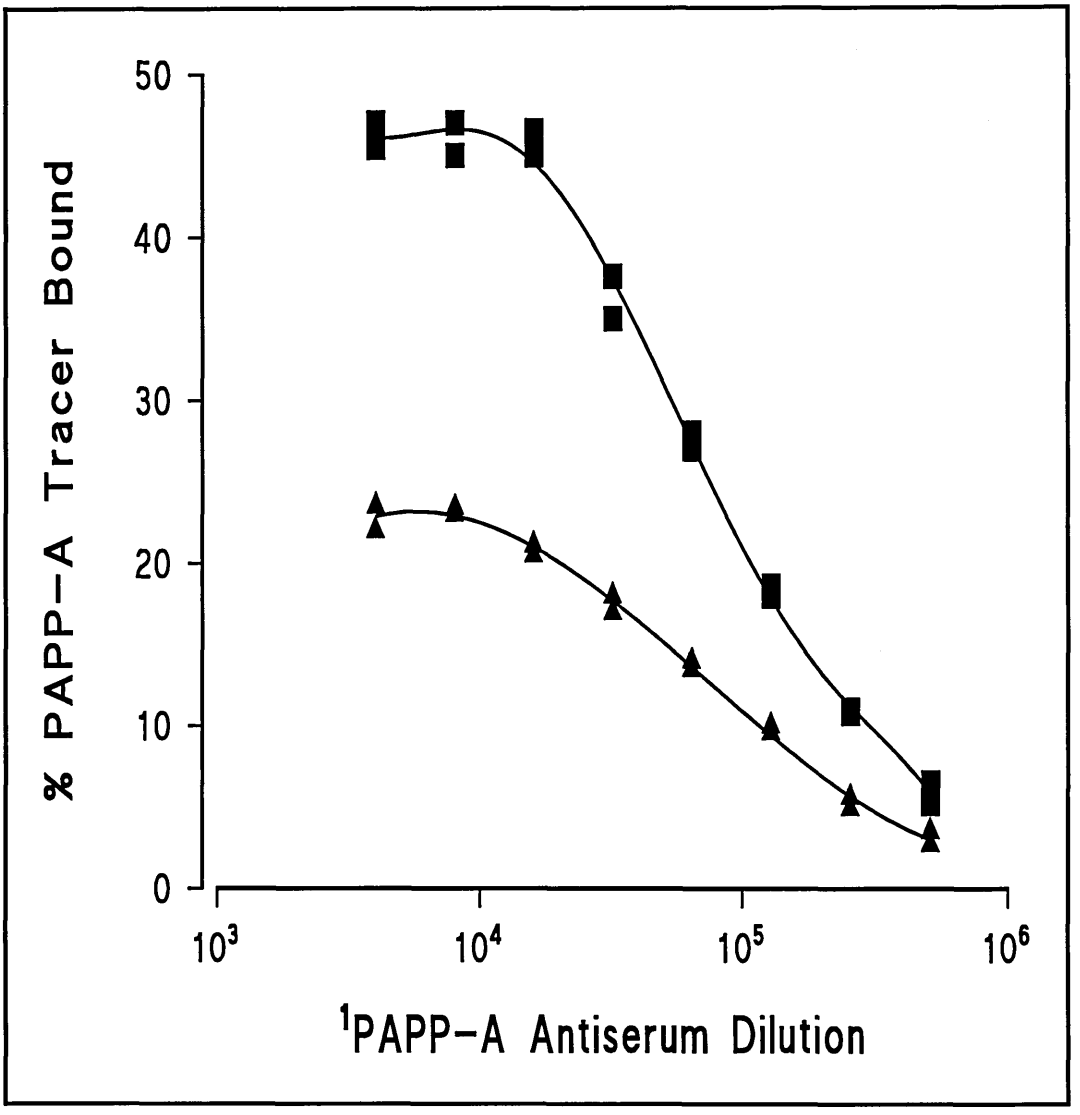


Figure 2.9

PAPP-A antiserum titration curve.

[Legend: (▲): 2 ng/ml crude PAPP-A tracer that had not been affinity purified. (■): 2 ng/ml affinity purified tracer. [1](10^3 represented a 1:1000 dilution of anti-PAPP-A antiserum (DAKO)). %Tracer bound was plotted as (Bound CPM - NSB CPM)/(Total CPM - NSB CPM). The titration curve was produced as outlined in section 2.6.4.3]

The assay was performed in duplicate and non-specific binding was assessed using assay diluent in the place of the PAPP-A antiserum. All the tubes were vortexed, covered and left to incubate overnight at room temperature. Separation of the bound and free antigen was by a solid phase magnetic system (Amerlex-M anti-rabbit, Appendix 1) with the bound/free phases separated by centrifugation and aspiration.

2.6.4.4 The PAPP-A RIA

Affinity purified tracer was used at a concentration of 2 ng/ml and a working anti-PAPP-A antibody dilution of 1:45 K. At this antibody dilution approximately 35% of the PAPP-A tracer was bound. 100 µl of tracer and antibody was added to 100 µl of standards (see above) or samples. Except the non-specific binding (NSB) control in which the PAPP-A antisera was replaced by assay diluent. All tubes were vortexed, covered and incubated overnight at RT. Separation was achieved as described in section 2.6.4.3. The percentage tracer bound plotted against the PAPP-A concentration. The RIACALC (Pharmacia) system was used to plot the results using a smoothed spline function, results were interpolated from the standard curve (Figure 4.7, Chapter 4).

2.6.5 Densitometry

It has been noted by Merrill (**1990**) that a linear relationship exists between the band intensity of stained proteins as measured by densitometry when compared to the protein concentration of these proteins separated by SDS-PAGE and visualised by protein staining. The stained band intensity was obtained using a BioRad model 620 video densitometer (Appendix 1) and 1D analyst capture software. By plotting the intensity of the band stained as a function of the area of the band (Absorbance per mm^2) against the protein concentration, a calibration curve was obtained (Figure 4.8, Chapter 4). High molecular weight markers (BDH, Appendix 1) or purified myosin were used as standards against which the concentration of PAPP-A and other proteins were compared.

2.7 Materials and Methods Section for The Isolation Of PAPP-A for Microsequencing

2.7.1 The FPLC System used in the isolation of PAPP-A

The FPLC system (Pharmacia) was composed of two solvent delivery pumps (P-500), sample injector valve (MV-8) fitted with a 10 ml supa-loop, a UV-M detector and system controller (LC-500) connected to an IBM compatible PC running the FPLC manager software (Appendix 1). The starting equilibration buffer was always in buffer reservoir A, referred to in Figure legends as buffer A. A gradient was obtained by the controller mixing pump B buffer with pump A buffer, it was assumed that the final mixture given as a percentage of Pump B buffer was as stated by the FPLC controller. The individual running conditions for each step are given with the appropriate Figure legend illustrating that step.

2.7.2 The Preparation Of Source Biological Material

Blood samples were obtained by either venupuncture from pregnant women in their 2nd/3rd trimester of pregnancy and collected as EDTA or Citrate Plasma. Blood was also collected into EDTA when lost via the vagina at delivery, this material underwent a degree of haemolysis and clotting prior to collection. Many thanks are due to the nurses working on the maternity ward at Jessop hospital for Women who collected and pooled samples of blood lost at delivery. The pre-treatment of samples was a modification of that originally described by Sutcliffe et al, (**1980**) for the preparation of filtered serum. All plasma samples were centrifuged at 40K x g. for 30 minutes at 4^0C. This removes particulate matter and allowed the lipid content to be reduced, as it forms a white scab layer that after centrifugation was separated from the plasma by careful decanting and filtering through glass wool. The filtered plasma was stored at - 70^0C.

2.7.3. Ammonium Sulphate Precipitation And Dialysis

As illustrated in Table 1.2 (Chapter 1), PAPP-A in term maternal blood is present at 0.07 - 0.16% of the initial protein. When the protein of interest

is less than 1% of the starting material, methods based on a protein's differential solubility are frequently used. Salt fractionation was chosen using ammonium sulphate precipitation, with a guide to its concentration taken from work originally described by Lin et al, (**1974a**) and Sutcliffe et al, (**1980**). A description of the technique that was used is given below:

Precipitation was carried out at 4°C. The concentration of ammonium sulphate (pH 7.1) was adjusted to 28% saturation. Then it was gently rocked for 45 minutes and centrifuged at 35K x g for 30 minutes. The supernatant was adjusted to 60% saturation with ammonium sulphate, gently rocked for 45 minutes and the precipitate collected by centrifugation (as above). The precipitate was resuspended in 10 mM potassium phosphate, pH 7.2. This was then dialysed against 5 litres of 10 mM potassium phosphate, pH 7.2 containing 50 mM NaCl at 4°C. Prior to dialysis, the buffer was pre-incubated for 1/2 hour with PMSF at a concentration of 0.1 mM and then just prior to the dialysis of the resuspended precipitate another aliquot of PMSF was added. After 3 changes of the dialysis buffer, the dialysed sample was centrifuged at 35K xg for 60 minutes and the supernatant was used immediately or stored at -70°C. The combined application of centrifugation with pre-treated filtered plasma/serum was advantageous as it removed particulate material that was likely to clog chromatographic columns and shorten the life of chromatographic separation media.

2.7.4 Heparin Affinity Chromatography

Heparin is a naturally occurring mucopolysaccharide that is composed of equimolar quantities of glucosamine and glucuronic acid, linked by α-1,4 glycosidic bonds. The common repeat unit is illustrated in Figure 2.10.

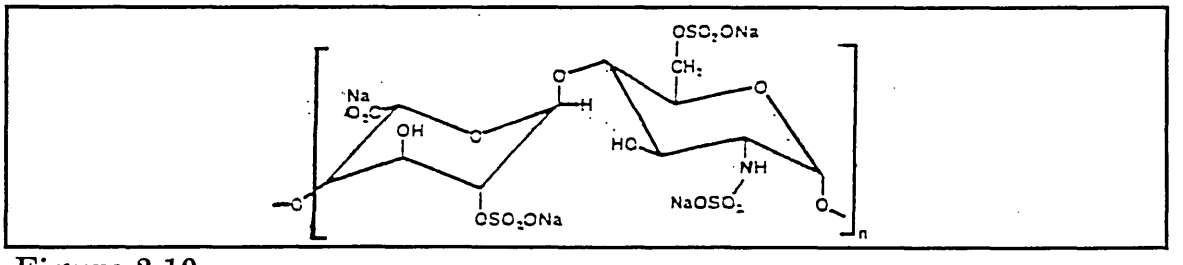


Figure 2.10

The common repeat unit found in heparin.

The main characteristic of heparin is the large number of sulphated amino groups that allow heparin to act as a high capacity cation exchanger or by virtue of its structural form acts as affinity ligand for coagulation factors (Walton, **1955**). Since the initial observation by Sinosich et al, (**1981**) that PAPP-A interacted with heparin this has subsequently been used as a method to affinity purify PAPP-A from other blood components (Davey and Teisner, **1982**).

Heparin Affi-gel (Bio-Rad, Appendix 1), heparin-Sepharose (Pharmacia: Pre-1984 and Post-1988) and heparin-Ultrogel A4R (Reactifs IBF, Appendix 1) were obtained and packed into a column (0.8 x 10 cm). Elution was monitored by absorbance at 280 nm. Initial work was carried out using the heparin-Sepharose (Pharmacia) and running conditions were as described by Sinosich et al (**1987**) except that 10 mM potassium phosphate (pH 7.2) was used as the buffer.

Initial work with the heparin Affi-gel (Figure 5.3) demonstrated that little or no protein bound when a salt concentration higher than 0.3M NaCl was used. This situation was not improved by using Tris-based buffers that Sinosich (**1987**) demonstrated improved the binding capacity of PAPP-A to this affinity matrix.

Information received from the supplier (Pharmacia) noted that heparin affinity gels post - 1984 were produced by a different supplier with two key differences noted in that the heparin was:

- From a different species.
- The linkage chemistry used to attach heparin to the support was different to the matrix that was produced pre - 1984.

The supplier of the original affinity gel (pre - 1984: Reactivs-IBF, Appendix 1) was located and this affinity matrix produced the expected elution profile, with enrichment of PAPP-A in the material eluted at 0.6 M. The high affinity (>0.6M NaCl) heparin bound PAPP-A (Figure 2.12) was used as part of a heparin based scheme, purification scheme 2 (Figure 5.3, Chapter 5).

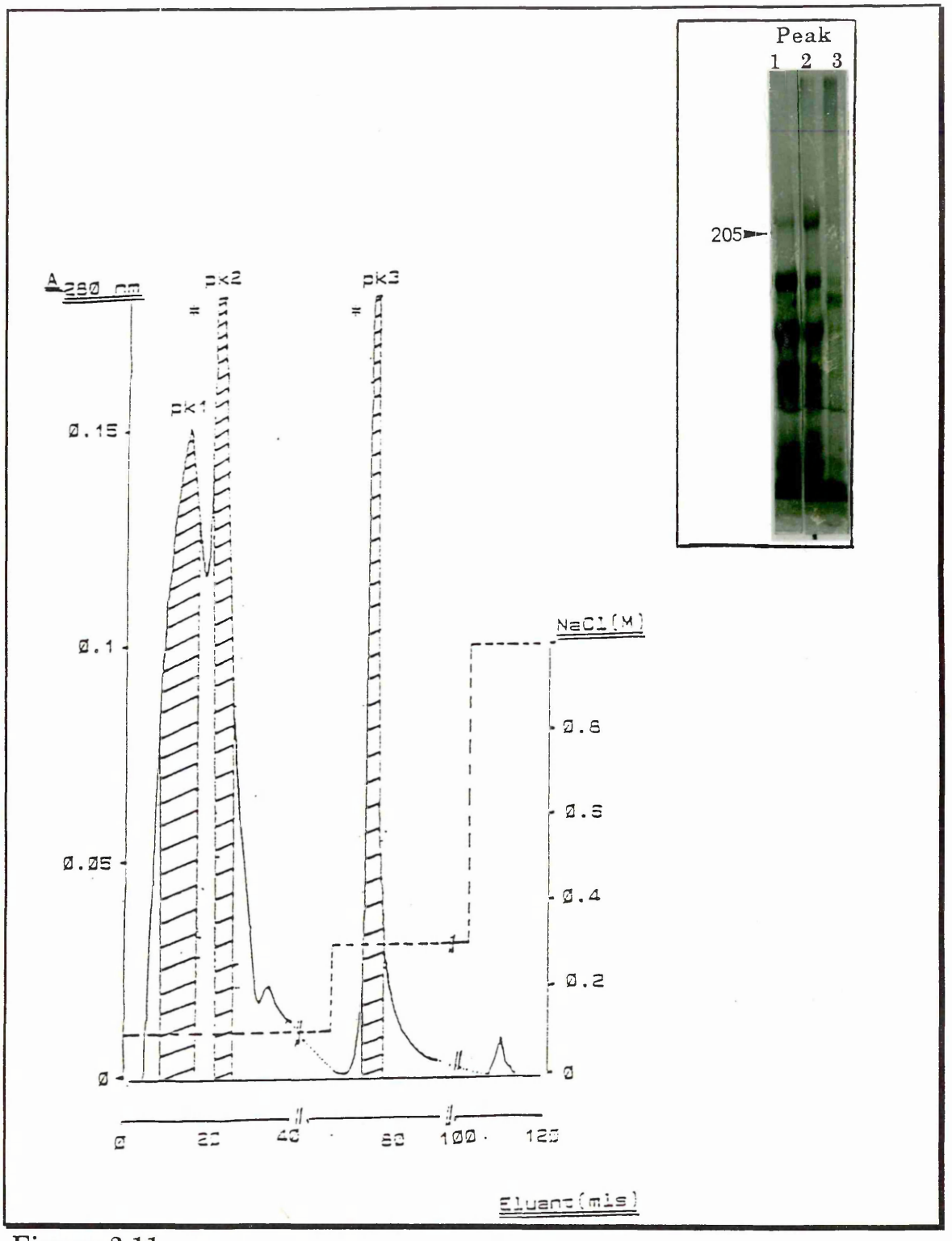


Figure 2.11

Elution profile from heparin-Affigel affinity column (**Plate inset 2.11:** represented a 5% SDS-PAGE of fractions indicated).

[Legend: *: indicated off-scale absorbance. Size markers were as indicated by arrows, the number represented the size in KDa. **Note:** Buffer A was 10 mM potassium phosphate, pH 7.2; Buffer B was as buffer A but included 1 M NaCl. The column used was as outlined in section 2.7.4]

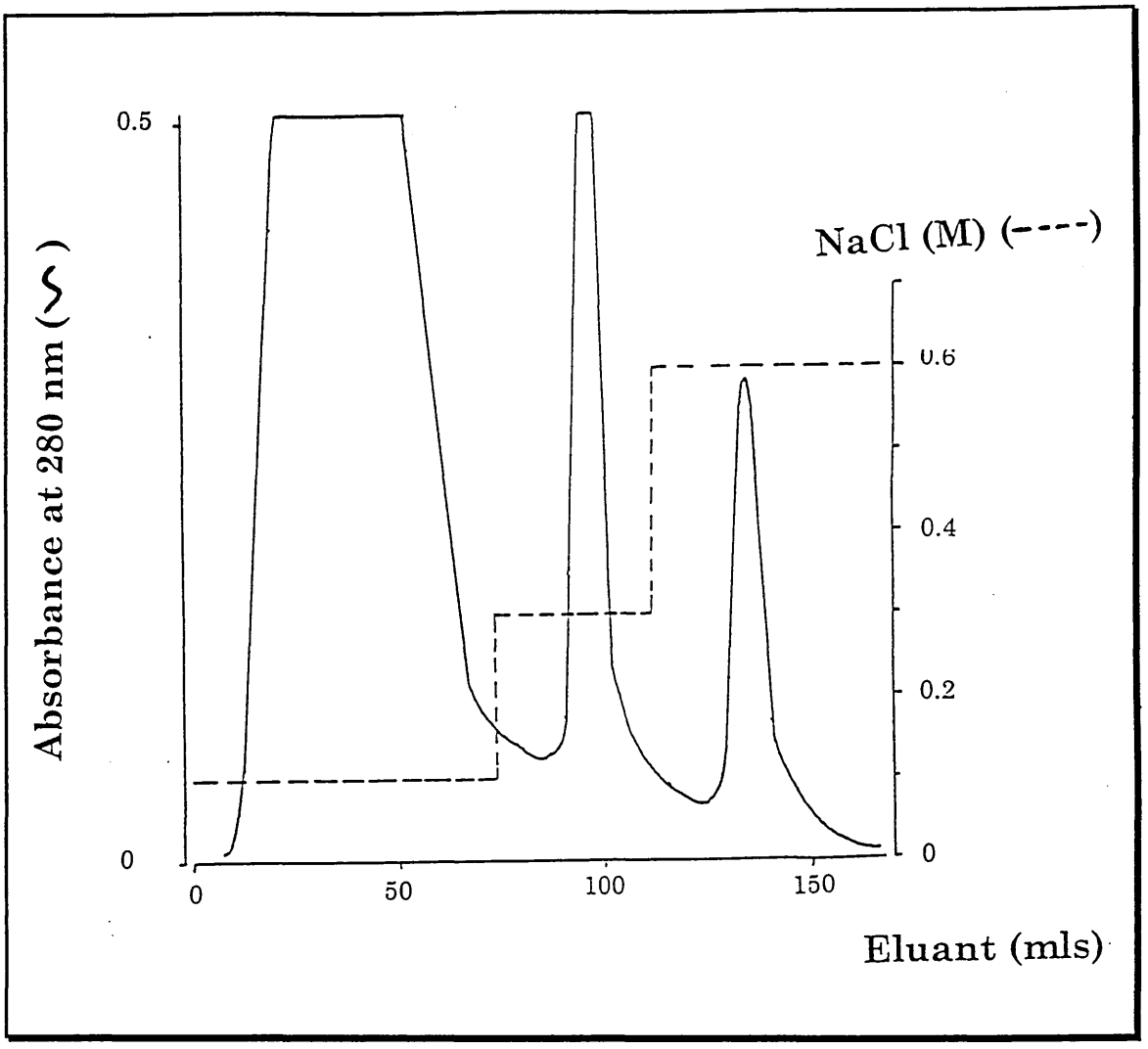


Figure 2.12

Elution profile from heparin-affinity column (Reactivs-IBF)

[**Legend:** *: indicated off-scale absorbance. Buffer conditions were as described in Figure 2.11. The column was loaded with an aliquot from the DEAE bound material as described in section 2.7.6. The bound material that was eluted from this column at 0.3 and 0.6 M NaCl was loaded onto the gel filtration column as described in section 2.7.11].

2.7.5 Sulphated Dextran Chromatography

Sulphation of polysaccharides has been shown to confer on them some heparin-like activity (Miletich et al, **1980**). Therefore this could offer another alternative to heparin. A column, 0.8 x 10 cm was loaded with sulphated dextran that had been equilibrated with 10 mM potassium phosphate (pH 7.2) containing 0.1 M NaCl at a flow rate of 1 ml/min. A step gradient elution system was used. This procedure enriched PAPP-A considerably (Figure 2.13)

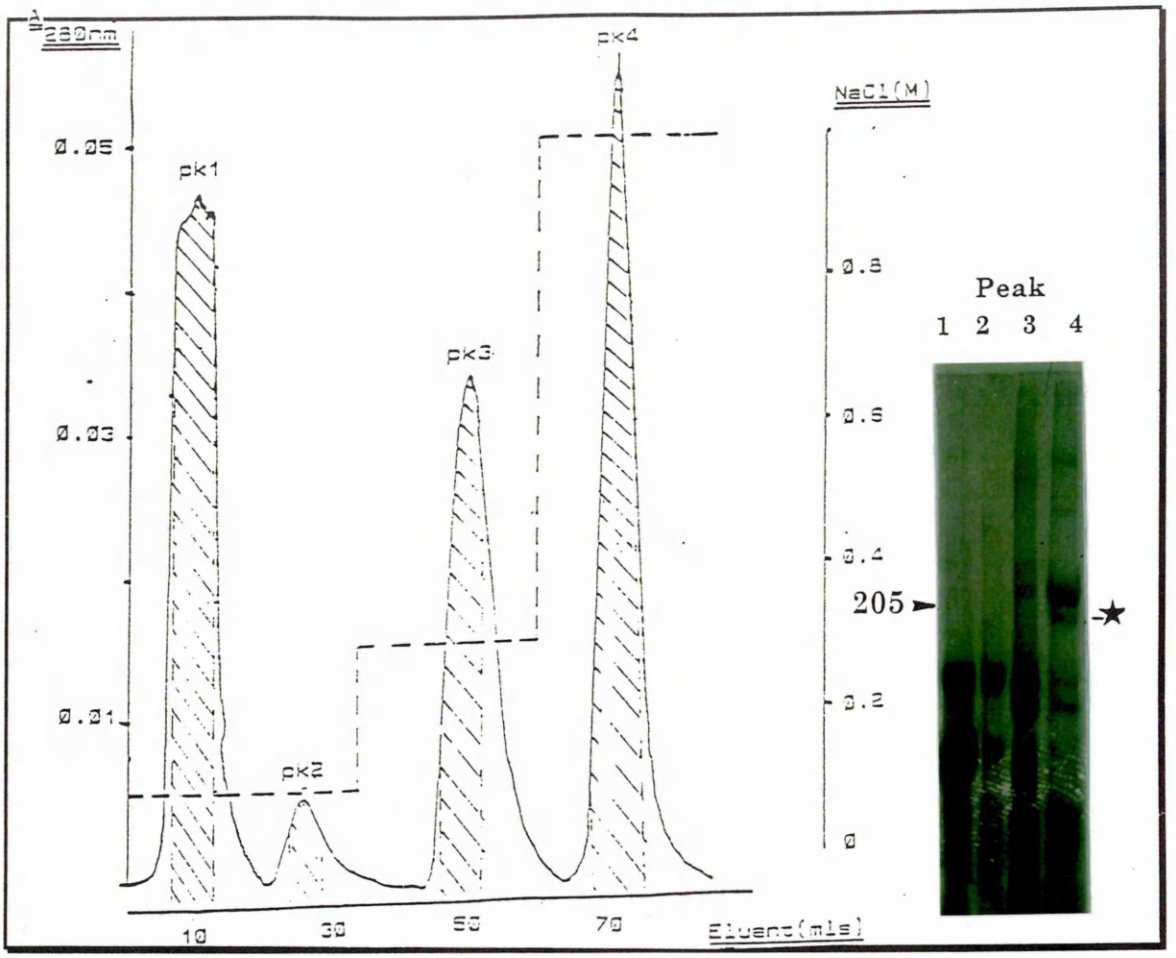


Figure 2.13

Elution profile from a sulphated dextran affinity matrix. **Plate Inset 2.13** represented a 5% SDS-PAGE gel of the fractions indicated

[Legend: *: indicated off-scale absorbance. Buffer A and B were as described in Figure 2.13. The size markers were as indicated by arrows and represented the size in KDa. The PAPP-A marked was determined by its size and Western blotting followed by immuno-detection with a polyclonal anti-PAPP-A.]

Although this affinity step did enrich PAPP-A, changes in the salt concentration produced dramatic fluctuations in volume of matrix that often resulted in the column being blocked or the increased pressure resulting in the matrix material being pushed through the frit of the column. The matrix effect that was seen with changing salt concentrations made this an unsuitable matrix for further work. However this affinity compound attached to a more rigid support such as Cellufine sulphate (Amicon) would probably have produced a usable matrix but this was not required as the heparin affinity matrix from Reactifs IBF was seen to enrich PAPP-A (Figure 2.12).

2.7.6 DEAE Ion Exchange Chromatography

Ion exchange chromatography has been used by a number of investigators as part of their purification schemes (Lin et al,**(1974a, 1974b)**, Bischof, **(1979a,1979b)**, McIntyre et al,**(1981)** and Sutcliffe et al, **(1980)**. A column of 2.4 x 15 cm was packed with DEAE-Trisacryl (Reactivs IBF, Appendix 1), equivalent to a bed volume of approximately 70 mls. Buffer A was 10 mM potassium phosphate, pH 7.2. Buffer B was buffer A but also containing 1M NaCl. A gradient was obtained by a pump mix of reservoirs, A and B. The column was loaded and run at 2 ml/min. Initially the column was equilibrated in 10 mM potassium phosphate buffer containing 50 mM NaCl. Gradients salt elution was tried as shown in Figure 2.14. The DEAE bound fraction was eluted with buffer containing 0.5 M NaCl.

As shown in Figure 2.14 and also as found by the investigators listed above, PAPP-A was eluted from the column over a wide range of salt concentrations, possibly as a result of heterogeneity in the carbohydrate portion of the PAPP-A molecule. A factor that has been seen to produce a similar heterogeneity in other proteins (Gerrard, **1990**). The salt elution profile was optimised to give maximum binding of PAPP-A binding to this column using a simple isocratic gradient (Table 2.5). The optimal conditions for loading the ion exchange column were achieved using 10 mM potassium phosphate containing 0.075 M NaCl, elution was with a salt increase to 0.5 M NaCl.

Table 2.5 Optimisation to yield maximum recovery of PAPP-A from DEAE ion exchange column using an isocratic gradient elution.

[1]Column loading conditions	%PAPP-A bound (detection by an RIA)	%Protein Bound (Absorbance at 280 nm)
50 mM NaCl	99.5%	28%
75 mM NaCl	99%	24%
100 mM NaCl	84%	13%

[Legend: [1] Column loading conditions were represented by the sample loaded in 10 mM Buffer A (section 2.7.6) containing x mM Salt. Elution was with Buffer A containing 500 mM Salt. [2]% Protein bound was determined from the integration of the elution profile of the absorbance at 280 nm using the FPLC manager software. The sample loaded was a dialysed resuspended aliquot of a 28/60% ammonium sulphate precipitate prepared as described in section 2.7.3.**]**

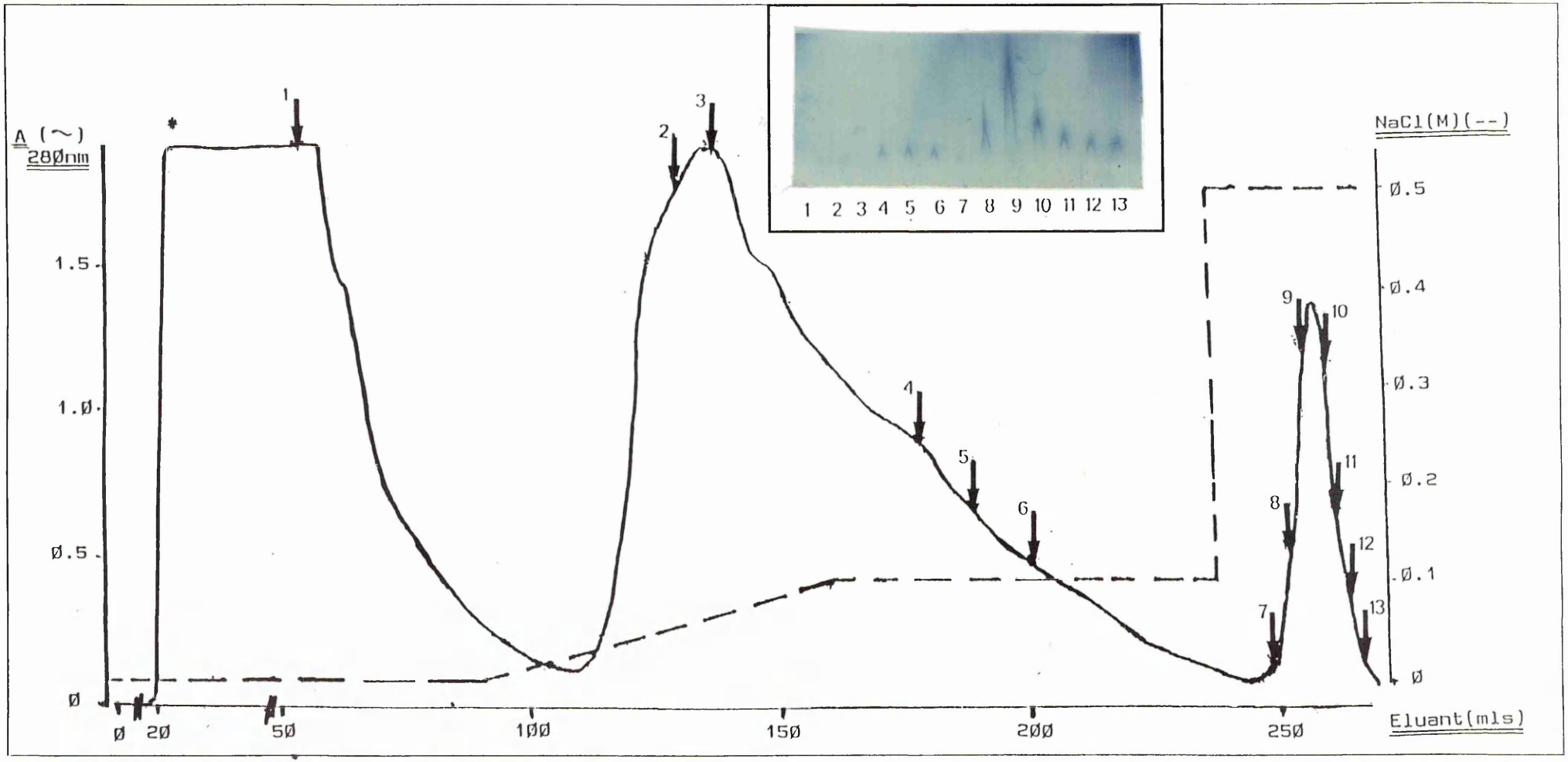


Figure 2.14 Elution profile from a DEAE-Trisacryl ion exchange column. (**Plate Inset 2.14:** PAPP-A detected by RIE as described in section 2.6.1. 5 µl aliquots of numbered fractions were loaded).

[Legend: *: Indicates off-scale absorbance. Buffer conditions were as described in section 2.7.6. A dialysed resuspended aliquot of a 28/60% ammonium sulphate precipitate as described in section 2.7.3 was loaded onto this column.]

2.7.7 Dye Affinity Chromatography

It was noted by Gore and Sutcliffe (**1984**) that PAPP-A did not bind to the dye, Cibachron-Blue. Sinosich et al, (Cited from **1988b**) also found that the inclusion of the dye, Red-120 in an intermediate gel in crossed immunoelectrophoresis resulted in the PAPP-A immunoprecipitate being abolished. Thus suggesting that PAPP-A was interacting with this dye. Using these observations a tandem Cibachron blue to Red-120 dye column system was tried. A Column of 2.4 X 15 cm was packed with Cibachron-Blue 3GA (Appendix 1) and Read-120 (Appendix 1) was packed to a column height of 4 cm. The columns were equilibrated in 10 mM potassium phosphate (pH 7.2) containing 0.075M NaCl (Buffer A). The sample was loaded onto the columns at a flow rate of 2 ml/min. The flow through from the Cibachron-Blue column was loaded directly onto the Red-120 column. The Blue-dye column was removed and the protein was eluted from the Red-dye column with Buffer A that contained 1 M NaCl.

The Cibachron-Blue column did not significantly separate PAPP-A from its contaminants (Figure 2.15) and the red dye column significantly increased the Contaminant X-group of molecules (Figure 5.4, Chapter 5). This group of molecules exist as single chain entities running at approximately 210 - 260 kDa as determined by SDS-PAGE (as described in section 2.3.2.2) and from the volume they elute from a calibrated gel filtration column (as described in section 2.7.11).

2.7.8 L-Arginine Agarose Affinity Chromatography

The rationale behind the use of L-Arginine as an affinity matrix for the purification of PAPP-A was that PAPP-A is a glycosaminoglycan (GAG). GAG's have been shown to interact with basic proteins (Sampson et al, **1985**). Therefore PAPP-A should interact with the basic amino acid, Arginine. A column, 0.9 x 1.5 cm was packed with L-Arginine agarose (Appendix 1) and equilibrated in 10 mM potassium phosphate (pH 7.2) containing 0.075M NaCl. The sample was loaded and eluted from the column by increasing the salt concentration to 1 M NaCl. (Figure 2.16).

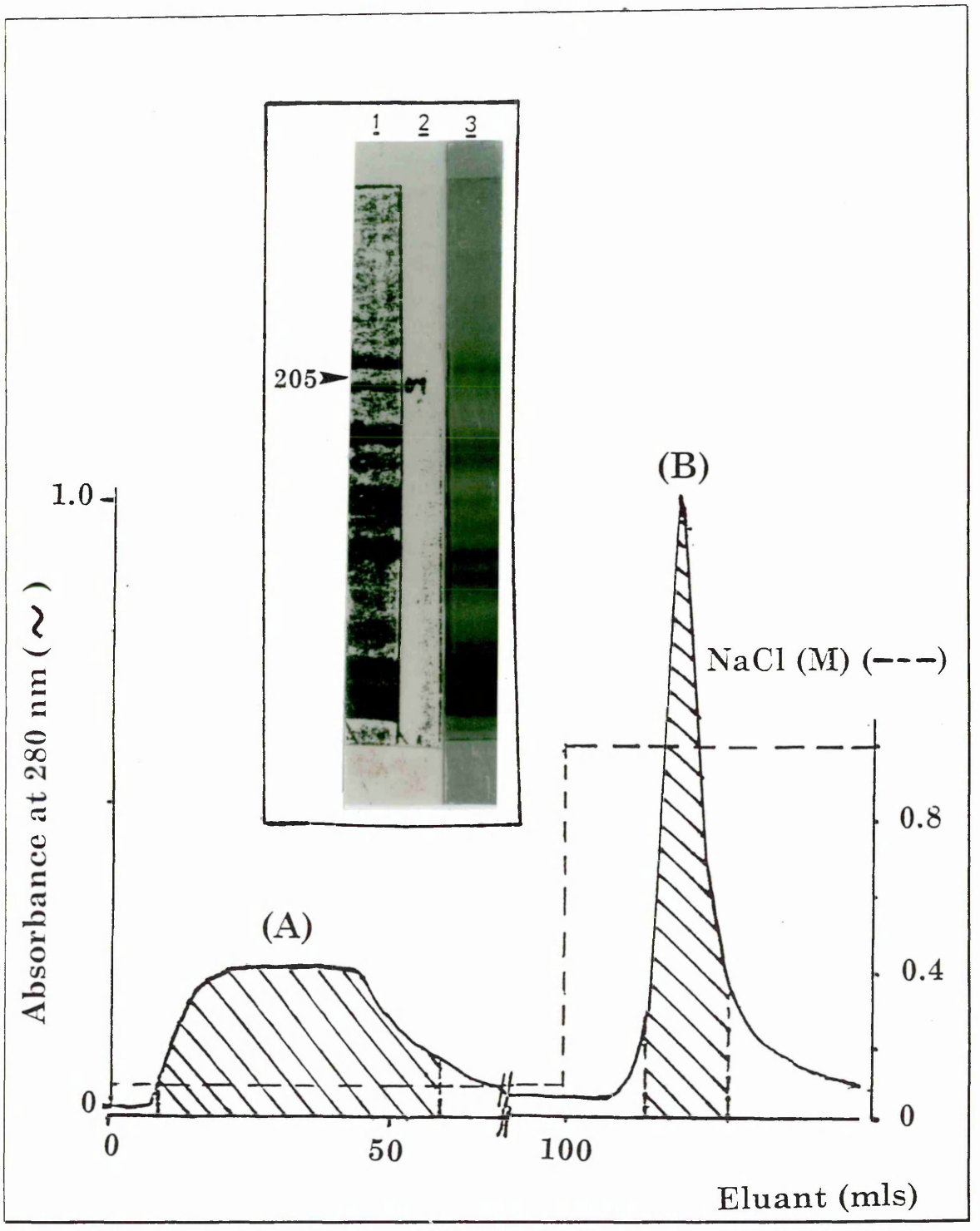


Figure 2.15

Elution profile from Cibachron-blue affinity column

(**Plate Inset 2.15: 1.** PVDF CBB stained sample from peak A run on a 5% SDS-PAGE gel. **2.** Western of peak A with immunodetection using polyclonal anti-PAPP-A. **3.** Silver stained 5% SDS-PAGE gel of the bound material, peak B.)

[Arrow indicated molecular weight marker with the size in kDa. Buffers and the sample loaded were performed as described in section 2.7.7]

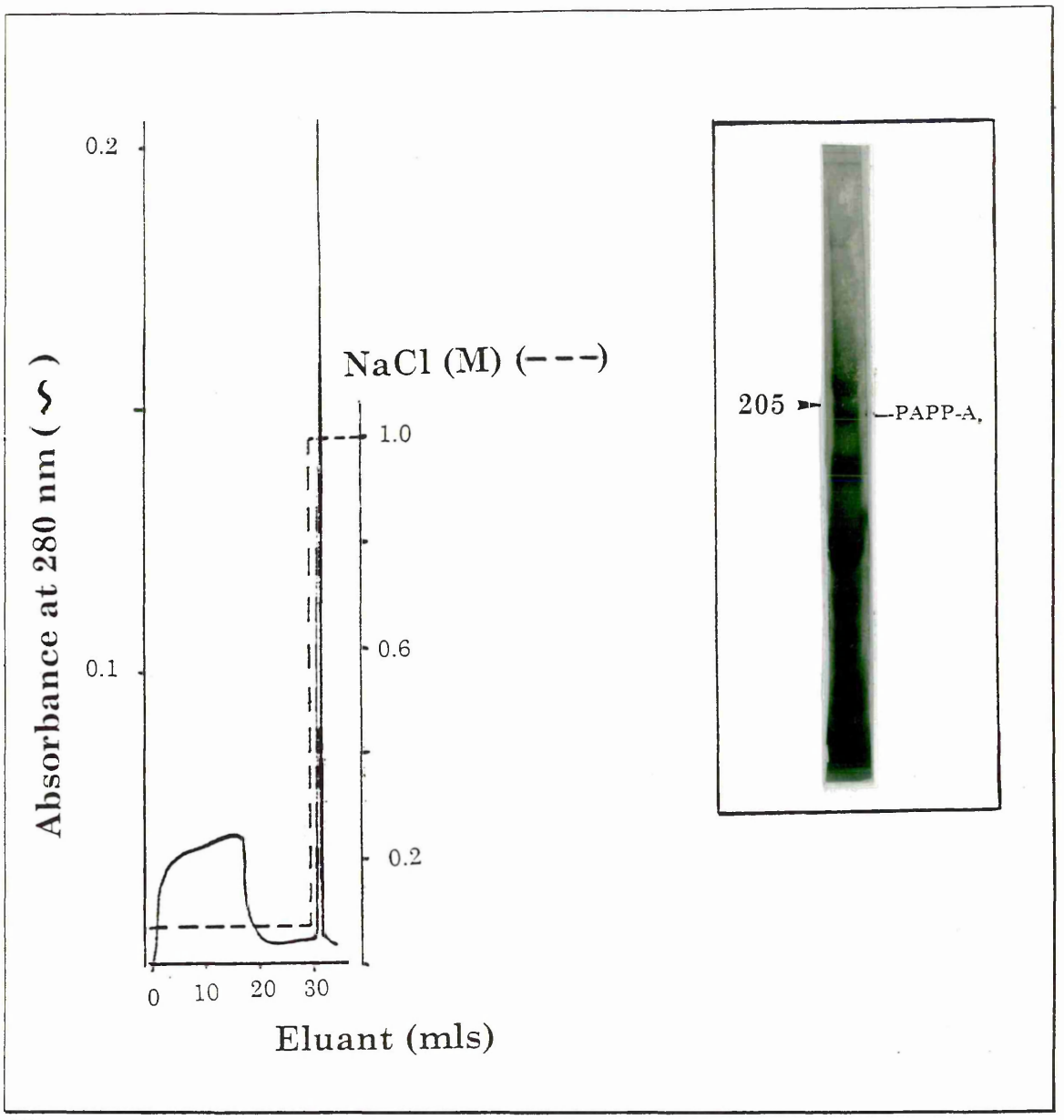


Figure 2.16

Elution profile from a L-Arginine affinity column (**Plate Inset 2.16:** 5% SDS-PAGE gel of bound fraction)

[Legend: The arrow indicated the molecular weight marker with the size in kDa. The band marked as PAPP-A was determined by Western blotting followed by immuno-detection with polyclonal anti-PAPP-A. Buffer conditions were as described in section 2.7.8, the sample loaded was an aliquot from the DEAE bound fraction, section 2.7.6]

2.7.9 Lectin Affinity Chromatography

The extent and type of glycosylation can be used as an aid in the purification of a protein (Gerrard, **1990**). PAPP-A is glycosylated (Chapter 1, section 1.4) there was therefore the possibility that the carbohydrate groups present could be used to separate PAPP-A from its contaminants. Lectins

are a class of molecules that have defined specificities for particular carbohydrate side chains. Bischof (**1979b**) used Con-A as an affinity matrix to aid in the purification of PAPP-A. Experiments that used PAPP-A electroblotted onto a membrane with subsequent incubation with lectin and appropriate carbohydrate (Chapter 7) revealed that the lectin that seemed to offer the best specificity for PAPP-A compared to its contaminants was *Lycopersicon esculentum*. A 2 ml column was packed agarose bound *Lycopersicon esculentum* lectin (Appendix 1). The lectin/PAPP-A interaction on the solid phase blots was not transferable to a chromatographic method as in the absence of detergents used on the blots the Lectin did not demonstrate the same specificity for PAPP-A that was observed on the blots.

2.7.10 Metal Chelate Chromatography

Immobilised metal ion chromatography (MCAC) separates proteins according to the presence of histidine, tryptophan and cysteine on the protein surface. These amino acids bind to metal ions that are complexed to chelating groups on the chromatographic medium. Sinosich et al,(**1983**) that PAPP-A interacts with Zinc and found that elution from a Zn MCAC column using EDTA did not resolve PAPP-A from its main plasma contaminants (Alpha -2- macroglobulin, PZP and fibronectin). A decreasing pH gradient did not separate these proteins, but PAPP-A was immunologically denatured as to be no longer immunologically measurable by crossed immunoelectrophoresis. This is in contrast to the stated, pH stability of PAPP-A by Lin (**1974b**) who observed immunological stability at a pH range of 2 - 12. I also observed that a physico-chemical change occurred to PAPP-A after Zn-MCAC, in that the protein separated tended to precipitate and was difficult to re-solubilise after elution from this column. Although detectable by RIA after elution (Figure 2.19) with >95% recovery of the PAPP-A loaded onto the column, in the indicated peak fraction (●). Because of the difficulties in keeping the protein in solution after elution from this column, it was not used as a step in the isolation of PAPP-A. The MCAC matrix (Appendix 1) was loaded to a bed volume of approximately

5 mls, and the matrix charged by running 20 mls of 0.1 M Zinc Sulphate in distilled water through the column at a flow rate of 0.5 ml/min. It was then equilibrated with 100 mM acetate buffer (pH 6.3) containing 0.5 M NaCl (Appendix 2), PAPP-A was eluted with a step gradient to 30% of 100 mM acetate buffer (pH 4) containing 0.5 M NaCl (Appendix 2) at a flow rate of 2 ml/min.

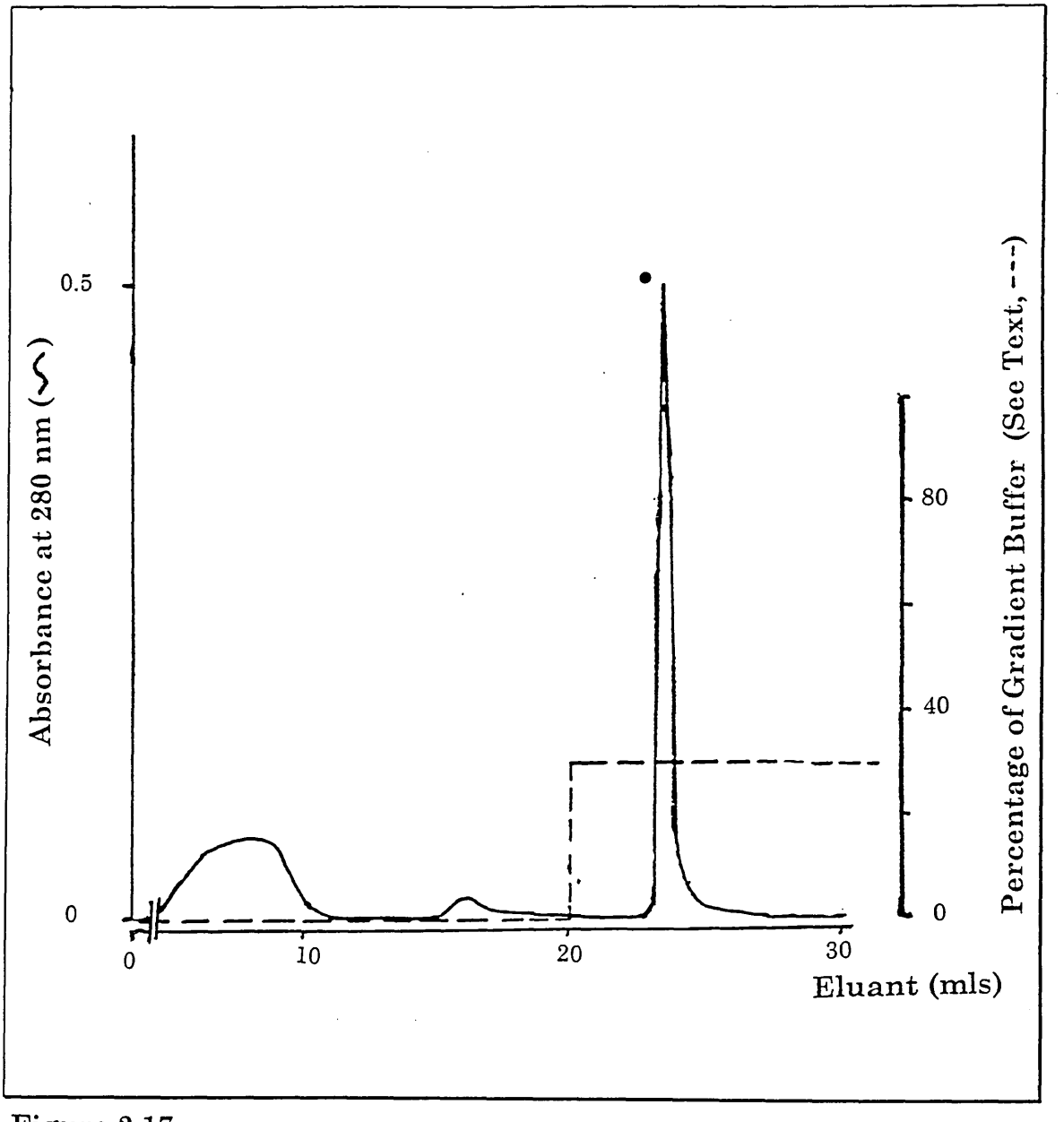


Figure 2.17

Elution profile from Zinc-MCAC column

[Legend: An aliquot of the DEAE bound fraction from section 2.7.6 was loaded onto this column, buffer conditions were as described in section 2.7.10. PAPP-A was detected by the RIA as previously described in section 2.6.4.5]

2.7.11 Gel Filtration

Gel permeation chromatography (gel filtration) is a form of partition chromatography that separates molecules by partitioning them between the solvent and a defined porous stationary phase. Protein aggregates can elute before the void volume, by being sterically hindered from diffusing between beads. Smaller molecules can enter the stationary phase and move through the column at a slower rate than larger molecules that will then elute first. Separation is then on the basis of shape and mass of a particular molecule. A pre-packed superDex-200 (Pharmacia, Appendix 1 with a bed volume of 305 mls) was equilibrated in 10 mM potassium phosphate buffer (pH 7.2) containing 0.15 M NaCl. The column was run at 4 ml/min and 5 ml fractions were collected (Figures 2.18 and 2.19).

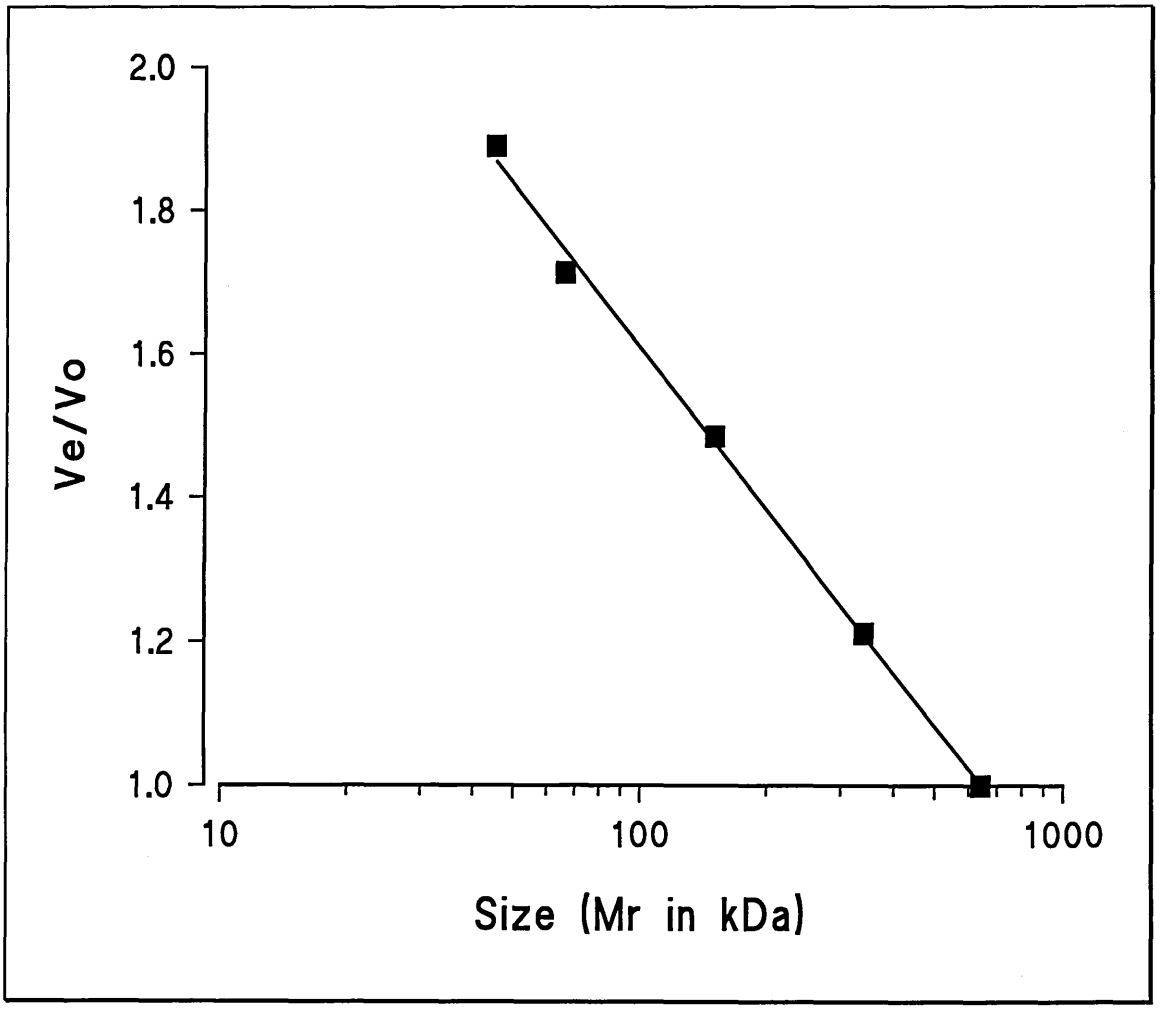


Figure 2.18

Calibration curve for the SuperDex-200 gel filtration column.
[Legend: Volume eluted/Void volume plotted against proteins of a known molecular weight (Appendix 1) taken to be Blue dextran (2000 kDa), Fibrinogen (340 kDa), IgG (150 kDa), Albumin (66 kDa) and Ovalbumin (45 kDa). $r^2 = 0.997$.]

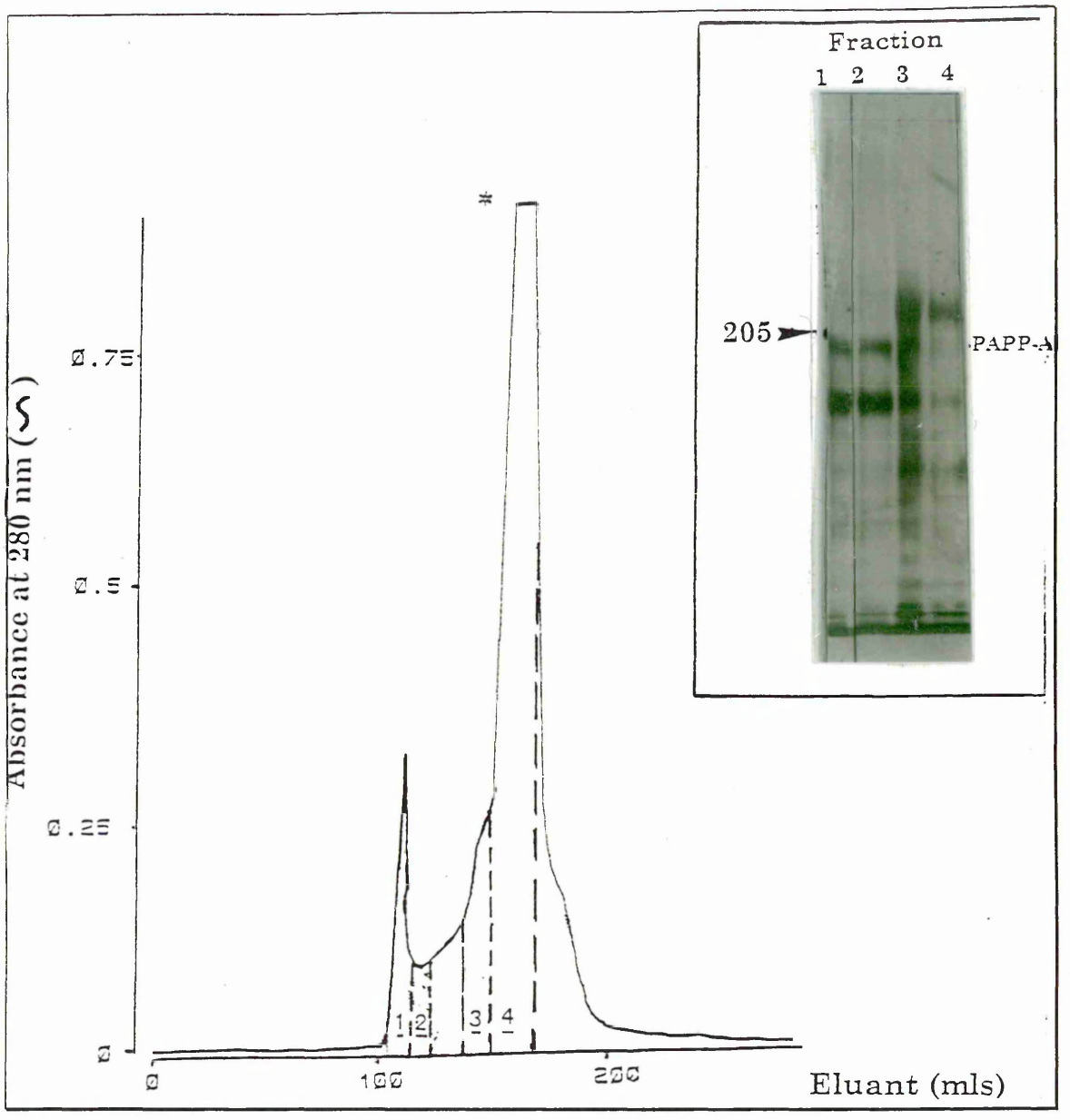


Figure 2.19

A typical elution profile from the superDex 200 gel filtration column (**Plate inset 2.19**, represented a silver stained 5% SDS-PAGE gel of fractions indicated.)

[Legend: *: indicated off-scale absorbance. The arrow indicated molecular size markers with the number in Kda. The material indicated as PAPP-A in the plate were confirmed by western blotting and immunodetection with anti-PAPP-A antibodies. An aliquot from the material bound on the L-arginine matrix was loaded onto this column, buffer conditions were as described in section 2.7.11**]**

2.7.12 Elution of Proteins From Gel Slices

Samples were reduced with 2-mercaptoethanol and run on an SDS-PAGE gel as described in section 2.3.2.2. Experiments indicated that major losses occurred at the post-gel staining/destaining stage using the standard CBB

staining method (section 2.3.2.2d). If the protein was to be recovered from the gel, 1.5 mls of 2% CBB-R250 in methanol was added to the top electrophoresis reservoir. This allowed in-gel staining of proteins and the recovery of protein from the gel by excising the visualised band (It was frozen at -70^{0}C). After thawing the gel slice was cut into small pieces and incubated for 3 hrs at 37^{0}C with 300 µl of gel elution buffer (Appendix 2). This was then spun through siliconised glass wool and the gel fragments were washed with 150 µl aliquots of elution buffer until the elution buffer had no traces of CBB. The fractions were pooled, freeze dried and finally reconstituted in sterile distilled water. Recoveries were calculated by re-running sample on an SDS-PAGE gel and video-densitometry as described in section 2.6.5. Recoveries for the in-gel staining were approximately 40% compared to a recovery of only 10% when post gel staining/destaining was used prior to the recovery of protein from the gel slices.

General concentration of samples (if required) was achieved using Centriprep-100 (Amicon, Appendix 1) as described by the manufacturers.

2.8 Materials and Methods Section For A Molecular Approach: Towards The PAPP-A Gene-II

2.8.1 Assessment of Protein Elution Methods from Blotting Membranes

Protein ((standard proteins, α_2M or purified myosin) and PAPP-A) were electroblotted onto nitrocellulose, immobilon-P as described in section 2.4.1. This material was then eluted from these membranes using a variety of solvents (Appendix 2) as described by these investigators:

- 5% Tween (Leykan and Strahler, **1992**).
- 20 - 100% Methanol (Charbonneau, **1991**).
- Hexafluoroisopropanol, HFIP (Morris, **1992**).

The eluted material was recovered by freeze-drying the material and reconstituted in sample loading buffer (Appendix 2). It was subsequently analysed by SDS-PAGE followed by densitometry as described in section

2.6.5). More than 80% of the standard protein was recovered from the membranes but PAPP-A could not be detected after using any of the suggested elution methods.

2.8.2 Solid-Phase Cyanogen Bromide (CNBr) Digestion

Cyanogen bromide specifically cleaves proteins at the methionyl peptide bond (cited from Gross et al, **1962**). Methionine residues are less frequently present in proteins than other amino acids, so cleavage at this site usually produces a limited number of large peptides.

The procedure described in the probe-design kit was followed (Promega, **1990**). The PAPP-A monomer was electroblotted onto immobilon-P membrane or nitrocellulose (Luo et al, **1990**). The blotted material was incubated overnight at RT with 50 - 100 µl of 70% formic acid containing 10 mg/ml CNBr. The CNBr solution was then transferred to a new sterile screw-top Eppendorf tube (eluted peptide vial). The peptides were eluted from the membrane by submerging it in 200 ul of peptide elution solvent (Appendix 2) for 2 hours. This process was repeated again and the eluted peptides combined in the eluted peptide vial and dried down using a nitrogen stream directed at the liquid in a well ventilated fume hood. The peptides were reconstituted in distilled water and loaded onto the RP-HPLC column .

2.8.3 RP-HPLC Separation of Peptides

The mechanism by which proteins/peptides adsorb to a RP column as proposed by Geng and Regnier (**1984**) is that the hydrophobic amino acids in a peptide or protein are retained on a reverse-phase surface by single or multi-point adsorption and the retention time on this surface can be predicted from the ratio of polar/non-polar amino acids in the molecule. The protein/peptide entering the column displaces the mobile phase organic modifier on the surface of the reversed phase support. The protein remains adsorbed until a critical concentration of mobile organic modifier is reached and is then desorbed. Large molecules interact with the surface of the

support by an adsorption type mechanism, which is highly dependent upon their protein conformation. Thus separation of a protein/peptide mixtures can be achieved. The use of TFA in the solvent protonates carboxyl groups on peptides thus increasing their affinity for the RP surface.

Briefly, the CNBr peptide digest was loaded onto a 250 x 4 mm RP-18 column (5 μm Merck) linked to a Pharmacia HPLC system (Appendix 1). It was eluted at a flow rate of 0.5 ml/min with a four-step gradient as illustrated in Figure 6.3, Chapter 6.

Solvent A: distilled water containing 0.06% TFA.

Solvent B: 80% Acetonitrile containing 0.06% TFA.

Detection was by absorbance at 214 nm, or using a diode-array detector (Appendix 1) and amino-acid analysis (Appendix 3) was used to determine if peaks contained protein that was suitable for microsequencing (Figure 6.3, Chapter 6).

2.8.4 Vapour Phase CNBr Cleavage Of PAPP-A In Gel Slices

This was performed by a modification of the method originally described by Zingde et al, (**1985**). TFA under reduced pressure was used instead of formic acid as this encouraged vapour formation and led to the production of an acidic CNBr atmosphere. The vapour phase method ensured that cleavage was likely to occur at methionine residues and not at the acid labile, Asp-Pro bonds. The use of a vapour also meant that extensive equilibration procedures were not needed prior to peptide separation thus reducing protein losses that occur at these stages. All procedures involving CNBr were carried out in a well ventilated, externally vented fume hood.

In-gel stained PAPP-A bands as described in section 2.7.12 that had been separated on 5% SDS-PAGE gels as described in section 2.3.2.2a were incubated in a quik-fit chamber containing 3 mls of a 20 mg/ml cyanogen bromide in 70% TFA. The gel slices were suspended above the CNBr containing TFA, care was taken that liquid did not splash gel slices. The vessel was flushed with nitrogen and placed under a vacuum until the TFA boiled. It was then sealed, covered in foil and left overnight at RT. The gel

slices were then freeze dried and washed in sample buffer (Appendix 2) until the buffer remained a blue colour (dye sensitive to pH changes). The gel slices were boiled in 50 µl of sample buffer for 3 minutes and loaded onto a 5 - 20% peptide separating gel as described in section 2.3.2.2c). After electrophoresis the proteins present in the gel were electroblotted onto a PVDF membrane as described in section 2.4.1 and sent for automatic sequencing.

Table 2.6

Proteolytic enzymes suitable for limited proteolytic cleavage in the Cleveland SDS-PAGE gel environment

Proteolytic Enzyme	[1]Cleavage Site	Reaction conditions	Other Information
Endoproteinase Glu-C (from *Staphylococcus aureus*; V8 protease.)	-Glu [2]X	50 mM Ammonium bicarbonate (pH 7.8) Or 50 mM Ammonium acetate (pH 4.0)	Active in presence of : - 0.2% SDS. - 2M Urea, - 2M Guanidine-HCl.
	-Glu [2]X or -Asp [2]X	50 mM Sodium phosphate, (pH 7.8)	50% activity in 4M urea. Mr 28,000 d.
Endoproteinase Lys-C (Type XXXIII-A from *Lysobacter enzymogenes*)	Lys-[3]X	100 mM Ammonium bicarbonate (pH 8.5)	Active in presence of: - 2M urea - 0.1% SDS Mr33,000 d.

[Legend: [1]Cleavage site: Specific cleavage site as determined by enzyme and reaction conditions. [2]**X:** Where X is any amino acid but not glutamic acid or proline. [3] **X:** Where X is any amino acid.**]**

2.8.5 Limited Proteolytic Cleavage in-gel Slices (Cleveland)

The Cleveland method (Cleveland et al, **1977)** enables in-gel proteolytic digestion of proteins. The method that was used was a modification of the Cleveland technique described by Andrews (**1989**). In-gel stained PAPP-A slices (Chapter 5) were cut in squares and equilibrated for 15 minutes at RT in stacking layer buffer (Appendix 2), they were then gently pushed into the wells of a 5 - 20% gradient SDS-PAGE system as described in section 2.3.2.2c. These gels were cast with a 5 cm stacking gel layer to allow the

protease and protein to be stacked together and facilitate proteolytic digestion. The gel slices were overlaid with stacking layer buffer containing 10% glycerol, a proteolytic enzyme (see Table 2.6, the concentration and type of protease that were used can be seen in the legends in Figures 6.4 and 6.5, Chapter 6) and 0.001% Bromophenol blue as dye marker. The gel was then run at 55 V (constant) until marker dye was 2 cm into stacking layer. Then the power was turned off and the samples were left to digest for 60 to 90 minutes. Subsequently electrophoresis was resumed, the fractionated digest was then electroblotted onto a PVDF membrane as previously described (section 2.4.1).

2.8.6 N-terminal microsequencing

The material produced on PVDF blots was sent to commercial sequencing services (services used were described in Chapter 2) which used an Applied Biosystems 477A Protein Sequencer. The sequences produced by these services are identified as part of the legend to Figures 6.4 and 6.5, Chapter 6. A full outline of the sequencing results can be found in Appendix 3.

2.8.7 Oligonucleotide Synthesis and Post Synthesis Processing

Oligonucleotides were produced in-house by Mr P. Loxley. The phosphoramidite version of the phosphite triester method was carried out on a solid phase support. De-protection and concentration of oligonucleotides were as described in Appendix 2. The following oligonucleotides were used as PCR primers (Table 2.7).

Table 2.7

Oligonucleotides synthesised from PAPP-A sequences obtained in this study.

5'- AAA GCI AGA GGI GCI ACI GAA **-3' :N-PAPP-1.**
5'- G C G G **-3' :N-PAPP-2.**
5'- GA(A/G) GCI (A/C)G(A/G) GGI GCI ACI GA(A/G) GA(A/G) C **-3' :PAPP-4**
5'- ICC ICC (G/A)TC IGC (A/G)TT (A/G)AA (A/G)TG **-3' :cPAPP-3**

[**Legend**: Bracketed figure indicates 50% wobble with nucleotides indicated at that position. N-PAPP-1/2 were two oligonucleotide pools produced from the first N-terminal sequence obtained for PAPP-A (K. Lilley, Appendix 3). PAPP-4 was the consensus N-terminal region for PAPP-A obtained from sequencing information (Table 6.3, Chapter 6) and PAPP-3 was a complementary strand to a putative internal stretch obtained from a V8 digestion of PAPP-A, Figure 6.5, Chapter 6. The synthesis of these primers is further outlined in Appendix 3.]

2.8.8 Polymerase Chain reaction (PCR)

The PCR reaction is an *in-vitro* DNA amplification process that occurs during a series of repeated cycles. Each cycle consists of three temperature dependent steps:

- Denaturation carried out at 90 - 95°C.
- Primer annealing carried out at 35 - 65°C.
- Extension at 70 - 75°C.

The temperature at which denaturation is performed is somewhat dependant upon the DNA template. The annealing temperature is dependent upon the individual primers present in the assay and the extension temperature is a function of the thermostable DNA polymerase. By cycling these three steps the polymerase extends the primers in a 5' → 3' direction and allows amplification of the original template. A more complete description of the PCR reaction and factors that influence this reaction can be found in Ehrlich, (**1987**) and Bej et al, (**1991**).

Highly degenerate primers were used (Table 2.7) and as suggested by Williams (**1989**) in such cases, it is best to use a low annealing temperature in the first cycle and then gradually increase it, giving a ramped PCR cycle. This type of program (Table 2.8) was the one that worked best at amplifying cDNA from the placental library these degenerate primers.

Table 2.8 Ramped PCR program for use with primers listed in Table 2.7:

5.1	95°C	Program paused after 9 minutes and 2.5 Units of Taq added, (**Hot-Start**) Restart, 1 minute at 95°C.
5.2	50°C	1 minute
5.3	72°C	2 minutes
5.4	95°C	1 minute
5.5	55°C	1 minute
5.6	72°C	2 minutes
5.7	95°C	1 minute
5.8	60°C	1 minute
5.9	72°C	2 minutes (Repeat x 28 from step 5.7).
5.10	72°C	10 minutes

The standard PCR was performed using 2.5 U of thermostable DNA polymerase (from *Thermus aquaticus,* strain YT2, (Appendix 1)). A 10X reaction buffer was used that consisted of 100 mM Tris-HCl, pH 8.3 (at 25^{0}C), 500 mM KCl and 15 mM $MgCl_2$. The standard PCR reaction volume was 50µl , with substrate dNTP's that were used at a final concentration of 200 µM each. The reaction mixture also contained 100 pmoles of each primer and 5µl of the cDNA library template (Appendix 1) at a 1:100 dilution. The PCR products were analysed (see Plate 6.1, Chapter 6) on a 2% agarose gel in TAE as described section 2.3.2.1.

2.9 Materials and Methods Section for Studies on An Interaction Between Reduced Monomeric PAPP-A and The Endoproteinase Arg-C.

Limited in-gel slice digestion was performed as described in section 2.8.5. Purified monomeric PAPP-A (see Plate Inset 5.3, Chapter 5) was used. A reduced SDS treated BSA (Appendix 1) which was prepared and eluted from gel slices in the same way as PAPP-A as described in section 2.7.12 was used as a control (a similar molar concentration of BSA and PAPP-A were used assuming that monomeric PAPP-A has a molecular weight of 195 kDa and BSA, 66 kDa). Protein sequencing grade endoproteinase Arg-C (Appendix 1) was used. The effect of PAPP-A or the control protein (BSA) on the esterolytic activity of endoproteinase Arg-C was measured. Briefly, 30 µl of endoproteinase Arg-C (for concentration see Figure legends 7.2 and 7.3, Chapter 7) were pre-incubated with 15 µl of buffer A (Appendix 2) and 15 µl of protein (PAPP-A, BSA or buffer A for the protein concentration see legends in Figures 7.2 and 7.3, Chapter 7) for 1/2 hour at RT and then 0.44 ml of pre-warmed (37^{0}C) buffer A and 1 ml of pre-warmed (37^{0}C) TAME substrate (Appendix 2) were added. After 30 minutes of incubation at 37^{0}C, 50 µl aliquots were removed and the TAME was measured as described in section 2.3.6.

CHAPTER THREE

Chapter Three

A Molecular Approach: Towards The PAPP-A Gene I

3.1 Introduction

The main classical strategies by which a gene may be cloned are:

- Direct selection is an approach whereby the cloned gene product confers a recognisable phenotype on the host cells. However in the case of PAPP-A the gene is a part of a complex genome and its product does not have a known function.
- Screening a DNA library in which the gene (or cDNA) itself or its translated product is identified.

The screenings of DNA libraries require the use of an efficient host/vector system. Molecules of DNA with one origin of replication function as units of replication and are called replicons. So if replication of a DNA fragment is required then the obvious solution is to attach it to a suitable replicon. Such replicons are called vectors and examples are plasmids and bacteriophages in which the exogenous DNA can be either:

- Inserted at a unique restriction site within the vector (Insertion Vectors).
- Used to replace phage DNA at a specific site as it contains a pair of specific restriction sites flanking both sides of a particular region (Replacement Vectors).

The original wild type bacteriophage lambda is not suitable as a cloning vector because it contains too many restriction sites, some of which are located within regions essential for the replication of the phage. However by a series of manipulations mutant bacteriophage strains have been produced that are suitable cloning vehicles for cDNA inserts. The use of bacteriophage vectors requires an understanding of the phage structure and its interaction with its bacterial host. To illustrate this interaction I have chosen the bacteriophage λgt11 and its bacterial host, *E. coli.* (a

derivative of K12, strain: Y1090). This system was used to screen for the PAPP-A clone using an antibody directed against PAPP-A as a probe and is described in this Chapter (Figure 3.1).

E. coli has the following advantages when used as the host organism:

- Gene expression is largely controlled at the level of initiation of transcription.
- It is a well studied organism that can be easily grown in a well defined media.
- The work can be carried out safely using standard microbial techniques as described by Bainbridge (**1991**) and needed no special precautions as *E. coli* is classified as a low risk category organism.

The double stranded DNA of the lambda phage genome is encapsulated in an icosahedral head from which a single tail fibre is projected. Infection is initiated by the adsorption of the tail fibre to the receptor sites in the outer membrane of the bacterium (this site is encoded by the *E. coli,* lamB gene). Receptors are produced when *E. coli* cells are grown in a medium that contains maltose as the host's carbon source. The adsorption is facilitated by the presence of magnesium ions.

After infection the phage can follow a productive (lytic) or temperate (lysogenic) pathway depending upon physiological conditions. In a lytic cycle, early to late transcription events take place that result in the production of virion proteins and the phages genomic material is packaged to generate bacteriophages. This type of replication eventually leads to the host cell lysis with the release of phages that can infect other host cells..

A lysogenic cycle results in the integration of the bacteriophage genome into the host chromosome and results in the phage (prophage) being replicated as part of the host, *E. coli* chromosome.

The Host/Vector System Used In This Study

Bacterial Host: *Escherichia coli* , strain: Y1090 with the following genotype:

- lacU169 ..... Deletion that prevents endogenous generation of functional β-galactosidase protein product.
- lon⁻ ..... Deletion of lon protease, which reduces proteolysis of expressed fusion proteins.
- araD139 ..... Cannot use arabinose as a carbon source.
- strA ..... Resistance to the antibiotic, streptomycin.
- supF ..... Results in the insertion of Tyrosine at UAG codons.
- trpC22 ..... Requires Tryptophan for growth.
- (pMC9) ..... Contains a plasmid that is a derivative of pBR322 which has the LacI gene that encodes the lac repressor. It also confers resistance to ampicillin and tetracycline.
- hsdR (rk-, mk+) Restriction minus, modification positive: Thus allowing material to be cloned into the host without cleavage of DNA by endogenous restriction endonucleases that are present.

Vector: The temperate bacteriophage, λg11 has the following stated features with the insertion site for exogenous DNA in a unique EcoRI site, 53 bp from the 3' terminus of lacZ gene; thus proteins are expressed as fusion products with β-galactosidase.

- Sam100 ..... Mutation renders the phage lysis-defective, so it cannot make plaques in hosts that lack the SupF supressor gene product.
- Nin5 ..... A deletion that removes a transcription terminator.
- cI857 ..... Encodes a temperate repressor protein (see below)

Figure 3.1 Vector/host interactions.

The Y1090 strain contains the pMC9 plasmid that carries the LacI gene which encodes a protein repressing the Lac promoter carried by the λgt11 phage so enabling a control of the production of a protein specified by the DNA insert. Y1090 is also lon- and so lacks a protease that is responsible for degrading abnormal proteins. The cI857 gene encodes a repressor protein,

which is inactive at 420C (active at 300C), and unable to shut off early phage promoters, this leads to a lytic response. The nin5 deletion (Figure 3.1) allows enough leaky readthrough to occur that when the amber mutation is suppressed it enables plaques to be formed.

Insertion of foreign DNA within the LacZ gene, results in a phenotypic change from LacZ+ to LacZ- , thus when plated onto an appropriate host which is LacZ- in the presence of IPTG (a synthetic inducer of the lac repressor) and X-Gal (a chromogenic substrate for β-galactosidase) the parental λgt11 produces blue plaques. Recombinant phage produce colourless plaques as the inserted DNA interrupts the coding sequence of the LacZ gene.

The described host/vector system makes it possible to synthesise a foreign protein in the host, *E. coli* thus enabling this host to produce a polypeptide that is specified by the cDNA present (Young and Davis, **1983**).

Screening of this cDNA library for a clone of interest can then be achieved by one of two methods:

- Nucleic acid hybridisation; with the cDNA itself being identified, but in the case of PAPP-A, no primary sequence information was available therefore this method could not be used initially to identify a PAPP-A clone.
- Immunological detection; with the translated product of the gene being immunologically identified, hence the use of an antibody directed against PAPP-A being used as an immunological probe.

This chapter describes the initial studies that were undertaken to study the structure and expression of the PAPP-A gene by searching for its cDNA in a bacteriophage expression library that had been prepared commercially from mRNA derived from placental tissue. Placental tissue has been shown to be a likely source of PAPP-A (Chapter 1, section 1.4.3) and thus a viable source of PAPP-A mRNA.

3.2 Materials and Methods

The reagents, methods and techniques that were used to generate the results described in this chapter are described fully in Chapter 2, section 2.5.

3.3 Results and Discussion

An antibody to PAPP-A was needed for the screening of a cDNA expression library that would recognise a denatured form of this protein. The host, *E. coli* has an internal cellular environment that does not allow the formation of disulphide bridges (Young and Davis, **1983**). Thus proteins produced by this system are not able to fold into their native state. The ability of a particular antibody to detect a denatured protein can be assessed in Western blotting where reduced and unfolded proteins are studied. The monoclonal and polyclonal (DAKO) antibodies directed against PAPP-A that were available in this study were assessed for their ability to detect a denatured monomeric PAPP-A protein (Plate 3.1)

The monoclonal PAPP-A antibody did not recognise the monomeric reduced form of PAPP-A but did recognise the PAPP-A dimer (Plate 3.1(**B**)) or the proposed PAPP-A/proMBP dimer (Oxvig et al, **1993**). This is discussed further in Chapter 7 , section 7.2. This monoclonal antibody's paratope was thus directed at a PAPP-A epitope that was related to the molecule's tertiary structure and so was unlikely to be useful for isolation of a cDNA clone for PAPP-A from this type of expression library.

The polyclonal antibody recognised many more epitopes on the unreduced PAPP-A than on the reduced sample as it was demonstrated by the intensity of staining (Plate 3.1(**A**)) when the same amount of this protein that was loaded into each lane. The polyclonal antibody did recognise PAPP-A in a denatured and unfolded form, so it was decided to use this antibody as a probe to isolate a PAPP-A clone from the placental cDNA expression library.

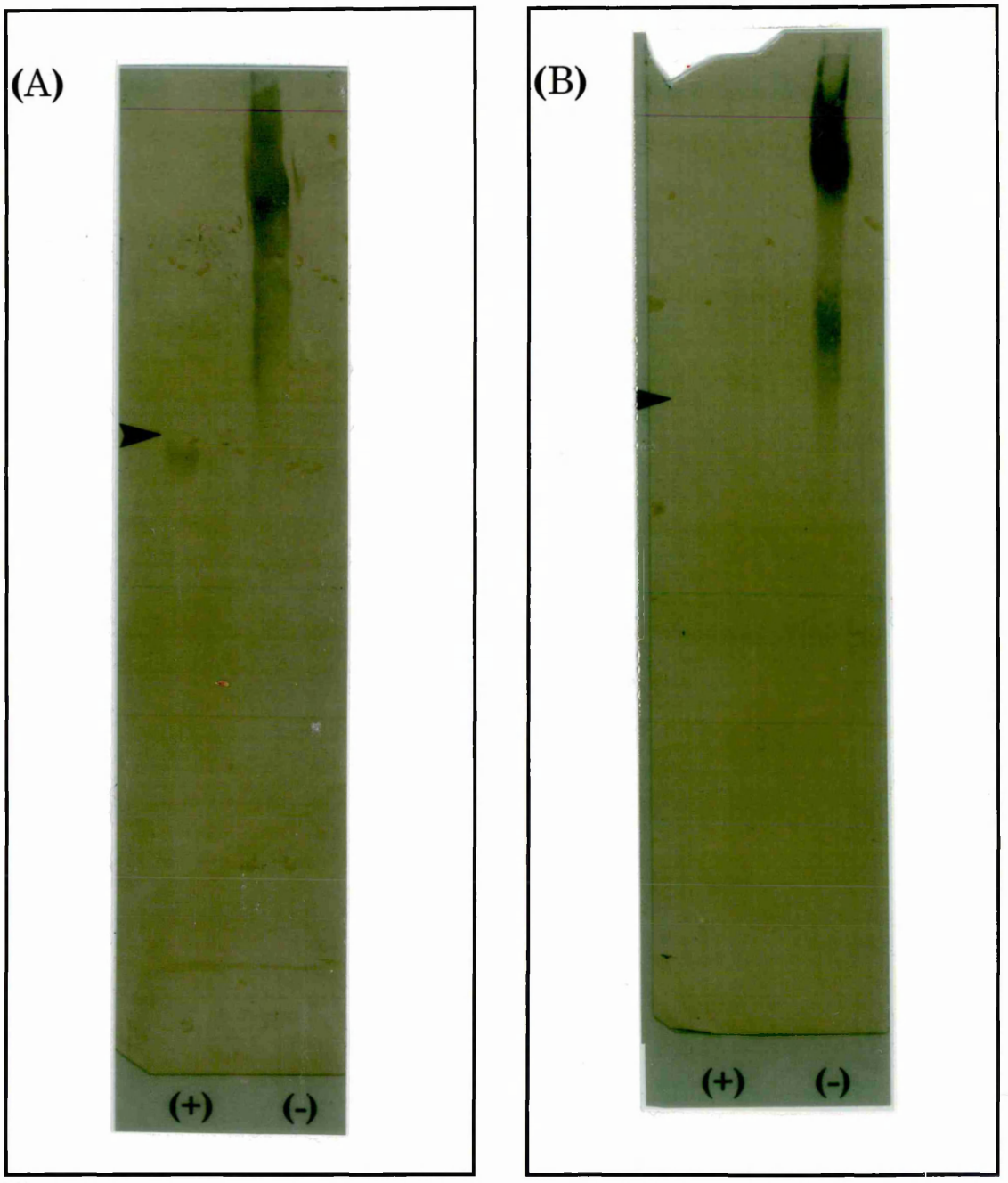


Plate 3.1

Western blots of PAPP-A prepared using DAKO polyclonal antibody (**A**) and anti-PAPP-A monoclonal (**B**).

[Legend: Western Blotting was performed as described in chapter 2, section 2.4.1. The polyclonal antibody was used at a 1:600 dilution and the monoclonal ascites tap at a 1:25 dilution. **(+):** Indicates that the protein sample was reduced with 2-mercaptoethanol prior to SDS-PAGE. **(-):** Indicates that protein sample was not reduced prior to SDS-PAGE. SDS-PAGE was carried out as described in Chapter 2, section 2.3.2.2a. The arrow indicates the position of the 205 kDa molecular weight size marker (Appendix 1) on the blotted membrane.**]**

Initial screening was performed on 90 mm plates, however this was soon changed to 140 mm plates as it allowed a larger number of clones to be screened at one time. If we assume that PAPP-A is encoded by a relatively infrequent species of mRNA then assuming a 99% probability of isolating this clone, a library must contain at least 9 x 10^5 independent clones to identify a clone that is represented by a relatively rare species of mRNA (Sambrook et al, **1989**). The library was titrated and it was estimated that 3.4 x 10^{10} clones were present in 1 ml of this library (Table 3.1).

Table 3.1 Titration of the placental cDNA library.

Volume of a 10^{-7} Dilution of library used	Number of plaques/plate
10 µl	20
20 µl	78
30 µl	130

[Legend: Results of cDNA library titration performed as described in Chapter 2, section 2.5.1.4.]

This was close to the figure obtained from Clontech of 9 x 10^9 clones/ml, with the difference being probably due to the error in diluting the library from neat to a 10^{-7} dilution. From this value of the number of plaque forming units (PFU's), a dilution was taken in subsequent work to give 8000 (20000) PFU's per 90 (140) mm plate. This number of plaques per plate enabled large numbers of clones to be screened and also allowed the identification of individual plaques that could be picked out and re-screened when a putative positive clone was found. Therefore to identify a rare clone it is necessary to screen a large numbers of clones. To this end an estimated 1.2 million plaques were screened in an attempt to identify a clone for PAPP-A. No clone for PAPP-A was identified using the DAKO polyclonal antibody. A further assessment of the characteristics of the library and the polyclonal antibody used was now made.

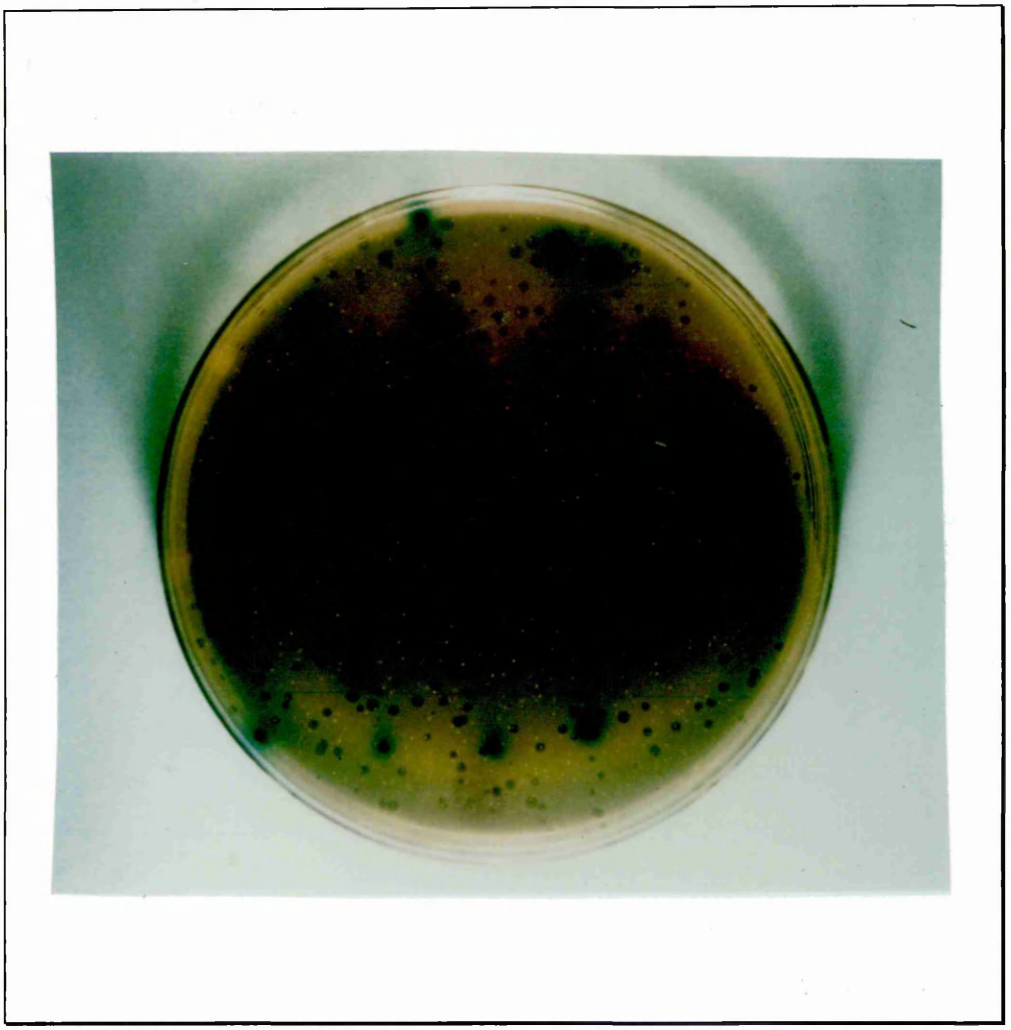


Plate 3.2

An Assessment of Vector/Recombinant Phages present in the placental cDNA library used in this thesis.

[Legend: The plate was prepared as described in Chapter 2, section 2.5.1.4. The small white dots were bacterial contamination that were seen on extended storage of the plate and were not present on the plates that were used to screen for the PAPP-A clones.**].**

One possibility was that the library contained a large number of non-recombinant vector bacteriophages as this would have reduced the number of recombinants that had been screened. As can be seen from Plate 3.2 less than 5% of the plaques were blue in the presence of X-Gal (a chromogenic substrate for the β-galactosidase gene product). This indicates that the placental library had a large number of recombinant bacteriophages and is well above the widely accepted 60% level for expression libraries (Huynh et al, **1985**).

A positive control for the screening system used was obtained by infecting the host, *E. coli* cells with recombinant phage containing an ovalbumin insert and detecting the products of the expression with an anti-ovalbumin antibody. The ovalbumin cDNA clones were detected very efficiently using this system demonstrating that the host/vector system was capable of transcription of the cDNA insert with subsequent production of a polypeptide (Plate 3.3). Therefore the inability to identify a clone that was representative of PAPP-A using the DAKO polyclonal anti-PAPP-A as a probe was not due to a failure of:

- The avidin/biotin end-detection system.
- Or the host/vector system that produced an expressed polypeptide.

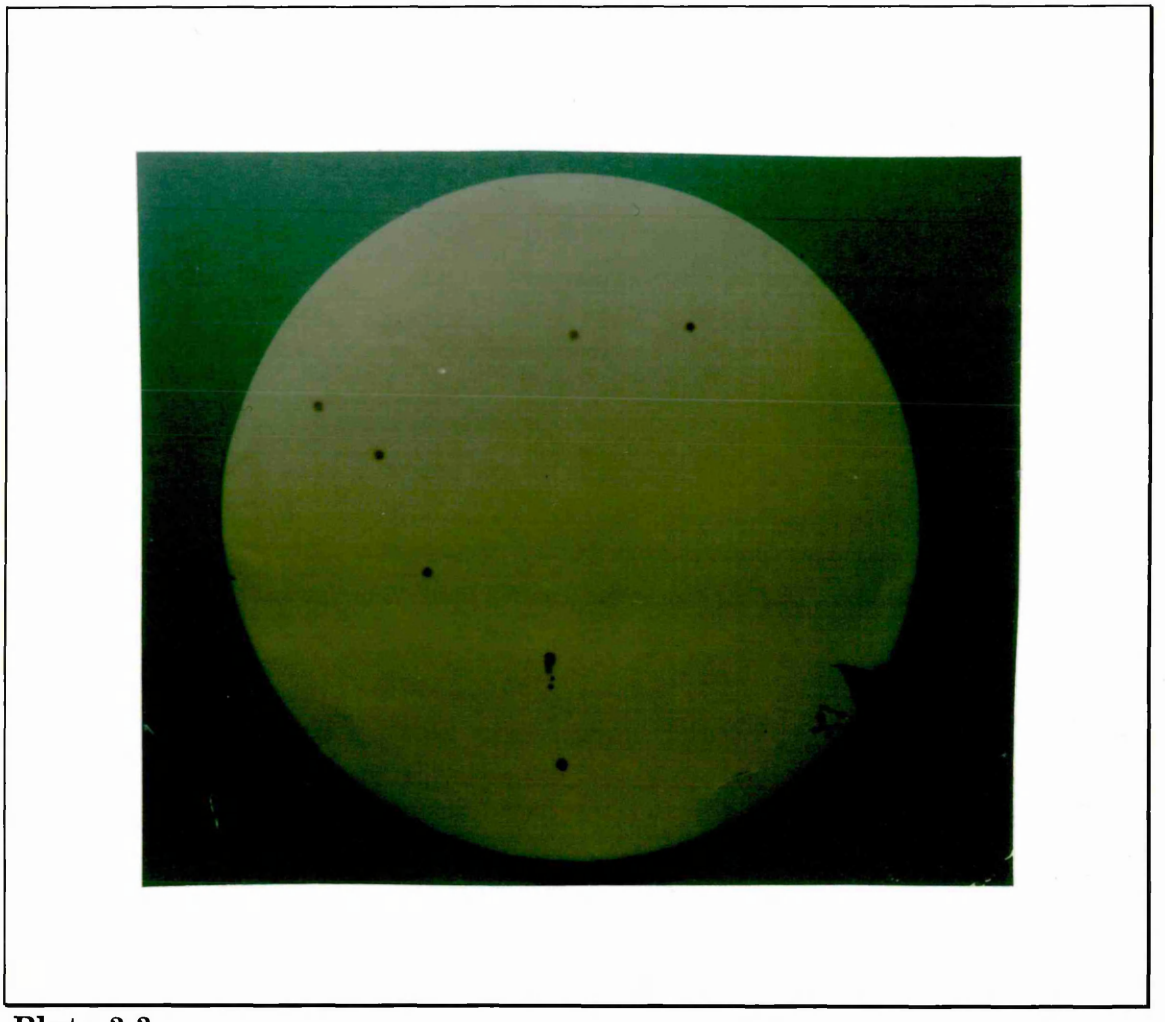


Plate 3.3

Positive control of the expression library/screening system

[Legend: Control ovalbumin cDNA clones were used to infect the host, *E. coli*. as described in Chapter 2, section 2.5.1.3 The clones were detected with a 1:5000 dilution of rabbit (Appendix 1) anti-ovalbumin antibody, detection was as described in Chapter 2, section 2.5.1.7].

Assuming that PAPP-A was present in the placental cDNA library the negative result could have been due to another characteristic of the prokaryotic system, that is the lack of post-translational modifications (e.g. glycosylation and phosphorylation) of proteins. The PAPP-A molecule has a significant carbohydrate component that would not have been added to the polypeptide chain produced in this system. The expression of a partial PAPP-A monomer was also likely as incomplete cDNA molecules would have been present in this library (insert size quoted as being 0.8 - 3.6 kb) which would also result in less epitopes being present for PAPP-A. To investigate the effect of removing the carbohydrate moiety from native PAPP-A was achieved by incubating PAPP-A that was blotted onto a nitrocellulose membrane with an enzyme preparation that would remove the glycan component of PAPP-A.

The enzyme preparation that was chosen was a crude chitinase preparation from *Aspergillus niger* as this has been shown to contain a mixture of glycosidic enzymes capable of de-glycosylating proteins (Maley et al, **1989**). Although this preparation was efficient at removing the glycan moiety it had a disadvantage in that it was shown to be contaminated with a limited amount of proteolytic enzymes (Figure 3.2) and any change in ability of the antibody to recognise PAPP-A could have been due to proteolytic digestion, rather than the removal of the glycan component. The subsequent treatment with a protease inhibitor cocktail has however demonstrated that the contaminating proteases were successfully inhibited (Figure 3.2) and that this protease inhibitor cocktail did not affect the activity of the glycosidic enzymes of this preparation (Figure 3.3).

Further treatment of the blot from an SDS-PAGE gel with chitinase and subsequent probing with the DAKO anti-PAPP-A antibody revealed that the band previously detected was no longer visible (Plate 3.4). This suggested that the polyclonal serum used to screen the library for the PAPP-A clone has a number of paratopes that are directed at epitopes on the glycosylated part of the

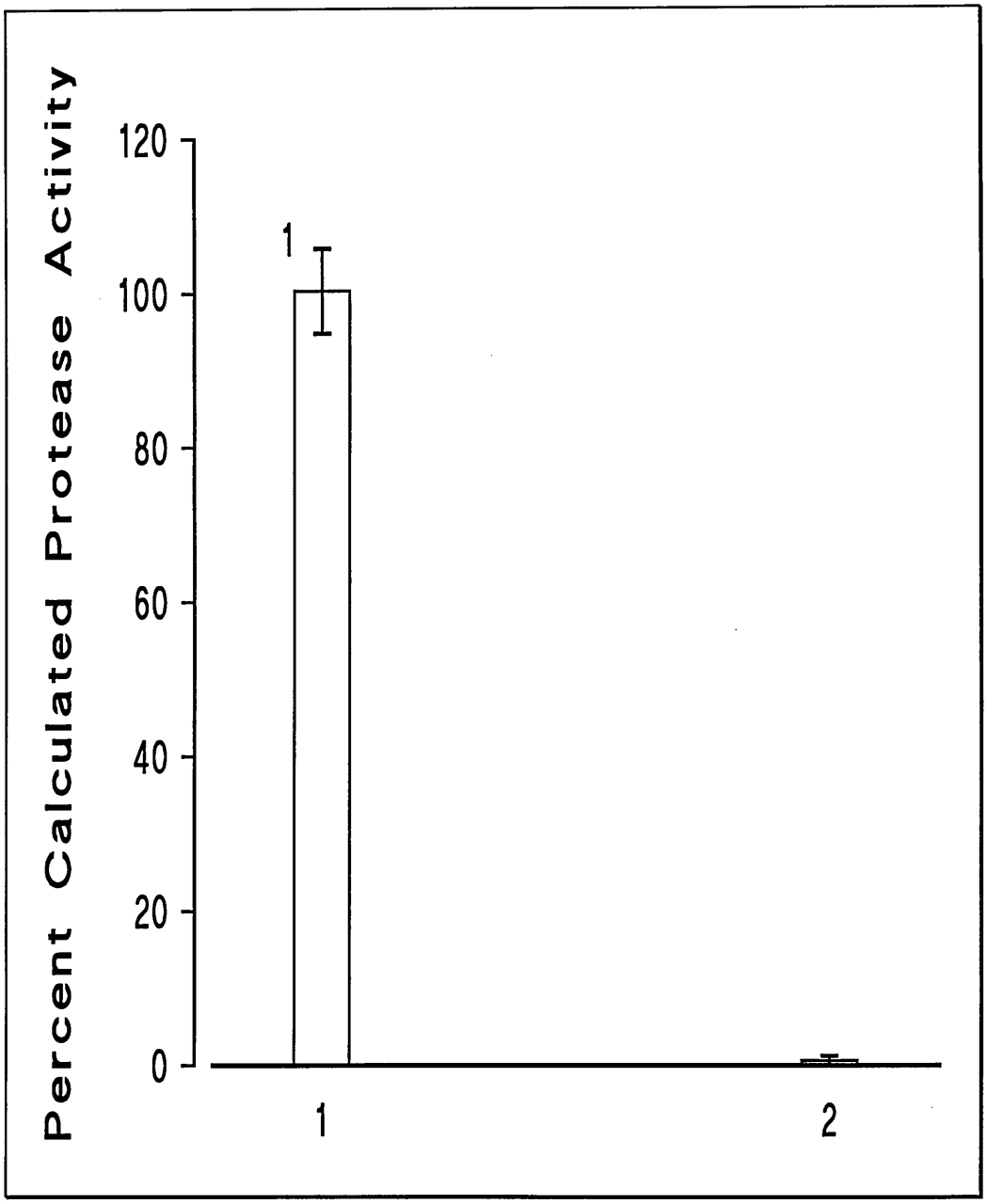


Figure 3.2

Calculated general proteolytic activity present in the chitinase enzyme preparation (before/after incubation of this preparation with a protease inhibitor cocktail).

[Legend: Percent calculated protease activity was obtained using the general protease determination as described in Chapter 2 , section 2.3.5) and these values were related to the mean level found in the chitinase preparation (1) after 16 hours at 37°C. [1]: Results were expressed as mean calculated activity +/- range for samples in triplicate. (1): 0.5 units (Appendix 2) of a chitinase preparation pre-incubated in TBS prior to assessment of proteolytic activity. This preparation of chitinase had been shown to be contaminated with an estimated 0.75% of proteolytic enzymes as measured by the general proteolytic assay described and compared to a standard protease preparation. (2): 0.5 units of the chitinase preparation pre-incubated for 1 hour with a protease inhibitor cocktail in TBS prior to the assessment of general protease activity present.]

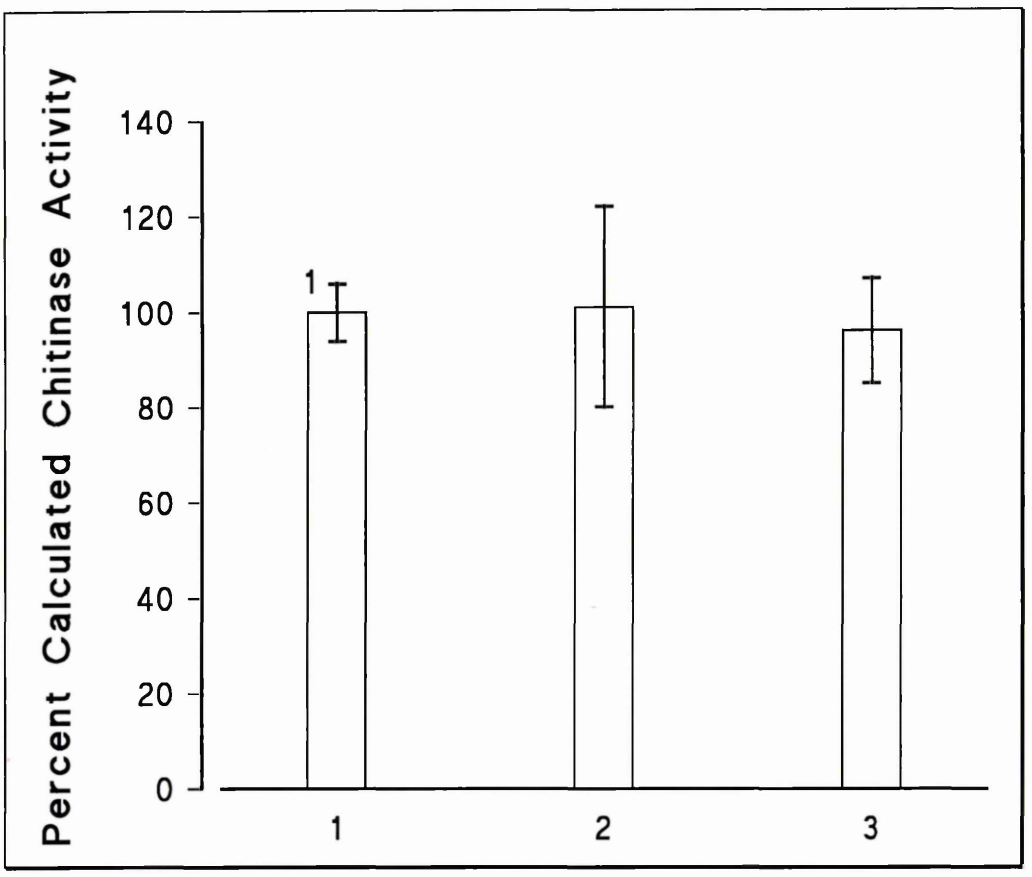


Figure 3.3

The calculated chitinase activity before and after incubating this enzyme preparation with a protease inhibitor cocktail.

[Legend: Percent calculated chitinase activity was obtained by comparing the values of the mean activity for the chitinase preparation (**1**) after 19 hours at 30°C as 100% and others plotted as a percentage of this value. **[1]:** all values were expressed as mean +/- range for each preparation in triplicate. (**1**): 0.5 units (Appendix 2) chitinase preparation in TBS (Appendix 2). (**2**): 0.5 units chitinase preparation (pre-incubated for 1 hour with a protease inhibitor cocktail in TBS). (**3**): 0.5 units chitinase preparation (pre-incubated for 1 hour and supplemented with another aliquot of protease inhibitor cocktail in TBS, just prior to being assessed for chitinase activity as described in Chapter 2, section 2.3.3).**]**

PAPP-A molecule or to the tertiary structure that is held in place by the glycan component of the PAPP-A molecule. The hypothesis that the lack of recognition of PAPP-A by this antibody was due the removal of glycan and not proteolysis of PAPP-A by proteases that were present in this crude enzyme preparation was further strengthened by experiments with electroblotting a control protein BSA, that is not glycosylated. Incubation of the BSA blots with the chitinase enzyme preparation did not diminish the intensity of immunodetection with an anti-BSA antibody when compared to the intensity on blots which had not been treated with this enzyme.

Subsequently a culture supernatant from *Allesheria terrestris* grown in conditions giving a limited proteolytic component (the general protease component present was determined as described in Chapter 2, section 2.3.5 and was shown to have 37 fold less protease than the chitinase preparation) was used to treat the Western blots for PAPP-A and BSA. Similar results to that found with the chitinase preparation with regard to immunodetection of these proteins were observed.

It has been shown that the polyclonal anti-PAPP-A antibody used under the described conditions detects a single band on the Western blot from an SDS-PAGE gel. This band corresponds in size to values attributed to PAPP-A by other investigators (Bischof, **1979**. Sinosich et al, **1990**). It also had a disulphide bridged tertiary structure as reported. It was however noted that a higher concentration of salt was required to produce a specific single band on a Western blot. Non-specific interactions of antibodies with other macromolecules are often electrostatic (Kabat,**1976**) therefore at an increased ionic strength these non-specific interactions are reduced as electrostatic charges are shielded by the increased concentration of ions. This thus limited the immunodetection to PAPP-A and may explain why a haptoglobulin related protein was not isolated in this study, in contrast to Kuhajda et al (**1989**) who isolated a cDNA encoding this protein using the DAKO antibody that was used in this study. Therefore it is likely that the failure to isolate a PAPP-A clone from this placental cDNA library using the DAKO polyclonal antibody was a function of the PAPP-A structure in that the antibody used under the described conditions did not have measurable levels of paratopes with a strong enough affinity to interact solely with PAPP-A's polypeptide chain or part of that chain that was available to form recognised epitopes. It was therefore decided to isolate the PAPP-A clone(s) using nucleic acid probes. No such primary sequence information was available for PAPP-A at the time, so it was necessary to obtain the PAPP-A protein in a form suitable for N-terminal amino acid sequencing (Chapter 5) and in sufficient quantities for limited proteolytic digestion to produce peptides for sequencing from which nucleic acid probes could be designed.

CHAPTER FOUR

Chapter Four

The Quantitative Measurement of PAPP-A

4.1 Introduction

An assay is a system of measurement for a defined material, which is based around a materials physico-chemical properties. The defined material in this case was PAPP-A and an assay was required to monitor the purification of PAPP-A from other blood plasma components. Assays that are based around a materials structure can be divided into two distinct sub-types (Ekins, **1981**):

- Non-competitive, reagent excess (Figure 4.1A).
- Competitive, reagent limited (Figure 4.1B).

A class of this type of assay are the immunoassays that use an antibody as the structurally specific reagent. The main problem in these types of assay is how to distinguish the free from the bound state and this is depicted in Figure 4.1. One method to resolve this problem is to use a labelled component (a tracer) in the depicted reaction that can be used to monitor the free/bound state. The types of tracer and label commonly used in immunoassays are illustrated in Table 4.1.

A) Reagent (Antibody) Excess

Analyte	+	Antibody	→	[Analyte-Antibody Complex]	+	Residual Antibody
(Free)		(Free)		(Bound)		(Free)

[The bound complex is directly proportional to the amount of analyte present.]

B) Reagent (Analyte) Limited

Analyte	+	Antibody	→	[Analyte-Antibody Complex]	+	Residual Analyte
(Free)		(Free)		(Bound)		(Free)

[The ratio of bound complex to free unreacted analyte is proportional to the original amount of analyte present.]

Figure 4.1
The principle of reagent excess (**A**) and reagent limited assays (**B**)

Table 4.1

Features of standard immunoassays that have been adapted for use in the measurement of PAPP-A.

Name (Acronym)	Nomenclature *Assay Type* (Mechanism)	Material Required (Type Of Detection)	Other Comments
Single Radial Immunodiffusion(SRID)	**Immunodiffusion** ***Immuno-precipitation*** (Diffusion, Fick's law and Preciptin Reaction)	**Antibody** (Visualisation of Precipitin Rings)	• [1] **Time:** 24 - 48 hours. • [2] **Sensitivity:** mmol/l. • [3] **Technical Level:** S.
Rocket Immunoelectrophoresis (RIE)	**Immunelectrophoresis** ***Immuno-precipitation*** (Electrophoretic separation and Preciptin Reaction)	**Antibody** (Visualisation of Preciptin rockets)	• **Time:** 24 hours. • **Sensitivity:** mmol/l. • **Technical Level:** S/M.
Enzyme Immunoassay (EIA/ELISA)	**Immunoassay** ***Reagent Limited/Excess*** (Antigen/Antibody Interaction)	**Varied dependent upon assay design** (Usually photometric end point detection)	• **Time:** 6 - 24 hours. • **Sensitivity:** µmol/l. • **Technical Level:** M/C.
Radioimmunoassay(RIA)	**Immunoassay** ***Reagent Limited*** (Antigen/Antibody Interaction)	**Labelled Antigen** (Radioactive detection)	• **Time:** 24 hours. • **Sensitivity:** nmol/l. • **Technical level:** M/C.
Immunoradiometric Assay (IRMA)	**Immunoassay** ***Reagent Excess*** (Antigen/Antibody Interaction)	**Labelled Antibody** (Radioactive detection)	• **Time:** 6 - 8 hours. • **Sensitivity:** nmol/l. • **Technical level:** M/C.

[NOTE: [1]**Time:** Time required from setting up the assay to obtaining a quantitative result. [2]**Sensitivity:** Approximate level of assay sensitivity. [3]**Technica Level:** **S** (simple), **M** (moderate) and **C** (Complex), includes factors such as assay development and laboratory equipment required. A mor detailed examination of these assays can be found in Bolton and Hunter (**1986**), Axelsen (**1983**) and Tijssen (**1985**)]

A number of types of immunoassay for the measurement of PAPP-A were available, but the choice of assay used was limited by a number of factors. The immunometric assays require a constant pure source of specific antibodies directed against PAPP-A that could be labelled, this supply was unavailable at the time when this work was being performed, so the IRMA was not considered a viable choice of an assay to measure PAPP-A. The radioimmunoassay requires a supply of pure antigen that obviously was unavailable at the start of a purification, a RIA was eventually used and this was as described by Pinto - Furtado et al, (**1984**). The immuno--precipitation methods of RIE and SRID only required a specific antibody; of which one was commercially available (DAKO). It was also possible to design a sandwich ELISA for PAPP-A using this antibody and it was these techniques that were used to monitor the PAPP-A purification procedure. A further technique of Western blotting and densitometry could also be used to semi-quantify PAPP-A but only when it was enriched to a level as to be visible as a distinct band on an SDS-PAGE stained gel.

4.2 Materials and Methods

An individual description of the Materials and Methods that were used in this chapter can be found in Chapter 2, section 2.6.

4.3 Results and Discussion

As has been seen in Chapter 1 the analysis of the PAPP-A protein produced during pregnancy has produced an array of conflicting information with investigators during the past 20 years finding little common ground. These results seem to be due to a variety of factors with differing results due to artefacts in the assay system (Pledger and Nicol, **1983**), the quality of the primary reagents e.g. The pure PAPP-A preparations containing

contaminants and the antisera used not being monospecific (Chemnitz et al, **1986**. Bueler and Bersinger, **1989**). Coagulation of the sample used to measure PAPP-A has also been seen to play a role, with detection in male patients and non-pregnant females being dependant upon where the components used to make the immuonoassay system were acquired (Bischof and Meisser, **1988**).

4.3.1 The PAPP-A ELISA

The ELISA used in this thesis (Figure 4.2B) differed from the ELISA described by Pledger and Bellfield (**1983,** Figure 4.2A) in that:

- The microtitre plate was coated with $F(ab')_2$ fragments instead of complete antibody molecules.
- The PAPP-A bound was detected by utilising the affinity of Protein-A for a binding site on the Fc portion of the C_H2 and C_H3 domains of IgG molecule (Langone, **1982** and Lian et al, **1991**) as illustrated in Figure 4.2B.

This ELISA design removed the need to specifically label the PAPP-A antibody and so reduced any damage that may have occurred to the antibody during enzyme labelling. It also meant that a lot less time was required to prepare assay components, as the protein-A conjugate was available commercially. Surprisingly, the amount of the material bound by the capture $F(ab')_2$ and directly detected with the Protein-A conjugate was the same as that detected when the second stage PAPP-A antibody was included. A significant level of PAPP-A was found in male serum (Figure 4.3) using this assay, it could not be determined whether PAPP-A or a plasma contaminant was binding to the protein-A enzyme conjugate.

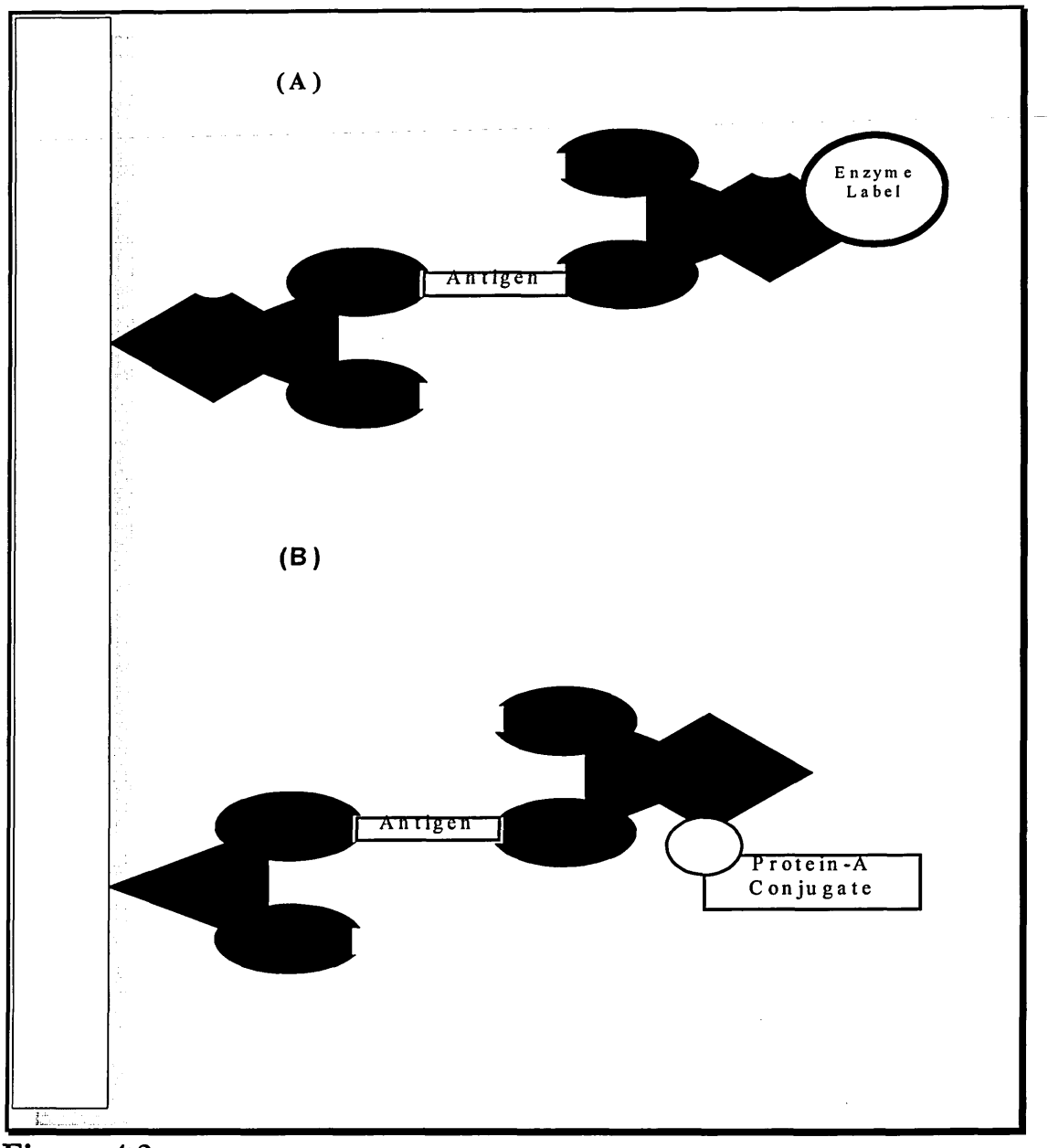


Figure 4.2

Schematic representation of PAPP-A ELISA design

[Legend: (A) Design used by Pledger and Bellfield (1983). DAKO PAPP-A antiserum was used as the capture phase and detected with HRP conjugated to DAKO PAPP-A antiserum. (B) Design for PAPP-A ELISA used in this thesis. DAKO PAPP-A antiserum $F(ab')_2$ fragments were used as the capture phase. DAKO PAPP-A antiserum was used as the second phase antibody with detection by a protein-A HRP conjugate.**]**

However the results that were found using the ELISA procedure described detected PAPP-A in male plasma, therefore it was likely that either the antiserum was detecting a normal serum component to the same extent as PAPP-A or that PAPP-A is in a complex with a serum component that binds protein-A. It is not known whether the recent observation that

PAPP-A is complexed with proMBP (Oxvig et al, **1993**) has an affect, or whether it was proMBP present in the male serum was the component that binds protein-A.

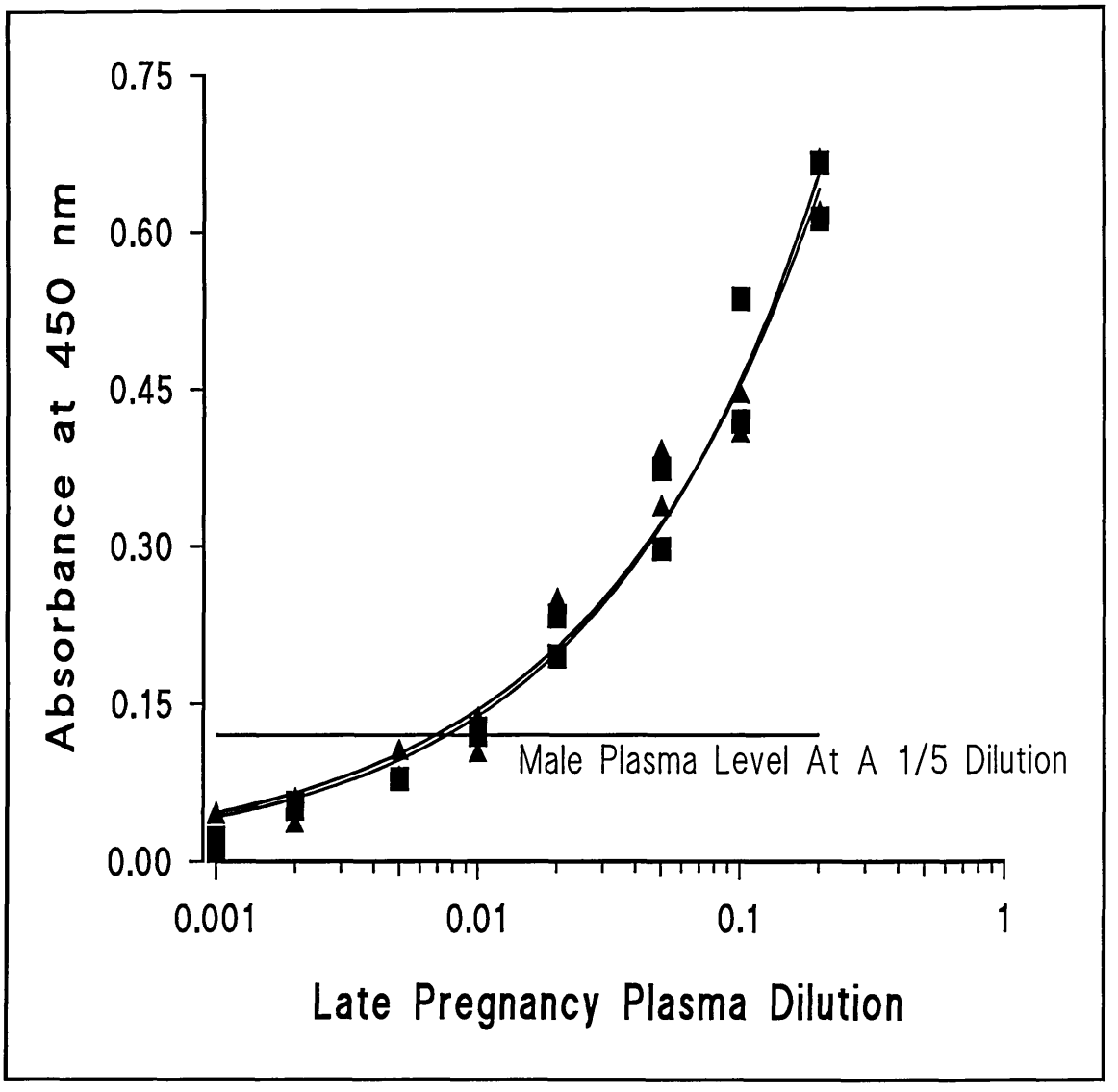


Figure 4.3
Unmodified ELISA +/- 2nd phase PAPP-A antibody with detection by a Protein-A enzyme conjugate
[Legend: (■) Wells were incubated with 1:2.5K dilution of DAKO Anti-PAPP-A antibody. (▲) Wells were incubated with PBS in place of the second stage antibody. All wells then incubated with a 1:1000 dilution of protein-A enzyme conjugate. Other conditions were as outlined in Chapter 2, section 2.6.3. **Note:** 0.1 represented a 1:10 dilution of a late 3rd trimester plasma pool prepared as outlined in section 2.1 and 2.6 (Chapter 2). Initial experiments used Male (Lithium Heparin) Plasma as the negative control as illustrated above, this was replaced with Male (Citrate) Plasma in the subsequent experiments.**]**

In an attempt to use this ELISA to measure PAPP-A several modifications were made:

- The standards and unknown samples were incubated in diluent that contained male plasma (Initially Li-Heparin was used but this was changed to the Citrate anticoagulant in subsequent experiments).
- The second antibody was pre-incubated in diluent that contained horse serum.

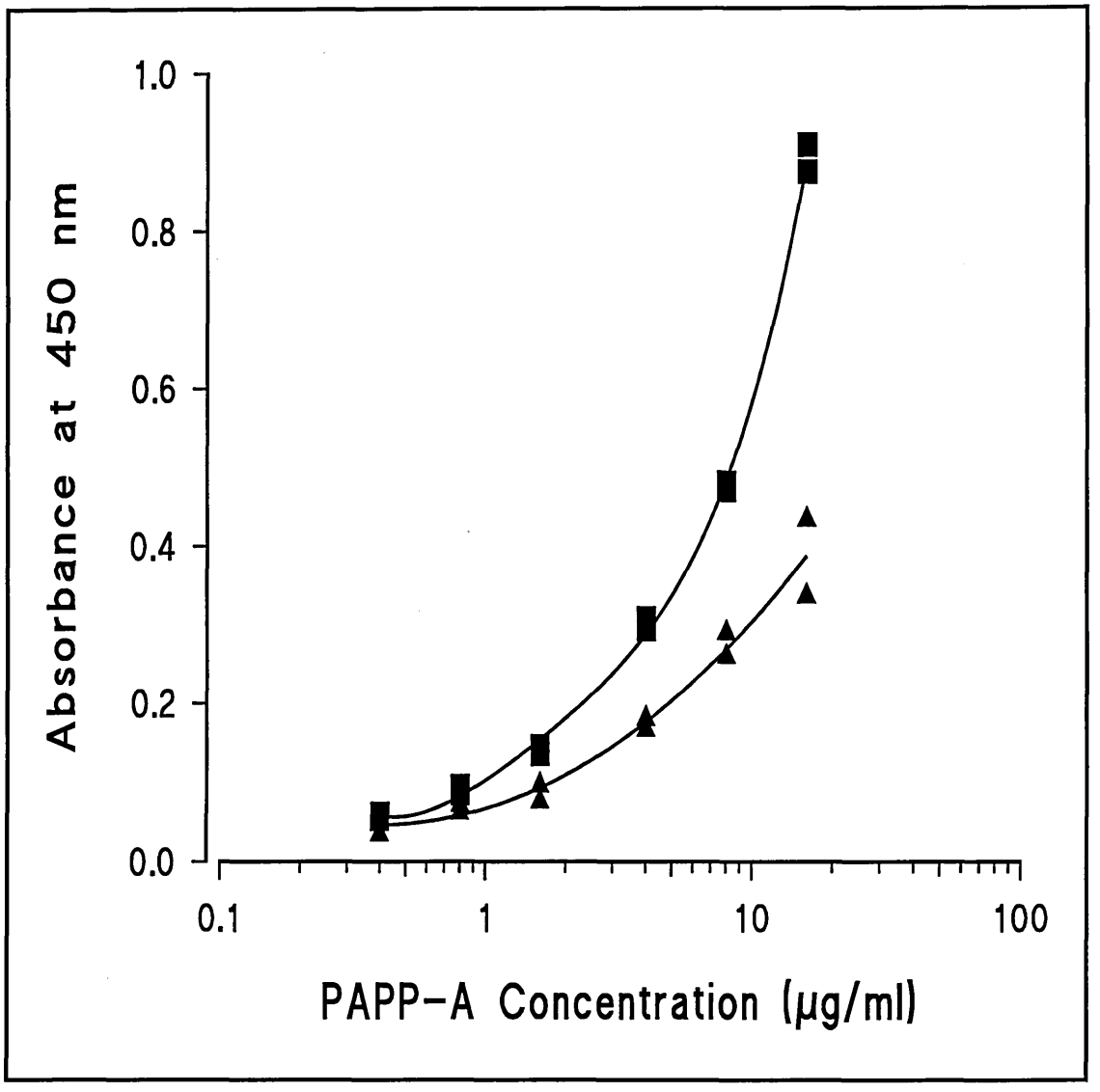


Figure 4.4

Modified PAPP-A ELISA with detection via a protein-A enzyme conjugate.

[Legend: Standards/Unknowns were incubated in ELISA diluent containing 5% Male Plasma (Citrate). A parallel duplicate set were run for each sample, one set (■) was incubated with a 1:2.5K dilution of PAPP-A antiserum in ELISA diluent containing 5% horse serum and the parallel set (▲) were incubated in ELISA diluent containing 5% horse serum. Detection was with a 1:1000 dilution of Protein-A enzyme conjugate. Other conditions were as outlined in Chapter 2, section 2.6.3. The PAPP-A concentration given was calculated from an in-house late 3rd trimester plasma pool that had been calibrated against the WHO 78/610 reference pool as described in section 2.1 (Chapter 2).]

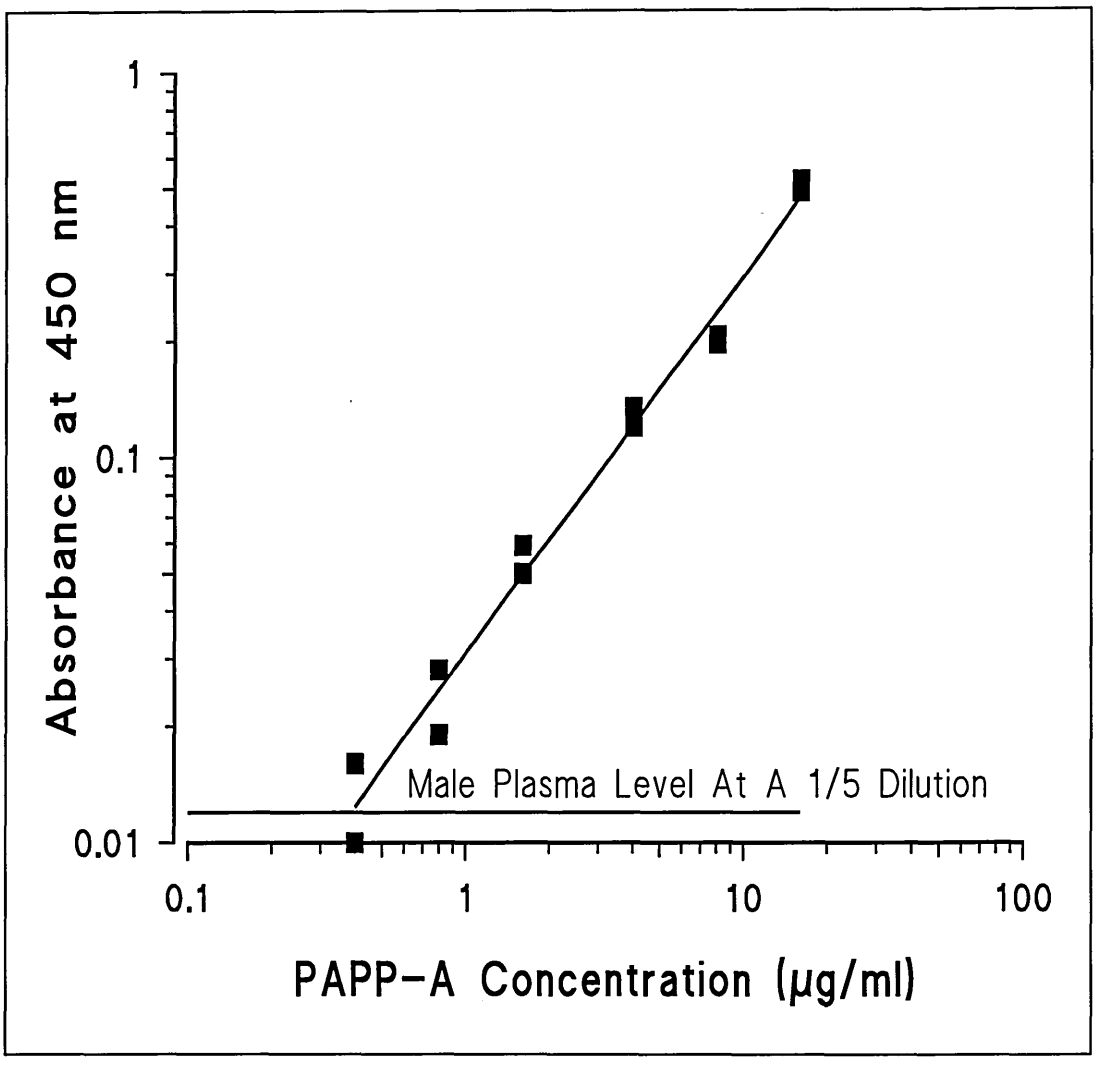


Figure 4.5

The modified PAPP-A ELISA

[Legend: (■) The modified Absorbance was plotted as values that were obtained from (■, Figure 4.4) minus their mean corresponding values, (▲, Figure 4.4). Male (Citrate) plasma was used as the negative control as illustrated above. All other conditions were as described in the legend for Figure 4.4]

Using these modifications, a difference in levels was observed when the second antibody was used (Figure 4.4). Therefore by incorporating these modifications an observable difference was seen in the presence (or absence) of second phase antibody. Thus these modifications enabled PAPP-A to be distinguished from the undefined material that was interfering with this assay. This undefined material did also seem to increase during pregnancy, but by comparing matched samples with/without the second phase antibody,

an assay was produced which did not detect PAPP-A in male plasma (Figure 4.5).

Because of the non-specific effects that were seen during the development of this assay and the lack of knowledge as to what non-specific component of blood was being measured, or what the effect on the ELISA would be of varying ratios of PAPP-A to this non-specific cross reactant that would be seen during a purification procedure, this assay was not used to monitor PAPP-A concentrations. It is interesting to note that Pledger and Bellfield (**1983**) also found increased levels in haemolysed samples with a suggestion that their assay may also be measuring a red cell component. Pro-MBP has also been shown to be linked to PAPP-A (Oxvig et al, **1993**), however it is unknown if either of these two factors is responsible for the non-specific effect that was observed during the development of this assay.

4.3.2 Immunoprecipitation Methods

These methods are dependant solely on the quality of the antiserum used with migration of antigen via electrophoresis in RIE and diffusion in SRID, both types of assay have similar levels of sensitivity and precision.

As seen from the results represented in Figures 2.6 (Chapter 2) and Figure 4.6, the composition of the matrix has a distinct affect on the level of PAPP-A detected. These effects have also been observed by Bjerrum (**1983**) who found they were produced by the dissociation of sub-units, aggregation or denaturation of the antigen due to exposure to conditions such as high temperature, high salt, or extremes of pH. The immunoprecipitate that was produced being increased (or decreased) dependant upon which of these described factors was present. An increase in the size of precipitin ring (or rocket height) is probably due to a loss of antigenic determinants, so that migration through the gel is over a longer distance before the complexes are large enough to be retained within the agarose network. A decrease can be due to partial unfolding of the protein or loss of antigenicity by some of the molecules involved. However it has been established that using the RIE and SRID under constant conditions for all

the samples measured then a level of PAPP-A could be determined when monitoring the purification scheme.

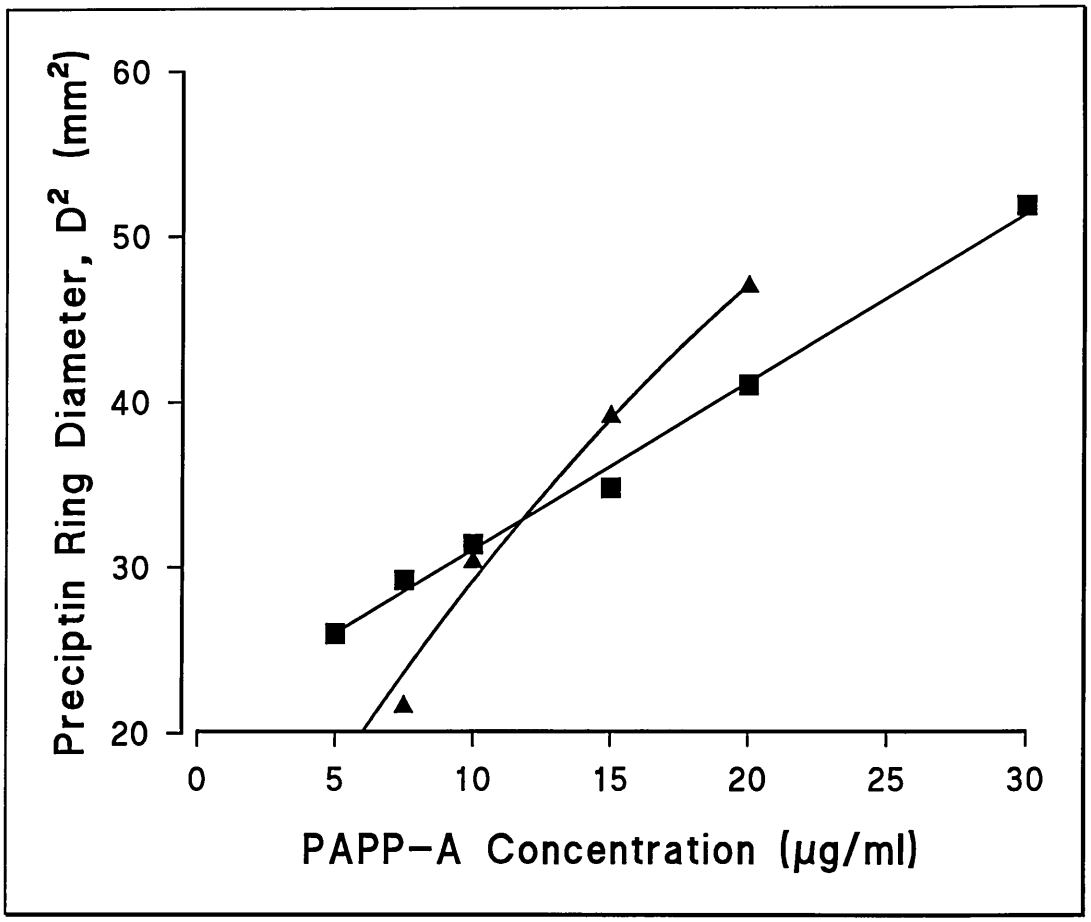


Figure 4.6

PAPP-A SRID illustrating the effect of salt concentration on the assay.
[Legend: (■: The samples were loaded in assay diluent containing male plasma, the plate was made in assay diluent containing a final concentration of 1 M salt. $r^2 = 0.997$). (▲: The samples were loaded in assay diluent at a final concentration of 1M salt (containing male plasma, Appendix 2), the plate was made in assay diluent containing a final concentration of 1M salt. $r^2 = 0.994$). Other conditions were as outlined in Chapter 2, section 2.6.2. The PAPP-A concentration was calculated against the WHO reference pool, 78/610.].

4.3.3 The PAPP-A RIA

The PAPP-A assay described by Pinto-Furtado et al (**1984**) was less prone to effects of anti-coagulants on the assay and it also gave a linear correlation of values obtained when compared against an IRMA that used the DAKO polyclonal and a monoclonal antibody directed against PAPP-A (Mowles et al, **1986**) thus imparting to this assay a greater specificity for PAPP-A. However this does not exclude the possibility that the monoclonal antibody is directed at an antigenic component other than PAPP-A as it has

been shown that it only binds to un-reduced PAPP-A (Chapter 3, Plate 3.1). PAPP-A has been shown to be linked to pro-MBP (Oxvig et al, **1993**).

The advantage to this assay of affinity purifying the PAPP-A tracer on a heparin column was illustrated in Figure 2.9 (Chapter 2) with less than 23% of the tracer bound to excess antibody prior to chromatography. This compares favourably with a level of 30% observed by Pinto-Furtado et al, (**1984**). After affinity chromatography a greater than two fold increase in tracer bound in excess antibody was observed. The low level of tracer binding to PAPP-A antiserum could have been due to damage produced during the iodination procedure or to the presence of other proteinaceous material in the iodination grade PAPP-A preparation (Chapter 6, Table 6.2).

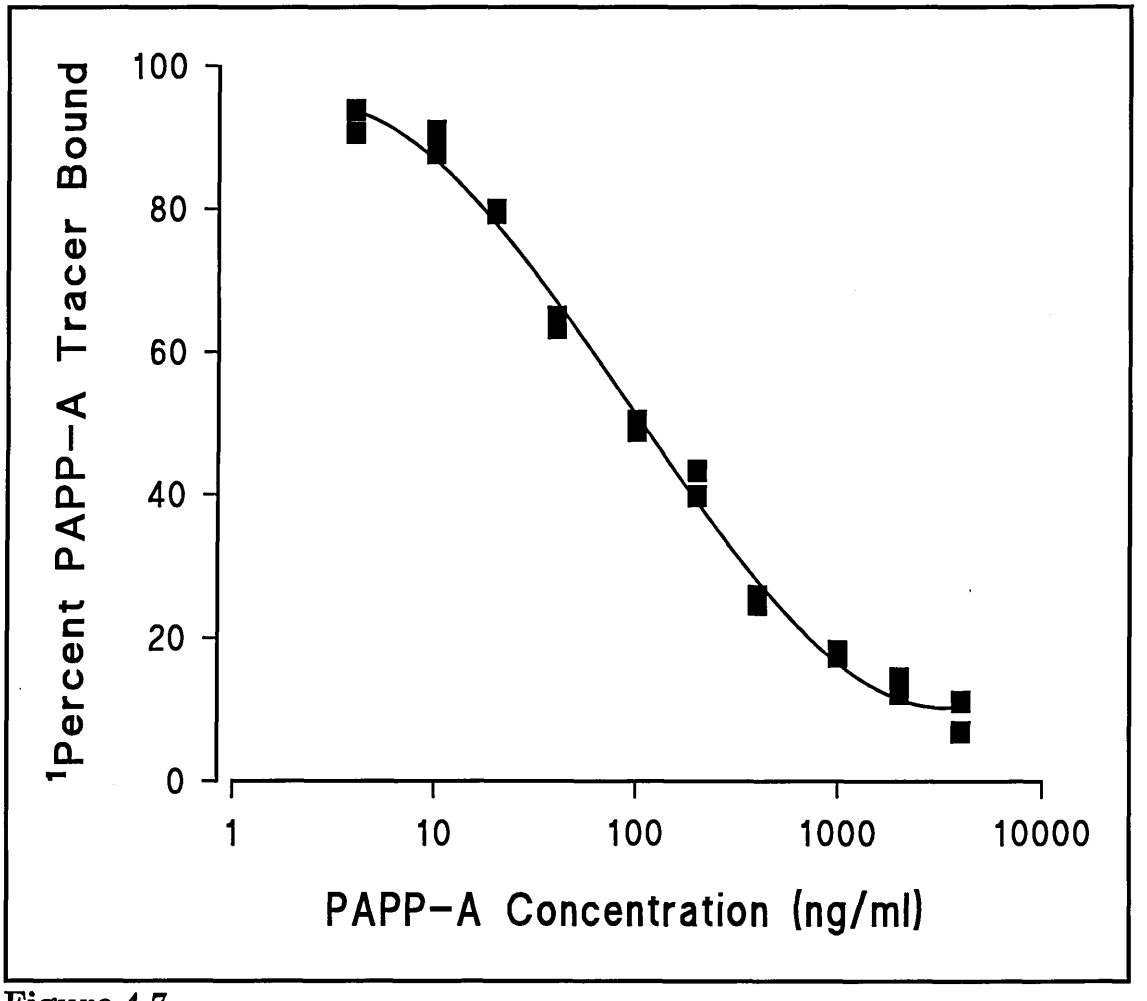


Figure 4.7
A typical PAPP-A RIA calibration curve.
[Legend: Results were expressed as [1] Percent tracer bound: (Bound CPM - NSB)/(B_o - NSB) Vs PAPP-A standard, the curve was fitted using RIACALC as described in Chapter 2, section 2.6.4.5]

The PAPP-A RIA (Figure 4.7) had a detection limit as defined from its precision profile of 15 ng/ml and an upper limit of 500 ng/ml at a level of precision level as measured by a coefficient of variance of 10% or less. This compared favourably to a detection limit of 10 - 15 ng/ml and an upper limit of 600 ng/ml that was seen by Pinto-Furtado et al, (**1984**) using the described RIA procedure.

As seen from Table 4.2, the PAPP-A tracer undergoes changes with time that reduce its ability to bind heparin. This reduction in binding is the factor that limits the life of the tracer to about four weeks. As seen from Figure 2.9 (Chapter 2) it was essential to use the heparin bound fraction of PAPP-A tracer for the RIA. This assay was used to monitor development of a PAPP-A purification scheme.

Table 4.2 Percentage of PAPP-A tracer bound to a heparin affinity column.

	Time After Iodination (days)			
	0	**7**	**14**	**21**
[1]Percentage of Total PAPP-A tracer Bound	47%	25%	13%	10%

[Note: [1]: The table represents the proportion of PAPP-A tracer bound Vs The age of PAPP-A tracer when separated using a heparin affinity column. The results are expressed as a percentage of total tracer applied that were bound to this affinity matrix under the conditions described in section 2.6.4.2 (Chapter 2).**]**

4.3.4 Quantification by Densitometry

This method can be used once the PAPP-A has been enriched to a level where it can be visualised by protein staining and a discernible band could be distinguished from other serum/plasma components. It should be noted however that the levels found using this method are for the monomeric chain of PAPP-A (as seen under reducing conditions of SDS-PAGE).

A linear relationship was found to exist between the intensity of staining and the protein concentration for CBB staining from 0.5 - 20 μg and 0.02 - 2.0 ng/mm^2 for silver staining. (Merril, **1990**).This compared favourably with the results that were obtained in this thesis of a linear relationship between 1 - 8 μg/mm^2 for CBB staining as was illustrated in Figure 4.8.

Therefore this method was used subsequently to calculate the recovery of monomeric PAPP-A protein levels from SDS-PAGE gels (Chapter 5).

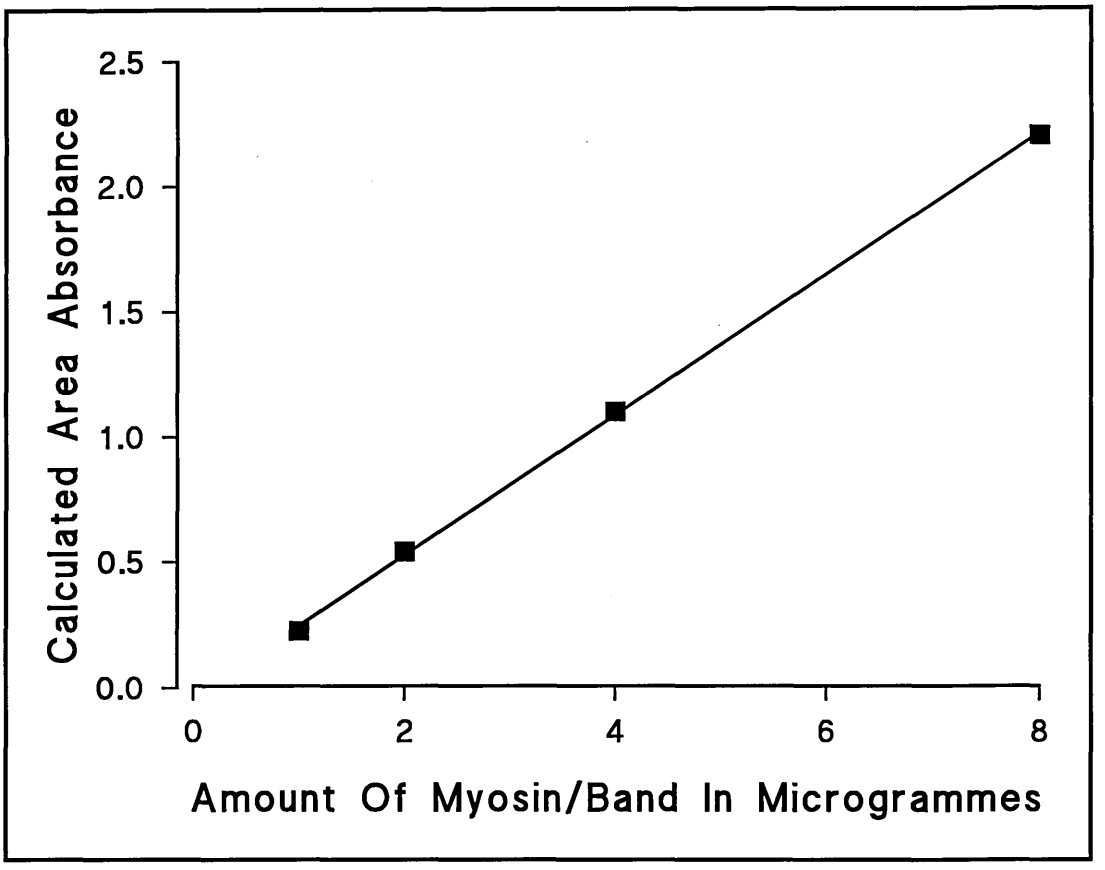


Figure 4.8
A typical calibration curve of protein levels seen using video densitometry.
[Legend: The calculated area/absorbance, (Chapter 2, section 2.6.6) was plotted Vs the amount of myosin loaded onto an SDS-PAGE gel. The gel was stained with CBB, as described in Chapter 2, section 2.3.2.2d.]

To summarise, the PAPP-A assays were found to be affected by a variety of factors from:

- The primary quality of the individual assay components.
- The individual assay design with affects due to molecular characteristics of PAPP-A.

These factors have undoubtedly affected the interpretation of the various clinical studies that were undertaken to see if PAPP-A could be used to monitor foetal well-being (Chapter 1, section 1.4.7). The assays developed and illustrated here were however sufficient for the purpose of monitoring a PAPP-A purification/enrichment procedure that is outlined in Chapter 5.

CHAPTER FIVE

Chapter Five

The Isolation of PAPP-A for Microsequencing

5.1 Introduction

The immunological approach to screening for the cDNA encoding PAPP-A did not give the expected results for the reasons that were discussed in Chapter 3. The biological activity of PAPP-A is unknown, so the only other approach to the identification of the PAPP-A cDNA required some primary sequence information of the PAPP-A protein. Thus allowing the preparation of nucleic acid probes that could be used for screening cDNA libraries (Huynh et al, (**1985**) and Sambrook et al, (**1989**). This information was unavailable at the time the practical work of this thesis was undertaken, therefore it was necessary to purify, or enrich PAPP-A so it would be in a form suitable for microsequencing. Such a purified product should then give an unambiguous N-terminal sequence, if it was not N-terminally blocked. This would then provide primary sequence information that would aid the isolation of PAPP-A cDNA (and hence the gene) and define its exact reading frame. It has been estimated that between 50 - 90% of eukaryotic proteins are N-terminally blocked and are thus refractory to the Edman chemistry (Brown and Roberts, (**1976**) and Stone et al, (**1989**). Taking this factor into account along with the large size of the PAPP-A protein (monomeric polypeptide chain is 160 - 180 kDa), it was also probable that internal stretches of amino acid sequence would be needed, not only to verify any putative clones identified but also to aid the isolation of this cDNA.

The design of a purification scheme for PAPP-A needs to take several factors into consideration and these are summarised in Figure 5.1. The purification schemes that have been used to isolate PAPP-A over the past two decades are illustrated in Table 1.1 (Chapter 1).

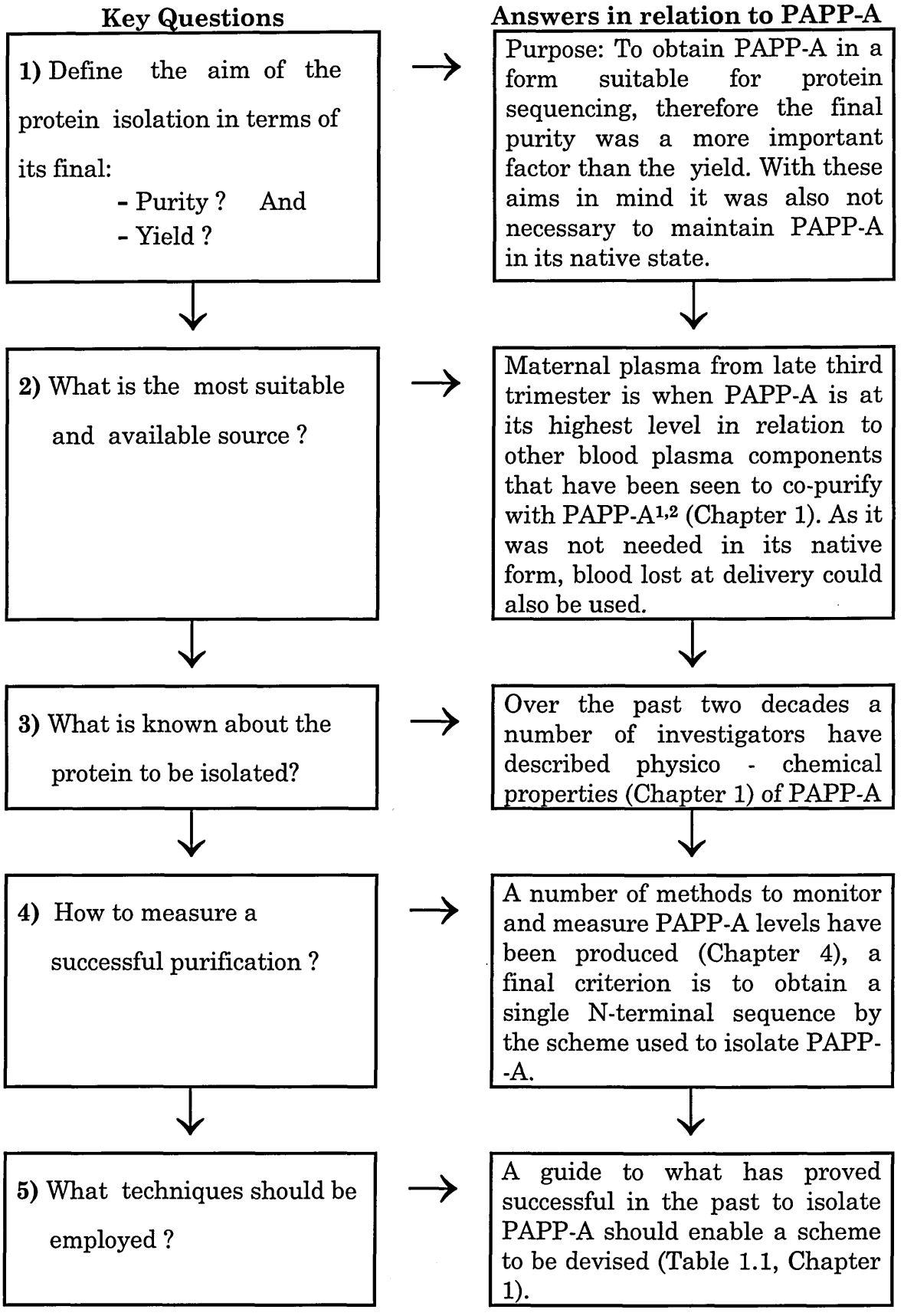


Figure 5.1
Factors involved in the design of a purification scheme with particular reference to PAPP-A.
[Note: [1]: Sutcliffe et al, (1980). [2]: Sottrup-Jensen and Sand (1985).]

The schemes outlined in Tables 1.1 and 1.2 (Chapter 1) represent the major purification schemes for PAPP-A and other published procedures were based around these described schemes. The previous work by other investigators in this field (discussed in Chapter 1) helped in the development of a scheme to isolate PAPP-A in a form that was suitable for microsequencing.

A complication arose in that there appeared to be an apparent change in the binding of PAPP-A to heparin which was shown to enrich PAPP-A and separate it from it's major contaminants, (Sinosich et al, **1987**) PZP and α_2-macroglobulin. This change in binding to heparin was investigated and it also made necessary a more thorough examination of the methods that could be used to purify PAPP-A. The development of purification schemes that yielded a single N-terminal sequence for PAPP-A are described in this chapter.

The isolation of a protein in a homogenous form suitable for Edman degradation has frequently been achieved by utilising a final step of RP-HPLC or separation under reducing conditions on SDS-PAGE and electroblotting onto a membrane suitable for solid phase sequencing (PVDF). These approaches have been shown to be successful in producing amino acid sequence information from a variety of different proteins (Charbonneau, (**1991**), Cleveland et al, (**1977**), Matsuidaira, (**1990**) and Stone et al, (**1989**).

5.2 Materials and Methods

All chromatographic procedures unless otherwise stated were carried out using a Pharmacia FPLC system. The methods used to monitor the purification are described in Chapter 4 of this thesis. The measurement method that was used to monitor PAPP-A levels is stated in the appropriate Figure legend. The overall purification schemes produced that were used to isolate PAPP-A are summarised in the following Results and Discussion section. Individual chromatographic steps are described in the appropriate section of the Materials and Methods in Chapter 2 (section 2.7).

5.3 Results and Discussion

A wide variety of methods were employed to separate PAPP-A from other blood components. Not all methods were successful but they did however serve to illustrate some of the biochemical features of PAPP-A and blood contaminants that co-purified with PAPP-A. They have been included for this purpose and the schemes described in this section are a reflection of the most successful routes that yielded an enriched form of PAPP-A.

It was beneficial to start with a biological source that contained PAPP-A in increased amounts, therefore maternal blood at term was used as the source material. The major contaminants that investigators have found difficult to separate from PAPP-A (Chapter 1, Table 1.2) were α_2-macroglobulin and pregnancy zone protein that are present at a level of 2 - 4 mg/ml (Sutcliffe et al, **1980**) and 1 - 1.4 mg/ml respectively in term plasma (Sottrup-Jensen and Sand, **1985**). So by using term/late 3rd trimester blood the concentration ratio of PAPP-A to α_2M/PZP was maximised.

As it was shown in Chapter 1 the use of the affinity of PAPP-A for heparin was a useful way to separate PAPP-A from other contaminants that often had been seen to contaminate PAPP-A preparations produced using the classical methods of protein purification. Without the use of the heparin affinity matrix, PAPP-A was frequently not separated from other maternal blood components such as α_2M, PZP and fibronectin. The heparin/PAPP-A interaction and purification of PAPP-A are discussed further in Chapter 7, section 7.4.

5.3.1 Purification Schemes That Were Successful in Enriching PAPP-A

As stated in the introduction to this chapter, the aim of the isolation of PAPP-A was to use that material for N-terminal microsequencing, A scheme (Figure 5.2) that utilised an affinity of PAPP-A for the amino acid L-arginine combined with steps described by other investigators (Table 1.1, Chapter 1) yielded PAPP-A enriched to approximately 30% of the proteins present in the final preparation. The only other major protein

As illustrated in the plate inset 2.19 (Chapter 2), PAPP-A could not easily be separated from an unidentified contaminant that was present as a single chain form with an Mr of approximately 210 - 240 kDa. This contaminating material (hereafter named group-X) was immunodetected using an antiserum directed against normal serum components. It also co-eluted with PAPP-A in all the methods used to isolate PAPP-A which are described in Chapter 2, section 2.7. The only method that was successful in removing it, was on a basis of its size by gel filtration. The material prepared by this scheme was separated from α_2M by SDS-PAGE under reducing conditions. It was electroblotted onto a PVDF membrane that is suitable for use in microsequencing (Plate inset 5.2). The PAPP-A band indicated by (★) yielded a single N-terminal sequence (Chapter 6, Table 6.2) thus confirming that the PAPP-A isolated had been separated from other contaminating serum proteins.

The second scheme that was successful in enriching PAPP-A utilised the described affinity of PAPP-A for heparin and is illustrated in Figure 5.3. The heparin bound fractions (0.3 and 0.6 M) shown in Figure 2.12 (Chapter 2) were subjected to gel filtration (Figure 7.4, Chapter 7) and the fractions containing PAPP-A were identified as described (Plates 5.1 and 5.2).

The heparin based scheme (Figure 5.3) as illustrated in Plates 5.1 and 5.2 significantly enriched the PAPP-A present and separated PAPP-A from its main contaminant seen in scheme 1 (Figure 5.2), that of α_2M. It however failed to separate PAPP-A from the unidentified X-contaminant. This contaminant has also been observed by Davey et al, (**1983**) who noted that it could be removed if a 0.4 M NaCl step was substituted between the 0.3 and 0.6 M NaCl steps during the heparin affinity chromatography stage. This did however result in a considerable reduction in the yield of PAPP-A and the material was still contaminated with other serum proteins. Sinosich et al, (**1990**) removed these by negative affinity immunoadsorption. In this study it was chosen to purify a single chain PAPP-A by elution from a gel slice as described in Chapter 2, section 2.7.12. This method yielded a pure monomeric PAPP-A molecule (Plate inset 5.3).

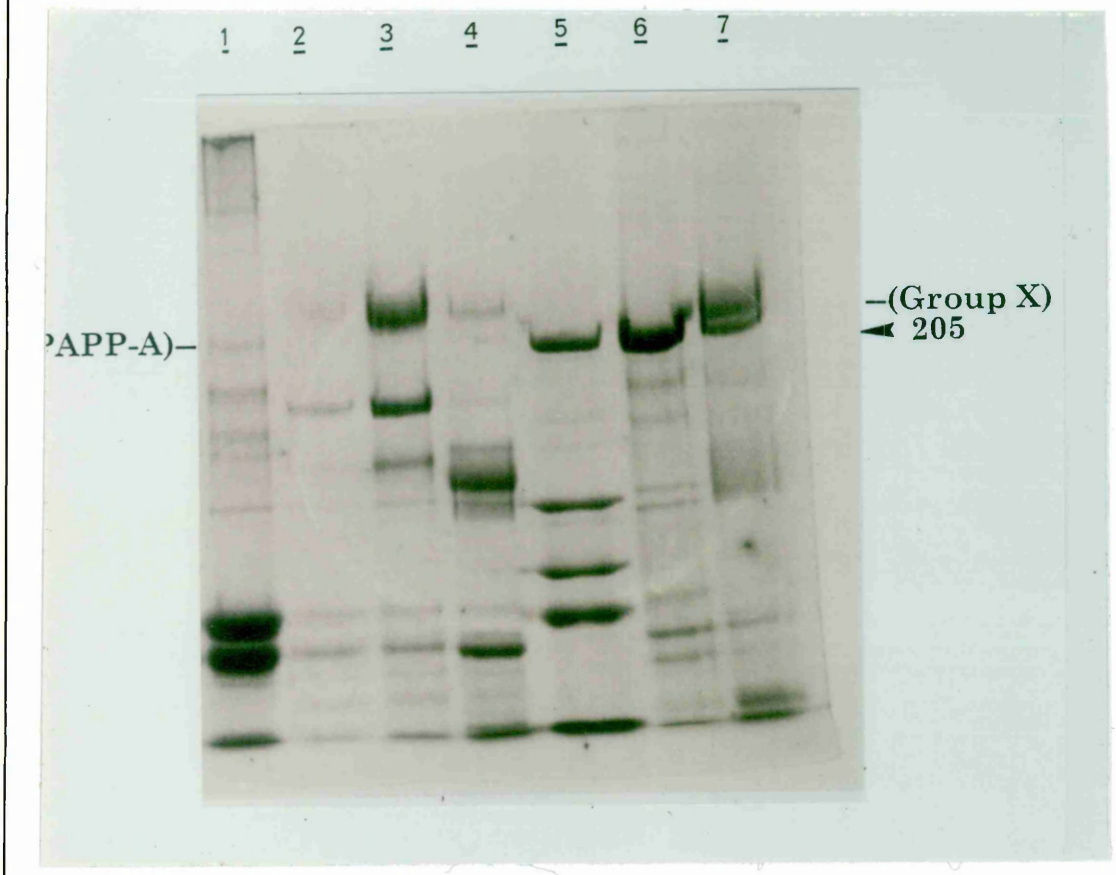


Plate 5.1

A CBB stained 5% SDS-PAGE gel of fractions from heparin based purification, Scheme 2.

[Legend: All samples were reduced with 2-mercaptoethanol before separation on this gel. The 0.3 and 0.6 M bound fractions from the heparin affinity column were loaded onto a gel filtration column (see text). The 0.3 M heparin bound fractions separated on the gel filtration column were (**1**): Fractions 1 - 4. (**2**): Fractions 5-6. (**3**): Fractions 7-8. (**4**): Fractions 11 - 12. (**5**): The high molecular weight markers (Appendix 1) with the arrow indicating the 205 kDa marker. The 0.6 M heparin bound fractions separated on the gel filtration column (see text) were (**6**): Fractions 1 - 4. (**7**): Fractions 5 - 8. The PAPP-A band marked was determined by western blotting and subsequent detection with anti-PAPP-A antibodies. The group-X contaminant was also marked.**]**

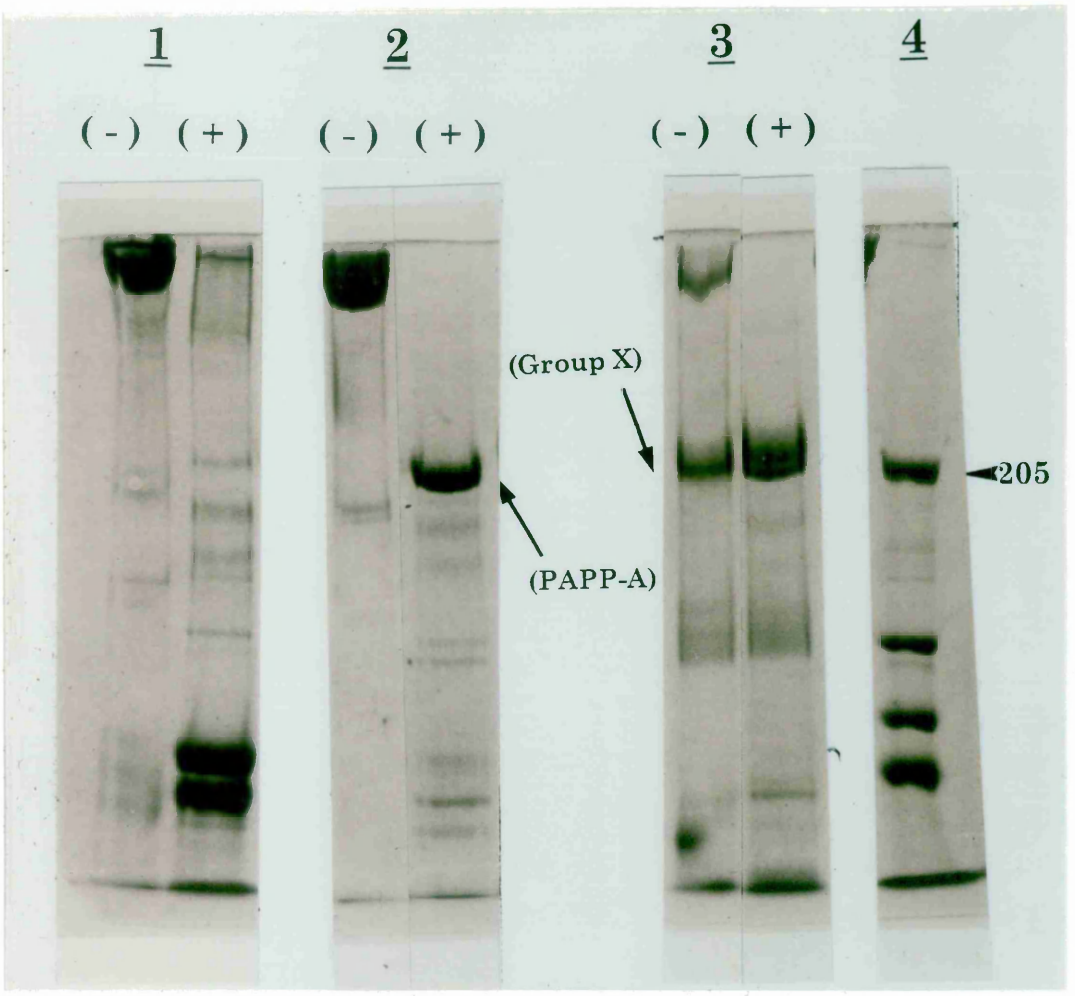


Plate 5.2

A CBB stained 5% SDS-PAGE gel illustrating the disulphide bridged structure of the proteins separated by the heparin based purification scheme

[Legend: (**+**) indicated samples were reduced with 2-mercaptoethanol, (**-**) indicated samples were treated with sample buffer that did not contain 2-mercaptoethanol. The samples separated were from the heparin affinity column and then loaded onto the superDex gel filtration column (see text): (**1**): 0.3 M bound heparin column, fractions 1 - 4 from gel filtration column. (**2**): 0.6 M bound heparin column, fractions 1-4 from gel filtration column. (**3**): 0.6M bound heparin column, fractions 5 - 8 from gel filtration column. (**4**): The high molecular weight markers (Appendix 1) with the arrow indicating the 205 kDa marker. The PAPP-A marked band was determined by western blotting and subsequent detection with anti-PAPP-A antibodies. The group-X contaminant was also marked.**]**

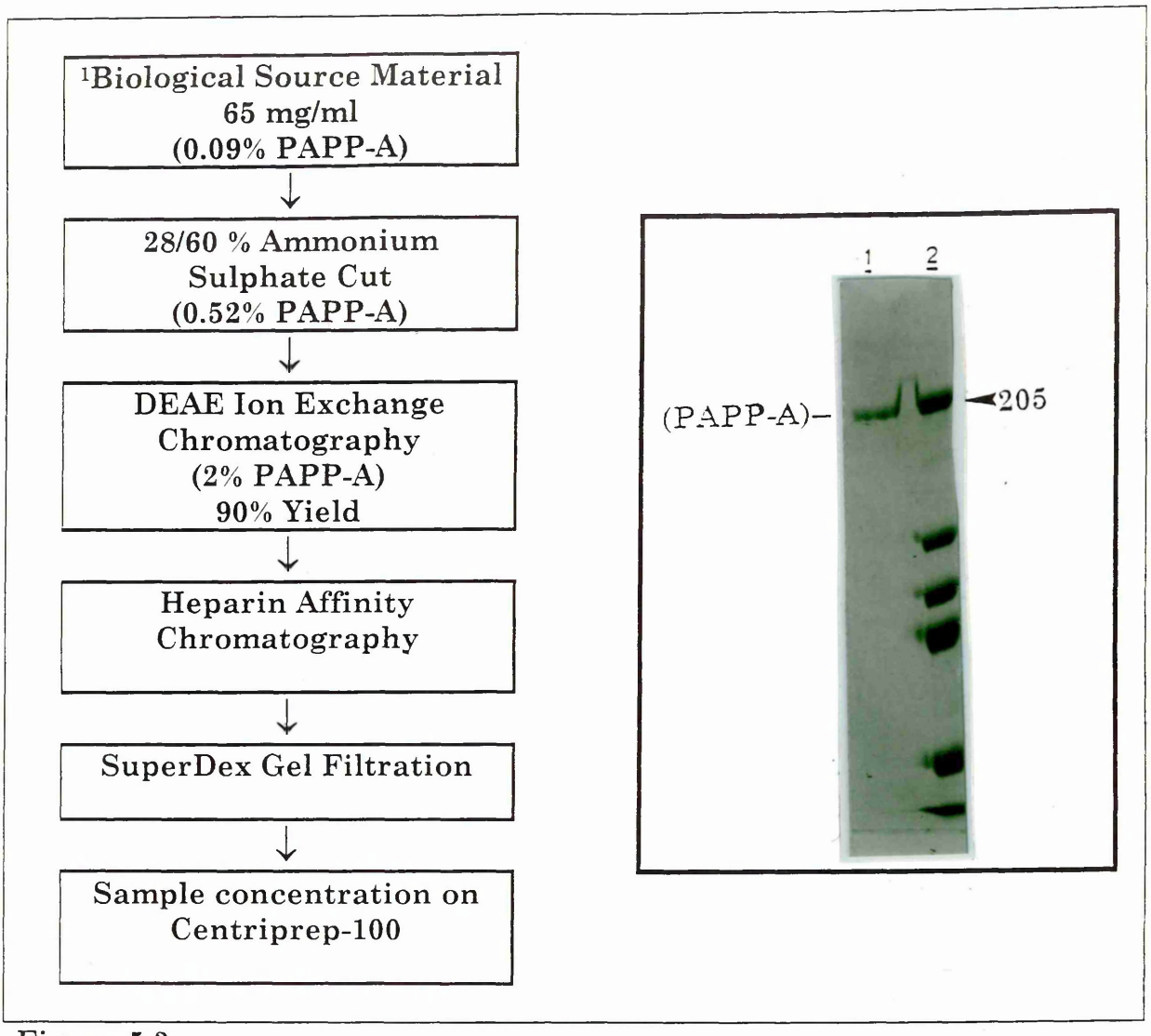


Figure 5.3

The heparin based purification, scheme 2. **Plate Inset 5.3**, Pure monomeric chain PAPP-A.

[**Legend:** [1]: Biological source material was composed of a pool of late 3rd trimester plasma and blood lost during delivery at term that had been treated as described in section 2.7.2, Chapter 2. The plate inset was a CBB stained 5% SDS-PAGE gel as described in section 2.3.2.2.a of the gel-slice eluted material (described in Chapter 2, section 2.7.12) from this purification scheme was illustrated in Plate 5.1. This preparation yielded a pure monomeric chain, PAPP-A. **Note:** (1): Represented eluted pure monomeric PAPP-A. (2): Represented high molecular weight markers (Appendix 1) with size illustrated in kDa.]

5.3.2 A Scheme That Significantly Enriched the Undefined (Group-X) Contaminant

Sinosich (1988) using CAIE with the dye, Red-120 observed that the PAPP-A immunoprecipitate was removed if this dye was included in an intermediate gel, suggesting that it might be possible to isolate PAPP-A by dye affinity chromatography. A scheme incorporating this dye was devised (Figure 5.4).

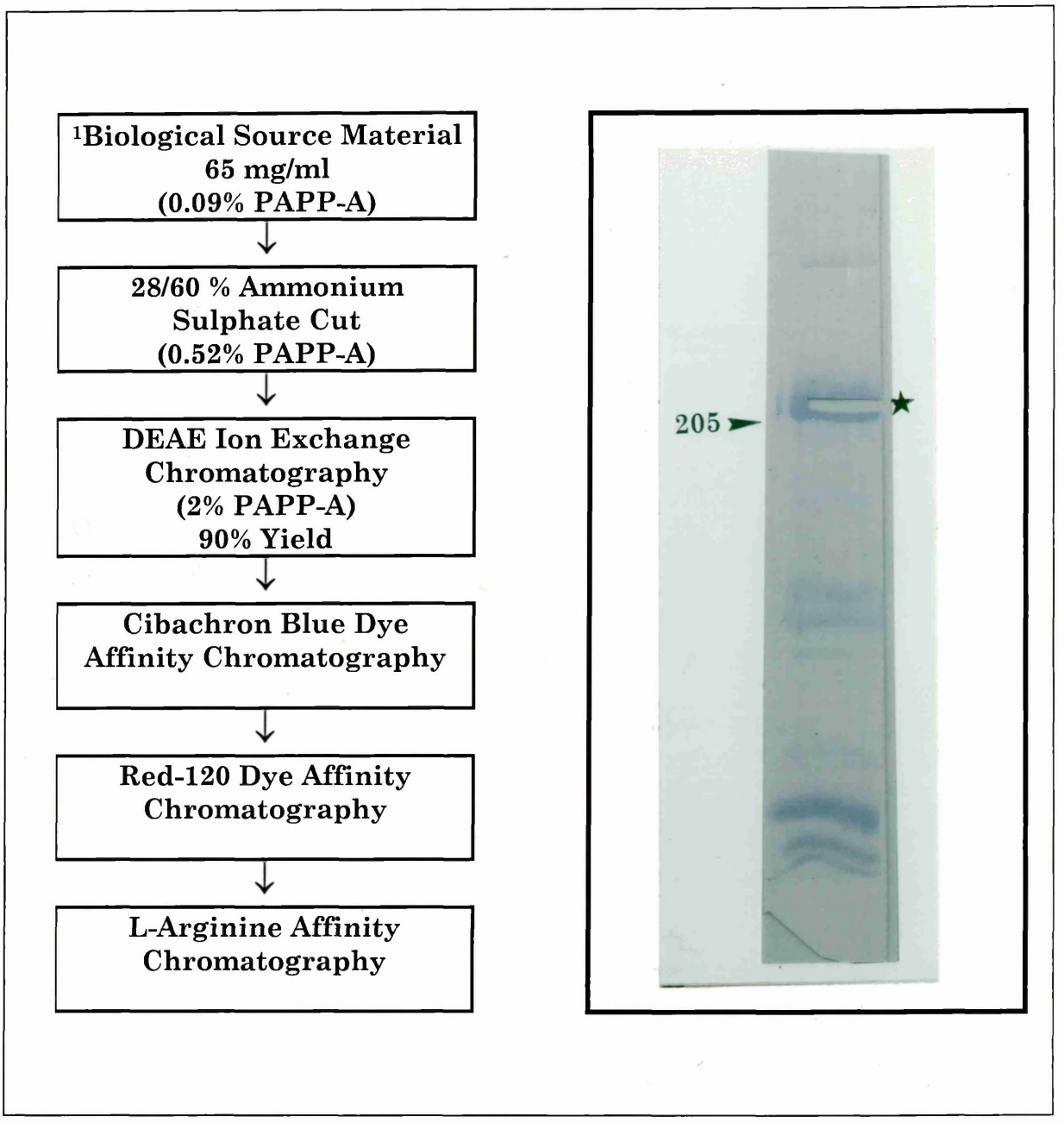


Figure 5.4

Enrichment scheme for group-X contaminant. (**Plate Inset 5.4** represented electroblotted material purified using this scheme.)

[Legend: [1]**:** Biological source material was composed of a pool of late 3rd trimester plasma and blood lost during delivery at term that had been treated as described in section 2.7.2, Chapter 2. The steps used in this purification were carried out as described in Chapter 2, section 2.7. The material in the final step was reduced with 2-mercaptoethanol and separated on a 5% SDS-PAGE gel, it was electroblotted on a PVDF membrane as previously described in Chapter 2, sections 2.3.2.2c and 2.4.1 and stained with CBB. **Note:** The arrow indicated molecular weight marker with the size in kDa. The Western blotted marked band (Group -X) did not demonstrate an affinity for anti-PAPP-A antibodies using the conditions previously described. The protein present in this band was sequenced by Dr A. Moir (Appendix 3).]

Surprisingly the Red-120 dye did not enrich the PAPP-A component but the group-X contaminant. This was an uncharacterised group of proteins, which in N-terminal microsequencing generated more than one N-terminal amino acid. The amino acids present did not correspond to the N-terminal sequence that had been obtained for PAPP-A prepared by the purification scheme 1 (Chapter 6). That the CAIE technique yields different results to that obtained by chromatography was also seen when the LEL lectin was used for purification. A further analysis of the implications of this contaminating group is given in Chapter 7.

The PAPP-A isolated and defined by the schemes described in this chapter produced a monomeric form of PAPP-A that was free of pro-MBP and yielded a single N-terminus (Chapter 6, Table 6.3). This material was thus suitable both as a substrate for limited chemical/proteolytic cleavage to obtain further primary sequence information (Chapter 6) and provided a source of pure monomeric PAPP-A that was used to study some of the physico-chemical properties of the PAPP-A molecule (Chapter 7).

CHAPTER SIX

Chapter Six

A Molecular Approach: Towards the PAPP-A Gene II

6.1 Introduction:

The possible strategies for identifying a cDNA encoding PAPP-A have been described in Chapters 3 and 5 and as it was shown the best available approach was to produce nucleic acid probes using the primary sequence of the PAPP-A protein. The primary sequence of PAPP-A was unknown at the time the practical work of this thesis was undertaken. In order to obtain it, PAPP-A was enriched by the methods described in Chapter 5 and finally isolated as a monomeric chain by SDS-PAGE. Its identity was confirmed by immunological detection on Western blots using polyclonal antibodies directed against PAPP-A. The reduced monomeric PAPP-A was prepared on membranes, or cleaved (enzymatically or chemically) and microsequenced. PAPP-A in gel slices was cleaved using the method described by Cleveland et al, (**1977**) to produce a limited proteolytic digest needed to obtain amino acid sequence of the internal regions of the PAPP-A molecule. The approaches that were applicable to the PAPP-A material that was produced are listed in Figure 6.1 and described in the relevant sections in this Chapter.

Primary protein sequence information can be obtained by N-terminal sequencing of proteins using the method described by Edman (cited from Edman, **1950**). Briefly the chemical sequencing cycle consists of the protein fragment being allowed to react with PITC to yield a phenylthiocarbormoyl peptide, then under anhydrous HCl conditions the labelled amino acid undergoes cyclisation to yield a free PTH-amino acid and a protein polypeptide chain that is n-1 residues shorter. The PTH labelled residue is identified and the n-1 polypeptide chain is put through another cycle (Figure 6.2).

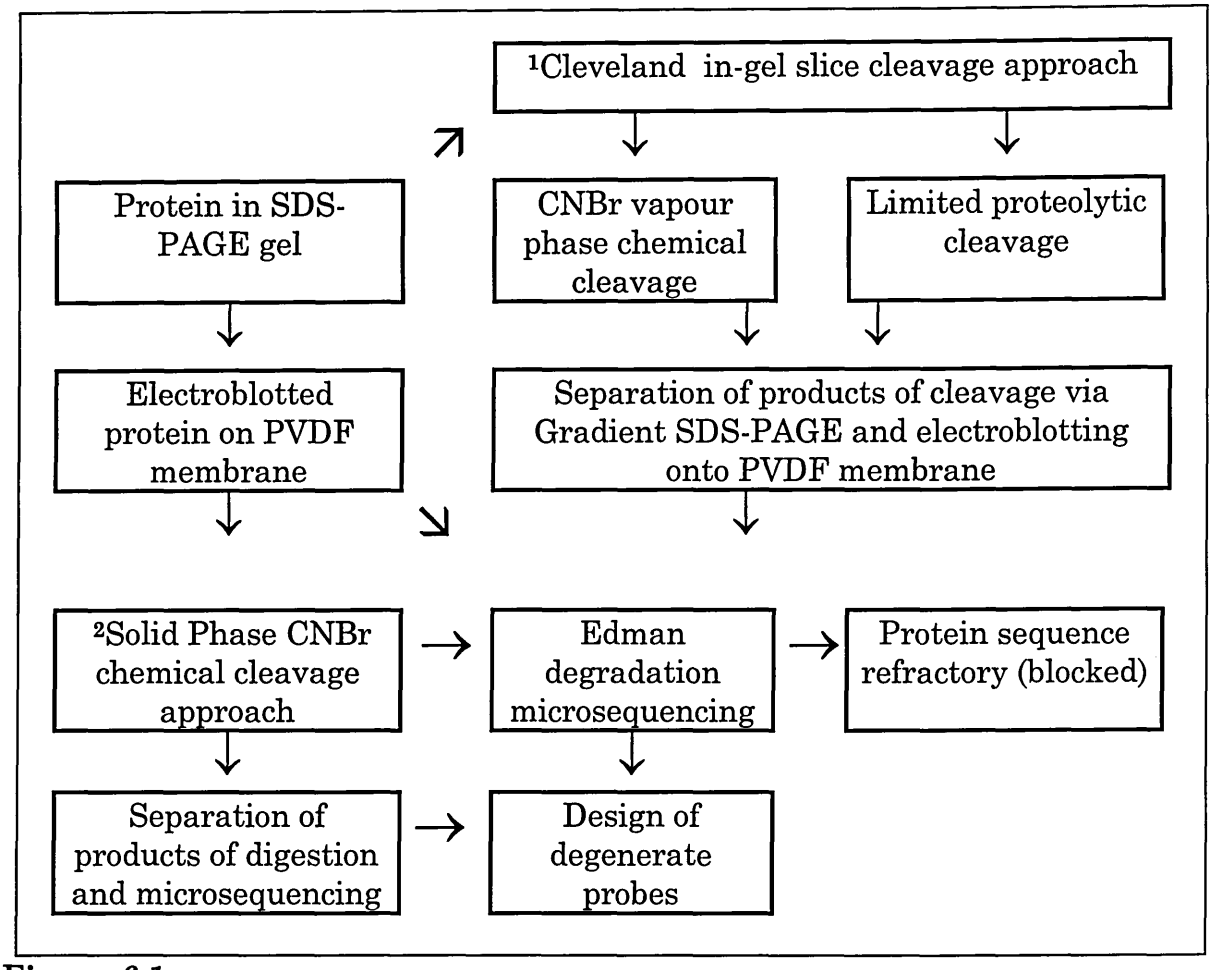


Figure 6.1

Approaches to producing primary sequence information with identified outcomes.

[Legend: [1] Cleveland in gel slice approach was as originally described by Cleveland et al, **(1977)** and discussed in more detail in Chapter 2, section 2.8.4: [2]Solid phase CNBr digestion was as described by Promega probe design kit (Promega) in Chapter 2, section 2.8.2.**]**

The manual process originally described by Edman has now been automated (Findlay and Geisow, **1990**) and techniques for protein transfer from a gel onto a thin uniform support matrix (usually a membrane) has been developed. This transfer procedure is referred to as blotting. (Bers and Garfin, (**1985**) and Stott (**1989**). A polymer based membrane prepared from polyvinylidene difluoride (PVDF) and introduced in 1987 has the advantage of being mechanically strong, easy to handle and chemically inert; so the Edman chemistry can be applied to a protein blotted onto it (Matsuidaira, **1989**).

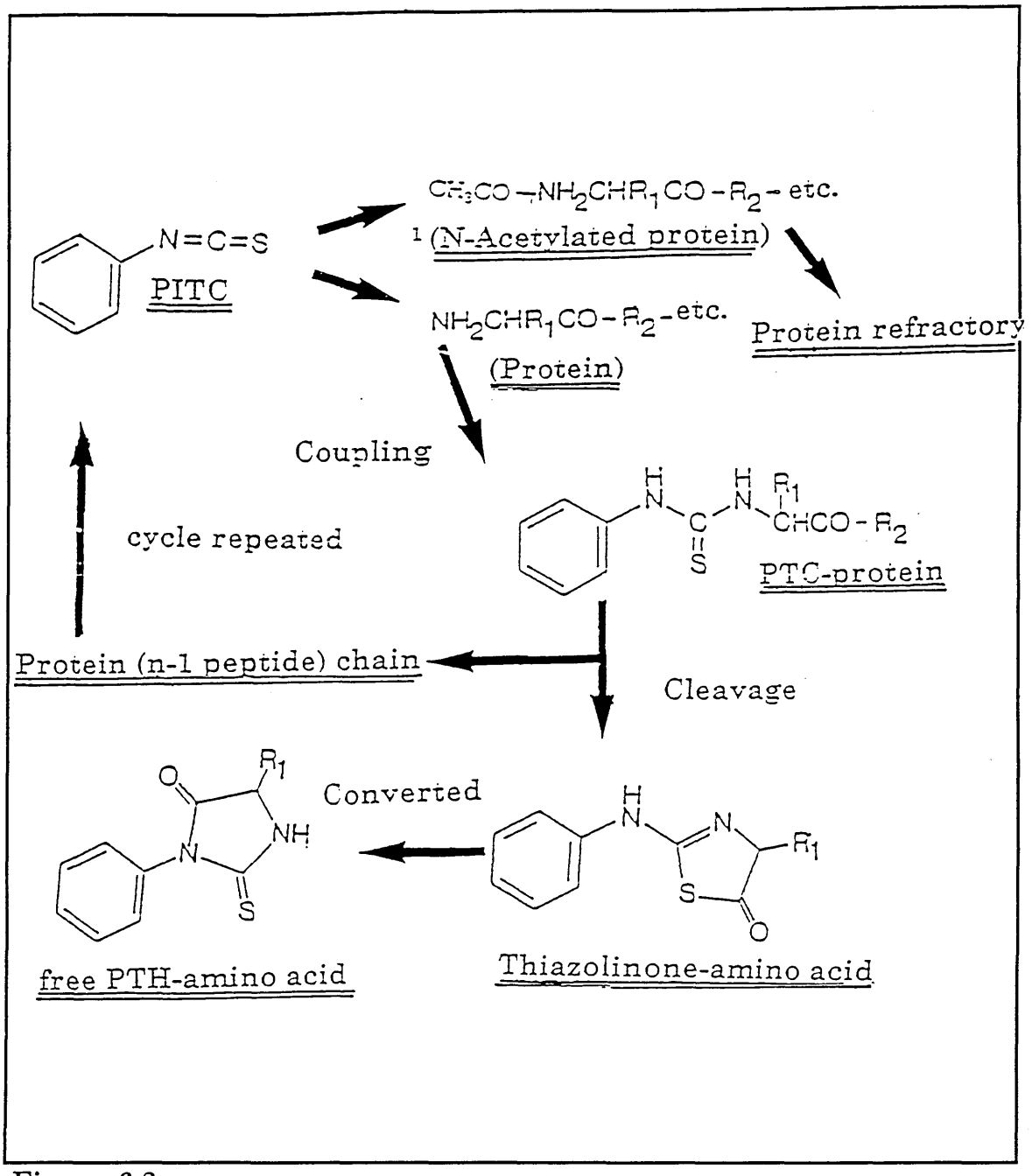


Figure 6.2

Principle of Edman degradation

[**Legend:** [1] The most common N-terminal block of N-Acetylation that occurs in eukaryotic proteins, further described by Walsh and Sasagawa(1984). **Note:** N-gycosylated residues are not blocked but fail to extract and thus present as a blank sequence cycle, O-glycosylated residues can involve a modified cyclisation reaction that blocks further sequencing, thus yielding a reduced yield of sequencable material beyond this type of residue (pers. comm. Dr A. Moir.)]

The amino acid sequence obtained by Edman degradation has to be reverse translated into a nucleotide sequence. However as it is illustrated in Table 6.1, the genetic code is degenerate in that a number of codons specify

one amino acid. The design of nucleic acid probes from this information will thus necessitate the production of a highly degenerate primer set, if the reverse translated sequence is to share 100 % homology with that found within the authentic gene. The design of this pool of degenerate oligonucleotides or guessmer's followed the guidelines listed below and is summarised in Table 6.1.

Grantham et al, (**1980**) demonstrated that organisms have a codon preference for the same gene. This information combined with the approaches suggested below by Lathe (**1985**) resulted in an increase in probe homology to the gene from 76% when a purely random choice of codon was used for a guessmer to 86% when the approaches described and codon usage of the species of interest were taken into account. The three amino acids with 6 codon choices were avoided if possible. CpG sequences formed by adjacent codons were eliminated as this dinucleotide is significantly under-represented in mammalian DNA and only occurs with about half of the expected frequency. If the most commonly used codon for a particular amino acid in a given species produces the CpG dinucleotide then the next most common choice is selected. If there is an unavoidable choice of 2,3 or 4 bases then a neutral base, such as inosine is used as it forms stable base pairs with all four conventional bases.

The above information relates to the design of degenerate pools of oligonucleotides. It was planned in this work that these pools would be initially used as PCR primers. Care was also taken so that the primers synthesised did not contain any complementary structures longer than 2 bases, especially at the 3' end that can lead to the formation of a primer-dimer complexes. This is where the primers anneal to each other and so lead to the production of a short duplex product (Williams, **1989**). Somner and Tautz (**1989**) also demonstrated the importance of the last 3 bases at the 3' end of primers that act as anchors to the DNA template, therefore it is important that the neutral base, inosine was not included at this position in any of the primers synthesised.

Table 6.1

Reverse translation methods for the production of degenerate primer pools

Amino Acid[1] (*)		Lathe Method[2] Optimal codon choice when following base is		Inosine Method[3] Optimal codon choice when following base is	
		A, C, T	G	A, C, T	G
(1) Methionine:	Met. (M)	ATG	-	ATG	-
(1) Tryptophan:	Trp. (W)	TGG	-	TGG	-
(2) Phenylalanine:	Phe. (F)	TTC	TTT	TTC	TTT
(2) Tyrosine:	Tyr. (Y)	TAC	TAT	TAC	TAT
(2) Histidine:	His. (H)	CAC[4]	CAT	CAC	CAT
(2) Glutamine:	Gln. (Q)	CAG	-	CAI	-
(2) Aspartic acid:	Asp. (D)	GAC	GAT	GAT	-
(2) Asparagine:	Asn. (N)	AAC	AAT	AAC	AAT
(2) Lysine:	Lys. (K)	AAG	-	AAI	-
(2) Cysteine:	Cys. (C)	TGC	TGT	TGC	TGT
(2) Glutamic acid:	Glu. (E)	GAG	-	GAI	-
(3) Isoleucine:	Ile. (I)	ATC	ATT	ATI	-
(4) Glycine:	Gly. (G)	GGC	-	GGI	-
(4) Alanine:	Ala. (A)	GCC	GCT	GCI[5]	-
(4) Valine:	Val. (V)	GTG[6]	-	GTI	-
(4) Proline:	Pro. (P)	CCC[7]	CCT	CCI	-
(4) Threonine:	Thr. (T)	ACC	ACA	ACI	-
(6) Leucine:	Leu. (L)	CTG	-	CTI	-
(6) Serine:	Ser. (S)	TCC	TCT	TCC	TCI
(6) Arginine:	Arg. (R)	CGG	-	CGI	-

[**Legend:** Amino acid[1]: Denoted by 3(1) letter code as illustrated by Cohn (1984). Reverse translation methods as suggested by Lathe (1985)[2] and the inosine substitution method from Sambrook et al. (1989)[3]. [4]CAT when followed by C. [5]I: Represents neutral base inosine.. [6]GTC when followed by T. [7]CCA when followed by T **Note:** (*) numbers in brackets indicating the number of codons that code for that specific amino acid.]

This chapter describes the methods used to produce primary sequence information for the PAPP-A protein that was subsequently used to design and synthesise degenerate nucleic acid PCR primers for screening the placental cDNA library.

6.2 Materials and Methods

The reagents, methods and techniques that were used to generate the results described in this chapter are described fully in Chapter 2, section 2.8.

6.3 Results and Discussion

The PAPP-A that had been enriched by a method based on the procedure described by Sinosich et al (**1983**) yielded material that could be used as a tracer in a RIA , Figure 4.7 (Chapter 4). It was not suitable for obtaining the primary sequence for PAPP-A (Table 6.2) as this material contained at least 6 polypeptide chains. Further purification of PAPP-A was therefore required to produce PAPP-A in a form suitable for microsequencing (Chapter 5).

Table 6.2
Microsequencing of PAPP-A material used for iodination

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
D	Q	E	L	N	D	K	E	F	E	E	N	F	N	V	V
	L	N	G	A	L	V	R	I	K	M	P	R	L	Q	D
	V	R	P	V	K	F			G	A	G	D	W	F	L
	M	W	F	I	R	A			A	P	H			A	
						G			Y		S			G	
						I			T					I	
E	A	R	G	A	T	E	E	P	S	P	P	S	R	A	L	Y	(1)

[Legend: Amino acids using 1 letter nomenclature, numbers indicate amino acid identified after number of Edman degradation cycles, the order listed indicating level of amino acid present with highest concentration at top of table. **Note (1):** indicates N-terminal protein region for PAPP-A from Kristensen et al, (**1994**) The enriched material sequenced was that used for iodination and was a PAPP-A enriched sample that was a gift of Ms K. Price. The material was microsequenced by the Leeds University central sequencing service, Appendix 3].

6.3.1 Results Of CNBr Chemical Cleavage of PAPP-A

The solid phase CNBr digest and RP-HPLC method for separating peptides did not generate material suitable for Edman degradation. Control material isolated from the RP-HPLC system was sequencable, indicating

that peptides were recoverable from RP-HPLC system and separation and collection procedure did not block the N-terminus. The elution of the cyanogen bromide digest as monitored by absorbance at 215 nm from the RP-HPLC did not yield material that was sequenceable (Figure 6.3).

As shown in Chapter 2, section 2.8.1 it was not possible to elute PAPP-A protein from a membrane (nitrocellulose or PVDF) using any of the methods described to elute protein from a membrane. This was in contrast to the control proteins which could be eluted using methods that other investigators have found successful for this purpose (Leykan and Strahler, (**1992**), Charbonneau, (**1991**) and Morris, (**1992**)). This difficulty in eluting PAPP-A may explain why the solid-phase CNBr digest approach did not yield any further information on the primary protein structure of PAPP-A.

The vapour phase in-gel digestion produced material that could be sequenced but as can be seen in Appendix 3, the separation technique used did not adequately resolve the peptides produced to give any sequence information that could be used to design oligonucleotide primers.

6.3.2 Limited Proteolytic Digestion of PAPP-A with Lys-C

The results of a typical digestion with Lys-C are illustrated in Figure 6.4 with the individual method performed as outlined in Chapter 2, section 2.8.5 yielded N-terminal information from the peptide Lys-C1 (Table 6.3). All the other bands that were sequenced represented peptide mixtures and did not yield any further primary sequence information.

6.3.3 Limited Proteolytic Digestion of PAPP-A with Glu-C

The results of a typical digestion with the endoproteinase Glu-C are illustrated in Figure 6.5 with the individual method performed as outlined in Chapter 2, section 2.8.5. The endoproteinase Glu-C can generate a smeared bands with some substrate proteins (Fischer, **1983**). PAPP-A as a substrate also generated a smeared band pattern, the significance or why some proteolytic enzymes generate this smearing effect is unknown but was true of the PAPP-A/V-8 interaction.

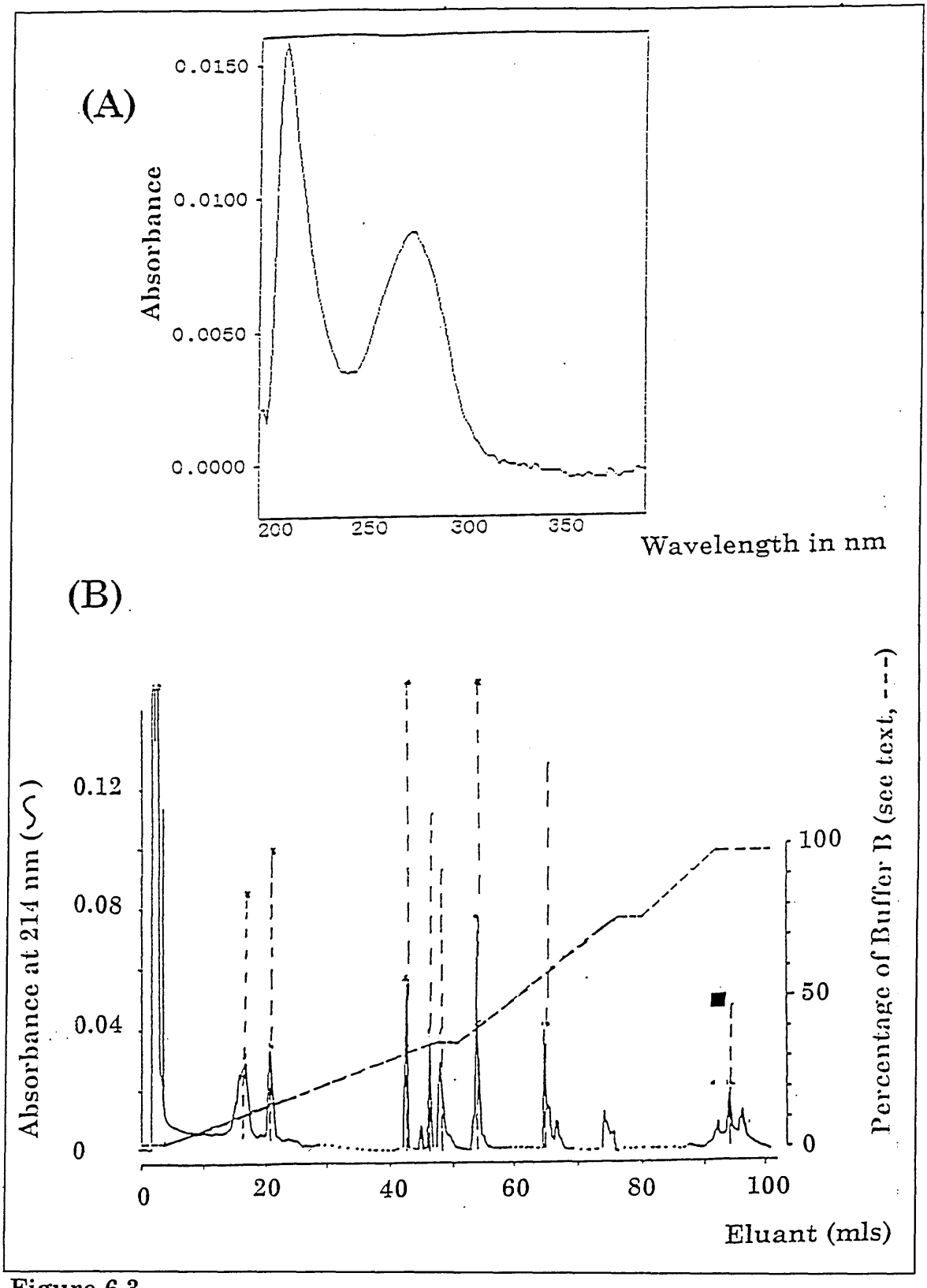
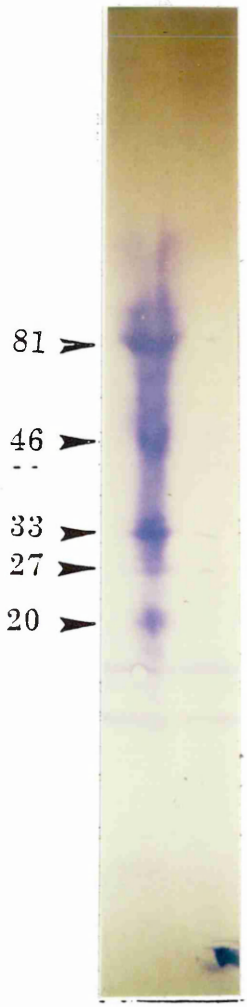


Figure 6.3

RP-HPLC elution profile of solid-phase CNBr cleavage of PAPP-A

[Legend: (A): Represented marked peak as detected using a diode-array detector, Amino acid analysis of the indicated peak (■), was a service provided by Dr A. Moir (Appendix 3). **(B):** Elution profile of CNBr cleavage on the reverse phase HPLC system as described in Chapter 2, section 2.8.3. The dotted lines indicated peaks that eluted in a second solid-phase CNBr digest.]



Lys-C Digestion

a) Lys-C1 produced an extended N-terminal region (Table 6.3)

b) Lys-C2
Mixture of at least 4 peptides (Appendix 3)

c) Lys-C3
EAR(Por G)
?Start of PAPP-A N-terminus but yield too low to sequence. (Appendix 3)

d) Lys-C4
Peptide mixture (Appendix 3).

e) Lys-C5
Mixture of at least 2 peptides. (Appendix 3).

Figure 6.4

Limited proteolytic digestion of PAPP-A with Lys-C. (**Plate inset 6.4:** PVDF electroblotted sample as sent for sequencing).

[Legend: Arrows on left of blot were size of fragments in kDa calculated as described in Chapter 2, section 2.3.2.2.c. Bands that were sequenced are indicated by * and number that identifies the given sequence (Appendix 3) Material was sequenced by Mr J. Gilroy, Durham. A limited digest was performed with 1 µg of endoproteinase Lys-C (Appendix1)]

The internal peptide identified in this study (K-2, Figure 6.5) was not found in the published sequence but did share some homology with part of the V8 molecule. (Appendix 3). This could have been due to a number of factors:

- The monomeric band identified as PAPP-A was not pure. However this was unlikely because another protein that could have co-purified with PAPP-A would have to have the same Mr as the monomeric PAPP-A chain and also have existed as a disulphide bridged dimer and share the same physico-chemical characteristics of PAPP-A (Chapters 5 and 7).
- Sequencing of material added to digestion (i.e. the proteolytic enzyme or a protein contaminant added in this enzyme preparation).

The degenerate primer produced from this sequence (cPAPP-3, Chapter 2, Table 2.6) did produce a specific PCR product with the primers produced from the N-terminal region of PAPP-A (Plate 6.1). This demonstrated that the placenta contained mRNA that codes for PAPP-A and thus demonstrated that PAPP-A is synthesised by the placenta.

Table 6.3

Comparison of PAPP-A N-Terminal Sequence information obtained during this thesis against the published sequence[1]

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
E	**A**	**R**	**G**	**A**	**T**	**E**	**E**	**P**	**S**	**P**	**P**	**S**	**R**	**A**	**L**	**Y**	(1)
E	**A**	**R**	**G**	**A**	**T**	**E**	**E**	**P**	**S**	I	D	I	**R**	**A**	**L**	I	(2)
E	**A**	**R**	**G**	**A**	**T**	**E**	**E**	**P**									(3)
K	**A**	**R**	**G**	**A**	**T**	**E**	?	v									(4)
E	**A**	**R**	**G**	**A**	**T**	**E**	**E**	**P**									(5)

[Legend: Amino acids using 1 letter nomenclature, numbers indicate amino acid position for mature protein chain starting at N-terminus. Bold lettering indicates agreement with sequence for mature N-terminal region described by Kristensen et al, **(1994)[1]. ?:** indicated blank sequence indicative of cysteine or post-translationally modified N-linked amino acid. **2:** Lys-C digestion, own blot band K-1. Sequencing via **3:** V-8 digestion, own blot, peptide band K-1. Sequencing via **4/5:** PVDF blot, N-terminal sequence by Dr K. Lilley **]**

Surprisingly the use of asymmetric PCR as described in Chapter 2, section 2.8.8 confirmed that using the PCR primers N-PAPP-1/2 on their own generated a specific PCR product > 1200 bps. Similar results were

obtained if the N-terminal primer, PAPP-4 was used. The use of the cPAPP-3 with the N-terminal primer generated a specific product of approximately 500 bps (Plate 6.1).

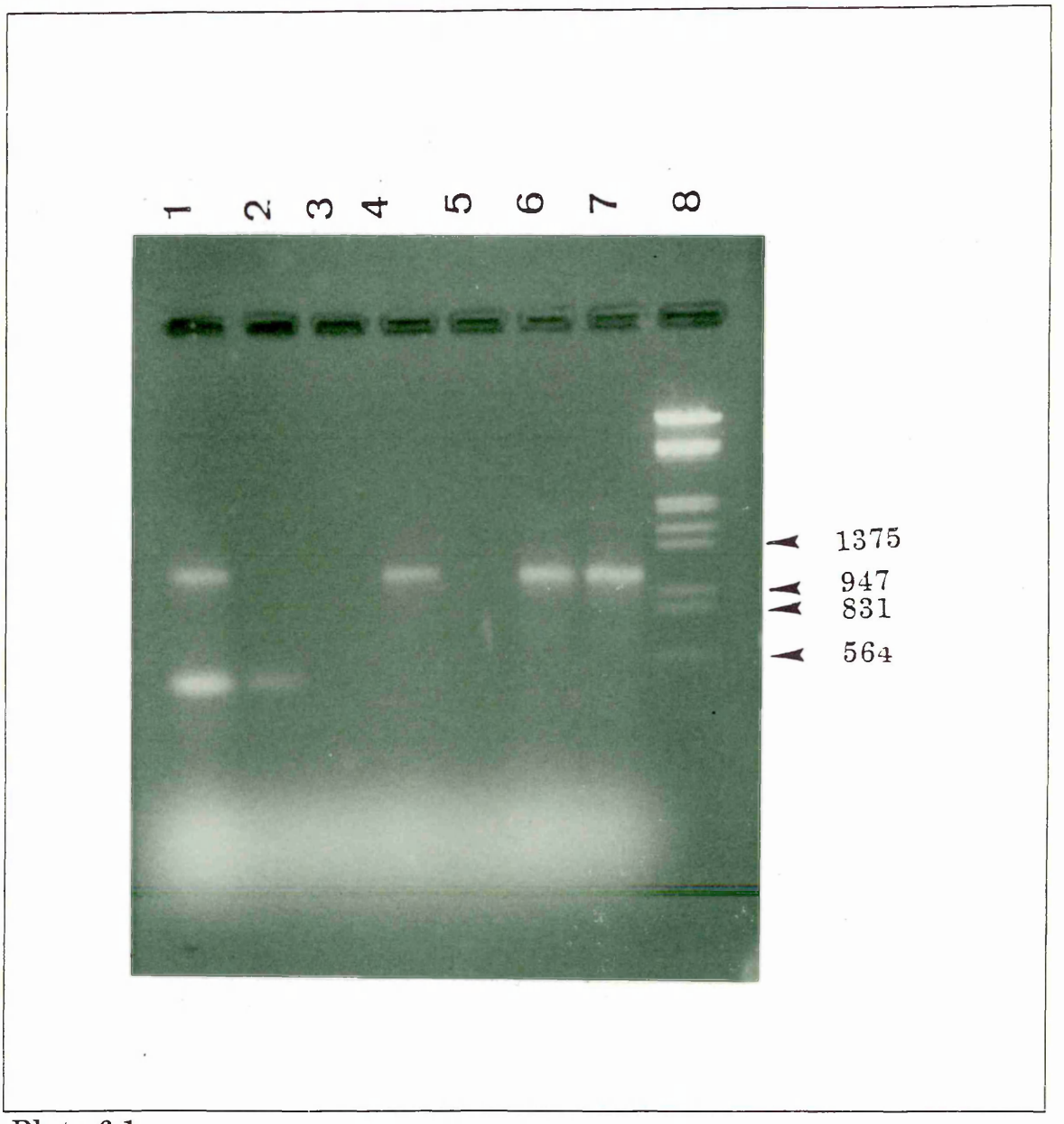


Plate 6.1

PCR using primers N-PAPP-1/2 and cPAPP-3 (an internal PAPP-A stretch, complementary primer)

[**Legend:** 7 ul of PCR product was run on a 2% agarose gel containing ethidium bromide in TAE (as described in Chapter 2, section 2.3.2.1).**Note:** All other conditions were as described in Chapter 2, section 2.8.8 (**1**): 100 pmol cPAPP-3 and N-PAPP-1/2. (**2**): 100 pmol cPAPP-3 and 1 pmol N-PAPP-1/2 . (**3**): 100 pmol cPAPP-3 and 0.1 pmol N-PAPP-1/2. (**4**): 0 pmol cPAPP-3 and 100 pmol N-PAPP-1/2. (**5**): 100 pmol cPAPP-3 and 0 pmol N-PAPP-1/2. (**6**): 0.1 pmol cPAPP-3 and 100 pmol N-PAPP-1/2. (**7**):. 1 pmol cPAPP-3 and 100 pmol N-PAPP-1/2. (**8**): Double stranded DNA size markers (Appendix 1), numbers in Plate represented size in bp's.]

A search of the GENBANK/EMBL databases with the sequences described in Table 2.6 (Chapter 2) using the GCG package at DARESBURY demonstrated that the no two PCR primer pairs shared homology with any of the published sequences. This did not exclude the possibility that these sequences were hybridising to something other than PAPP-A. But an analysis of the published sequence demonstrated that the 500 and >1200 bp PCR products could have been generated from the PAPP-A cDNA. Sequences present in the PAPP-4 primer shared 100% homology with the N-terminus region and a 10 bp complementary sequence was located at the 3' prime end of this primer that would give a >1200 bp product using the cDNA sequence as a template. A similar partial homology was observed for the degenerate cPAPP-3 primer that would produce a 500 bp product with the N-terminus primer using the described cDNA sequence as a template.

The solid phase chemical method of cyanogen bromide cleavage did not yield sequenceable material by Edman degradation. The vapour phase CNBr cleavage and limited proteolytic digestion using an adapted Cleveland method yielded sequence information, usually in the form of peptide mixtures that were not well enough separated by the gradient SDS-PAGE method that was described in this thesis. Further work using RP-HPLC or 2D-PAGE (and electroblotting) would have led to methods that would have resolved these peptide mixtures would have yielded single internal peptide sequence information. However the time taken to generate this sequence information precluded a further examination of the cDNA. After the practical aspects of this work were completed a Danish group published work describing the isolation of PAPP-A cDNA (Kristensen et al (**1994**) and Oxvig et al, (**1993**) that confirmed the N-terminus obtained for PAPP-A.

The presence of sequences other than that described in the published cDNA (Kristensen et al, **1994**) is likely to be resolved when the entire gene sequence for PAPP-A has been obtained. It will also resolve why there are two distinct mRNA species of 9 Kb and 12 Kb in size which are much larger than that expected to produce a PAPP-A polypeptide chain with an estimated size of 172 kDa (Oxvig et al, **1994**).

CHAPTER SEVEN

Chapter Seven

The Molecular Characterisation of PAPP-A

7.1 Introduction

During the time that this thesis was undertaken it became apparent that the there were a number of areas that concerned PAPP-A that were controversial in that contrasting results were described by different investigators. This chapter is involved with what was found in this study about the PAPP-A molecule with regards to:

- Glycosylation with an investigation of the glycan component in terms of extent and type of glycosylation (section 7.2).
- Studies on an a previously undescribed interaction that was observed in this thesis between PAPP-A and the endoproteinase Arg-C, which is discussed with the implications this interaction has on a suggested function for PAPP-A (section 7.3).
- The tertiary protein structure of PAPP-A was examined (section 7.4).

7.2. Studies on The Carbohydrate Component of PAPP-A

7.2.1 Introduction

Glycoproteins are proteins which have been post-translationally modified, with the addition of carbohydrate residues (glycans) to the polypeptide chain. They may contain between 4 - 60% (w/w) of the glycan component (Lee et al, **1990**). The glycan can be linked to the polypeptide chain in one of two major ways by:

- N-glycosidic bonds through the amido nitrogen group of asparagine.
- O-glycosidic bonds through the hydroxyl oxygen group of serine or threonine, but also 5-hydroxy-lysine or 4-hydroxy-proline.

The glycan component can perform a variety of functions in Glycoproteins including:

- Protection of the polypeptide chain from proteolytic attack. This is probably due to a simple steric blocking of access of the enzyme to protein's peptide core.
- Induction and maintenance of a protein's active form, e.g. de-glycosylation of the cysteine proteinase, thiostatin results in it's inactivation (Rusiniak et al, **1991**).
- Cellular signalling as it has become apparent that many differentiation antigens on normal and neoplastic cells are the oligosaccharide determinants of glycoproteins rather than the peptide. (Feizi et al, **1987**).
- Masking of cell surface antigens from immune surveillance. It has been reported that modification of the glycan component by addition of further carbohydrate residues can have a masking affect on antigens present on the cell surface (Childs et al, **1983**).
- Receptor interaction, with the observation by Alexander (**1989**) that the ligand for the sperm receptor on the ovum is an 0-linked oligosaccharide and it is the glycan component rather than the polypeptide chain of the protein that is the recognition factor in this interaction.

Changes in the glycosylation pattern of serum proteins occur during a variety of pathological conditions, e.g. alpha-$_2$-macroglobulin has different patterns of glycosylation in patients with the autoimmune (rheumatoid arthritis and chronic inflammation) diseases than that found in healthy individuals (Silvestrinin et al, **1989**). It has been reported that the pattern and extent of glycosylation play an important role in cell-cell signalling and changes in them have been observed in neoplasia (Saso et al, **1992**).

Methods that can be used to characterise the glycan constituent of glycoproteins have been extensively reviewed by Lee et al, (**1990**), Faye and Salier (**1989**), Montreuil et al, (**1986**). Lectins can be used in a number of ways to study the glycan component of glycoproteins but the two main approaches have been described by Faye and Salier (**1989**):

- Crossed affino-immunoelectrophoresis (CAIE).

- Probing of Western blots with lectins.

Both methods allow a quick determination of glycan composition of a glycoprotein. CAIE allows an examination of the oligosaccharide chains linked to a protein if the tertiary structure of the protein does not make them inaccessible for interaction with the lectin, therefore a lack of interaction does not exclude the presence of a particular carbohydrate group. A disadvantage of the CAIE approach is that if a studied protein forms a complex with another protein then this method cannot differentiate between components of the complex, thus a positive result with a particular lectin could be due to the carbohydrate group being present on the complexed protein.

The advantage of probing a blot with lectins is that glycoproteins are in a denatured form and thus the glycan is accessible for interaction with the lectin and any interaction is with the protein of interest and not a complex (as described above). However it is not capable of detecting heterogeneity in individual oligosaccharide chains that may be present. The lectin probing of Western blots combined with chemical or enzymatic deglycosylation was thus chosen to investigate the glycan component of PAPP-A. The affinity of lectins used in this thesis for carbohydrate residues is summarised in Table 7.1.

The glycan component of PAPP-A has been studied by various investigators (Table 1.3, Chapter 1). The glycan component of PAPP-A has been estimated to be 13 - 19% (w/w) of the molecule. A number of investigators have studied (Bischof, (**1979a**), Sinosich et al, (**1985,1990**) Sutcliffe et al, (**1980**) and Oxvig et al, (**1994**) the overall amount and type of carbohydrate residues present; although this is valuable information, it does not however determine the nature and type of oligosaccharide chains that were present on the PAPP-A molecule. The studies using CAIE (as undertaken by Sinosich et al, **1985,1990**) analysed the native PAPP-A molecule and in the light of the recent finding that PAPP-A is a complex with proMBP (Oxvig et al, **1993**) it is unknown whether the glycan assessed

Table 7.1 Specificities of lectins for specific carbohydrate residues.

	Carbohydrate Specificity[2]														
Lectin[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
BSL-1	$+^{\alpha}$	$+^{\alpha}$	-	-	-	-	-	-	-	-	-	-	-	-	-
BSL-IB	-	$+^{\alpha}$	-	-	-	-	-	-	-	-	-	-	-	-	-
BSL-II	-	-	$+^{tn}$	-	-	-	-	-	-	-	-	-	-	-	-
Con-A	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
DSL	-	-	-	-	-	-	$+^{ol}$	$+^{ol}$	-	-	-	-	-	-	-
DBA	$+^{\alpha}$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EBL	-	+	-	-	-	-	-	-	$++^{t}$	-	+	-	-	-	-
ECL	-	$+^{s-}$	-	-	-	-	$++^{s-}$	-	-	-	-	-	-	-	-
Jacalin	-	-	-	-	-	-	-	-	-	$+^{o-l}$	-	-	-	-	-
LCA[3]	-	-	$+^{\alpha}$	$++^{\alpha}$	$+++^{\alpha t}$	-	-	-	-	-	-	-	-	-	-
LTL[4]	-	-	-	-	-	-	-	-	-	-	-	$+^{\alpha}$	-	-	-
LEL	-	-	$+^{ol}$	-	-	-	-	-	-	-	-	-	-	-	-
PHA-E4	-	$+^{l.n-l}$	$+^{l.n-l}$	-	-	-	-	-	-	-	-	-	-	-	-
PNA	+	$+^{\alpha}$	-	-	-	-	-	-	-	$++^{s-}$	-	-	-	-	-
PSA[5]	-	-	-	-	$+^{l.n-l}$	-	-	-	-	-	-	$+^{l.n-l}$	-	-	-
PWN	-	-	$+^{ol.n-l}$	-	-	-	-	-	-	-	-	-	-	-	-
RCA-I[6]	$+^{t.s}$	$+^{t.s}$	-	-	-	-	-	-	-	-	-	-	-	-	-
STL	-	-	$+^{ol}$	-	-	-	-	-	-	-	-	-	-	-	-
SJA	$+^{t}$	$+^{t}$	-	-	-	-	-	-	-	-	-	-	-	-	-
SBA[7]	$++^{t}$	+	-	-	-	-	-	-	-	-	-	-	-	-	-
UEA-I	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
UEA-II	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
VVA[8]	$+^{t\alpha}$	-	-	-	-	-	-	-	-	+	-	-	-		-
WGA[9]	-	-	$+^{ol.t.s}$	-	-	-	-	-	-	-	-	-	-	-	-
WGA-S	-	-	$+^{ol.t}$	-	-	-	-	-	-	-	-	-	-	-	-
VAA	-	-	-	-	-	-	-	-	-	-	-	-	$+^{t}$	-	-
GNA	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
MAA[10]	-	-	-	-	-	-	-	$+^{t}$	$+^{t}$	-	-	-	-	-	-
ABA	$+^{tn}$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LPA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
CAA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
WFA	$+^{t}$	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[**Legend:** **Lectin[1]:** Abbreviations for lectins and carbohydrate specifities adapted from those found in Lis and Sharon(**1986**), Wu et al,(**1988**), Lee et al,(**1990**) and as used by Vector Laboratories (Appendix 1). **Carbohydrate specificity[2]:** (**1**) N-Acetyl-galactosamine. (**2**) Galactose. (**3**) N-Acetyl-β-D-glucosamine. (**4**) Glucose. (**5**) Mannose. (**6**) Mannose, branched structure. (**7**) Galactosyl (β-1,4)N-acetyl-glucosamine. (**8**) N-Acetyl-lactosamine. (**9**) Sialic acid terminal to galactose. (**10**) Galactosyl(β-1,3)N-Acetyl-Galactosamine. (**11**) Lactose. (**12**) L-Fucose. (**13**) Galactosyl residues. (**14**) N,N-di-Acetyl-Chitobiose. (**15**) Sialic acid. (**+,++**: Affinity for specific carbohydrate group, if more than one (+) then indicates relative affinity. **Abbreviations:** x^{α} , Alpha linked residue. x^{tn} , Non-reducing terminal residue. x^{ol} , oligomers of residue. x^{o-l}, O-linked residue. x^{n-l}, N-linked residue. x^{s-}, Only will bind residue if de-sialated. x^{l}, only binds if both residues present. x^{t}, binds if terminal residue. x^{s}, residue needs to be sialated for lectin to bind. **Notes:** [3]: More specific than Con-A, fucose also increases affinity. [4]: Similar to UEA-I but has different affinities for carbohydrate residues. [5]: Increased affinity if terminal mannose. [6]: Greater affinity for β-D than α-D galactose. [7]: Binding blocked by substitutions in penultimate sugars. [8]: Two isolectins, A4 binds terminal α-linked and B4 binds to single residue bound to serine/threonine. [9]: Increased affinity for higher oligomers of N-Acetyl-glucosamine. [10]: Increased affinity for α-2,3 linked sialic acid to galactose.]

using this method was present on the PAPP-A monomer or on the proMBP/PAPP-A complex. The investigation carried out in this thesis used an isolated reduced monomeric chain of PAPP-A that was free of proMBP and the glycan component of the PAPP-A monomer is assessed in this section.

7.2.2 Materials and Methods

The materials and methods used in the section can be found in Chapter 2, section 2.4.

7.2.3 Results and Discussion

7.2.3.1 The Antigenicity of PAPP-A

Western blotting of PAPP-A reduced into a monomeric form, followed by immunodetection with a polyclonal antibody (DAKO) against PAPP-A yielded a specific band at approximately 195 kDa (Chapter 3, Plate 3.1A). A single band was however produced with this antibody only when a high concentration (0.6M) of salt was used to increase the specificity of the interaction with the immune serum. At this salt concentration the serum also had to be used at a high concentration to produce a detectable band (a 1:600 dilution for polyclonal anti-PAPP-A). Thus it was possible to make this antiserum specific for PAPP-A, but this specificity was achieved at a cost of using only a sub-group of paratopes present in this antiserum, some of which were directed against the glycan component of PAPP-A (as determined by deglycosylation experiments which revealed that the recognised antigenic determinants had a carbohydrate component, Chapter 3, Plate 3.4). To further characterise the antigen (PAPP-A)- antibody interaction, a chemical method for cleaving the carbohydrate groups was used as described in section 2.4.5 (Woodward et al, **1985**, see Plate 7.1)

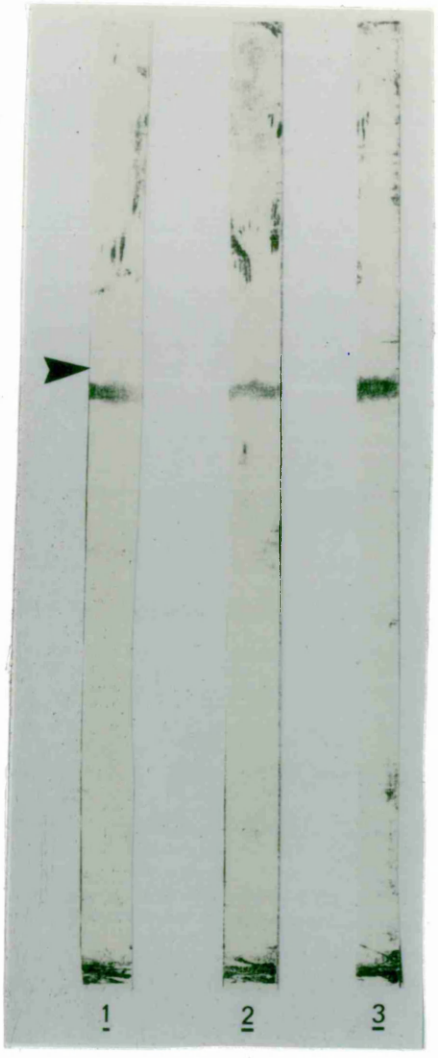


Plate 7.1

A chemical assessment of the glycan epitopes detected by polyclonal anti-PAPP-A paratopes at high salt concentration (0.6 M NaCl).

[Legend: The Western blots were prepared and treated as described in Chapter 2, section 2.4. Development of the blot with antibody was as described in Chapter 2, section 2.4.3. but before development the blots were pre-incubated with**:** (**1**) TBS. (**2**) 2 mM sodium periodate. (**3**) 10 mM sodium periodate. The arrow indicated the 205 kDa molecular weight marker.**]**

It has been reported that not all carbohydrate groups are sensitive to this type of chemical treatment (Woodward et al, **1985**), and as it can be seen from Plate 7.1; the carbohydrate components of the antigenic determinants detected by the PAPP-A antiserum were not sensitive to this form of chemical treatment. Kabat (**1976**) has shown that the glycans which are to the chemical cleavage with periodate are a groove type of determinant that have a repeat linear structure. Thus it is likely that such a structure is

detected by this sub-group of PAPP-A paratopes. It is also likely that this glycan is O-linked (Plate 7.4) as PAPP-A was still immunodetected after the removing the N-linked carbohydrate chains. The lectin analysis (Table 7.2) also supports the presence of a repeat unit present as a linear carbohydrate structure.

7.2.3.2 The Carbohydrate Structure of The Glycan Component of PAPP-A

The N-terminal sequence of the PAPP-A monomer determined in this study has been confirmed by Kristensen et al, (**1994**). However there is a difference in the carbohydrate structure reported in this study and that found by Oxvig et al, (**1994**). These authors did not detect N-acetyl-galactosamine in the PAPP-A monomer (Table 1.3, Chapter 1), the PAPP-A monomer demonstrated a weak interaction was observed with VVA (Table 7.2) thus indicating the presence of N-acetyl-galactosamine.

The interaction with the LEL lectin (Plate 7.2) which indicated the presence of oligomers of N-acetyl-glucosamine was further (Plate 7.2) investigated as a possible means of PAPP-A purification. Only one band was detected on blots probed with this lectin. Regardless of this, it was not possible to separate PAPP-A from its major contaminants (e.g. α_2M) using an LEL-column (Chapter 2, section 2.7.9).

The interaction of PAPP-A with Con-A has been described by Bischof (**1979**) who also used it as part of a purification procedure. The PAPP-A present on the blot reacted strongly with the lectin, Con-A (Table 7.2). Treatments with its respective sugar (in the presence of detergents) failed to remove the Con-A bound to PAPP-A. Thus suggesting that the PAPP-A protein and not the carbohydrate groups may be binding this lectin.

This explains the observations by Bischof et al, (**1983**) who demonstrated that PAPP-A appeared to be inhibit lymphocyte transformation, this was probably due to a sequestering of the lectin used to stimulate the lymphocytes.

Table 7.2 Reduced monomeric chain PAPP-A's reactivity with various lectins as determined from work in this thesis.

[1]Lectin:	[2]Reactivity with Monomeric PAPP-A	Lectin has specificity for the following carbohydrate residues
LEL	***	Oligomers of N-Acetyl-β-D-Glucosamine
STL	*	Similar specifities to that of LEL
VVA	**	Terminal alpha linked N-Acetyl-Galactosamine or a single residue of this bound to Ser/Thr. Galactosyl (α-1,3) N-acetylgalactosamine.
DSL	**	Branched Mannose structure, Oligomers of Galactosyl (β-1,4) N-acetylglucosamine and N-Acetyl-lactosamine.
ECL	-	Galactose, Galactosyl (β-1,4) N-acetylglucosamine but will not bind if sialated
BSL-II	-	N-Acetyl-β-D-Glucosamine as a non-reducing terminal residue.
JACALIN	[3]**?Unk.**	O-linked Galactosyl (α-1,3) N-acetyl-galactosamine
Con-A	[4]**** **(?)**	Presence of glucose/mannose residues.

[Legend: [1]Lectins: Lectins that were used on the blots (see text), abbreviations were as found in Table 7.1. [2]Reactivity with monomeric PAPP-A determined with Western blotting and immunodetection of PAPP-A with polyclonal anti-PAPP-A antibodies. [3]**?Unk:** Unable to determine from material used for blotting if Jacalin reacts with PAPP-A as a strong interaction (****) with the > 205 kDa protein contaminant was seen (Chapter 6). [4]**:** The interaction with Con-A was not able to be compared to the other lectins as this interaction was monitored using a Con-A-HRP conjugate (Appendix 1) **Note:** The relative intensity of staining of the blot was indicated by *, **, etc. with increased number indicating a stronger interaction, **-:** Indicated no observable interaction with lectin under the described conditions, see text of Chapter 2, section 2.4.**]**

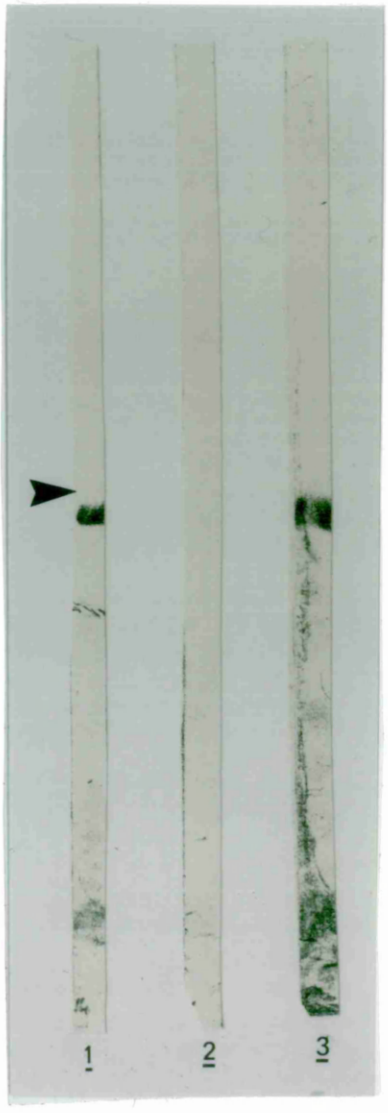


Plate 7.2

The reactivity of reduced monomeric PAPP-A with the LEL lectin

[Legend: After incubation of the blots with biotinylated lectin as described in Chapter 2, section 2.4.4. They were pre-incubated with (**1**)**:** TBS and (**2**): 0.5 M glucosamine in TBS. End detection was with streptavidin-HRP conjugate as described in Chapter 2, section 2.4.4. (**3**): Represented a Western blot with subsequent immunodetection with polyclonal anti-PAPP-A antibodies. **Note:** The Arrow indicated the 205 kDa molecular weight size marker.**]**

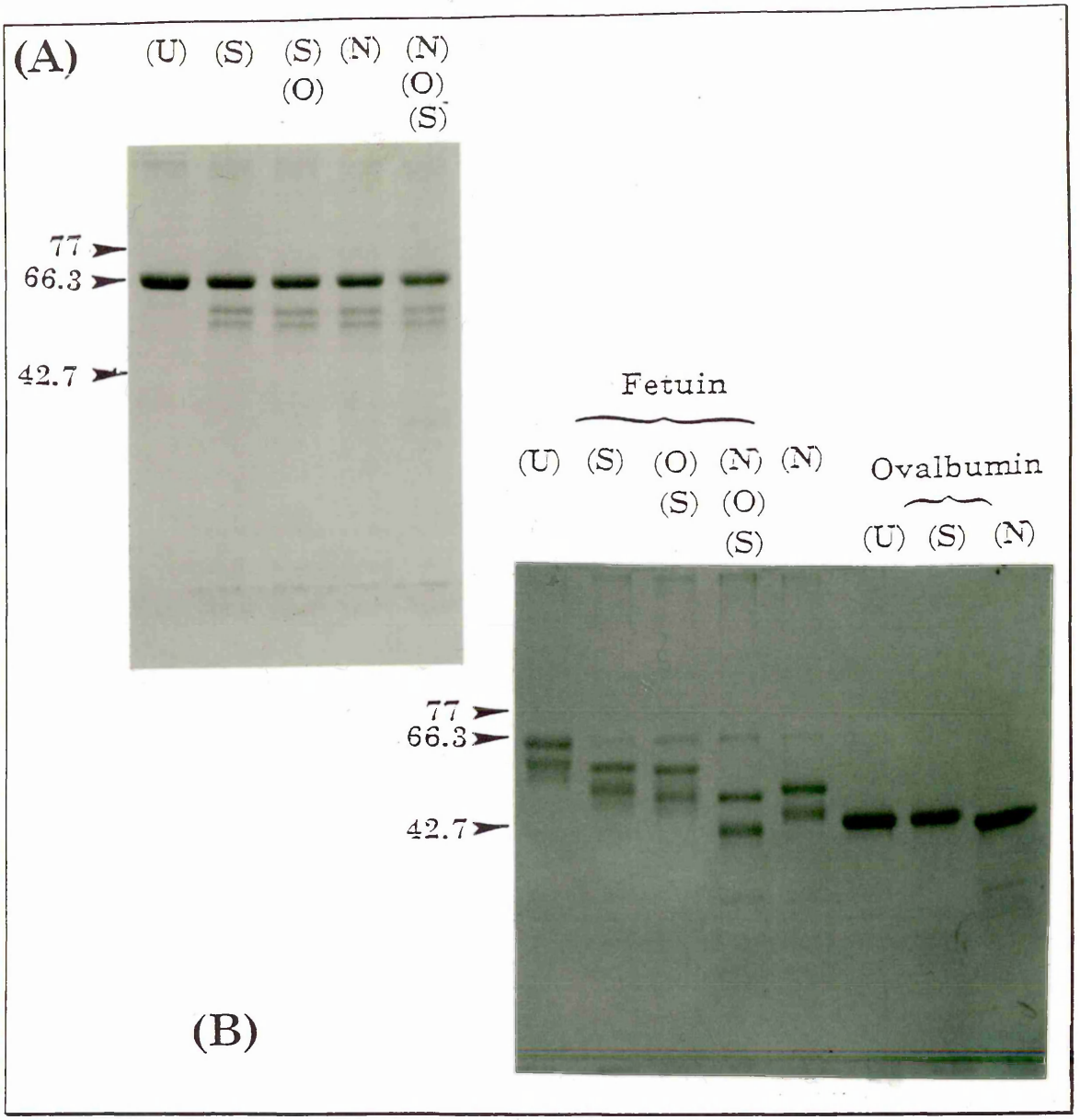


Plate 7.3
Enzymatic deglycosylation of control proteins, human serum albumin (**A**), fetuin and ovalbumin (**B**).
[**Legend:** (**U**): Untreated native protein, (**O**): Protein treated with O-glycanase (**N**): Protein treated with N-Glycanase (**S**): Protein treated with neuraminidase. **Note:** **A** and **B** were separated on a 10% SDS-PAGE gels. Arrows indicated positions of molecular weight markers with size in kDa as indicated, SDS-PAGE conditions were as described in Chapter 2.].

The control protein, Human Serum Albumin (HSA) contains a proportion of contaminating serum globulins (as indicated by the manufacturer). It was these that are deglycosylated and appear as the faint bands under the HSA band (Plate 7.3, **A**). The HSA band as indicated on this gel was not itself affected by enzymatic deglycosylation as HSA itself is not glycosylated.

The control protein, ovalbumin has N-linked sugars as indicated by the enzymatic treatment illustrated in Plate 7.3 (**B**). The control, fetuin has N and O-linked chains that were removed by the enzymatic treatment as indicated by reduction in size of the fetuin bands that were seen in Plate 7.3 (**B**). Thus the control proteins reacted as expected to the enzymatic treatment that was capable of removing the N and O-linked glycans that are known to be present in these proteins.

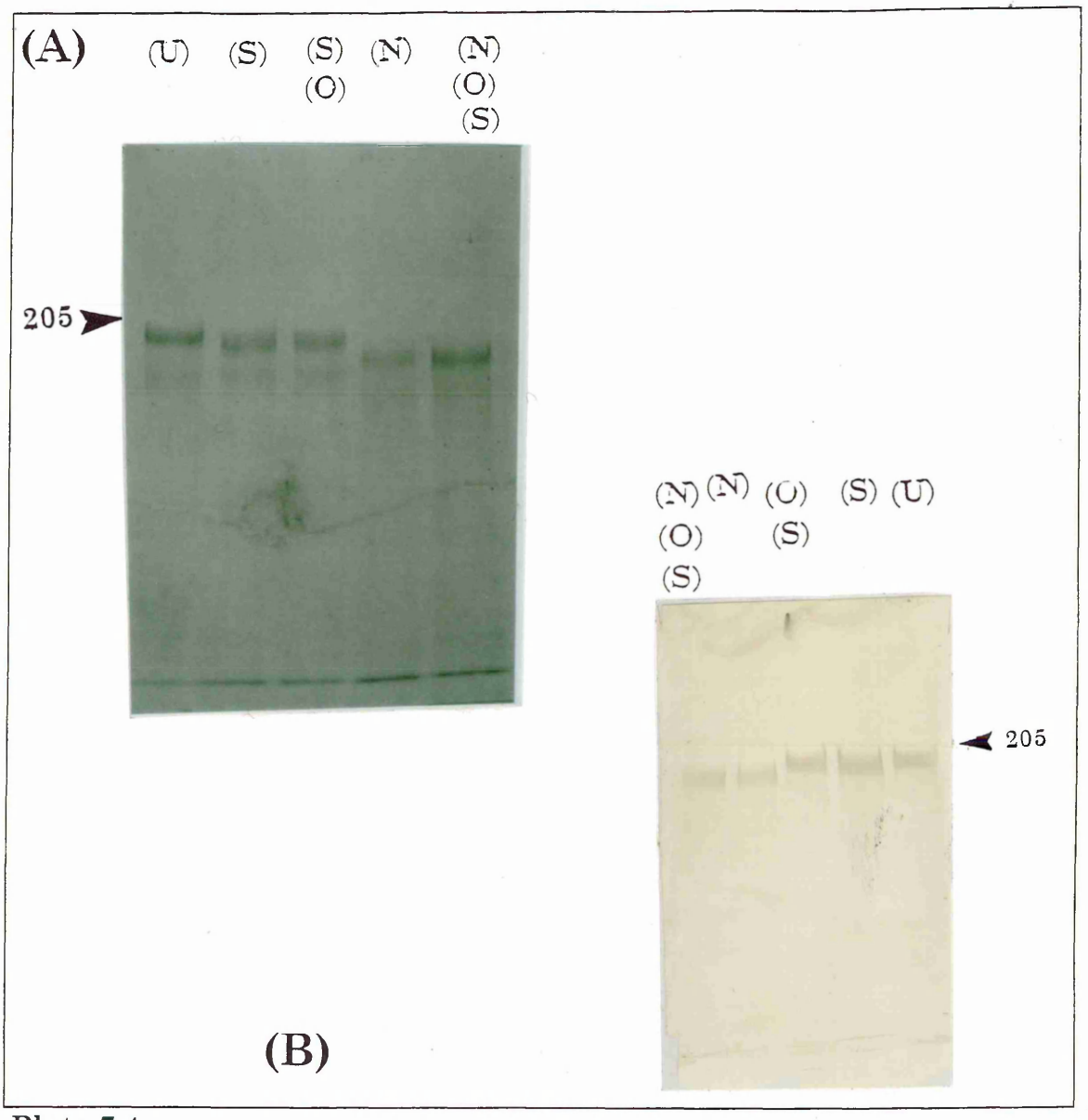


Plate 7.4

Enzymatic deglycosylation of an enriched PAPP-A fraction (**A**).

[Legend: All samples were treated as described in section 7.2.2.3. Samples were separated on a 5% SDS-PAGE gel. **Note:** (**U**): Untreated native protein, (**O**): Protein treated with O-glycanase (**N**): Protein treated with N-glycanase (**S**): Protein treated with neuraminidase. **Note:** Western Blot and immunodetection of samples with Polyclonal (DAKO) anti-PAPP-A antibodies (**B**). Arrow indicated position of molecular weight marker with size in kDa. SDS-PAGE and Western blotting conditions were as described in Chapter 2, section 2.3.3.2. and 2.3.].

Experiments on the deglycosylation of PAPP-A using this approach (Plate 7.4, A) demonstrated removing of N-linked sugar with an estimated reduction in size compared to original PAPP-A of approximately 8%. However further treatment to remove the O-linked sugar groups did not result in a reduction in size. Immunodetection of deglycosylated PAPP-A with the polyclonal antibody demonstrated that a part of the glycan component was still present as this had been shown to be necessary for the binding of this antibody. This could have been due to partial removing of N-linked groups, but the denaturation in the presence of detergents and N-glycanase made this unlikely. It was more likely that O-linked groups were present on the monomeric PAPP-A isolated and that these groups were not removed following neuraminidase treatment. As the O-glycanase is sensitive to any substitutions present on the O-linked chains, this might indicate that they were likely to be present in a complex substituted O-linked form. An analysis of the types of glycan present from whole PAPP-A by probing with lectins and the periodate chemical treatment suggested the presence of linear repeat glycan groups.

This work was performed on the monomeric PAPP-A and therefore did not include the proMBP molecule that may have been present in studies by other investigators, except those studies described by Oxvig et al, (**1994**). The PAPP-A/proMBP content of carbohydrates found by these investigators (Sinosich et al, (**1990**) and Sutcliffe et al, (**1980**)) to be 19.2 - 19.4% (w/w) and agrees well with that of 17.4% (w/w) obtained by Oxvig et al, (**1994**). However differences with the reported carbohydrate constituents of PAPP-A have been shown (Table 1.3, Chapter 1). The differences could have been due to the method of analysis or the purity of the PAPP-A preparations. Possible reasons for these discrepancies are considered further in section 7.4 of this Chapter.

As described in this section, PAPP-A is extensively glycosylated. This work suggests that PAPP-A contains N- and O- linked glycan groups. The presence of such an extensively post-translationally modified protein located at the placental interface is another indicator that suggests an important

role for PAPP-A at this interface. Because of the complex nature of the glycan component of PAPP-A, it is unlikely that recombinant PAPP-A expressed in bacteria will have the same biological activity as the native fully glycosylated PAPP-A that is produced during pregnancy. The function of the carbohydrate component could be further studied by comparing a recombinant PAPP-A protein (that is not post-translationally modified and hence lacks its carbohydrate groups) against PAPP-A that has been purified from the placenta.

7.3 Studies on An Interaction Between Reduced Monomeric PAPP-A and The Endoproteinase Arg-C.

7.3.1 Introduction

Whilst a limited proteolytic digest of PAPP-A with endoproteinase Arg-C was performed (Chapter 2, section 2.8.5), an interaction between these proteins was observed which resulted in the formation of an SDS stable 1:1 stoichiometric complex. Further studies on this interaction were undertaken to assess any affect on the enzyme were carried out by measuring the esterolytic activity of endoproteinase Arg-C (Levy et al, **1969**).

7.3.2 Materials and Methods

A brief description of the method used to generate the results presented in this section can be found in Chapter 2, section 2.9.

7.3.3 Results and Discussion

When a limited proteolytic digest of PAPP-A with the endoproteinase Arg-C was performed using an in-gel slice technique (Cleveland method,

Chapter 2, section 2.8.5), a surprising observation was made that the reduced monomeric chain PAPP-A formed a stoichiometric complex (Figure 7.1) with this enzyme (as determined from the size of this complex when fractionated on a 5-20% modified SDS-PAGE gel).

It has been demonstrated that the endoproteinase isolated from mouse submaxillary glands is composed of two forms, A and D (Levy et al, **1967**). Since their initial work, the kinetics have been further studied (Boesman et al, **1976**) and the specificity of the enzyme has been analysed (Schenkein et al, **1977**). The characterisation of the forms A and D led to the identification of form D as epidermal growth factor binding protein (Taylor et al, **1974**). The D form's activity was also noted to be affected by the presence of glycine. Schenkein et al, (**1980**) have subsequently demonstrated that the A form can be converted into the D form of this enzyme.

Reduced monomeric PAPP-A in the absence of glycine did not inhibit the endoproteinase Arg-C. The control BSA protein also did not have any measurable effect on the esterolytic activity of the enzyme. (Figure 7.2). Pre-incubation of this enzyme with PAPP-A for 30 minutes in the presence of glycine resulted in approximately 30% inhibition (The control protein BSA had no effect, Figure 7.3).

It was unknown whether digestion of PAPP-A would have occurred if PAPP-A was incubated with this enzyme in the absence of glycine as the electrophoresis system used for the limited proteolytic cleavage (Figure 7.1) also contained glycine as part of the electrophoresis running buffer.

It is accepted that the PAPP-A used for this work was in a reduced monomeric form that was eluted from an SDS-PAGE gel and the results illustrated here may not be found in native PAPP-A. However when a PAPP-A:Arg-C complex was observed (Figure 7.1) and as was illustrated in Table 7.3 that the formation of this complex required pre-incubation of PAPP-A with the enzyme suggesting that slow association between these proteins. This resulted in the inhibition of the enzyme because the control protein BSA, did not affect the activity of this enzyme and this interaction seemed specific for PAPP-A in the presence of glycine.

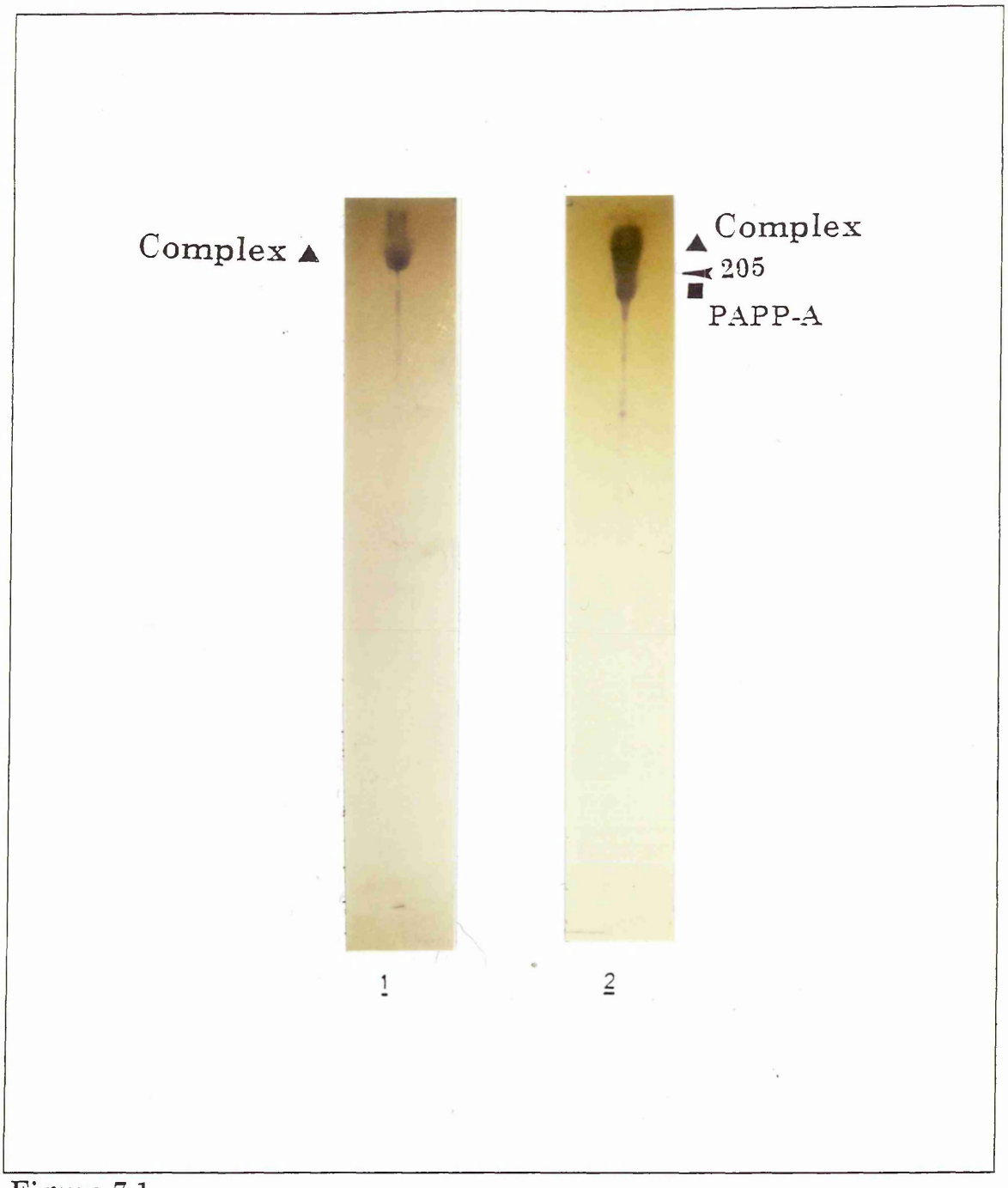


Figure 7.1

Incubation of PAPP-A containing gel slice with endoproteinase Arg-C

[**Legend:** Illustration of CBB stained electroblotted samples on a PVDF membrane from a 5-20% modified SDS-PAGE gel as described in Chapter 2, sections 2.3.2.2c, 2.3.2.2d and 2.4.1. **Note:** (1) and (2) were performed as limited proteolytic digestion in-gel slices as described in Chapter 2, section 2.8.5. PAPP-A enriched samples from the purification schemes described in Chapter 5 were prepared as gel slices containing PAPP-A and incubated with the enzyme, Endoproteinase Arg-C: (1): 1 µg and (2): 4 µg. The amount of PAPP-A present in the gel slices used in (1) and (2) was different (as assessed by densitometry). The arrow indicated the 205 kDa molecular weight marker, (▲), represented enzyme:PAPP-A complex and (■) represented excess, unreacted PAPP-A.]

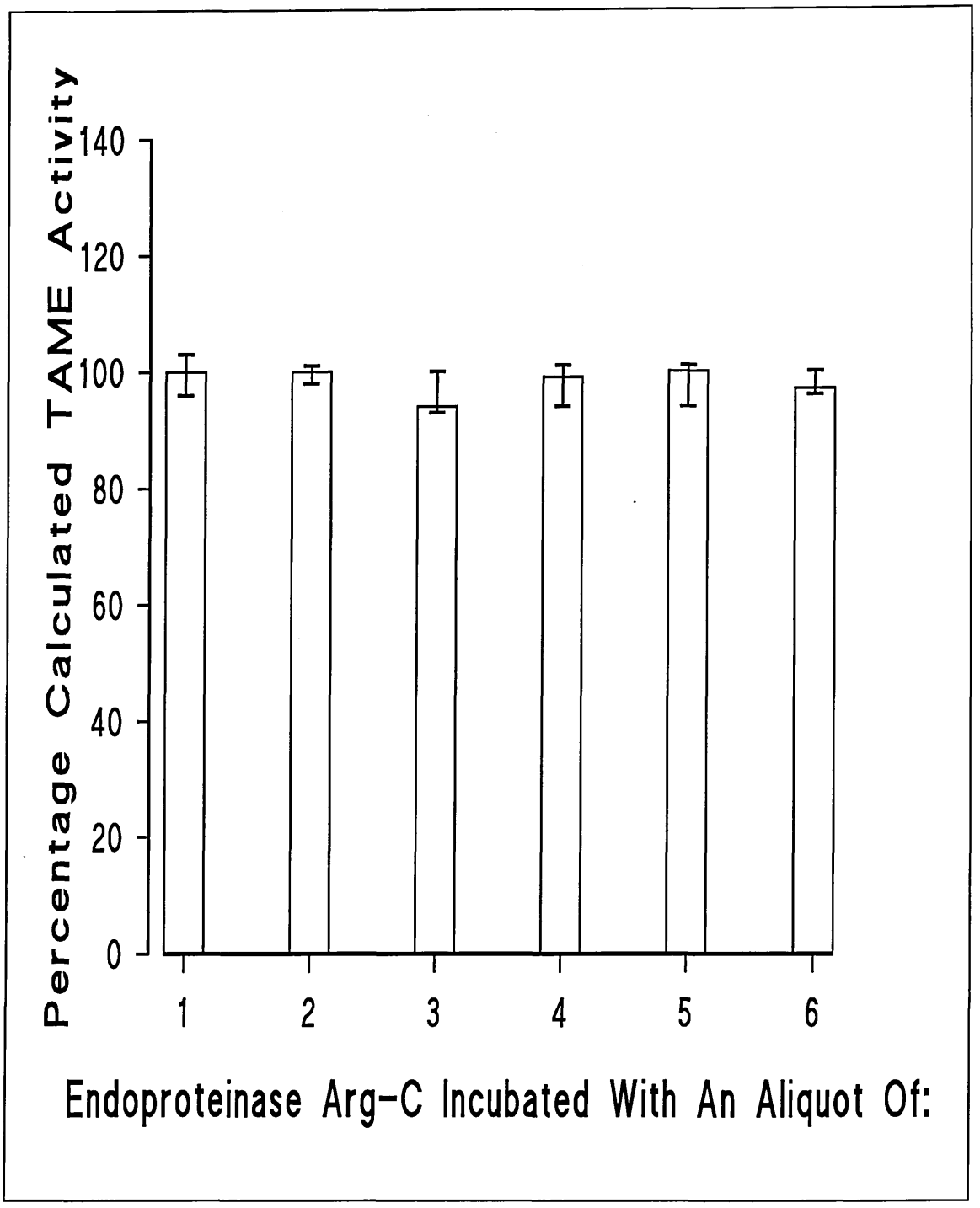


Figure 7.2

The calculated activity of the enzyme, endoproteinase Arg-C incubated with PAPP-A (non-specific control protein, BSA) in the absence of glycine.

[Legend: 5 µg of endoproteinase Arg-C was pre-incubated for 1/2 hour with: **(1)** Reaction buffer A, **(2)**: 5 µg of PAPP-A. **(3)**: 2.5 µg of PAPP-A. **(4)**: 1.25 µg of PAPP-A **(5)**: 15 µg of BSA and **(6)**: 5 µg of BSA. **Note:** The amount of protease was determined from specific activity of endoproteinase (Appendix 1), Eluted PAPP-A and BSA were calculated by densitometry as described in Chapter 4, section 4.3.4. Results were expressed as a mean activity of **(1)** +/- range for three determinations. The calculated % activity remaining in pre-treated enzyme aliquots **(2 - 6)** was calculated by comparing activity present in these aliquots to what was found in the mean aliquot **(1).]**

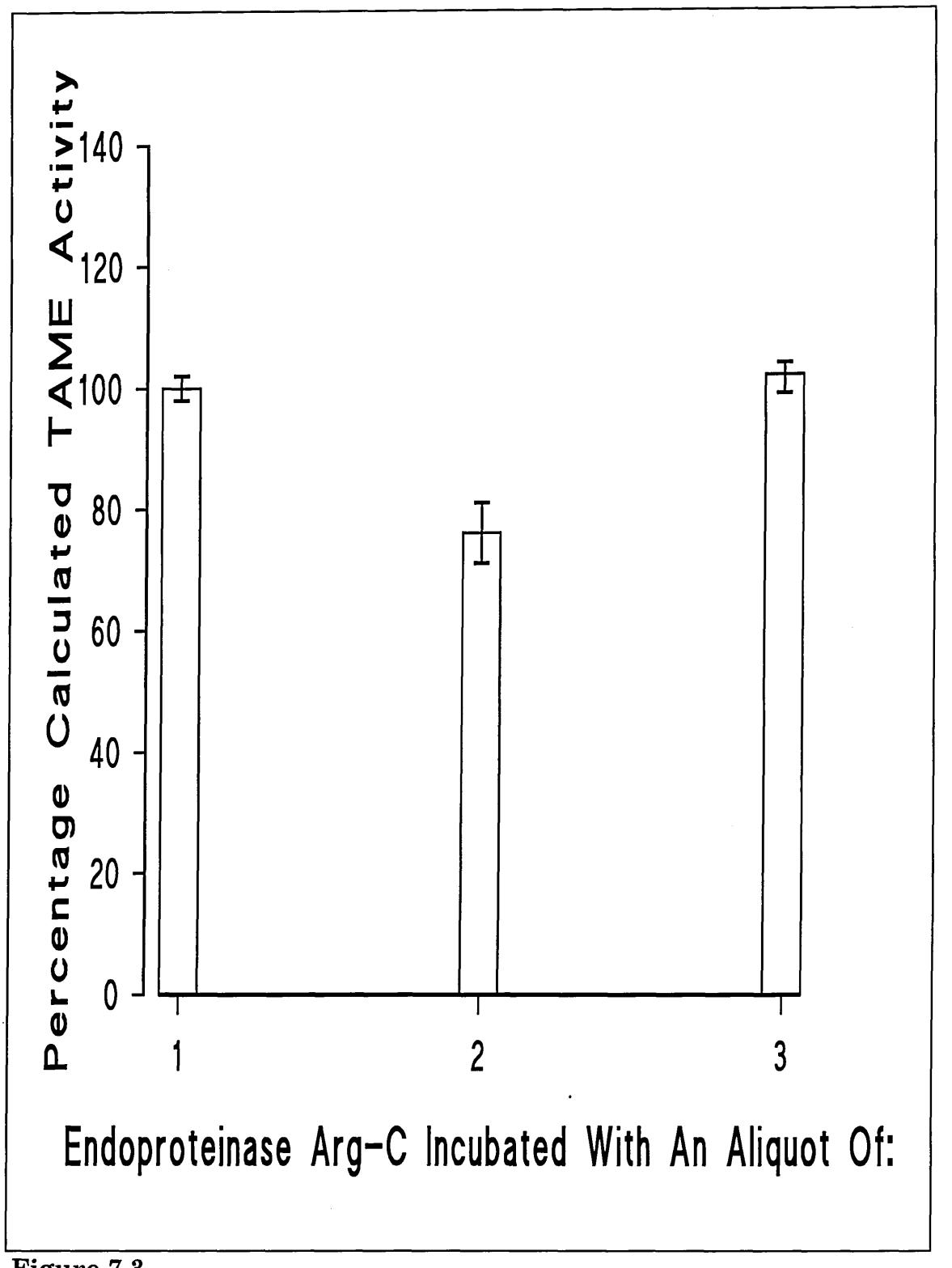


Figure 7.3

The calculated activity of the enzyme, endoproteinase Arg-C incubated with PAPP-A (non-specific control protein, BSA) in the presence of glycine.

[Legend: 5 µg of endoproteinase Arg-C was pre-incubated for 1/2 hour with: (1) Reaction buffer A containing 0.19 M glycine (Appendix 2) (2): 5 µg of PAPP-A in reaction buffer A containing 0.19 M glycine. (3): 15 µg of BSA in reaction buffer A containing 0.19 M glycine. **Note:** The amount of protein and the calculated TAME activity were estimated as described in Figure legend 7.2]

Table 7.3

The esterolytic activity of endoproteinase Arg-C after pre-incubation for various times with PAPP-A (or BSA) incubated in buffer A containing 0.19 M glycine.

Endoproteinase Arg-C pre-incubated with:	Time of Pre-incubation (Minutes)			
	0	5	15	30
PAPP-A	102 +/- 2	91 +/- 2	79 +/- 1	70 +/- 3
BSA	102 +/- 2	100 +/- 1	99 +/- 1	101 +/- 4

[Legend: 5 μg of endoproteinase Arg-C were incubated with buffer A containing 0.19 M glycine, 5 μg of PAPP-A or 15 μg of BSA. Results were expressed as % calculated TAME activity compared to the mean +/- range of esterolytic activity measured in the endoproteinase Arg-C incubated in buffer A containing 0.19 M glycine, 3 determinations were made for each time point. The esterolytic activity was determined after a 30 minute incubation with substrate as described in Chapter 2, section 2.3.8]

This inhibitory effect was only observed when a sequencing grade of endoproteinase Arg-C (Appendix 1) was used. However this inhibition was consistently observed with different batches of the enzyme. On a very speculative basis an explanation for this process could be that PAPP-A is interacting at the level of conversion of forms A to D that is known to occur (Schenkein et al, **1980**). The exact nature of this interaction will have to await further characterisation of the PAPP-A/Arg-C interaction. A specific inhibition of this enzyme was observed with the monomeric PAPP-A. Care should however be exercised in translating an *in-vitro* occurring reaction to what is observed *in-vivo.*

7.4 The Tertiary Structure Of PAPP-A

Information obtained during this study yielded data concerning the sub-unit structure of PAPP-A. Assuming a molecular weight of 205 kDa for myosin (Appendix 1), the reduced monomeric chain of PAPP-A has an estimated molecular weight of 195 +/- 5 kDa in the SDS-PAGE systems

that were employed in this thesis. This monomer was also disulphide bridged to form a dimer as it was detected in non-reducing conditions by the same gel system (Plate 5.2, Chapter 5).

Assuming that the monomer was 195 kDa, then a pure dimer not containing any pro-MBP should have a molecular weight of approximately 400 kDa. The proMBP has an estimated size of 29 kDa (Popken-Harris et al, **1994**) and Oxvig et al, (**1993**) has suggested an equimolar 2:2 complex of PAPP-A/proMBP therefore the proposed dimer should have a theoretical weight of at least 440 kDa. Therefore by looking at the PAPP-A separated by gel filtration, an assessment of the size and structure of this dimer can be made.The material that was eluted at 0.3 and 0.6 M NaCl from the heparin affinity matrix yielded PAPP-A that eluted in different fractions from the gel filtration column. The 0.3 M heparin eluted fraction had a molecular weight of approximately 480 kDa whereas the 0.6 M heparin eluted fraction had a molecular weight of approximately 400 kDa as determined by gel filtration chromatography (Figure 7.4). This suggests that the heparin affinity column is possibly separating different forms of PAPP-A. This is interesting in the light of the described proMBP/PAPP-A complex. A similar observation that PAPP-A purified from different sources is eluted from the heparin affinity matrix in different ways was made by others (Davey et al, (**1983**) and Davey and Teisner (**1982**). PAPP-A isolated from the placenta was eluted from the heparin affinity matrix at 0.6 M NaCl (it was also shown to have a very different pI to PAPP-A isolated from maternal serum, Chapter 1) but did not elute at 0.3 or 0.4 M NaCl. Whereas PAPP-A from maternal serum could be eluted from this matrix at 0.3, 0.4 and 0.6 M NaCl. Re-chromatography of the fraction eluted at 0.4 M resulted in a proportion of the protein which was eluted at 0.6M NaCl, thus suggesting that this affinity matrix allows separation of PAPP-A into fractions with different affinities for heparin. This could be due to molecular heterogeneity of PAPP-A. The differences in the ability of PAPP-A to inhibit HGE which have been observed between the material purified by Oxvig et al, (**1994**) and Sinosich

(1990) could also be due to a difference in the tertiary structure of the PAPP-A prepared by these two groups.

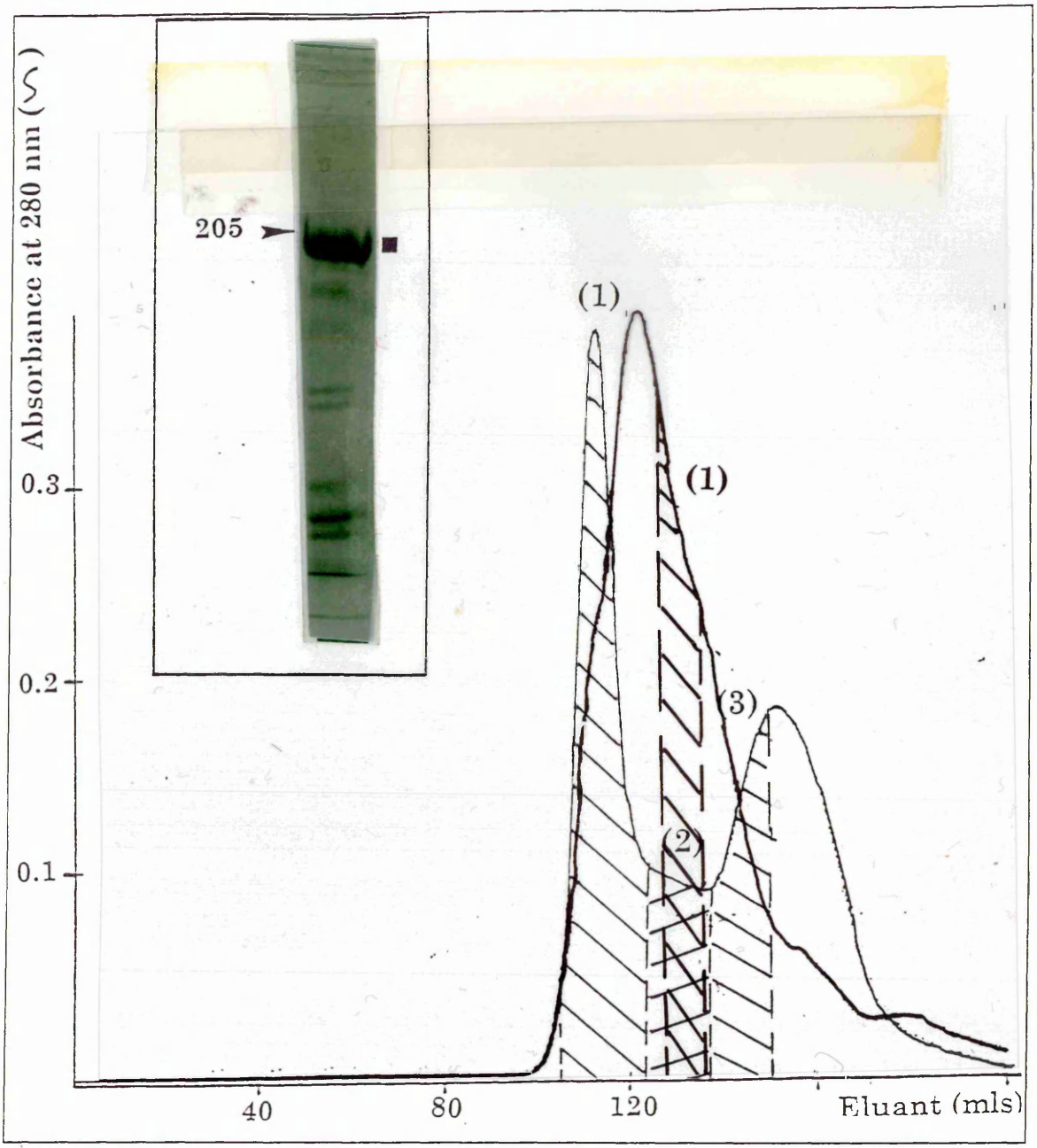


Figure 7.4

Elution profile of 0.3 and 0.6 M (Figure overlay) heparin eluted fractions on a SuperDex-200 gel filtration column (**Plate inset overlay 7.4**: A 5% SDS-PAGE gel of the fraction containing the peak PAPP-A of material eluted at 0.6 M from the heparin affinity matrix).

[**Legend:** PAPP-A was determined by SDS-PAGE as previously described, the indicated fractions were separated on a 5% SDS-PAGE gel, Plates 5.1 and 5.2). **Note:** SDS-PAGE of 0.6 M eluted heparin fraction and material separated on the gel filtration column **(1):** Fractions 4/5. The arrow represented the 205 kDa size marker and the PAPP-A marked was detected by immunodetection with anti- PAPP-A antibodies as previously described.]

The proposed PAPP-A/proMBP complex raises interesting questions about the observed relationship between these two proteins as the affinity of PAPP-A for L-arginine that is described in this thesis (Figure 2.16, Chapter 2) demonstrates that PAPP-A will bind to L-Arginine. proMBP is rich in arginine (Gleich et al, **1979**) and therefore may associate with PAPP-A. Once in close association proMBP has been shown to have a tendency to form SDS-stable oligomers. Recently Oxvig et al, (**1995**) have also demonstrated that proMBP also forms complexes with other plasma constituents. An understanding as to the specificity and affect of these interactions on the individual components will have to await further studies to characterise this interaction.

A unique feature of the placenta is that it continues to undergo differentiation throughout the gestation period (Boime et al, **1986**). This necessitates the regulated expression of specific genes at different stages, e.g. only 3 of 7 β-hCG genes are expressed in the placenta. The presence of these proteins in extraplacental sites suggest that they may be involved in other physiological processes such as cell growth. The multigene nature of proteins produced by the placenta has been demonstrated for other placental proteins, e.g. SP1 (Chan and Qui, **1988**). Bonno et al, (**1994a, 1994b**) demonstrated by *in-situ* hybridisation that proMBP and PAPP-A were synthesised by X-cells of the placental septa. In addition PAPP-A was also synthesised in syncytiotrophoblast cells of the placenta. The PAPP-A mRNA is present as two distinct species of 8 and 12 kbp (Kristensen et al, **1994**). Although a single N-terminus has been found by microsequencing of purified PAPP-A this does not exclude the possibility that N-terminally blocked chain(s) were also present in this material. Analysis of the proteolytic cleavage products demonstrated the presence of amino acid stretches that were not present within the published cDNA sequence for PAPP-A (Kristensen et al, **1994**). Thus the tertiary structure and forms of PAPP-A present still remain to be resolved. Suggestions from the work in this thesis are that different PAPP-A complexes may exist in the maternal serum but this requires further investigation.

CHAPTER EIGHT

8.1 Summary and General Discussion

The aim at the start of this work was to isolate and characterise the PAPP-A cDNA with the view to study the structure and expression of the PAPP-A gene. A commercially prepared library of placental cDNA's in an expression vector lambda phage gt11 was selected for this purpose. A polyclonal antibody against PAPP-A (DAKO) was used for screening. Characterisation of this antibody by Western blotting detected a single band which had size and tertiary structure as described for PAPP-A by other investigators (Chapter 1, section 1.4.2.1). However one clear band of PAPP-A monomer was only achieved when:

- The antibody was incubated in a high (0.6 M NaCl) salt concentration.
- The antibody was also used at a relatively high concentration, a 1:600 dilution.

Approximately 1.2 million plaques were screened and no putative positive clone was found. Western blotting with this antibody revealed that the sub-population of paratopes in the anti-PAPP-A antiserum which was used at this high salt concentration required a glycan component as part of the antigenic determinant(s). Polypeptides expressed in *E. coli* are not post-translationally modified e.g. glycosylated (Chapter 3). Thus an alternative approach to isolate the PAPP-A cDNA was undertaken, this utilised oligonucleotide probes. Such probes necessitated the knowledge of some primary amino acid structure for PAPP-A. No primary amino acid information was available for PAPP-A when this work was being undertaken. The PAPP-A available and suitable for use as a Tracer in a PAPP-A RIA was not pure PAPP-A and was composed of at least 6 different proteins (Table 6.2 , Chapter 6). Therefore purification of PAPP-A was undertaken to produce PAPP-A in a form that would yield the primary sequence information that was necessary for the production of PAPP-A specific oligonucleotide probes.

It was hoped that a purification scheme based on the described affinity of PAPP-A for heparin would yield material sufficiently enriched to facilitate its microsequencing. Heparin affinity matrices (Affi-gel and Pharmacia, post-1984) did not enrich PAPP-A as expected. The heparin affinity matrix produced post-1984 had lost its ability to bind PAPP-A at a salt concentration of 0.6 M NaCl. Other investigators had previously shown that it was this high affinity binding site that was necessary to enrich PAPP-A and separate it from other plasma protein contaminants (Davey et al, **1983**). The loss of this high affinity site necessitated the development of purification schemes to enrich PAPP-A.

Subsequent control experiments with a heparin affinity matrix produced pre-1984 confirmed that the heparin matrix was capable of enriching PAPP-A, it was determined that the provider of the matrix (Pharmacia) had changed its supplier of the affinity matrix. Information from this supplier revealed that the chemistry used to link the heparin to the affinity matrix and the type of heparin preparation had been changed. The original manufacturer of the heparin matrix pre-1984 was found (Reactiv's IBF) and it was found that this affinity matrix was capable of enriching PAPP-A as previously described (Davey et al, **1983**).

The use of the heparin affinity matrix (Reactiv's IBF) to purify PAPP-A demonstrated a heterogeneity of PAPP-A separated as distinguished by what bound and eluted at 0.3 M and 0.6 M NaCl from this column and was subsequently separated by gel filtration (Figure 7.4, Chapter 7). This difference that was seen in the tertiary structure of PAPP-A could explain the contradictory results regarding its activity on Human granulocyte elastase that have been found by different groups for PAPP-A purified by different groups (Sinosich and Zaker, (**1991**), Bischof and Meisser, (**1988**) and Oxvig et al, (**1994**).

Microsequencing of the PAPP-A purified during this thesis revealed a protein with a single N-terminal sequence (Table 6.3, Chapter 6) thus indicating that the material isolated as a monomeric chain was homogeneous. Analysis of this sequence against the Daresbury Database

demonstrated that the N-terminal region obtained did not share any homology with any published sequences at that time. The verification of any clones identified would have also required further primary sequence information. Therefore further limited partial proteolytic digests were performed (Chapter 6). These digests consistently yielded N-terminal amino acid sequence or a mixture of sequences that were not well enough separated to be useful in determining any internal primary sequence information for PAPP-A. One digest did however reveal a putative internal stretch for PAPP-A. Degenerate primers were designed and synthesised from this information (Table 2.7, Chapter 2). These degenerate primers yielded two PCR products (Approximately 500 and >1200 bps), when the placental cDNA library was used as the DNA template thus confirming that PAPP-A was expressed at a mRNA level in the placenta during pregnancy (Chapter 6, Plate 6.1).

An analysis of the carbohydrate structure of PAPP-A from work described in this thesis demonstrated the presence of O and N-linked carbohydrate residues. The N-linked residues were approximately 8% (w/w) of the monomeric PAPP-A. The O-linked chains were extensively modified and consisted of repeat oligomers of N-acetyl-glucosamine. It was this structure that was also responsible for binding to the antibody paratope subset that was shown to bind at a high (0.6 M NaCl) salt concentration (Chapter 7). PAPP-A is thus extensively glycosylated and was shown to contain O-linked groups that were post-translationally modified because these glycan groups could not be removed using standard enzymatic techniques (Chapter 7).

An interaction with endoproteinase Arg-C (or epidermal growth factor binding protein) was observed using reduced monomeric PAPP-A. These two proteins formed a complex that was stable in SDS. A slow association of PAPP-A to endoproteinase Arg-C was demonstrated that resulted in inhibition of the esterolytic activity of this enzyme (Chapter 7).

It was hoped that during this study to characterise the cDNA for PAPP-A, however because of the factors described above and the time that was

required to produce partial amino acid sequence information for this protein this was not achieved. The N-terminal sequence of the PAPP-A monomer produced in this thesis has subsequently been confirmed by Oxvig et al, (**1993**).

8.2 Hypothesis Of A Possible Function For PAPP-A

Many clinical and biochemical observations have been made about PAPP-A by various investigators since its discovery in the early '70s, some of them are listed in Figure 8.1. Exploiting the observations made by these investigators and what was seen for PAPP-A from work described in this thesis, I would like to speculate on a possible function for PAPP-A protein. I would like to propose that PAPP-A is involved in the regulation of growth hormones. These are necessary for the growth of some tissues; such as the developing foetus, but would cause inappropriate growth in adult tissues I propose that PAPP-A acts as a barrier to control the inappropriate secretion of growth hormones.

Taylor et al, (**1974**) suggested that EGF-BP converts epidermal growth factor (EGF) from an inactive to active form in a similar manner to that observed for Kallikrein. EGF has a carboxy terminal arginine and the esterase, EGF-BP removes this and converts it to its active form. The observed interaction described in this thesis (Chapter 7) between PAPP-A and EGF-BP may act to limit the activity of some form of the pro-enzyme/hormone.

If PAPP-A was performing this function then it would be expected to be found almost entirely in the maternal rather than foetal compartments, as is seen for PAPP-A. Other areas that exhibit growth patterns inappropriate to the adult tissues are those that are involved in development of gametes. PAPP-A has also been found in seminal plasma and follicular fluid (Figure 8.1)

Maternal serum PAPP-A levels are increased in Twin pregnancies and reduced in conditions that result in growth retardation, such as ectopic pregnancy. Reduced levels of Maternal serum PAPP-A are also observed in

Cornelia-de-Lange and Down's syndrome and could also be explained by this control hypothesis as they are both conditions which have elements of a growth disorder. One interesting observation reported is that the protease nexin-2 has been seen to form stable stoichiometric inhibitory complexes with EGF-BP that are stable after treatment with SDS (Van Nostrand et al, **1990**). This has been postulated to have an affect on cause of the neurodegenerative lesions that are seen in Downs syndrome. It was also noted that PAPP-A was very difficult to separate from the serpin, alpha-2-macroglobulin. A number of Kallikrein-like enzyme inhibitors have been demonstrated in the mouse, but relatively few have been described in the human, perhaps PAPP-A is part of this family.

A single amino acid replacement of arginine to valine in APP-KD, a member of this family results in the formation of a potent inhibitor of HGE (Sinha et al, **1991**), and may explain PAPP-A's inhibition of HGE.

Clinical Observations

PAPP-A levels were raised in:

- Twin pregnancies (Lin et al, **1974a**).

PAPP-A levels found to reduced in:

- Downs (Wald et al, **1992**).
- Cornelia-de-Lange syndrome (Westergaard et al, **1983c**).
- Ectopic pregnancy (Tornehave et al, **1987**).

Observed Localisation of PAPP-A

- Unilateral secretion into the maternal compartment during pregnancy.
- Present within granulosa cells, production was static in atretic follicles, levels were depressed in ovarian cysts where no egg was present but increased where two oocytes were growing compared to single follicular event.
- PAPP-A found in seminal plasma but absent from the Testes and proximal portions of the vas deferens. (Sinosich, **1985**)

Observed Biochemical Features:

- Interaction and inhibition of esterolytic properties of endoproteinase Arg-C, which was also shown to be epidermal growth factor binding protein. (Work described in this thesis).

Figure 8.1
Observations made for PAPP-A

The PAPP-A that was isolated and used in this study was in a reduced monomeric form (while it is present as a disulphide bridged dimer in the blood). It was also denatured, with the extent of refolding by the processes used to elute the protein being unknown. Therefore the described observations seen between PAPP-A and the endoproteinase Arg-C were with material that was dissimilar to that found under physiological conditions, more work will have to be done to confirm or refute these findings. It was however seen to bind to endoproteinase Arg-C in a very specific way, with control proteins and PAPP-A in the absence of glycine seen not to inhibit this enzyme, thus a specific physical interaction has been observed (Figure 7.1, Chapter 7) that was noted to have an inhibitory affect on the esterolytic properties of this enzyme (Figure 7.3, Chapter 7), in particular the D form that has subsequently been shown to be EGF-BP.

8.3 Conclusions and Possible Future Work for PAPP-A

PAPP-A has thus been confirmed to be a disulphide linked dimer. Studies on the purification of PAPP-A have revealed heterogeneity in size for the PAPP-A isolated. In light of the proposed PAPP-A/proMBP structure (Oxvig et al, (**1993**)) it is unknown whether all the PAPP-A is linked to pro-MBP. PAPP-A has shown to be linked to bind to L-Arginine, pro-MBP has been shown to bind other basic proteins in the serum (Oxvig et al, (**1995**)) thus it is unknown whether all the PAPP-A or that isolated using the purification schemes described in Chapter 5 contains pro-MBP bound to PAPP-A. At the time this work was being undertaken, serum components were seen to affect the measurement of PAPP-A (Chapter 4) but it was unknown what these components were. It would be interesting to determine the presence or absence of proMBP as bound to PAPP-A by Western blotting using a specific antibody for proMBP for PAPP-A isolated using the purification schemes described in this thesis. The PAPP-A monoclonal antibody produced by Mowles et al, (**1986**) was shown in this thesis to bind to the PAPP-A dimer (Chapter 3) but not to the monomeric form, it would be interesting to re-examine this binding in light of the proposed proMBP interaction to

evaluate whether the monoclonal antibody was binding to PAPP-A or the proposed PAPP-A/proMBP complex. As seen in Chapter 4, an ELISA was produced for PAPP-A that measured a component present in the plasma of men, it would be interesting to examine whether this component being measured was in fact proMBP. The PAPP-A tertiary structure has been seen to have an affect on PAPP-A measurements and has been observed by many investigators (Chapter 1, section 1.4.5). During the development of methods to measure PAPP-A different levels and effects were seen on the individual assay systems that also seemed to be linked to the tertiary structure of the PAPP-A molecule. An investigation into the affect proMBP has on immunoassay systems should be made as this may explain why different groups are obtaining contradictory results when trying to use PAPP-A as a biochemical marker for clinical conditions (Chapter 1, section 1.4.7).

The work described in this thesis has produced a series of new questions for PAPP-A but further confirms that PAPP-A is likely to be an important protein that is located at the border between maternal and foetal systems.

The further study of such a protein at the genetic level should answer some of the questions that have been raised about PAPP-A in this thesis.

The isolation of PAPP-A cDNA and expression of PAPP-A in heterologous systems will enable the generation of large amounts of pure PAPP-A, free of proMBP. However it has been demonstrated that PAPP-A is extensively glycosylated so this type of expression will have to occur in mammalian cell systems that are capable of mimicking the glycosylation found on the native protein. Protein expressed in this way could be used further to examine the interactions that have been described for endoproteinase Arg-C and HGE.

The use of probes with *in-situ* PCR in the clinical conditions of Down's and Cornelia-de-Lange syndrome should help clarify whether the levels of PAPP-A that are reduced in the blood at a protein level are also reduced at a mRNA level in syncytiotrophoblast cells. These approaches at a DNA level will help to confirm and possibly offer an insight into the control and allow a definition of the function of this intriguing protein.

CHAPTER NINE

Aaij, C. and Borst, P. (**1972**). " The Gel Electrophoresis of DNA. " **Biochim. Biophys. Acta.** Vol 241, pp 120 - 125.

Alexander, N.J. (**1989**). " Natural and induced immunological infertility. " **In:** Current Opinion in Immunology (Johnson, P.M.:**Ed.**) Volume 1, pp 1125 - 1130

Amaral, D., Kelly-Falcoz, F. and Horecker, B.L. (**1966**). " Galactose oxidase of Polyporus circinetus. " **Methods In Enzymology.** Vol. IX, pp 87 - 92.

Andrews, A.T. (**1989**). " Peptide Mapping. " **IN:** Gel Electrophoresis of Proteins: A Practical Approach (Hames, B.D. and Rickwood, D.:**Eds.**). IRL Press, Oxford. 2nd Edition, pp 301 - 318.

Anthony, F., Masson, G.M. and Wood, P.J. (**1983**) " Development of a radioimmunoassay for pregnancy - associated plasma protein - A and establishment of normal levels in the first trimester of pregnancy." **Ann. Clin. Biochem.** Vol 20, pp 26 - 30.

Axelsen, N.H. (**1983**). Handbook of immunoprecipitation in gel techniques. Blackwell Scientific Publishers, Oxford.

Bainbridge, B.W. (**1991**). " Microbiological techniques for molecular biology: Bacteria and Phages. " **IN:** Essential Molecular Biology: A practical approach Volume I. (Brown, T.A. :**Ed.**). IRL Press, Oxford, pp 13 - 45.

Barnea, E.A., Bischof, P., Page, C., Decherney, A.H., Herrmann, W. and Naftolin, F. (**1986a**). " Placental and circulating pregnancy - associated plasma protein - A concentrations in normal and pathological pregnancies. " **Obstet. Gynecol.** Vol 68, pp 3823 - 3886.

Barnea, E.R., Sanyal, M.K., Brami, C. and Bischof, P. (**1986b**). " *In-vitro* production of pregnancy - associated plasma protein - A (PAPP-A) by trophoblastic cells." **Arch. Gynecol.** Vol 237, pp 187 - 190.

Baron, D.N. **:Ed.** (**1994**). " Units, Symbols and Abbreviations: A guide for Medical and Scientific Writers. " The Royal Society Medical Press, London. 5th Edition.

Bej, A. K., Mahbubani, M.H. and Atlas, R.M. (**1991**). " Amplification of Nucleic Acids by Polymerase Chain Reaction (PCR) and other methods and their applications. " **Crit. Rev. Biochem. Mol. Biol.** Vol 26, pp 301 - 334.

Bers, G. and Garfin, D. (**1985**). " Protein and Nucleic Acid Blotting and Immunobiochemical detection. " **BioTechniques.** Vol 3, pp 276 - 288.

Bell, S.C.(**1988**). " Secretory endometrial proteins and hormones - toward a molecular basis for endometrial / fetoplacental paracrine interactions. " **IN:** Placental and endometrial proteins: Basic and Clinical apsects. Proceedings of the 6 th International Congress on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 27 - 34.

Bersinger, N.A. Keller, P.J., Naiem, A., Fischer, M. and Schneider, H. (**1987**). " Pregnancy - specific and pregnancy - associated proteins in threatened abortion. " **Gynecol. Endocrinol.** Vol 1, pp 379 - 384.

Bersinger, N.A., Malek. A., Benz. B., Keller, P.J. and Schneider, H.(**1988**) " Effect of protein synthesis inhibitors and metabolic blockers on the production of placental proteins by the *in - vitro* perfused human placenta. " **Gynecol. Obstet. Invest.** Vol 25, pp 145 - 151.

Bersinger, N.A., Brizot, M.L., Johnson, A., Snijders, R.J.M., Abbott, J., Schneider, H. and Nicolaides, K.H. (**1994**). " First trimester maternal serum pregnancy - associated plasma protein A and pregnancy - specific β1 - glycoprotein in fetal trisomies. " **Br. J. Obstet. Gynaecol.** Vol 101, pp 970 - 974.

Bischof, P. (**1979a**). "Purification of pregnancy associated plasma protein A (PAPP-A). " **Arch. Gynecol.** Vol 227, pp 315 - 326.

Bischof, P. (**1979b**). " Observations on the isolation of pregnancy - associated plasma protein - A. " **IN**: Placental proteins. (Chard, T. and Klopper, A. **:Eds.**) Springer-Verlag, Berlin, pp 105 - 119.

Bischof, P., Bruce, D., Cunningham, P. and Klopper, A. (**1979c**). " Measurement of pregnancy associated plasma protein A (PAPP-A). " **Clin. Chim. Acta**. Vol 95, pp 243 - 247.

Bischof, P., DuBerg, S., Herrmann, W. and Sizonenko, P.C. (**1981a**). " Pregnancy - associated plasma protein-A (PAPP-A) and hCG in early pregnancy. " **Br. J. Obstet. Gynaecol.** Vol 88, pp 973 - 975.

Bischof, P., Gervaix, A., Meisser, A. and Suter, S. (**1990**) " Pregnancy associated plasma protein -A induced inhibition of human leukocyte elastase: An artifact. " **Gynecol. Obstet. Invest.** Vol 29, pp 169 - 172.

Bischof, P. and Megevand, M. (**1986**). " Pregnancy - associated plasma protein - A concentrations in men with testicular and prostatic tumors. " **Arch. Androl.** Vol 16, pp 155 - 160.

Bischof, P. and Tseng, L. (**1986**). " In Vitro release of pregnancy-associated plasma protein - A (PAPP-A) by human endometrial cells. " **Am. J. Reprod. Immunol. Microbiol.** Vol 10, pp 139 - 142.

Bischof, P., Haenggli, L., Sizonenko, M.T., Herrmann, W.L. and Sizonenko, P.C. (**1981b**). " A radioimmunoassay for the measurement of pregnancy-associated plasma protein - A (PAPP-A) in humans." **Biol. Reprod.** Vol 24, pp 1076 - 1081.

Bischof, P.. Martin - du - Pain, R., Lauber, K., Girard, J., Herrmann, W. and Sizonenko, P. (**1983**). " Human seminal plasma contains a protein that shares physicochemical, immunochemical and immunosuppressive properties with pregnancy - associated plasma protein-A (PAPP-A). " **J. Clin. Endocr. Metab.** Vol 58, pp 359 - 362.

Bischof, P.,Mignot, T.M. and Cedard, L (**1989**). " Are pregnancy-associated plasma protein - A (PAPP-A) and CA 125 measurements after IVF-ET possible predictors of early pregnancy wastage ? " **Placenta. (Abstract).** Vol 10, pp 493 - 494.

Bischof, P. and Meisser, A. (**1988**). " Immunological and Biochemical heterogeneity of pregnancy - associated plasma protein-A (PAPP-A) : A possible explanation of ongoing controversies." **IN:** Placental and Endo -metrial proteins: Basic and Clinical aspects. Proceedings of the 6th International Congress on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 19 - 26.

Bischof, P., Sizonenko, M.T. and Herrmann, W.L. (**1986**). " Trophoblastic and decidual response to RU486 effects on human chorionic gonadotrophin, human placental lactogen, prolactin and pregnancy - associated plasma protein - A production *in - vitro.* " **Hum. Reprod.** Vol 1, pp 3 - 6.

Bjerrum, O.J. (**1983**). " Electroimmunopreciptation Artefacts. " **Scand. J. Immunol.** Vol 17(suppl. 10), pp 333 - 342.

Bjorkman, N. (**1986**). " Comparative Stuctural and Functional Features Of The Mammalian Placenta. " **IN:** Pregnancy Proteins in Animals. (Hau, J.:**Ed.**). Walter de Guyter Press, Berlin, pp 1 - 8.

Blazer, F.W., Blair, C., Clark, D.A., Coulham, C., Gill, T.J. and Wegmann, T. (**1991**). " Materno-foeto-placental interactions. " **J. Reprod. Fertil.** Vol 92, pp 231 - 244.

Boesman, M., Levy, M. and Schenkein, I. (**1976**). " Esteroproteolytic Enzymes from the Submaxillary Gland: Kinetics and other Physicochemical properties. " **Archiv. Biochem. Biophys.** Vol 175, pp 463 - 476.

Bohn, H. (**1971**). Detection and characterization of pregnancy proteins in the human placenta and their quantitative immunochemical determin--ation in sera from pregnant women." **Arch. Gynaekol.** Vol210, pp 440.

Bohn, H. (**1973**). " Isolerung des Plazenta - Proteins PP2 und seine Identifizierung als Ferritin. " **Arch. Gynaekol.** Vol 215, pp 263 - 275.

Bohn, H. and Winckler, W. (**1988**). " Isolation and Characterisation of placental proteins with special reference to the proteins solubilized by Triton X - 100. " **IN:** Placental and Endometrial Proteins: Basic and Clinical aspects. Proceedings of the 6 th International Congress on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 3 - 10.

Boime, H., and W einmann, E. (**1986**). " Structure and expression of human placental hormone genes. " **IN:** Advances in experimental medicine and biology: Molecular and cellular aspects of reproduction. (Dhindsa, D.S. and Bahl, O.P.**: Eds**). Plenum Press, London. **Vol 205**, pp 267 - 280.

Bolton, A.E. (**1981**). " The Preparation And Assessment Of Radioiodinated Tracers For Immunoassays. " **Irish Veter. J.** Vol 35, pp 230 - 237.

Bolton, A.E. and Hunter, W.M. (**1986**) " Radioimmunoassay and Related Methods. " **IN:** Handbook of Experimental Immunology. Volume 1. (Weir, D.M., Blackwell, C.C. and Herzenberg, L.A. **:Eds.**). Blackwell Scientific Publishers, Oxford and Edinburgh. Chapter 26.

Bolton, A.E., Pinto-Furtado, L.G., Andrew, C.E. and Chapman, M.G. (**1986**). " Measurement of the pregnancy - associated plasma protein A in human seminal plasma." **Clin. Reprod. Fertil.** Vol 4, pp 233 - 40.

Bonno, M., Kephart, G.M., Carlson, C.M., Loegering, K.K. and Gleich, J. (**1994a**). " Expression of Eosinophil - Granule Major Basic Protein Messenger Ribonucleic Acid in Placental X Cells. " **Laboratory Invest.** Vol 70, pp 234 - 241.

Bonno, K., Oxvig, C., Kephart, G.M., Wagner, J.M., Kristensen, T., Sottrup-Jensen, L. and Gleich, G.J. (**1994b**). " Localization of Pregnancy-Associated Plasma Protein - A and Colocalization of Pregnancy-Associated Plasma Protein - A Messenger Ribonucleic Acid and Eosinophil Granule Major Basic Protein Messenger Ribonucleic Acid in Placenta. " **Laboratory Invest.** Vol 4, pp 560 -566.

Bradford, M. (**1976**). " A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. " **Anal. Biochem.** Vol 72, pp 248 - 254.

Brambati, B., Macintosh, M.C.M., Teisner, B., Maguiness, S., Shrimanker, K., Lanzani, A. Bonacchi, I., Tului, L., Chard, T. and Grudzinskas, J.G. (**1993**). " Low maternal serum levels of pregnancy associated plasma protein A (PAPP-A) in the first trimester in association with abnormal fetal karyotype. " **Br. J. Obstet. Gynaecol.** Vol 100, pp 324 - 326.

Brambati, B., Tului, L., Bonacchi, I., Shrimanker, K., Suzuki, Y. and Grudzinskas, J.G. (**1994**). " Serum PAPP-A and Free β-hCG are First Trimester Screening Markers For Down Syndrome. " **Pren. Diagn.** Vol 14, pp 1043 -1047.

Brown, J.L and Robert, W.K. (**1976**). " Evidence that approximately eighty percent of the soluble proteins from Ehrlich ascites cells are N^{α}-Acetylated. " **J. Biol. Chem.** Vol 251, pp 1009 - 1014.

Bruner, J.P. and Hsia, Y.E. (**1990**). " Prenatal findings in Brachmann-de Lange syndrome. " **J. Obstet. Gynecol.** Vol 76, pp 966 - 968.

Bueler, M.R. and Bersinger, N.A. (**1989**). " Antiserum to pregnancy-associated plasma protein A (PAPP-A) recognizes human haptoglobulin. " **Br. J. Obstet. Gynaecol.** Vol 96, pp 867 - 869.

Buyse, M.L.:**Ed** (**1990**). **IN:** Birth Defects Encyclopedia. Blackwell Scientific Publishers. Cambridge, Massachusetts.

Castellano, F.J. and Sodetz, J.M.(**1969**). " Rabbit Plasminogen and plasmin isozymes. " **Methods in Enzymology**. Vol 19, pp 273 - 283.

Chard, T.(**1976**). " Normality and abnormality." **IN:** Plasma hormone assays in evaluation of fetal wellbeing. (Klopper, A. **:Ed.**). Churchill Livingstone Press, Edinburgh, pp 1 - 11.

Chard, T.(**1986**). " Pregnancy proteins in the human - biological and clinical significance. " **IN:** Pregnancy Proteins in Animals. (Hau, J. **:Ed.**). Walter de Guyter Press, Berlin, pp 9 - 20.

Charbonneau, H. (**1991**). " Strategies for obtaining partial amino acid sequence data from small quantities (5 nmol) of pure or partially purified protein. " **IN:** A practical guide to protein and peptide purification for microsequencing. (Matsudaira, P.T. **:Ed.**). Academic press, San-Diego, pp 15 - 30.

Chemnitz, J., Folkersen, J., Teisner, B., Sinosich, M.J., Tornehave, D., Westergaard, J.G., Bolton, A.E. and Grudzinskas, J.G. (**1986**). " Comparison of different antibody preparations against pregnancy-associated plasma protein - A (PAPP-A) for use in localization and immunoassay studies. " **Br. J. Obstet. Gynaecol.** Vol 93, pp 916 - 923.

Childs, R.A., Pennington, J., Uemura, K., Scudder, P., Goodfellow, P.N., Evans, M.J. and Feizi, T. (**1983**). " High - molecular weight glycoproteins are the major carriers of the carbohydrate differentiation antigens I, I and SSEA-1 of mouse teratocarcinoma cells. " **Biochem. J.** Vol 215, pp 491 - 503.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (**1977**). " Peptide mapping by limited proteolysis in Sodium Dodecyl Sulfate and analysis by Gel Electrophoresis. " **J. Biol. Chem.** Vol 252, pp 1102-1106.

Cohn, W.E. (**1984**). " Nomenclature and Symbolism of α-Amino acids. " **Methods In Enzymology.** Vol 106, pp 3 - 21.

Compton, S.J. and Jones, C.G. (**1985**). " Mechanism and Dye Response to Interferences in the Bradford Protein Assay. " **Anal. Biochem.** Vol 151, pp 369 - 374.

Cuckle, H.S., Wald, N.J. and Thompson, S.G. (**1987**). "Estimating a woman's risk of having a pregnancy associated with Down syndrome using her age and serum alpha - fetoprotein level. " **Br. J. Obstet. Gynaecol.** Vol 94, pp 387 - 402.

Cuckle, H.S. and Wald, N.J. (**1992**). " hCG, Estriol, and other Maternal Blood Markers of Fetal Aneuploidy. " **IN:** Maternal Serum Screening for Fetal Genetic Disorders. (Elias, S. and Simpson, J.L.: **Eds**). Churchill Livingstone Inc., New York, pp 87 - 107.

Davey, M.W., Teisner, B., Sinosich, M.J. and Grudzinskas, J.G. (**1983**). " Interaction between Heparin and Human pregnancy - associated plasma protein - A (PAPP-A): A simple purification procedure. " **Anal. Biochem.** Vol 131, pp 18 - 24.

Davey, M.W. and Teisner, B. (**1982**). " Purification and characterisation of pregnancy associated plasma protein A. " **IN:** Pregnancy Proteins. Biology, chemistry and clinical application. (Grudzinskas, J.G., Teisner, B. and Seppala, M.:**Eds**). Academic press, Sydney, pp 313-319.

Davis, B.J. (**1964**). " Disc electrophoresis - II: Method and application to human serum proteins. " **Ann. N.Y. Acad. Sci.** Vol 121, pp 404 - 427.

DuBerg, S., Bischof, P. Schindler, A.M., Beguin, F., Herrmann, W. and Sizonenko, P.C. (**1982**). " Tissue and plasma concentrations of pregnancy - associated plasma protein - A (PAPP-A): Comparison with other fetoplacental products. " **Br. J. Obstet. Gynaecol.** Vol 89, pp 352 - 357.

Duenas, J.L., Silvan, J.M. and Navarro, J. (**1988**). " Immunohistochemical location of PAPP-A and PP12 in normal and pathological female sex organs." **IN:** Placental and Endometrial Proteins: Basic and clinical aspects. Proceedings of the 6 th International Congress on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 213 - 216.

Edman, P. (**1950**). **Acta. Chem. Scand.** Vol 4, pp 283.

Ekins, R.P. (**1981**). " Merits and Disadvantages of Different Labels and Methods of Immunoassay. " **IN:** Immunoassays for the 80s. (Voller, A., Bartlett, A. and Bidwell, D. **:Eds.**). MTP press, Lancaster, pp 5 - 16.

Ehrlich, H.A. (**Ed: 1989**). " PCR technology: Principles and amplifications for DNA amplification. " Stockton Press, New York.

Faye, L. and Salier, J.P. (**1989**). "Crossed affino-immunoelectrophoresis or affino - blotting with lectins: Advantages and limitations for glycoprotein studies. " **Electrophoresis.** Vol 10, pp 841 - 847.

Fay, T.N., Price, K., Teisner, B., Jacobs, I. and Grudzinskas, J.G. (**1989**). "Simultaneous autoradiography and line immunoelectrophoresis (ARLIE): a novel combination to identify *de-novo* protein synthesis by pregnancy tissues. " **Placenta.** Vol 10, pp 543 - 551.

Feizi, T. and Childs, A. (**1987**). " Carbohydrates as antigenic determinants. " **Biochem. J.** Vol 245, pp 1 - 11.

Findlay, J.B.C. and Geisow, M.J. **:Eds.** (**1990**). Protein sequencing: A Practical Approach. IRL Press, Oxford.

Folkersen, J., Westergaard, J., Hindersson, P. and Teisner, B. (**1979**). " Affinity chromatographic purification of a new high molecular weight pregnancy specific protein, SP_4. " **IN:** Carcinoembryonic proteins. (Lehmann, F.G. **:Ed.**). Elsevier Press, Amsterdam. Vol. 2, pp 503 - 508.

Folkersen, J., Grudzinskas, J.G., Hindersson. P., Teisner, B. and Westergaard, J.G. (**1981**). " Pregnancy - Associated plasma protein A: circulating levels during normal pregnancy. " **Am. J. Obstet. Gynecol.** Vol 139, pp 910 - 914.

Geng, X. and Regnier, F.E.(**1984**). " Retention model for proteins in reverse-phase liquid chromatography. " **J. Chromatogr.** Vol 296, pp 15 - 30.

Gerrard, C. (**1990**). " Purification of glycoproteins. " **Methods In Enzymology.** Vol 182, pp 529 - 539.

Gleich, G.J., Frigas, E., Loegring, D.A., Wassom, D.L. and Steinmuller, D. (**1979**). " Cytotoxic properties of the eosinophil Major basic protein. " **J. Immunol.** Vol 123, pp 2925 - 2927.

Goka, A.K.J. and Farthing, M.J.G. (**1987**). " The use of 3, 3', 5, 5'-Tetra-methylbenzidine as a peroxidase substrate in microplate enzyme linked immunosorbent assay. " **J. Immunoassay.** Vol 8, pp 29 - 41.

Gore, C.H. and Sutcliffe, R.G. (**1984**). " Pregnancy - associated plasma protein - A. Purification under mild conditions, peptide mapping and test for possible interactions with Trypsin,plasmin and complement. " **Placenta.** Vol 5, pp 293 - 314.

Grantham, R., Gautier, C., Guoy, M., Mercier, R. and Pave, A. (**1980**). " Codon catalog usage and the genome hypothesis. " **Nucl. Acid Res.** Vol 8, pp r49 - r62.

Gross, E. and Witkop, B. (**1962**). **J. Biol. Chem.** Vol 237, pp 1856 - 1860.

Halbert, S.P. and Lin, T.M. (**1979**). " Pregnancy-associated plasma proteins: PAPP-A and PAPP-B. " **IN:** Placental proteins. (Chard, T. and Klopper, A. **:Eds.**). Springer-Verlag Press, Berlin, pp 89 -103.

Haselbeck, A. and Hosel, W. (**1988**). " Studies on the effect of the incubation conditions, various detergents and protein concentration on the enzymatic activity of N - Glycosidase F (Glycopeptidase F) and Endoglycosidase F. " **Topics in Biochem.** No 8, pp 1 - 4.

Hernandez, D. and Fisher, E.M.C. (**1996**). " Down syndrome genetics: unravelling a multifactorial disorder. " **Hum. Mol. Gen.** Vol 5 Review, pp 1411 - 1416

Hurley, P.A., Ward, R.H.T., Teisner, B., Iles, R.K., Lucas, M., and Grudzinskas, J.G. (**1993**). " Serum pregnancy measurements in first trimester screeening of Down's syndrome. " **Prenat. Diagn.** Vol 13, pp 903 - 908.

Huynh, T.V., Young, R.A. and Davis, R.W. (**1985**). " Constructing and Screening cDNA libraries in λgt10 and λgt11. " **IN:** DNA cloning, volume 1: A practical approach. (Glover, D.M. **:Ed.**). IRL press, Oxford, pp 49 - 78.

Ikarashi, T. and Takeuchi, S. (**1987**). " Immunohistochemical localization of placental proteins and tumour - associated antigens in endometrial cancer and endometrial hyperplasia. " **Nippon. Sanka. Fujinka. Gakkai. Zasshi.** Vol 39, pp 1634 - 1640.

Isaka, K. and Bischof, P. (**1986**). " Binding of pregnancy - associated plasma protein-A (PAPP-A) to placental subfractions. " **Arch. Gynecol.** Vol 237, pp 117 - 126.

Khyse-Andersen, J. (**1984**). " Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. " **J. Biochem. Biophys. Meth.** Vol 10, pp 203 - 209.

Kabat, E.A.:**Ed.** (**1976**). Structural concepts in immunology and immunochemistry. Holt, Rienhart and Winston Publishers, London, pp 143-197.

Kijimoto - Ochiai, S., Katagiri, Y.U. and Ochiai, H. (**1985**). " Analysis of N-linked Oligosaccharide Chains of Glycoproteins on Nitrocellulose Sheets using Lectin - Peroxidase Reagents. " **Anal. Biochemistry.** Vol 147, pp 222 - 229.

Klopper, A.(**1987**). " Fetal monitoring:- Biochemical methods." **Baillieres Clin. Obstet. and Gynaecol.** Vol 1, pp 1 - 16.

Knight, G.J, Palomaki, G.E., Haddow, J.E., Miller, W., Bersinger, N.A. and Schneider, H. (**1993**). " Pregnancy associated plasma protein A as a marker for Down Syndrome in the second trimester of pregnancy. " **Prenat. Diagn.** Vol 13, pp 222 - 223.

Kristensen, T., Oxvig, C. Sand, O., Moller, N.P.H. and Sottrup-Jensen, L. (**1994**). " Amino Acid Sequence Of Human Pregnancy-associated Plasma Protein - A Derived from Cloned cDNA. " **Biochemistry.** Vol 33, pp 1592 - 1598.

Kuhajda, F.P., Katamuluwa, A.I. and Pasternack, G.R.(**1989**). " Expression of haptoglobulin- related protein and its potential role as a tumour antigen. " **Proc. Natl. Acad. Sci. USA.** Vol 86, pp 1188 - 1192.

Laemmli, U.K.(**1970**). " Cleavage of structural proteins during the assembly of the head of bacteriophage T4. " **Nature.** Vol 227, pp 680.

Langman, J. (**1975**). Medical Embryology. Williams and Wilkins Publishers. Baltimore and London.

Langone, J .J. (**1982**). " Protein - A of Staphylococcus aureus and related immunoglobulin receptors produced by Streptococci and Pneumococci. " **Adv. Immunol.** Vol 32, pp 157 - 252.

Lathe, R. (**1985**). " Synthetic oligonucleotide probes deduced from amino acid sequence data; theoretical and practical considerations. " **J. Mol. Biol.** Vol 183, pp 1 - 12.

Lee, K.B., Loganathan, D., Merchant, Z.M. and Linhardt, R.J. (**1990**). " Carbohydrate Analysis Of Glycoproteins: A review. " **App. Biochem. Biotechnol.** Vol 23, pp 53 - 80.

Levy, M., Schenkein, I. and Fishman, L. (**1969**). " Mouse Submaxillary Gland Proteases. " **Methods in Enzymology.** Vol 19, pp 672 - 681.

Leykam, J.F.and Strahler, J.R. (**1992**) " High Efficiency Protein Extraction From PVDF Membranes For Internal Sequence Analysis." The American Protein Society. (**Abstract**). S91.

Lian, L.-Y., Yang, J.C., Derrick, J.P., Sutcliffe, M.J., Roberts, C.K., Murphy, J.P., Goward, C.R. and Atkinson, T. (**1991**). " Sequential ^{1}H NMR Assignments and Secondary Structure of an IgG-Binding Domain from Protein-G." **Biochemistry.** Vol 30, pp 5335 - 5340.

Lin, T.M., Halbert, S.P., Kiefer, D. and Spellacy, W.N. (**1974a**). " Three pregnancy - associated human plasma proteins: purification, monospecific antisera and immunological identification. " **Arch. Allergy. Appl. Immunol.** Vol 47, pp 35 - 53.

Lin, T.M., Halbert, S.P., Kiefer, D., Spellacy, W.N. and Gall, S. (**1974b**). " Characterization of four human pregnancy - associated plasma proteins. " **Am. J. Obstet. Gynecol.** Vol 118, pp 223 - 226.

Lin, T.M. and Halbert, S.P. (**1976a**). " Placental localization of human pregnancy - associated plasma proteins. " **Science.** Vol 193, pp 1249 - 1252.

Lin, T.M., Halbert, S.P. and Kiefer, D. (**1976b**). " Quantitative analysis of pregnancy - associated plasma proteins in human placenta. " **J. Clin. Invest.** Vol 57, pp 466 - 472.

Lis, H. and Sharon, N. (**1986**). " Lectins as molecules and tools." **Ann. Rev. Biochem.** Vol 55, pp 35 - 67.

Lowry, O.H., Rosebrough, N.J., Farr, A. L. and Randall, R.J. (**1951**). " Protein measurements with the folin phenol reagent." **J. Biol. Chem.** Vol 193, pp 265 - 275.

Luo, K., Hurley, T.R. and Sefton, B.M. (**1990**). " Transfer of proteins to membranes facilitates both cyanogen bromide cleavage and two dimensional proteolytic mapping." **Oncogene.** Vol 5, pp 921 - 923.

MacLaren, J., Thornes, R.D., Poby, C.C. and Reid,D.E. (**1959**). " An immunological characteristic of serum of normal pregnancy." **Am. J. Obstet. Gynecol.** Vol 78, pp 939 - 946.

Maley, F., Trimble, R.B., Tarentino, A.L. and Plummer, T.H. Jr. (**1989**). " REVIEW: Characterization of Glycoproteins and Their Associated Oligosaccharides through the use of Endoglycosidases. " **Anal. Biochem.** Vol 180, pp 195 - 204.

Mancini, G., Vaerman, J.P., Carbonara, A.O. and Heremans, J.F. (**1964**) " A single radial diffusion method for the immunological quantitation of proteins. " **Protides Biol. Fluids** Vol 11, pp 370 - 373.

Matsuidaira, P. T. (**:Ed.**). (**1989**). A practical guide to protein and peptide purification for microsequencing. Academic press, San Diego.

Martin - du - pan, R.C., Bischof, P., Bourrit, B., Lauber, K., Girard,J.P. and Herrmann, W.L. (**1983**). " Immunosuppressive activity of seminal plasma and pregnancy - associated plasma protein - A (PAPP-A) in men. " **Arch. Androl.** Vol 10, pp 185 -188.

Matsudaira, P.T. (**1990**). " Limited N - terminal sequence analysis." **Methods in Enzymology.** Vol. 182, pp 602 - 613.

McIntyre, J.A., Hsi, B., Faulk, W.P., Klopper, A. and Thomson, R. (**1981**). " Immunological studies of the human placenta: functional and morphological analysis of pregnancy - associated plasma protein-A (PAPP-A) . " **Immunology.** Vol 44, pp 577 - 583.

Meisser, A., Geinoz, A. and Bischof, P. (**1985**). " Interaction of pregnancy associated plasma protein-A (PAPP-A) with coagulation: a bioassay for PAPP-A. " **Biol. Reprod.** Vol 33, pp 1073 - 1077.

Meisser, A., Geinoz, A.and Bischof, P (**1988a**). " *In-Vitro* effects of pregnancy - associated plasma protein - A: Artifacts due to heparin. " **Biol. Reprod.** Vol 39, pp 373 - 378.

Meisser, A., Geinoz, A and Bischof, P.(**1988b**). " Facts and Artefacts About The Biological Effects Of Pregnancy - Associated Plasma Protein - A (PAPP-A). " **IN:** Placental and endometrial proteins: Basic and Clinical apsects. Proceedings of the 6 th International Congress on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 407 -410.

Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (**1981**). " Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. " **Science.** Vol 211, pp 1437 - 1438.

Merrill,C.R. (**1990**). "Gel Staining Techniques." **Methods In Enzymology.** Vol 182, pp 477 - 488.

Miletich, J.P., Broze, Jnr, G.J. and Majerus, P.W.(**1980**). " The synthesis of sulfated dextran beads for isolation of human plasma coagulation factors II, IX and X. " **Anal. Biochem.** Vol 105, pp 304 - 310.

Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G. and Streckey, G. (**1986**). " Glycoproteins " **IN:** Carbohydrate analysis: A practical approach (Chaplin, M.F. and Kennedy, J.F. **:Eds.**) IRL Press, Oxford, pp 143 - 168.

Morris, P.W.(**1992**). " Hexafluoroisopropanol: An Efficient aid in binding and elution of proteins from PVDF. " The American Chemical Society. (**Abstract**). S85.

Mossman, H.W. (**1987**) Vertebrate Fetal Membranes. Macmillan Press Ltd, Basingstoke.

Mousa, M.A., El - Mackawi, T., Abu-Salem, A. and El - Sayed, A. (**1988**). " Pregnancy associated plasma protein - A (PAPP-A) in the decidua of the first trimester abortion. " **IN:** Placental and Endometrial Proteins. Basic and Clinical apsects. Proceedings of the 6 th International Congress on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 229 - 233.

Mowles, E.A., Pinto - Furtado, L.G. and Bolton, A.E. (**1986**), " A two-site immunoradiometric assay for human pregnancy - associated plasma protein- A (PAPP-A) using monoclonal antibodies. " **J. Immunol. Methods.** Vol 95, pp 129 - 133.

Opitz, J.M. (**1985**). " Brachmann - de Lange Syndrome. " **Am. J. Med. Genet.** Vol 22, pp 89 - 102.

Ornstein, L.(**1964**) " Disc electrophoresis-I: Background and theory. " **Ann. N.Y. Acad. Sci.** Vol 121, 321 - 349.

Oxvig, C., Sand, O., Kristensen, T., Gleich, G.J. and Sottrup - Jensen, L. (**1993**). " Circulating Human Pregnancy-associated Plasma Protein-A Is Disulphide-Bridged to the Proform of Eosinophil Major Basic Protein. " **J. Biol. Chem.** Vol 268, pp 12243 - 12246.

Oxvig, C., Sand, O., Kristensen, K., Kristensen, L. and Sottrup-Jensen, L. (**1994**). " Isolation and characterisation of circulating complex between human pregnancy-associated plasma protein-A and proform of eosiniphil major basic protein. " **Biochim. Biophys. Acta.** Vol 1201, pp 415 - 423.

Oxvig, C., Haaning, J., Kristensen, L., Wagner, J.M., Rubin, I., Stigbrand, T., Gleich, G.J. and Sottrup - Jensen, L. (**1995**). " Identification of Angiotensinogen and Complement C3dg as Novel Proteins Binding the Proform of Eosinophil Major Basic Protein in Human Pregnancy Serum and Plasma. " **J. Biol. Chem.** Vol 270, pp 13645 - 13651.

Panigel, M., Duenas, J.L., Nguyen Son, H. and Wegmann, R. (**1985**). " Distribution in the Human Placenta of the enzymes of protein metabolism. " **IN:** Proteins of the Placenta. 5th International Congress on Placental Proteins. (Bischof, P. and Klopper, A. **:Eds.**). Karger, Basel, pp 68 - 83.

Patterson, S.D. and Bell, K. (**1990**). " The Carbohydrate side chains of the major Plasma serpins of Horse and Wallaby: Analyses of enzymatic and chemically treated (including 'Smith degradation') Protein blots by Lectin binding. " **Biochem. Int.** Vol 20, pp 429 - 436.

Pepe, G.J., Waddell, B.J., Sinosich, M.J. and Albrecht, E.D. (**1994**). " Influence of Fetectomy on serum Pregnancy - Associated Plasma Protein - A Concentrations in the Baboon. " **Biol. Reprod.** Vol 50, pp 442- 448.

Pinto - Furtado, L.G., Bolton, A.E., Grudzinskas, J.G., Chapman, M.G., Sinosich, M.J. and Sharma, V. (**1984**). " The development of a radioimmunoassay for human pregnancy-associated plasma protein -A (PAPP-A). " **Arch. Gynecol.** Vol 236, pp 83 - 91.

Pledger, D.R. and Bellfield, A. (**1983**). " An ELISA for pregnancy-ssociated plasma protein A. " **Ann. Clin. Biochem.** Vol 20, pp 236 - 240.

Pledger, D.R. and Nicol, K.M. (**1983**). " The effect of hemolysis in the measurement of pregnancy - associated plasma protein - A (PAPP-A)." **Clin. Chim Acta.** Vol 135,, pp 309 - 311.

Popken - Harris, P., Thomas, L., Oxvig, C., Sottrup - Jensen, L., Kubo, H., Klein, J.S. and Gleich, G.J. (**1994**). " Biochemical properties, activities, and presence in biologic fluids of eosinophil granule major basic protein. " **J. Allergy Clin. Immunol.** Vol 94, pp1282 - 1289.

Price, K.M., Silman. R., Armstrong, P and Grudzinskas, J.G. (**1995**). " Abnormal amniotic fetal antigen 2 concentrations in trisomy 18 and trisomy 21. " **Hum. Reprod.** Vol 10, pp 2438 - 2440.

Reisig, J.L., Strominger, J.L. and Leloir, L.F. (**1955**). " A modified colorimetric method for the estimation of N-acetylamino sugars. " **J. Biol. Chem.** Vol 217, pp 956 - 966.

Reynolds, J.A. and Tanford, C. (**1970**). " The gross conformation of protein-sodium dodecyl sulfate complexes. " **J. Biol. Chem.** Vol 245, pp 5161- 5170.

Rosenbach, Y., Zahavi, I. and Dinari, G. (**1992**). " Gastroesophageal dysfunction in Brachmann - de Lange syndrome." **Am. J. Med. Gen.** Vol 42, pp 379 - 380.

Rusiniak, M.E., Bedi, G.S. and Back, N. (**1991**). " Role of carbohydrate in rat plasma thiostatin: deglycosylation destroys cysteine proteinase inhibition activity. " **Biochem. Biophys. Res. Comm.** Vol 79, pp 927 - 932.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (**1989**). Molecular cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sampson,P.M., Heimer, R. and Fishman, A.P. (**1985**). " Detection of glycos-aminoglycans at the one - nanogram level by ^{125}I - Cytochrome C. " **Anal. Biochem.** Vol 151, pp 304 - 308.

Sarath, G., de la Motte, R.S. and Wagner, F.W. (**1989**). " Protease Assay Methods. " **IN:** Proteolytic Enzymes: A Practical Approach (Beynon, R.J. and Bond, J.S.:**Ed.**). IRL Press, Oxford, pp 25 - 56.

Saso, L., Silvestrini, B., Guglielmotti, A., Lahita, R.G. and Cheng, C.Y. (**1992**). " Abnormal glycosylation of selected glycoproteins during chronic inflammatory conditions can be reversed by Bindarit, 2-[(1- Benzyl-Indazol-3-YL) Methoxy]-2-Methyl Propionic Acid. " **Clin. Chem.** Vol 38, pp 1073.

Sataloff, R.T., Spiegel, J.R., Hawkshaw, M., Epstein, J.M. and Jackson, L. (**1990**). " Cornelia-de-Lange syndrome. " **Arch. Otolaryngol.** Vol 116, pp 1044 - 1046.

Sauer, M.V., Sinosich, M.J., Yeko, T.R., Vermesh, M., Buster, J.E. and Simon, J.A. (**1989**). " Predictive value of a single serum pregnancy associated plasma protein - A or progesterone in the diagnosis of abnormal pregnancy. " **Hum. Reprod.** Vol 4, pp 331 - 334.

Schagger, H. and Von Jagow, G. (**1987**). " Tricine - Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis for the separation of proteins in the range from 1 to 100 kDa. " **Anal. Biochem.** Vol 166, pp 368 - 376.

Schenkein, I., Levy, M., Franklin, E.C. and Frangione, B. (**1977**). " Proteolytic Enzymes from the Mouse Submaxillary Gland. " **Archiv. Biochem. Biophys.** Vol 182, pp 64 - 70.

Schenkein, I., Franklin, E.C. and Frangione, B.(**1980**)." Proteolytic Enzymes from the Mouse Submaxillary Gland: A partial sequence and demonstration of spontaneous cleavages. " **Archiv. Biochem. Biophys.** Vol 186, pp 57 - 62.

Schindler, A.M., Dayer, A. and Bischof, P. (**1986**). " Immunohistochemical localization of pregnancy - associated plasma protein-A in the male genital tract. " **Hum. Reprod.** Vol 1, pp 55 - 59.

Silahtaroglu, A.N., Tumer, Z., Kristensen, T., Sottrup - Jensen, L. and Tommerup, N. (**1993**). " Assignment of the human gene for pregnancy - associated plasma protein A (PAPP-A) to 9q33.1 by fluorescence *in - situ* hybridization to mitotic and meiotic chromosomes. " **Cytogenet. Cell Genet.** Vol 62, pp 214 - 216.

Silvestrin, B., Guglielmotti, A., Saso, L. and Cheng, C.Y. (**1989**). " Changes in Concanavalin A- reactive proteins in Inflammatory Disorders. " **Clin. Chem.** Vol 35, pp 2207 - 2211.

Sinha, S., Knops, J., Esch, F. Moyer, E.D. and Oltersdorf, T. (**1991**). " Conversion of the Alzheimer's β - Amyloid Precursor Protein (APP) Kunitz Domain into a potent Human Neutrophil Elastase inhibitor. " **J. Biol. Chem.** Vol 266, pp 21011 - 21013.

Sinosich, M.J. (**1988a**). " Past, Present and Future Of Pregnancy-Associated Plasma Protein - A (PAPP-A). " **IN:** Placental and endometrial proteins: Basic and Clinical apsects. Proceedings of the 6th International Congress on Placental and Endometrial Proteins. (Tomoda,Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 11 - 18.

Sinosich, M.J. (**1988b**). " Physiology and Biochemistry of Pregnancy associated plasma protein - A (PAPP-A) in human pregnancy. " **PhD Thesis**. University of New England.

Sinosich, M.J. (**1988c**). " Pregnancy - Associated Plasma Protein - A: Fact, Fiction and Future. " **IN:** Implantation-Biological and Clinical Aspects (Chapman, M., Grudzinskas, J.G. and Chard, T. **:Eds.**). Springer-Verlag Press, Heidelberg. Chapter Five, pp 45 - 81.

Sinosich, M.J. (**1990**). " Molecular characterisation of pregnancy-associated plasma protein - A by electrophoresis. " **Electrophoresis.** Vol 11, pp 70 - 78.

Sinosich, M.J., Bonifacio, M.D. and Hodgen, G.D. (**1987**). " Affinity immunoelectrophoresis and chromatography for ioslation and characterization of a placental granulocyte elastase inhibitor. " **IN:** Protein Purification, Micro to Macro. (Burgess, R. **:Ed.**). Alan. R. Liss Publishers, New York, pp 225 - 238.

Sinosich, M.J., Davey, M.W., Ghosh, P. and Grudzinskas, J.G. (**1982a**). " Specific inhibition of human granulocyte elastase by human pregnancy associated plasma protein - A. " **Biochem. Int.** Vol 5, pp 777 - 786.

Sinosich, M.J., Teisner, B., Folkersen, J., Saunders, D.M. and Grudzinskas, J.G. (**1982b**). " Radioimmunoassay for Pregnancy - Associated Plasma Protein-A. " **Clin. Chem.** Vol 28, pp 50 - 53.

Sinosich, M.J., Davey, M.W., Teisner, B. and Grudzinskas, J.G. (**1983**). " Comparative studies of pregnancy associated plasma protein-A and α 2 - macroglobulin using metal chelate chromatography. " **Biochem. Int.** Vol 7, pp 33 - 42.

Sinosich, M.J., Ferrier, A. and Saunders, D.M. (**1985**). " Monitoring of post-implanatation embryo viability following successful *in - vitro* fertilization and embryo transfer by measurement of placental proteins. " **Fertil. Steril.** Vol 44, pp 70 - 74.

Sinosich, M.J., King, M., Bonifacio, M.D., Nelson, D. and Saunders, D.M. (**1984**). " Pregnancy - associated plasma protein -A in human seminal plasma. " **IN:** Protides of Biological Fluids. (Peeters, H., **:Ed.**). Pergamon Press, Oxford. Vol 32, pp 289 - 292.

Sinosich, M.J., Lee, J., Wolf, J.P., Williams, R.F. and Hogden, G.D. (**1989**) " RU486 induced suppression of placental neutrophil elastase inhibitor levels." **Placenta.** Vol 10, pp 569 - 578.

Sinosich, M.J., Sim, R.B. and Teisner, B. (**1990**). " Characterisation of Pregnancy - associated plasma protein - A: Comparison with α_2-Macroglobulin. " **Biochem. Int.** Vol 20, pp 579 - 589.

Sinosich, M.J., Teisner, B., Davey, M.W. and Grudzinskas, J.G. (**1981**). " Pregnancy associated plasma protein A: Interaction with heparin in crossed immunoelectrophoresis." **Aust. N.Z. J. Med.** Vol 11, pp 429- 433.

Sinosich, M.J. and Zacher, A. (**1991**) " Pregnancy - associated plasma protein A interaction with heparin: A critical appraisal. " **Gynecol. Obstet. Invest.** Vol 32, pp 72 - 77.

Smith, R., Bischof, P., Hughes, G., and Klopper, A. (**1979**). " Studies on pregnancy - associated plasma protein - A in the third trimester of pregnancy. " **Br. J. Obstet. Gynaecol.** Vol 86, pp 882 - 887.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (**1985**) " Measurement of Protein Using Bichinoninic Acid. " **Anal. Biochem.** Vol 150, pp 76 - 85.

Sommer, R. and Tautz, D. (**1989**). " Minimal Homology Requirements for PCR primers. " **Nucl. Acid. Res.** Vol 16, pp 6749.

Sorensen, S., Momosen, G., Ruge, S. and Pedersen, J.F. (**1995**). " Differential increase in the maternal serum concentrations of the placental proteins human chorionic gonadotrophin, pregnancy-specific - β_1 - glycoprotein, human placental lactogen and pregnancy-associated plasma protein - A during the first half of normal pregnancy, elucidated by means of a mathematical model. " **Hum. Reprod.** Vol 10, pp 453 - 458.

Sottrup - Jensen, L., Sand, O. and Folkersen, J. (**1986**). " Studies on the Structure and Function of Human Pregnancy Zone Protein, A close homolog of Human α_2 -Macroglobulin. " **IN:** Pregnancy Proteins in Animals. (Hau, J. **:Ed.**). Walter de Guyter Press, Berlin, pp 91 - 112.

Stabile, I., Campbell, S. and Grudzinskas, J.G. (**1988**). " Early pregnancy failure: diagnostic aspects. " **IN:** Placental and Endometrial Proteins: Basic and Clinical Apsects. Proc. of the 6th Int. Congr. on Placental and Endometrial Proteins. (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A. **:Eds.**). VSP, Utrecht, pp 607 - 614.

Stabile, I., Campbell, S. and Grudzinskas, J.G. (**1989**) " Ultrasound and circulating placental protein measurements in complications of early pregnancy. " **Br. J. Obstet. Gynaecol.** Vol 96, pp 1182 - 1191.

Stott, D.I. (**1989**). " Immunoblotting and Dot Blotting: A minireview. " **J. Immunol. Methods.** Vol 119, pp 153 - 187.

Stone, K.L., LoPresti, M.B., Williams, N.D., Crawford, J.M., DeAngelis R. and Williams, K.R. (**1989**). " Enzymatic digestion of proteins and HPLC peptide solation in the sub-nanomole range." **IN:** Techniques in Protein Chemistry. (Hugli, T.E. **:Ed.**). Academic Press, San Diego, pp 377 - 391.

Sutcliffe, R.G., Kukulska, B.M., Nicholson, L.V.B. and Paterson, W.F.(**1979**). " The use of affinity chromatography and other methods in the study of pregnancy associated proteins. " **IN:** Placental Proteins. (Klopper, A. and Chard, T. **:Eds.**). Springer-Verlag Press, Berlin, pp 55.

Sutcliffe, R.G., Kukulska-Langlands, B.M., Coggins, J.R., Hunter, J.B. and Gore, C.H. (**1980**). " Studies on human pregnancy-associated plasma protein A : Purification by affinity chromatography and structural comparisons with α_2 - Macroglobulin. " **Biochem. J.** Vol 191, pp 799 - 809.

Swank, R.T. and Munkres, K.D. (**1971**). " Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulphate. " **Anal. Biochem.** Vol 39, pp 462 - 477

Tatarinov, Y.S. and Masyukevich, V.N. (**1970**). " Immunochemical identification of a new $beta_1$ - globulin in the blood serum of pregnant women. " **Bull. Eksp. Biol. Med (USSR).** Vol 69, pp 66 - 69.

Taylor, J.M., Mitchell, W.M. and Cohen, S. (**1974**). " Characterization of the binding protein for Epidermal Growth Factor. " **J. Biol. Chem.** Vol 249, pp2188 - 2194.

Thomas, K.R. (**1981**). " Protoplast production and localization of cellulase in species of Aspergillus. " **PhD Thesis**. Sheffield City Polytechnic.

Tijssen, P. (**1985**). " Practice and theory of enzyme immunoassays. " **IN:** Laboratory techniques in biochemistry and molecular biology. (Burden, R.H. and Van-Knippenberg, P.H.**:Eds.**). Elsevier publishers, Amsterdam.

Toop, K. and Klopper, A (**1983**). " Effect of anticoagulants on the measurement of pregnancy associated plasma protein - A (PAPP-A). " **Br. J. Obstet. Gynaecol.** Vol 90, pp 236 - 240.

Tornehave, D., Chemnitz, J., Westergaard, J.G., Teisner, B., Poulsen, H.K., Bolton, A.E. and Grudzinskas, J.G. (**1987**). " Placental proteins in peripheral blood and tissues of ectopic pregnancies. " **Gynecol. Obstet. Invest.** Vol. 23, pp 97 - 102.

Tornehave, D., Folkersen, J., Teisner, B. and Chemnitz, J. (**1986**). " Immunohistochemical aspects of immunological cross - reaction and masking of epitopes for localization studies on pregnancy-associated plasma protein-A. " **J. Histochem.** Vol 18, pp 184 - 188.

Towbin, H., Staehelin, T. and Gordon, J. (**1979**). " Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. " **Proc. Natl. Acad. Sci. U.S.A.** Vol 76, pp 4350 - 4354.

Van Nostrand, W.E., Wagner, S.L., Farrow, J.S. and Cunningham, D.D. (**1990**). " Immunopurification and Protease Inhibitory Properties of Protease Nexin-2 / Amyloid β - Protein. " **J. Biol. Chem.** Vol 265, pp 9591 - 9594.

Voisin, G.A., Duc, H.T. and Bobe, P.(**1985**). " Immunomodulatory proteins of the placenta. " **IN:** Proteins of the placenta. 5th International Congress on Placental Proteins. (Bischof, P. and Klopper, A. **:Eds.**). Karger, Basel, pp 54 - 67.

Wahlstrom, T., Teisner, B. and Folkersen, J.(**1981**) " Tissue localisation of pregnancy associated plasma protein - A (PAPP-A) in the normal placenta. " **Placenta.** Vol 2, 253 - 258

Wald, N.J. (**1988**). " Maternal serum screening for Down's syndrome in early pregnancy. " **Br. Med. J.** Vol 297, pp 883 - 887.

Wald, N.J., George, L., Smith, D., Densem, J.W. and Petterson, K. (**1996**). " Serum screening for Down's syndrome between 8 and 14 weeks of pregnancy. " **Br. J. Obstet. Gynaecol.** Vol 103, pp 407 -412.

Wald, N.J., Kennard, A. and Hackshaw, A.K. (**1995**). " First Trimester Serum Screening For Down's Syndrome. " **Pren. Diagn.** Vol 15, pp 1227 - 1240.

Wald, N., Stone, R., Cuckle, H.S., Grudzinskas, J.G., Barkai, G., Brambati, B., Teisner, B. and Fuhrmann, W. (**1992**). " First trimester concentrations of pregnancy associated plasma protein A and placental protein 14 in Down's syndrome. " **Br. Med. J.** Vol 305, pp 28.

Walsh, K.A. and Sasagawa, T. (984). " High - Performance Liquid Chromatography Probes For Postranslationally Modified Amino Acids. " **Methods In Enzymology.** Vol 106, pp 22 - 29.

Walton, K.W. (**1955**) " Chemistry and mode of action of heparin and related compounds. " **Br. Med. Bull.** Vol 11, pp 62 - 69.

Ward, M.A. (**1994**). " Early prenatal biochemical screening for chromosome abnoramalities: Introduction of the programme in a 5 million population." **7th Int. Conf. on Early Prenatal Diagnosis** (Abstract).

Westergaard, J.G., Sinosich, M.J., Bugge, M., Madsen, L.T., Teisner, B. and Grudzinskas, J.G. (**1983b**) " Pregnancy- associated plasma protein - A in the prediction of early pregnancy failure. " **Am. J. Obstet. Gynecol.** Vol 145, pp 67 - 69.

Westergaard, J.G., Teisner, B., Chemnitz, J., Ipsen, L., Poulsen, H.K., Ipsen, L., Beck, B. and Grudzinskas, J.G. (**1983c**). " Pregnancy associated plasma protein ; a possible marker in the classification and prenatal diagnosis of Cornelia de Lange syndrome. " **Prenat. Diagn.** Vol 3, pp 225 - 232.

Westergaard, J., Teisner, B., Sinosich, M., Madsen, L.and Grudzinskas J.G. (**1985**) " Does ultrasonic exmination render biochemical tests obsolete in prediction of early pregnancy failure. " **Br. J. Obstet. Gynaecol.** Vol 92, pp 77 - 83.

Westergaard, J.G., Teisner, B., Stabile, I. and Grudzinskas, J.G. (**1988**) " Diagnostic apsects of ectopic pregnancy. " **IN:** Placental and endometrial proteins: Basic and clinical aspects. Proceedings of the 6th International Congress on placental and endometrial proteins (Tomoda, Y., Mizutani, S., Narita, O. and Klopper, A **:Eds.**) VSP, Utrecht, pp 615 - 622.

Williams, J.F. (**1989**). " Optimization Strategies for the polymerase chain reaction. " **BioTechniques.** Vol 7, pp 762 - 768.

Williams, P.L. and Warwick, R. : **Eds.** (**1980**). " Gray 's Anatomy. " Churchill Livingstone, Edinburgh. 36th Edition.

Wilson, K.J. and Yuan, P.M.(**1989**). " Protein and Peptide purification. " **IN:** Protein sequencing: A practical approach. (Findlay, J.B.C and Geisow, M.J.**:Eds.**). IRL press, Oxford, pp 1 - 41.

Winter, R.M., Knowles, S.A.S., Bieber, F.R. and Baraitser, M. **:Eds.** (**1988**). " The Malformed Fetus and Stillbirth." John Wiley and Sons, Chichester.

Woodward, M.P., Young, W.W. and Bloodgood, R.A. (**1985**). " Detection of monoclonal Antibodies Specific for carbohydrate Epitopes Using Periodate Oxidation. " **J. Immunol. Methods.** Vol 78, pp 143 - 153.

Wu, A.M., Sugii, S. and Herp, A. (**1988**). " A guide for carbohydrate specificities of lectins. " **Adv. In Exp. Med. And Biol.** Vol 228, pp 819 - 847.

Young, R.A., and Davis, R.W. (**1983**). " Efficient isolation of genes using antibody probes." **Proc. Natl. Acad. Sci. USA.** Vol 80, pp1194-1198.

Zingde, Z.M., Shirsat, N.V. and Gothoskar, B.P. (**1986**) " Peptide mapping of proteins in gel bands after partial cleavage with acidic Cyanogen Bromide vapours. " **Anal. Biochem.** Vol. 155, pp 10 - 13.

APPENDIX ONE

Appendix I

I.1 Materials

Antibodies

Rabbit Anti - Human Albumin (DAKO: A001)
Rabbit Anti - Human Alpha$_2$-Macroglobulin (DAKO: A033).

Rabbit Anti - Human Placenta(DAKO: A146. Lot 061A).

Rabbit Anti - Total Human serum (DAKO: A209)

Rabbit Anti - Human SP3 (Pregnancy zone protein, DAKO: A132).

Rabbit Anti - Human Pregnancy-associated plasma protein-A (DAKO: A230).

Donkey Anti- Rabbit immunoglobulins (IDS: A-PPT1).

Bacterial strains

Escherichia coli, strain: Y1090(Clontech; Cat. No. HL1008b).

Genotype: F' ,Δ(lacU169), proA$^+$, Δ(lon), araD139, strA, supF, [trpC22: Tn10(tet^r)], (pMC9). hsdR(r$_k^-$, m$_k^+$).

Buffer/Buffer Additives

Thimerosal(Sigma: T-5125)

Sodium azide(Koch Light: 61-15036).

Aprotinin(Koch Light).

Leupeptin(Sigma: L2884)

TPCK(Sigma: T-4367). - 20°C.

TLCK(Sigma: T-7254). - 20°C.

Phenylmethylsulfonyl Fluoride(Sigma: P7626).

0.5 M EDTA(Disodium salt) (Sigma: E7889)

Chromatography Supports

L-Arginine-Agarose(Sigma: A-8405)

DEAE-Trisacryl®M(IBF reactivs).

CM-Cibachron-Blue 3GA (Sigma: C-8658).

Agarose-Bound *Lycopersicon esculentum* Lectin(Vector: AL-1173)

LKB Heparin-Ultrogel A4R(LKB: 2214-610).

Heparin-Ultrogel® A4R(Sepracor)

Heparin Affi-gel®(Bio-Rad: 153-6173).

HiLoad 26/60 Superdex™ 200 'prep. grade' (Pharmacia: 17-1071-01)

Pre Column LiChrosorb RP-18(5 μm)(BDH: 15692 2T).

LiChrosorb RP-18(7 μm) 4mm x 250 mm(BDH: 15934 2R).

Sulphated Dextran Beads(Sigma: D-5650).

Detergents

Nonidet P40 'Lab. Reagent'(BDH: 56009)

Sodium deoxycholate 'Biochemical Gr.'(BDH: 43035)

Sodium Dodecyl Sulphate 'Specially purified Biochemical' (BDH: 44244).

Tween-20 (Sigma: P-1379).

Triton X-100(Sigma: T-6878).

MEGA-10 (ICN: 150657).

Electrophoresis(Acrylamide Supports/Cross-Linkers.)

Acrylamide 'Electran' (BDH: 44299).

Acrylamide 'Electran' Grade I (BDH: 44313).

Acrylamide/bis-acrylamide Mol biol. grade 19:1 (Sigma: A2917).

Piperazine di-Acrylamide(PDA)Bio-Rad (Cat. No. 161-0202).

N,N-methylenebisacrylamide 'Electran'(BDH: Cat. No. 44300).

N,N-Diallytartardiamide 'Electran'(BDH: 44224 2W).

Ammonium persulphate (Mol. Biol. Grade)(Sigma: A9164)

TEMED. Mol. Biol. Gr.(Sigma: T7024)

Electrophoresis (Agarose)

Litex HSA agarose(Park scientific)

Agarose 'Mol. Biol. Gr.(Sigma: A-7431).

Agarose(Type I: Low EEO)(Sigma: A-6013).

Agarose(Type II: Medium EEO, Sigma: A-6877).

Agarose(Type VIII: Special High EEO, Sigma: A-4905).

Electrophoresis (Buffer Components)

Boric Acid 'Analar'(BDH: 272052).

CAPS(Sigma: C-2632).

Diethyl Barbituric acid.

N-Ethylmaleimide(Sigma: E3876).

Glycine 'Electrophoresis grade'(Sigma: G-8898)

Calcium Lactate.

Taurine (Sigma: T-0625)

TRIZMA® Base'Mol. Biol. Gr.'(Sigma: T7149).

Tris-Borate-EDTA(5X concentrate)Mol. Biol. Gr.(Sigma: T-6400)

USB Urea 'Ultrapure'(Cambridge Bioscience: 23040A)

Electrophoresis (Markers)

Low M_r markers (BDH: Cat. No. 44247 2L).

Medium M_r markers (BDH: Cat. No. 44264 2L).

High M_r markers (BDH: Cat. No. 44358 2U).

α_2-Macroglobulin for SDS-PAGE (Sigma: M-3398)

Myosin 'Electrophoresis grade' (Sigma: M-3889).

Lambda DNA EcoRI & HindIII digest (NBL: 030604).

Enzymes

Chitinase(Sigma: C-6137).

α-Chymotrypsin (Type I-S: Sigma C 7762).

Endoproteinase Arg-C(Sigma: P-8402).

Endoproteinase Arg-C 'Sequencing grade' (P-6056).

Endoproteinase Glu-C 'Sequencing grade'(Sigma: P-6181).

Endoproteinase Lys-C 'Sequencing grade' (Sigma: P-3428).

N-Glycosidase F(Boehringer Mannheim UK: 903337).

Neuraminidase from *Clostridium perfringens* (Sigma: N-2876).

O-Glycanase®(Genzyme: O-ASE-25).

Endo-α-N-acetylgalactosaminidase (from cultures of *Diplococcus pneumoniae,* Sigma:).

Pepsin(Sigma: P-6887).

T_{aq} DNA polymerase(Advanced Biotechnologies: AB-0192).

T_{th} DNA polymerase(Advanced Biotechnologies: AB-0193).

Vent™ DNA polymerase(New England Biolabs).

General Chemicals

Ammonium sulphate 'Mol. Biol. Gr.'(Sigma: A-4418).

Ficoll type 400(Sigma: F-4375).

Glycerol M. Biol. Gr.(Sigma: G5516).

Human IgG (Sigma: I-2511)

Mineral oil M. Biol. Gr.(Sigma: M5904).

Poly-Vinyl Sulphuric acid-Potassium salt(Sigma: P-6000).

Growth media/components

Caesein Hydrolysate (NZ-Amine, ICN: 22692).

Tryptone 'Bacto' (DIFCO).

Yeast Extract Bacto® (DIFCO).

Bacto® - Agar (DIFCO).

Ampicillin Sodium Salt (NBL: 070609)

Kits/Kit components

Silver Stain kit (Bio-Rad: 161-0443).

Protein assay kit II(Bio-Rad: 500-0002).

Bicinchoninic acid solution(Sigma: B-9643).

CLIK® Clontech Lambda(λ) gt11. Human cDNA library(placenta, 34 wks) with 1 x 10^9 clones(Average insert size range of 0.8 - 3.6 kb. (supplied through Cambridge Bioscience: Cat. No: HL-1008b).

Isopropyl-β-D-thiogalactoside (NBL: 070206).

Geneclean II kit (Stratech scientific).

Gene Amp™ PCR amplification kit (Perkin-Elmer Cetus: N801-0043).

ExtrAvidin™ Biotin staining kit (Sigma: Extra-3(Rabbit)).

ExtrAvidin™ Biotin staining kit (Sigma: Extra-2(Mouse)).

Vectastain® Elite ABC Kit (Rabbit, Vector Laboratories: PK-6101).

Biotinylated lectin kit III(Vector: BK-3000)

Con-A, Peroxidase labelled(Sigma: L-4010)

Protein-A-Peroxidase conjugate(Sigma P-8651).

Membranes

Dialysis Visking Tubing(Size 8 32/32, BDH: 1270-05).

Nitrocellulose:

- 0.20 μm Biometra.
- 0.45 μm Schleicher and Schuell(Via Anderman).
- 0.20 μm NC plus(Sartorius: SM 12807).
- 0.45 μm 82 mm Circles (Whatman: Cat. No.7184008) WCN Type.
- 0.45 μm 132 mm Circles (Schleicher and Schuell, via Anderman)

PVDF:

- 0.2 μm Fluorotrans (PALL: Cat. No. PVM020C200S).
- 0.45 μm Immobilon-P (Millipore: Cat. No. IPVH 151 50).

Miscellaneous

Parafilm® PM-992(BDH: 2350416\02).

Molecular Sieves Type 3A(BDH: 54002).

Amerlex-M (Amersham).

Oligonucleotide Synthesis Reagents

Phenoxyacetyl dA β-cyanoethyl phosphoramidite(Pharmacia: 27-1223-01).

Phenoxyacetyl dG β-cyanoethyl phosphoramidite(Pharmacia: 27-1724-01).

Isobutyrl dC β-cyanoethyl phosphoramidite(Pharmacia: 27-1725-01).

dT β-cyanoethyl phosphoramidite(Pharmacia: 27-1236-01).

DMT-Biotin-C6-PA(CRB: DR-16-100A).

Protein Modifying Reagents

Chloramine-T 'GPR' (BDH: 27670).

Cyanogen Bromide (Sigma: C-6388)

DL-Dithiothreitol(Sigma: D-0632)

2-Mercaptoethanol(Sigma: M-6250).

Glutathione (Reduced, Sigma: G-4251)

Iodoacetamide(Sigma: I-6125).

Thioglycolic acid(T-0632)

Trifluoroacetic acid 'Sequencing grade' (Sigma: T-1647)

Radiochemicals

^{125}Iodine (Amersham: IMS.30)

^{125}Iodine (ICN: 63034)

Solvents

Acetonitrile Far UV HiPerSolv™ HPLC(BDH: 15251)

Ammonia solution HiPerSolv™(BDH: 15331)

Di-Ethyl Ether(M&B).

N,N-DimethylFormamide(Sigma: D-4254).

DMSO 'ACS' reagent(Sigma: D-8779).

Ethanol 'GPR'(BDH).

Hexafluoroisopropanol(Sigma H8508)

Methanol 'HPLC Gr. 205'(M&B: MF1087)

Methanol 'GPR'(BDH).

Propan-2-ol 'Analar'(BDH)

Stains

Brilliant Blue G(Sigma: B-0770)

Brilliant Blue R-250 65 % Dye content(Sigma: B-6630).

Bromophenol Blue 'ACS' free acid(Sigma: B-6896).

Ethidium Bromide (Sigma: E-8751)

Xylene cyanol FF(Sigma: X-0377)

Substrates/Substrate Components

Human serum albumin 'Fraction V' : 96 - 99% (Sigma: A-1653).

4-Chloro-1-Napthol(Sigma: C-8890).

Hydrogen Peroxide 30%(Sigma: H-1009)

Chitin(Sigma: C-3387).

TAME(Sigma: T-4626)

TMB(Sigma: T 2885).

Appendix I.2 Equipment

Centrifuges:

Beckman J2-21(Rotors: JA-20 and JA-10).

MSE Mistral 2L.

ALC® micro-centrifugette 4124(Supplied by CAMLAB)

Electrophoresis Equipment

LKB 2197 Power supply.

LKB 2117 Multiphor.

LKB 2201 Multitemp bath.

EC570 Power supply(Via Biometra).

Gallenkamp Vertical electrophoresis system

Minigel system(Biometra: Cat. No. G41)

Fastblot®(Biometra: Cat. No. B33)

FPLC system

Pharmacia LCC-500 Controller.

Pharmacia P-500 pumps.

Pharmacia UV-M monitor.

Pharmacia Frac-200 fraction collector.

Pharmacia FPLC manager(Ver 1.1) Software.

HPLC Equipment

Pharmacia HPLC 2152 Controller.

Pharmacia HPLC 2150 pump.

Pharmacia Low pressure Ultrograd 11300 mixer driver.

Pharmacia Frac-100 fraction collector.

Pye Unicam LC-UV Detector

Miscellaneous:

TA40 CRT Instant camera(GRI)

Gallenkamp SuperCold 85 (-70 ^{o}C Freezer).

Whatman No. 1 Filter paper (Whatman).

Whatman 3MM Filter paper (Whatman).

Glass immunoelectrophoresis plates (Sizes: 26 x 76. 84 x 94 mm)

Centricon -100 (Amicon: 4211).

Centriprep - 30 (Clear base)-100(Blue base):(Amicon: 4307).

NAP-5 column (Pharmacia).

Magic™ Minicolumns(Promega).

Pye Unicam 292 pH meter.

PREM™ PCR (LEP Scientific)

LKB Multiwell gamma counter (with software RIACALC).

Grant water Baths.

Gallenkamp Orbital Incubator

UV-Transilluminator(GRI).

UV-Safety faceshield (GRI).

FinnPipettes(variable 0.5 - 10, 40 - 200, 200 - 1000 µl and 1 - 5 ml).

Titertek 8 channel variable pipettes (Flow).

Microtitre plates: Falcon 3915 (Becton Dickinson)

Titertek Multiskan plus (Ver 2.0) microtitre plate reader (Flow).

Nalgene® Centrifuge tubes.

NICK™ spin columns (Pharmacia)

Bio-RAD Model 620 Video densitometer(Plus 1D analyst software).

4RT-Rocking table (GRI).

Gyrotory® Shaking water Bath (NBS).

Innova 2000 Platform shaker (NBS).

Pumps

LKB varioperpex® II pump.

LKB ReCyChrom® pump.

LKB MicroPerpex®S Peristaltic pump

Water Supply

Autostill IIplus.

Fi-Stream Cyclon™(Glass distilled water).

Barnstead NanopureII(Glass distilled/de-ionized/filtered water).

Appendix I.3 List of Suppliers

Applied Biosystems(ABI): Warrington, Cheshire, England.

Advanced Biotechnologies Ltd: West Hampstead, London.

Amersham International Plc: Amersham, Buckinghamshire, England.

Amicon Ltd: Stonehouse, Gloucester, England.

Anachem: Luton, Bedfordshire, England.

Anderman: Kingston-upon-Thames, Surrey, England.

BDH Ltd: Poole, Dorset, England.

Becton Dickinson & Co: Cowley, Oxford, England.

Biometra Ltd: Maidstone, Kent, England.

Bio-Rad Laboratories Ltd: Hemel Hempstead, Hertfordshire, England.

Boehringer Mannheim UK Ltd: Lewes, East Sussex, England.

The Binding Site Ltd: Edgbaston, Birmingham, England.

British Oxygen Co: Leeds, West Yorkshire, England.

Cambridge Bioscience, Cambridge, England.

Cambridge Research Biochemicals(CRB): Northwich, Cheshire, England.

Camlab Ltd: Cambridge, England.

DAKO Ltd: High Wycombe, Buckinghamshire, England.

Denley Instruments Ltd: Billinghurst, Sussex, England.

DIFCO Laboratories UK Ltd: Molesby, Surrey, England.

Fisons Scientific Ltd: Peterborough, England.

Flow Laboratories Ltd: Irvine, Ayrshire, Scotland.

Fluka: Glossop, Derbyshire, England.

Gallenkamp:(Fisons Plc. Sci. Equip. div. Loughborough, England).

Genzyme, West Malling, Kent, England.

Genetic Research Instruments Ltd(GRI): Dunmow, Essex, England.

ICN Biomedicals Ltd: High Wycombe, Buckinghamshire, England.

IDS: Washington, Tyne & Wear, England.

IBF reactivs: Villeneuve-la-Garenne, Paris,France.

Jones Chromatography Ltd: Hengoed, Mid-Glamorgan, Wales.

Koch-Light Ltd: Haverhill, Suffolk, England.

LEP Scientific Ltd: Milton Keynes, England.

May & Baker Ltd: Dagenham, Essex, England.

Millipore UK Ltd: Watford, Hertfordshire, England.

NEN® DuPont UK Ltd: Stevenage, Hertfordshire, England.

New Brunswick Scientific(NBS): North Mymms, Hatfield, England.

Northumbria Biologicals Ltd(NBL): Cramlington, Northumberland, England.

Pall Biosupport Division: Portsmouth, England.

Park Scientific Ltd: Northampton, England.

Perkin-Elmer Cetus: Chalfont St Giles, England.

Pharmacia LKB Biotechnology: Milton Keynes, Buckinghamshire, England.

Promega UK Ltd: Southampton, England.

Rathburn Chemicals Ltd: Walkerburn, Peeblesshire, Scotland.

Sarstedt: Leicester, England.

Sartorius Filtration Ltd: Epsom, Surrey, England.

Scigen Ltd: Sittingbourne, Kent, England.

Serva:(UK supplier Uniscience, London, England).

Sigma-Aldrich Chemical Co: Poole, Dorset, England.

Stratech Sciences Ltd: Luton, Bedfordshire, England.

Vector Laboratories: Bretton, Peterborough, England.

Whatman International Ltd: Maidstone, England.

APPENDIX TWO

APPENDIX II.

Preparation and Storage of Reagents/Buffers.

Section 2.3.2.1: Horizontal Agarose Slab Gel Electrophoresis of DNA

- **Electrophoresis buffer:** 20X (Stock) Tris-Acetate-EDTA. Stored at RT. 800 mM Tris-base (96.9 g), 400 mM Na acetate (32.8 g), 20 mM EDTA (7.45 g), was adjusted to pH to 7.4 with glacial acetic acid and made up to 1 litre with distilled water.

- **Sample buffer:** 6X (Stock). Stored at 4 °C.

  Composed of 1.5 ml Glycerol, 1 ml Bromophenol blue (1% (w/v) in distilled water), 1 ml Xylene cyanol (1 % w/v in distilled water) and 0.5 ml distilled water.

- **Ethidium Bromide:** (Appendix 1) Stored in foil wrap at 4 °C.

  Stock solution at 1 mg/ml (w/v) in distilled water. (**CARE !** Suspected carcinogen !).

Section 2.3.2.2: SDS-PAGE

- **Sample Buffer:** Stored at 4°C

  1 ml 10 % (w/v) SDS, 4.8 g Urea, 0.15 ml 1 M Tris-HCl (pH 6.8), 0.1 ml 2-mercaptoethanol and 0.2 mls Bromophenol Blue stock (3% w/v dissolved in ethanol and stored at 4°C), made up to 10 ml with distilled water.

- **Acrylamide/Bis-acrylamide Stock**: Stored at 4°C in the dark.

  Monomer solution (30 % T, 2.7% C_{bis}), 58.4 g acrylamide and 1.6 g Bis-acrylamide (Appendix 1) were made up to a final volume of 200 mls in distilled water.

- **20 % (w/v) ammonium persulphate**: Made up in distilled water, aliquoted and stored frozen at - 20°C.

- **Water-saturated n-Butanol:** Combine N-Butanol (Appendix 1) with and equal volume of distilled water , the top phase was used to overlay gels.

Section 2.3.2.2c: SDS linear gradient gel electrophoresis

- **Peptide Gel Buffer:** 5X stock.

3 M Tris-HCl (adjusted to pH 8.45), containing 0.3 % (w/v) SDS.

- **Anode Buffer:** 10X stock.

2M Tris-Base (adjusted to pH 8.9)

- **Cathode Buffer:** 10X stock.

1 M Tris-Base containing 1M Tricine and 1% (w/v) SDS.

Section 2.3.3: Measurement of chitinase activity

- **Preparation of chitin substrate.**

Crude chitin (Appendix 1) was purified by dissolving 20 g of chitin in 200 ml of concentrated HCl in an ice-bath. The residue was filtered through glass wool. The dissolved chitin was then precipitated by adding 200 ml of absolute ethanol. The purified chitin was washed with distilled water until a neutral pH was obtained, this was then air dried and resuspended in PBS. It was stored at 4^0C.

Section 2.3.4: Measurement of N-acetyl-glucosamine

- **Preparation of DMAB reagent**

10 g. of p-Dimethylaminobenzaldehyde was dissolved in 90 mls of glacial acetic acid and then made to 100 mls with 10 mls of concentrated hydrochloric acid. The 10 fold concentrated stock was stored at RT and just prior to use an aliquot was diluted 1:10 with glacial acetic acid.

Section 2.3.5: The general protease assay

- **BSA substrate solution:** A 1 %(w/v) was made in PBS, centrifuged at 10 K x g for 5 minutes and the supernatant was carefully removed and used as the protein substrate.

- **TCA Reagent:** 5% (w/v) solution of Trichloroacetic acid made in distilled water. Stored at RT.

Section 2.3.6: Measurement of Tosyl-arginine methyl ester (TAME)

- **TAME substrate:** 0.1 M solution of TAME was made in 0.1M Tris-HCl buffer (pH 8).
- **Reagent A:** Prepared immediately prior to use. Equal volumes of 3.5M NaOH solution and a 2M Hydroxylamine hydrochloride solution.
- **TCA reagent:** A 3% (w/v) Trichloroacetic acid solution made in 0.19M HCl.
- **Ferric Chloride Reagent:** 0.11 M Ferric Chloride made in 0.04M HCl.

Section 2.4.1: Semi-Dry Electrotransfer (Western Blotting)

- **Stock 10X Electro-transfer Buffer.** Stored at 4 ^{o}C.

480 mM Tris-base, 390 mM Glycine and 0.3 % (w/v) SDS. Made 1X just before use and containing 10 % (v/v) methanol.

- **Stock 10X Elecro-Transfer buffer II:** Stored at 4^{o}C.

100 mM CAPS, 22.13 g of CAPS in 900 ml of water. Titrated with 2 M NaOH to pH 11 and distilled water added to make the volume up to 1 L.

Section 2.4.2: Detection of Protein on PVDF Blots

- **CBB-Staining solution:** 0.1% CBB R-250 in 1% acetic acid/40% MeOH.

1g of CBB was dissolved in 400 ml methanol, Stirred for 1 hour then 10 ml of glacial acetic acid was added and made up to 1 L with distilled water. It was stirred for another 30 minutes then filtered. Stored at RT.

Section 2.4.3: Probing Western Blots

- **1X TBS** (Tris buffered saline)**:** 0.05 M Tris-HCl, 0.15 M NaCl at pH 7.5.
- **1X TBS-T** (Tris-Tween 20 Solution), as above but with 0.2% (v/v) Tween-20.

Section 2.4.4: Lectin Probing

- **TB-GT**: Tris buffer as above but containing 0.05% (w/v) Gelatin (Appendix 1) and 0.05% (v/v) Tween-20.
- **Incubation buffer:** Biotinylated Con-A solution.

10 μg of biotinylated Con-A/ml of a 2% (w/v) PVP-360 solution containing 1mM Ca^{2+} and Mn^{2+}.

Section 2.4.5: Periodate Chemical Treatment of Nitrocellulose Blots

- **Buffer A:** A 50mM Solution of Sodium Acetate, pH 4.5.
- **PBS-T:** As described in section 2.6.3 (Appendix 2).

Section 2.4.6: Enzyme Digest Treatment of Proteins Electroblotted onto Nitrocellulose

Protease Inhibitors (Stocks stored as aliquots at - 20°C).

- Aprotinin: 1 mg/ml (in dH_2O). 500X final concentration.
- Leupeptin: 1 mg/ml (in dH_2O). 500X final concentration.
- PMSF: 200 mM (in EtOH). 200X final concentration.
- TPCK: 3 mM (in EtOH). 250X final concentration.
- TLCK: 3 mM (in dH_2O). 250X final concentration.
- EDTA: 500 mM (in dH_20). 500X final concentration.

Section 2.4.7:

- **Sample Buffer:** As described in section 2.3.2.2 (Appendix 2).

Section 2.5: Screening A Placental cDNA Library

- **Tryptone Broth (TB) Medium:**
  - 10 g Tryptone(DIFCO)
  - 5 g NaCl

Distilled water to 1 litre, Autoclaved and then add to a final concentration, 10 mM Magnesium sulphate from 0.5 M Autoclaved stock

and 0.2% maltose from a 20 % filter sterilised stock. TB medium was stored at 4°C.

- **Plate Agar:**
  - 4 g NZ amine.
  - 2 g Bacto yeast extract.
  - 2 g Sodium Chloride.
  - 0.8 g Magnesium Sulphate
  - 6 g Bacto-Agar.

  Distilled water to 400 ml, Autoclaved prior to pouring plates.
- **Lambda Phage Diluent:** 10 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 0.1 mM EDTA. Autoclaved and stored at 4°C.
- **Top Agarose:**
  - 1 g NZ amine.
  - 0.5 g Bacto yeast extract.
  - 0.5 g Sodium Chloride.
  - 0.2 g Magnesium Sulphate
  - 0.7 g Agarose (Type 1, low EEO, Appendix 1)

  Distilled water to 100 ml, Autoclaved and stored at 4°C.
- **IPTG impregnated filters:** Sterile nitrocellulose filters soaked for 1 hour in a 10mM IPTG solution made in sterile distilled water.
- **Ampicillin Stock Solution:** Ampicillin (Appendix 1) was made to 10 mg/ml in distilled water, filter sterilised (0.22μm) and stored in aliquots at -70°C.
- **TBS-TB:** Tris Buffered Saline made as in section 2.4.3 (Appendix 2) containing 0.05% (v/v) Tween-20 and 20% (v/v) Bovine serum albumin.
- **TBS-T:** Tris Buffered Saline containing 0.05% (v/v) Tween-20.
- **Stock X-Gal:** 100 mg X-Gal (NBL) in 1 ml dimethylformamide.
- **Stock IPTG:** 1 M solution of IPTG.
- **TN buffer:** 25 mM Tris/HCl, pH 7.5 containing 0.5 M NaCl.
- **TNT buffer:** 0.1% (v/v) Triton X-100 in TN buffer.

α-CN-H_2O_2 solution: 6 mls of Reagent A added to 30 mls of Reagent B immediately prior to use. Reagent A: 3 mg/ml solution of α-CN made in

methanol. Reagent B: 150 µl of 1 M Imidazole and 15µl of a 30% solution of H_2O_2 made in 30 mls of TN buffer (Made just prior to use).

Section 2.6.1: Rocket Immunoelectrophoresis

- **Electrophoresis buffer: (**4X Stock) Tris-Barbital Buffer. Stored at RT.
  - 44.3 g of Tris
  - 22.4 g of Diethyl Barbituric acid
  - 0.533 g of Calcium Lactate

  Distilled water to a final volume of 1 litre with buffer stock having a final pH of 8.6.

Section 2.6.2: Radial Immunodiffusion (RID)

- **Preparation of agarose for RID**

1 g of agarose is dissolved in 100 ml of Tris-barbital running buffer and aliquoted into glass tubes (sealed with parafilm and stored at 4 °C.). The volume of agarose required for a 1.5 mm thick was calculated. An aliquot was liquefied in a boiling water bath and this stock agarose was diluted to a final concentration of agarose of 0.75 % (v/v) made to contain a final concentration of 2% (v/v) PEG 6000 solution (using a 40 % (w/v) PEG 6000 solution dissolved in Tris-barbital running buffer and stored at RT) with the difference in volume being made up with running buffer. This was left to equilibrate in a water bath at 55 °C and then the appropriate antibody was added (see plate captions).

- **Procedure for "Coating" glass plates**

Gently wipe a "hot" solution (approx. 70 °C) of 1 % (w/v) low EEO (Sigma) across the electrophoresis glass plate and allow to dry by convection. Repeat this procedure 2 - 3 times. The plate was then said to be coated.

- **Procedure for Pressing/Washing and Drying of Electrophoresis plates:**

1: The wells of the plate were filled with distilled water.

2: Carefully cover the gel with a damp Whatman filter.

3: Cover the plate with a wad of dry tissue.

4: Place a flat surface on top of the tissue and leave a 1 kg weight to press down on plate (leave for about 5 minutes).

5: Carefully remove wad of tissues and repeat from step 3. until tissues no longer appeared to be damp.

6: Carefully remove filter covering plate (from step 2) and place the plate in a 0.15 M NaCl solution for about 15 minutes.

7: Repeat step 6.

8: Repeat steps 2 - 5.

9: Carefully remove filter paper and place plate in distilled for about 15 minutes.

10:Repeat step 9.

11:Repeat steps 2 - 5.

12:Carefully remove filter paper and dry plate gently by leaving it on the lid of a water bath at 95 ^{o}C until the wet opaque agarose appears clear.

- **Staining/Destaining of electrophoresis plates**

The plate was immersed plate in stain (see below) for 10 - 45 minutes, then placed in destain and left until the desired contrast between immunoprecipitin and background on the plate had been reached.

- **Immunoelectrophoresis Stain(CBB-R250)/Destain.**

  - 0.5 % CBB(w/v).
  - 45 % EtOH(v/v).
  - 10 % Glacial Acetic Acid(v/v).

  Made to 100% with distilled water.

Destain consisted of the same ratio of chemicals, except no CBB-R250 was included.

Section 2.6.3: Sandwich ELISA for PAPP-A

- **ELISA Blocking Buffer: PBS-Block (plus BSA):** PBS containing 5 %(w/v) BSA. Prepared on day of use.
- **ELISA Assay Diluent: PBS-TB (plus BSA):**PBS-T containing 0.2 % (w/v) BSA. Prepared on day of use.
- **ELISA Wash Buffer: PBS-Tween (PBS-T):** PBS containing 0.05 %(v/v) Tween-20. Prepared on day of use.
- **Substrate development Buffer: Citric acid-Phosphate Buffer (pH 5.6):** A 0.1M solution of Citric acid monohydrate and a 0.2M solution of Na_2HPO_4, 12 H_2O were mixed until the pH was 5.6 at RT.
- **ELISA Substrate Stock** (as originally described by Goka and Farthing, **1987**). Essentially 10 mg/ml TMB (Appendix 1) was dissolved in DMSO, containing 8 mM Imidazole. It was stored in a foil wrapped container at RT.
- **ELISA colour reagent:** Just prior to use the ELISA substrate stock was made to a final concentration of 0.1 mg/ml and 0.005 % H_2O_2 (from a 30% (v/v) H_2O_2 stock) in substrate development buffer (see above) and used immediately.
- **ELISA stop reagent:** 1 M sulphuric acid in distilled water. Stored at RT.

Section 2.6.4:

- **RIA assay diluent:** 50 mM Phosphate Buffer, pH 7.4 containing 0.1% (w/v) BSA and 50 mM NaCl.
- **Column Blocking Solution:** 30 % BSA (v/v) made in RIA Assay Diluent (see above).

Section 2.8.1: Assessment of Protein Elution Methods from Blotting Membranes

- **Sample Buffer:** As described in section 2.3.2.2 (Appendix 2).

Section 2.8.4: Vapour Phase CNBr Cleavage of PAPP-A in Gel Slices

- **Sample Buffer:** As described in section 2.3.2.2 (Appendix 2).

Section 2.8.7: De-protection of PAC amidites

- Place cassette (flange up) in sterile 1.5. ml eppendorf tube.
- Pulse spin in microfuge.
- Remove cassette and place in sterile 2ml flat bottom tube.
- Add 1 ml of 30% Ammonia solution and pulse spin in microfuge.
- Ensure cap is sealed tightly with parafilm, place inside universal tube.
- Heat Universal at 70^{0}C for 1 hour.
- Let tube equilibrate to 4^{0}C before opening.
- Remove cassette and place flange uppermost in sterile 1.5 ml eppendorf.
- Pulse spin in microfuge and discard cassette.
- Pool ammonia and precipitate oligonucleotides.

Section 2.8.7: Precipitation of oligonucleotides

- Aliquot the 1 ml of ammonia containing oligonucleotides into 3, 330 ul in sterile 1.5 ml eppendorf tubes.
- Add 33 ul of 3M sodium acetate and 915 ul of absolute ethanol to each tube.
- Allow tubes to stand overnight at -20^{0}C.
- Spin at 13 k rpm for 15 minutes in a microfuge at 4^{0}C.
- Discard supernatant and resuspend pellet in 0.5 ml of 80% ethanol.
- Pool all 3 tubes into 1 sterile 1.5 ml eppendorf and spin at 4^{0}C as above.
- Discard ethanol, cover with perforated parafilm and freeze dry.
- Resuspend oligonucleotide pellet in 600 ul of sterile distilled water.

Section 2.9: The interaction between reduced monomeric PAPP-A and the endoproteinase Arg-C.

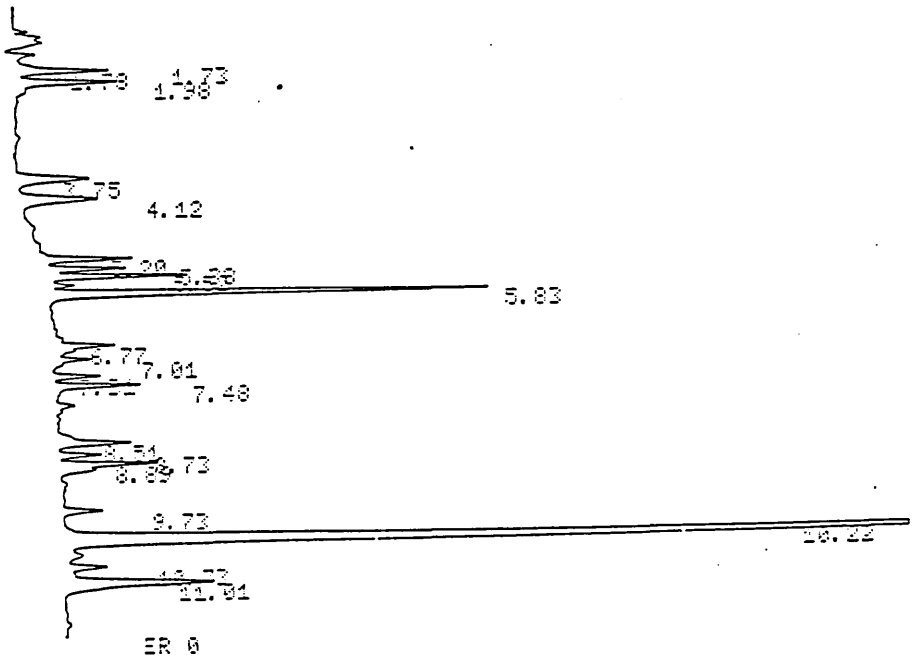
- **Buffer A:** 0.1M Tris-HCl, pH 8.

TAME substrate: Solution as in section 2.3.6 (Appendix 2).

APPENDIX THREE

Appendix III

AIII.1 Amino Acid Analysis



PICOTAG 07:03:92 17:27:09 CH= "A" PS= 1.

FILE 1. METHOD 5. RUN 14 INDEX 1

ANALYST: ART

NAME	PICOMOLES	RT	AREA	BC	RF
ASX	13.591	1.73	23522	02	1200.628
2	0.[illegible]	1.98	48273	03	
GLX	46.919	4.38	53061	01	1130.912
SER	43.65	5.75	50462	01	1156.072
GLY	48.199	4.12	55773	01	1145.26
ARG	31.966	5.83	36885	01	1151.376
THR	28.092	6.38	31692	02	1128.136
ALA	49.886	6.82	62559	03	1253.856
PRO	185.992	5.63	239084	01	1285.44
10	0.	6.77	19553	01	
TYR	7.893	7.01	10088	01	1278.12
12	0.	7.34	20054	01	
VAL	24.774	7.48	34850	01	1406.688
CYS	16.968	8.51	35148	01	2163.736
ILE	11.558	8.73	14762	02	1389.088
LEU	27.394	8.89	[illegible]	02	1330.024
PHE	14.027	[illegible]	[illegible]	01	1322.864
18	0.	10.22	96.390	01	
LYS	8.371	10.73	17578	01	2099.984
20	0.	11.01	123026	01	

PROBABLY X 6 SINGLE PEAK

$\times NH_3$

RUN 1527 AB II 18

Results of total Amino acid analysis on the selected peak from a CNBr digest (Figure 6.3). Service provided by Dr A. Moir.

AIII.2 Protein Microsequencing

AIII.2.1 Results of vapour phase CNBr cleavage of PAPP-A as described in Chapter 6, section 6.3.1. Sequencing was performed by Mr J. Gilroy, Durham University.

Band sizes in kDa of CNBr digest of PAPP-A

Fragment	Size
CNBr 1	70
CNBr 2	48
CNBr 3	42
CNBr 4	32
CNBr 5	21
CNBr 6	18
CNBr 7	13

Only CNBr4 gave a reasonable amount of material that could be sequenced, a summary of the information obtained from this peak is given below.

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	S	4.57	4.57	8.49	6.09	6.09	9.74	SER
2	A	7.86	7.85	127.45	78.73	72.73	45.87	ALA
3	A	7.85	7.85	172.32	126.59	126.59	73.75	ALA
4	A	7.88	7.85	157.96	115.21	115.21	67.13	ALA
5	S	4.60	4.57	7.40	4.53	4.53	7.24	SER
6	D	3.47	3.43	23.25	10.53	12.89	8.57	ASP
7	I	15.85	15.82	8.21	1.66	2.00	2.94	ILE
8	H	7.90	7.90	7.20	4.02	5.02	8.20	HIS
9	N	3.95	3.90	7.05	1.22	1.66	2.67	ASN
10	L	16.45	16.42	11.21	13.07		16.02	LEU

REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
S: 1, 5	92.68 %	1.000	:SER
A: 2, 3, 4	120.97 %	0.573	:ALA

Average AA Repetitive Yield: 111.91 %

Combined AA Repetitive Yield: 73.23 % 0.309

Theoretical Initial Yield: 82.99 pmol (82.99 %)

AIII.2.2 Initial N-terminus for PAPP-A obtained from material purified using scheme 1 as illustrated by a ★ in Figure 5.2 (Chapter 5). Sequencing was performed by Dr K. Lilley.

CYCLE No.	RESIDUE	AMOUNT(pmoles)
1	Lys	2.3
2	Ala	11.4
3	Arg	6.7
4	Gly	10.0
5	Ala	7.1
6	Thr	10.6
7	Glu	5.7
8	?	
9	Val	3.5

AIII.2.3 Consensus sequence for N-terminus of PAPP-A. Sequencing was performed by Mr J. Gilroy.

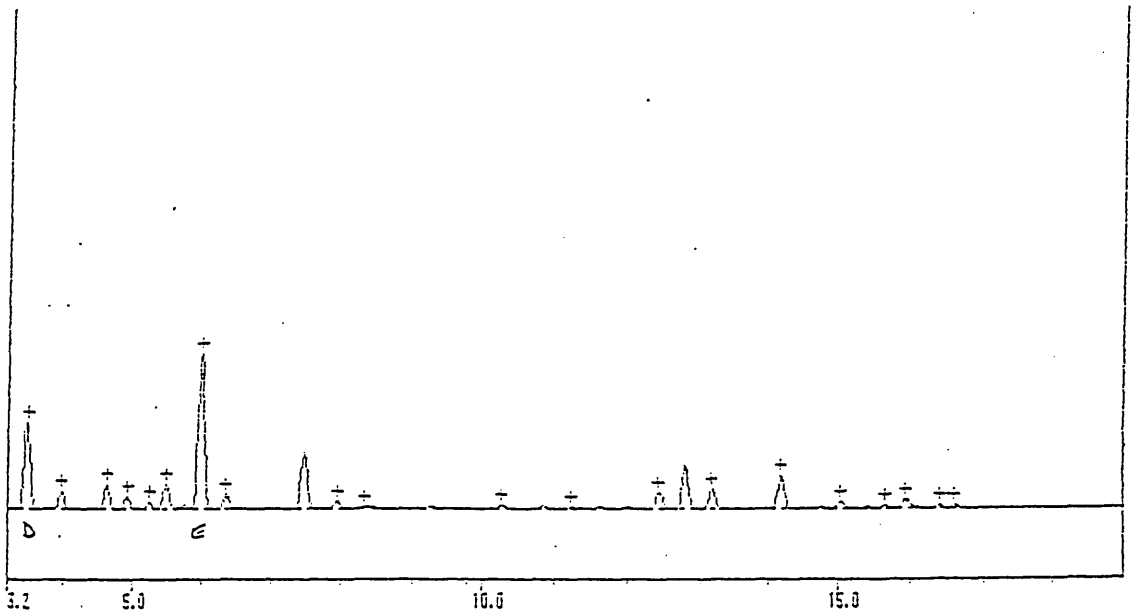
CYCLE No.	RESIDUE	AMOUNT(pmoles)
1	Glu	2.9
2	Ala	5.7
3	Arg	1.2
4	Gly Leu	3.6 1.6
5	Ala	2.6
6	Thr	2.6
7	Glu	2.6
8	Glu	4.6
9	Pro	2.8

AIII.2.4 PAPP-A N-terminal sequence obtained from Lys-C digestion of fragment (a) as seen in Figure 6.4 (Chapter 6). Sequencing was performed by Mr J. Gilroy.

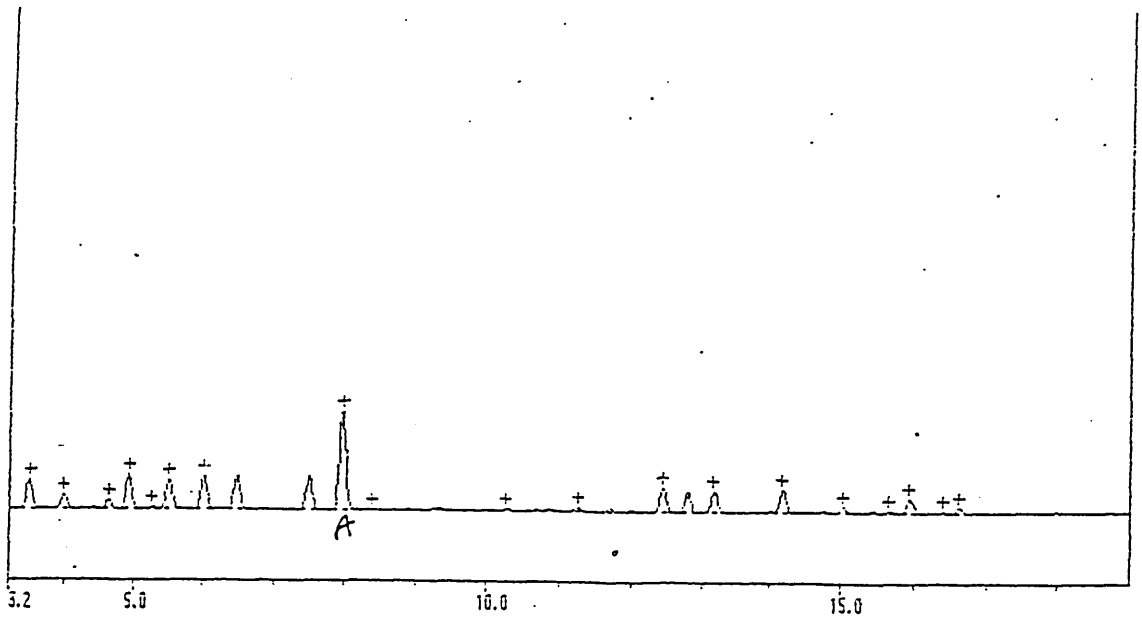
SAMPLE : PAPPA/LYS-C-1/MS
[Initiated 6 Nov 1992 8:43am]

CYCLE SUMMARY :
Reaction cycle : FBLOT
Conversion cycle : FBLOT
Gradient : FBLOT
Data collect time : 0.0 to 19.0 min
Data interval : 1.0 sec
Inject volume : 50 of 150 uL

AMINO ACID # 1 [6 Nov 1992 10:01am] 0.0100 FU
Filtered Data

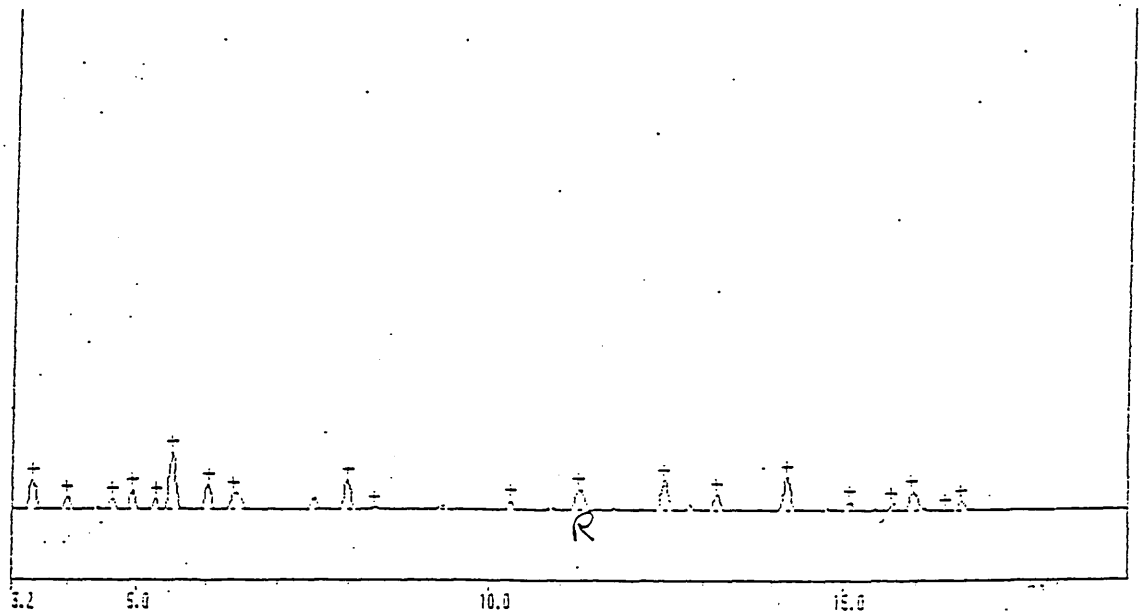


AMINO ACID # 2 [6 Nov 1992 10:33am] 0.0100 FU
Filtered Data



AMINO ACID # 3 [6 Nov 1992 11:05am] 0.0100 FU

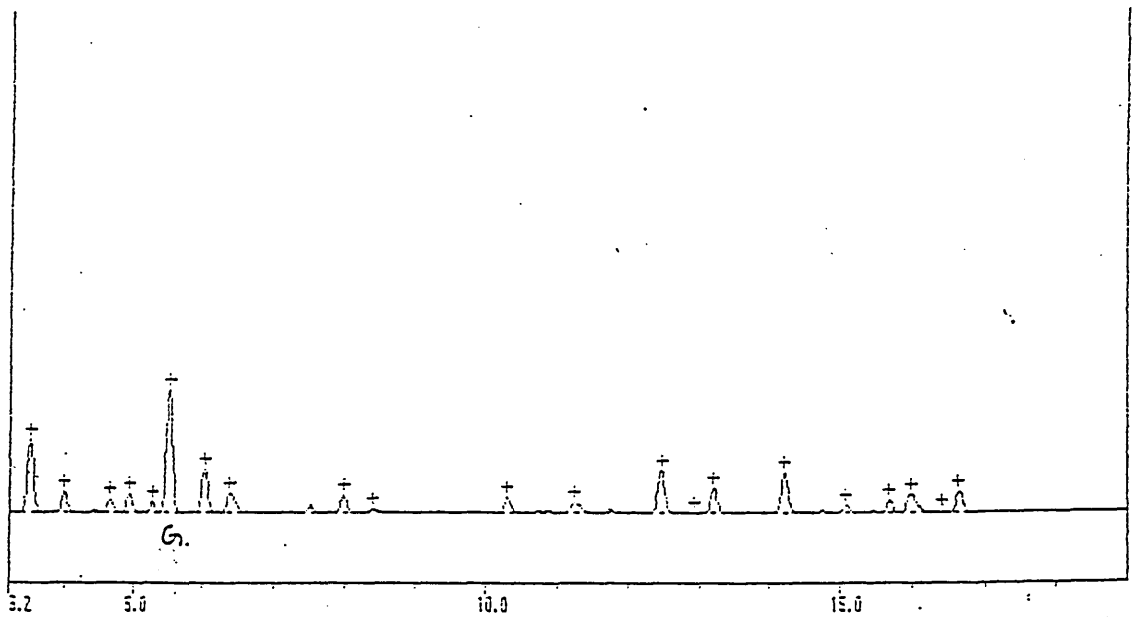
Filtered Data

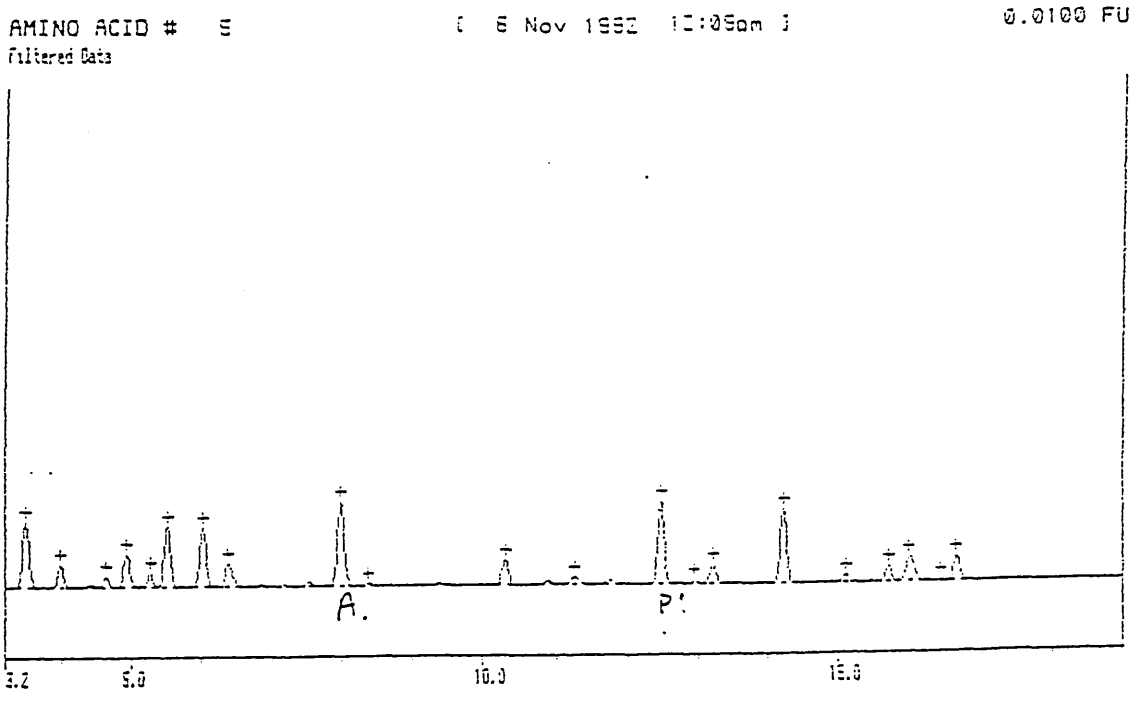


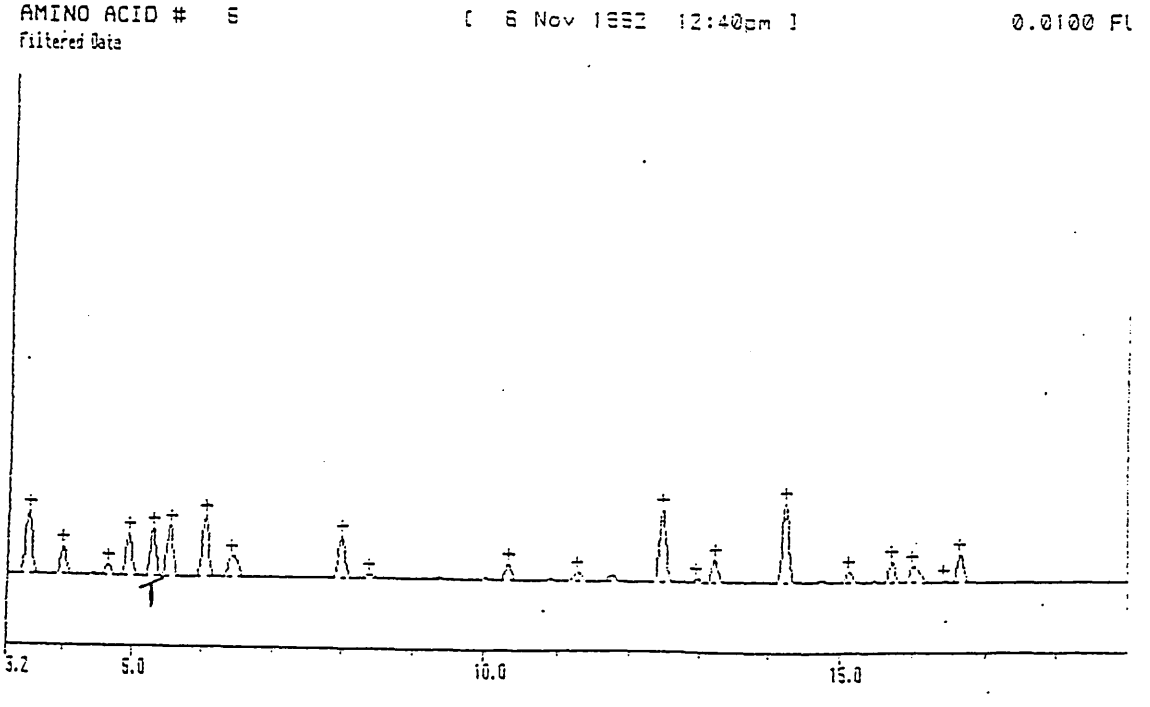
Retention Time: Minutes

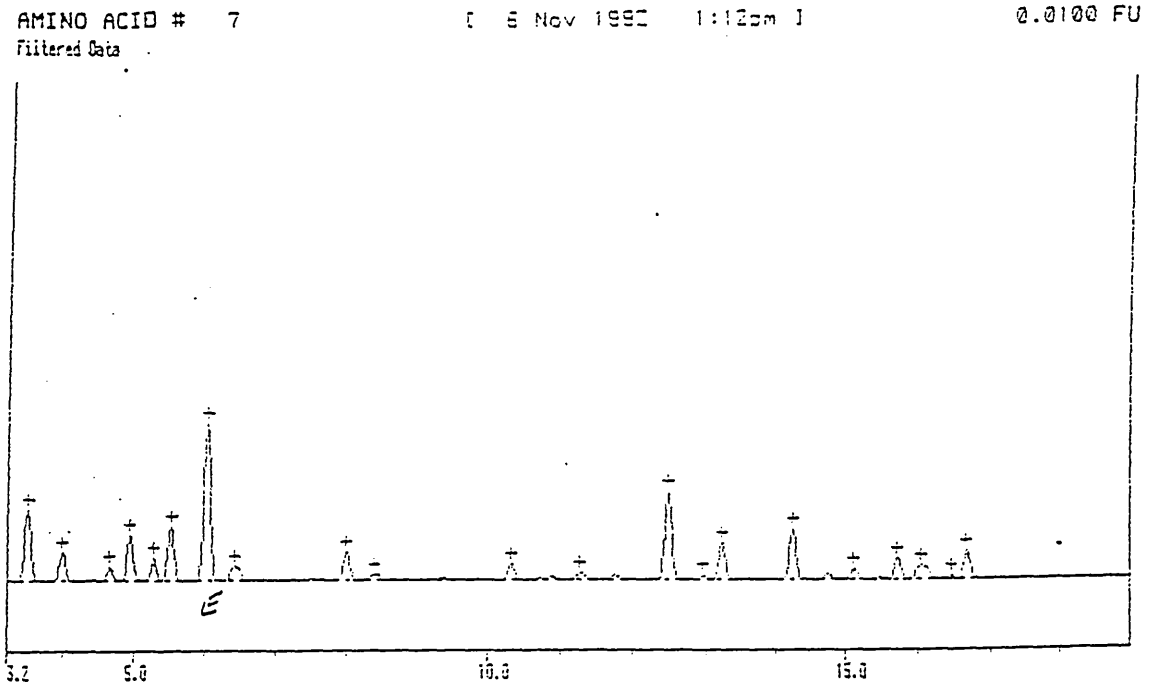
AMINO ACID # 4 [6 Nov 1992 11:37am] 0.0100 FU

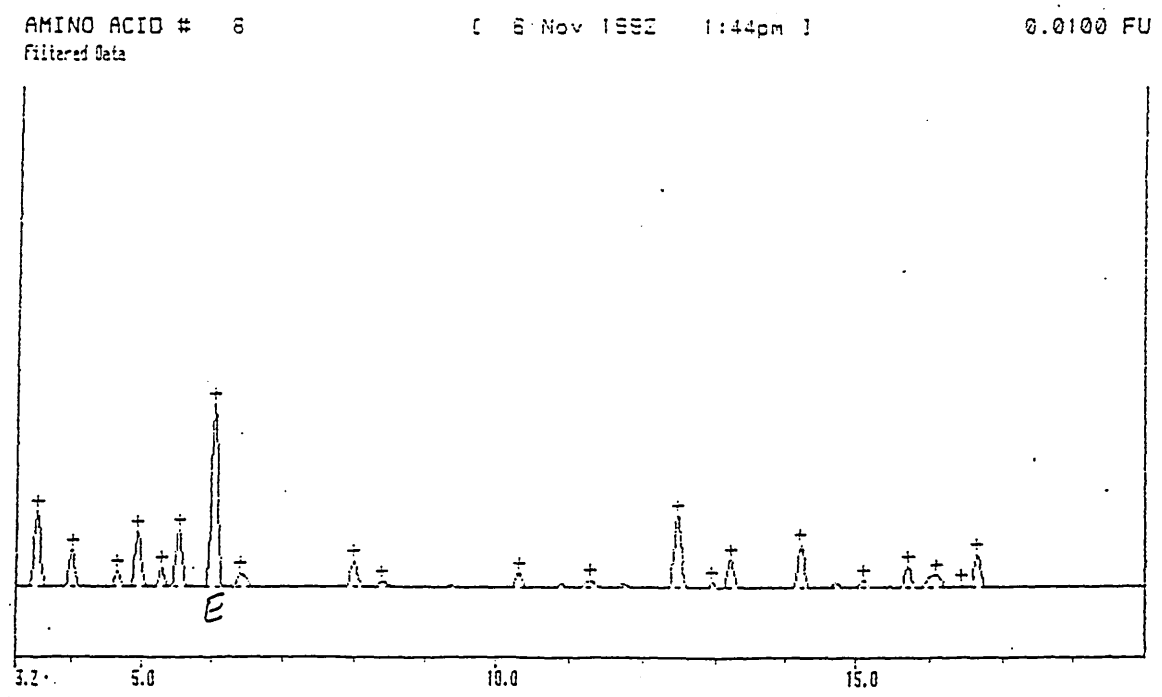
Filtered Data

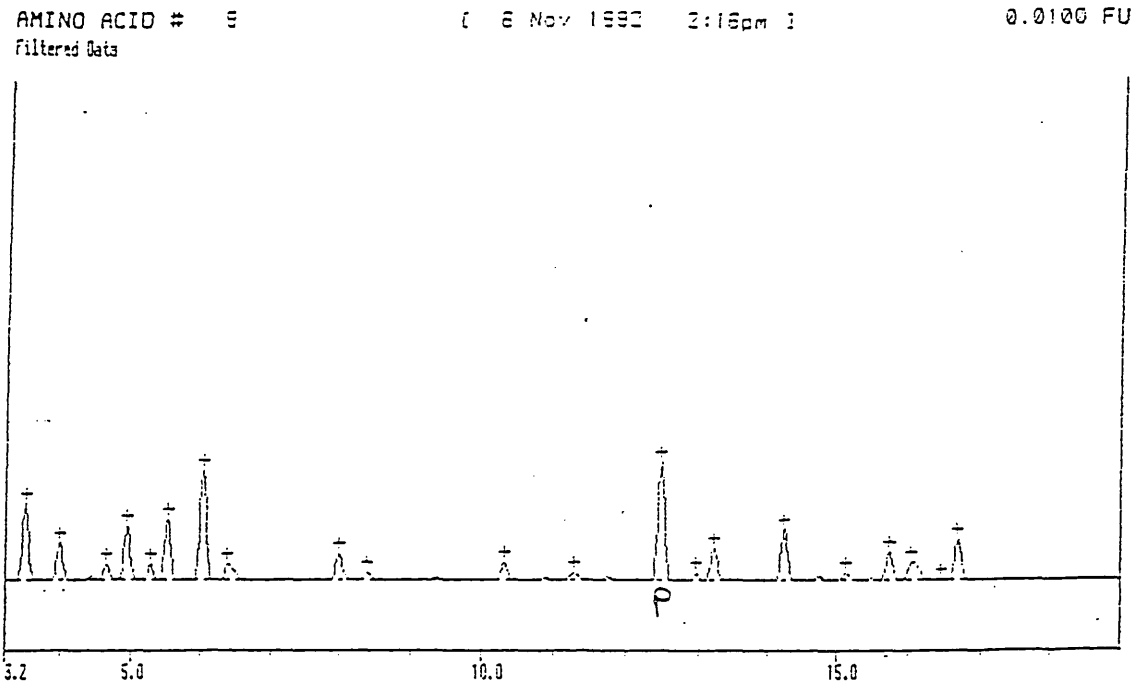


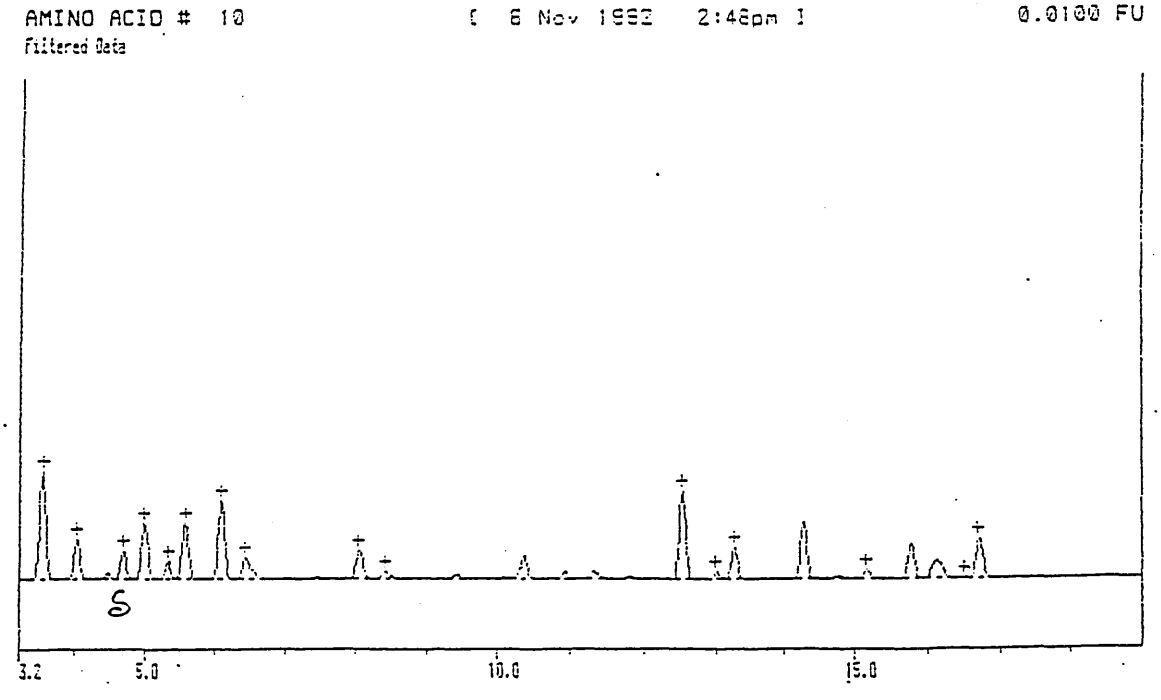

AMINO ACID # 5
[6 Nov 1992 12:09pm]
0.0100 FU
Filtered Data
A.
P'
3.2
5.0
10.0
15.0

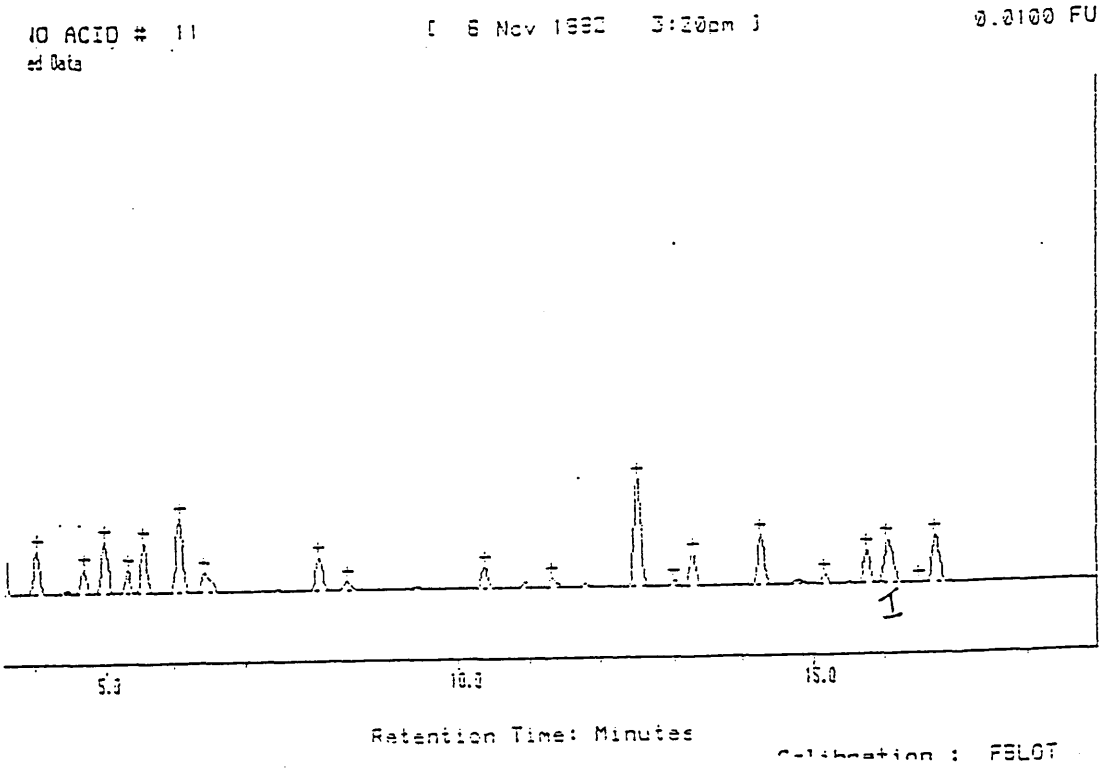

AMINO ACID # 5
[6 Nov 1992 12:40pm]
0.0100 FU
Filtered Data
T
3.2
5.0
10.0
15.0

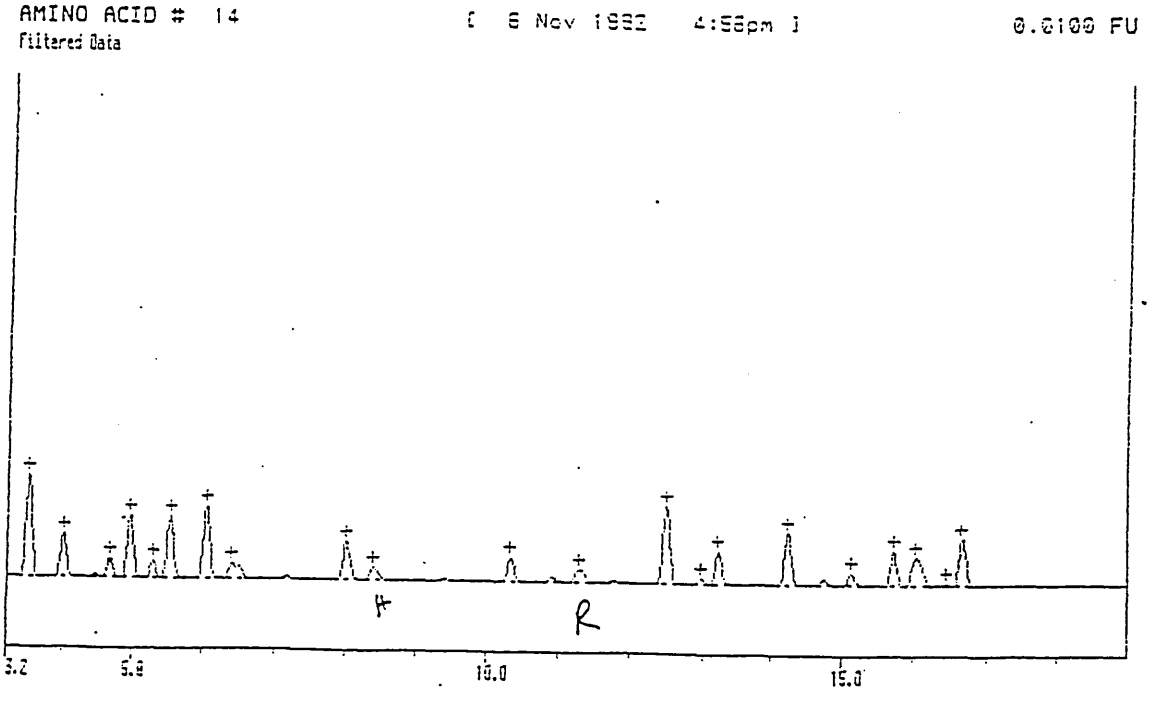

AMINO ACID # 7
[6 Nov 1992 1:12pm]
0.0100 FU
Filtered Data
E
3.2
5.0
10.0
15.0
Retention Time: Minutes


AMINO ACID # 6
[6 Nov 1992 1:44pm]
0.0100 FU
Filtered Data
E
3.2
5.0
10.0
15.0


AMINO ACID # 9
[6 Nov 1992 2:18pm]
0.0100 FU
Filtered Data
P
3.2
5.0
10.0
15.0


AMINO ACID # 10
[6 Nov 1992 2:48pm]
0.0100 FU
Filtered Data
S
3.2
5.0
10.0
15.0


0.0100 FU
1
5.0
10.0
15.0
Retention Time: Minutes


AMINO ACID # 14
Filtered Data
[6 Nov 1992 4:58pm]
0.0100 FU
H
R
3.2
5.0
10.0
15.0

SAMPLE : PAPPA/LYS-C-1/MS
[initiated 6 Nov 1992 8:43am]

Sample Amount: 100 pmol

	AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
(P)	1	E	6.00	6.00	35.57	32.25	34.95	47.32	GLU
	2	A	7.97	7.95	35.98	32.25	38.10	47.60	ALA
	3	G	5.52	5.50	22.13	9.20	11.34	21.54	GLY
	4	G	5.53	5.50	46.98	32.47	38.91	70.61	GLY
(P)	5	A	7.99	7.95	31.12	23.63	30.63	38.60	ALA
	6	T	5.28	5.30	20.58	14.46	18.02	37.28	THR
	7	E	6.03	6.00	36.02	22.48	30.03	41.12	GLU
	8	E	6.03	6.00	42.10	27.58	28.98	38.63	GLU
	9	P	12.45	12.40	33.32	10.73	13.25	11.64	PRO
	10	S	4.70	4.60	10.93	4.77	5.53	15.83	SER
	11	I	16.03	15.97	15.97	10.01	14.21	12.47	ILE
	12	D	3.52	3.45	23.66	4.34	4.97	8.48	ASP
	13	I	16.00	15.97	16.05	9.53	12.63	11.13	ILE
R.	14	(H)	8.38	8.30	10.21	3.19	4.17	10.24	HIS
	15	A	8.00	7.95	16.84	3.60	5.84	7.05	ALA
	16	L	16.67	16.60	25.98	5.13	8.53	13.60	LEU
	17	I	16.05	15.97	13.01	5.05	7.51	8.59	ILE
?	18	P	12.45	12.40	13.48	0.85	1.06	0.95	PRO
?	19	P	12.47	12.40	13.37	3.97	6.81	5.81	PRO
?	20	G	5.55	5.50	16.98	1.95		3.51	GLY

REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
E: 1, 7, 8	95.50 %	0.554	:GLU
A: 2, 5, 15	84.07 %	0.992	:ALA
G: 3, 4, 20	87.95 %	0.755	:GLY
P: 9, 18, 19	84.42 %	0.555	:PRO
I: 11, 13, 17	88.65 %	0.929	:ILE

Average AA Repetitive Yield: 99.32 %

Combined AA Repetitive Yield: 35.14 % 0.742

Theoretical Initial Yield: 40.11 pmol (40.11 %)

AIII.2.5 Sequencing obtained from Lys-C digestion of fragment (b) as seen in in Figure 6.4 (Chapter 6). Sequencing was performed by Mr J. Gilroy.

SAMPLE : PAPPA/LYS-C-2/MS
[initiated 11 Nov 1992 8:58am]

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	N	3.82	3.82	11.93	11.25	13.04	60.82	ASN
2	P	12.03	12.02	11.38	5.79	7.93	11.58	PRO
3	T	5.08	5.08	14.42	11.75	15.32	31.13	THR
4	V	12.86	12.87	11.59	7.70	9.01	43.98	VAL
5	P	12.03	12.03	20.85	12.84	16.58	24.18	PRO
6	G	5.32	5.32	34.19	16.89	22.35	8.87	GLY
7	E	5.78	5.78	17.20	9.33	11.96	25.04	GLU
8	Q	4.70	4.70	18.70	9.29	12.20	11.31	GLN
9	V	12.88	12.87	12.28	6.47	7.78	37.95	VAL
10	D	3.40	3.38	25.34	10.32	15.25	15.37	ASP
11	T	5.09	5.08	12.87	4.08	5.03	12.29	THR
12	T	5.09	5.08	14.22	5.23	4.65	9.46	THR
13	H	8.15	8.17	8.23	2.42	3.40	8.99	HIS
14	H	8.12	8.17	10.05	5.98	7.72	15.88	HIS
15	H	8.10	8.17	11.36	7.02	7.31	15.04	HIS
16	L	16.27	16.25	14.93	4.48	7.34	20.02	LEU
17	A	7.70	7.68	32.09	6.39	9.71	9.35	ALA
18	E	5.82	5.78	17.46	2.74	3.04	6.35	GLU
19	N	3.82	3.82	9.59	0.42	0.38	1.76	ASN
20	V	12.90	12.87	11.14	3.24		15.18	VAL

REPETITIVE YIELD ANALYSIS:

		Rep.Yield	Variance	
N:	1, 19	83.28 %	1.000	:ASN
P:	2, 5	130.42 %	1.000	:PRO
T:	3, 11, 12	88.93 %	0.895	:THR
V:	4, 9, 20	94.60 %	0.996	:VAL
E:	7, 18	89.48 %	1.000	:GLU
H:	13, 14, 15	170.29 %	0.860	:HIS

Average AA Repetitive Yield: 112.54 %

Combined AA Repetitive Yield: 91.13 % 0.462

Theoretical Initial Yield: 15.31 pmol (15.31 %)

AIII.2.6 Sequencing obtained from Lys-C digestion of fragment (d) as seen in Figure 6.4 (Chapter 6). Sequencing was performed by Mr J. Gilroy.

SAMPLE : PAPPA/LYS-C-4/MS
[initiated 12 Nov 1992 10:09am]

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	S	4.47	4.42	5.27	3.74	3.99	27.55	SER
2	Q	4.73	4.67	3.84	1.82	2.29	6.54	GLN
3	I	15.70	15.63	3.99	1.23	1.10	3.05	ILE
4	G	5.33	5.29	12.30	6.48	6.37	9.72	GLY
5	R	11.00	10.98	2.93	1.43	1.79	12.15	ARG
6	T	5.08	5.03	3.50	1.09		3.78	THR

REPETITIVE YIELD ANALYSIS:

Combined AA Repetitive Yield: 86.08 % 0.156

Theoretical Initial Yield: 3.54 pmol (3.54 %)

SAMPLE : PAPPA/LYS-C-4/MS

Sample Amount : 100 pmol
Date/Time of Run : 12 November 1992 10:09am
Cycles Run : 1 STD, 6 AA

Raw Data Tabulation ... (in pmols)

Cycle	A ALA	R ARG	N ASN	D ASP	C CYS	E GLU	Q GLN	G GLY	H HIS	I ILE	L LEU	K LYS	M MET	F PHE	P PRO	S SER	T THR	W TRP	Y TYR	V VAL
1	5.3	2.8	2.0	3.4	0.0	6.1	3.1	12.3	1.1	2.2	1.4	0.0	0.0	0.0	2.6	5.3	3.0	1.4	1.5	1.7
2	0.0	0.0	1.1	1.6	0.0	2.5	3.3	4.1	0.2	3.9	0.9	0.1	0.0	0.0	0.0	1.7	0.6	0.3	0.0	0.0
3	5.7	0.0	1.4	2.8	0.0	0.0	3.2	6.6	0.3	4.0	0.0	0.0	0.5	0.0	0.0	1.1	1.3	0.4	0.9	0.0
4	0.0	0.0	1.4	3.1	0.0	0.0	1.9	12.3	1.4	3.7	0.0	0.0	0.5	0.0	0.0	1.1	1.0	0.4	0.0	0.0
5	0.0	2.6	1.2	3.1	0.0	3.1	2.0	6.5	0.5	2.9	0.0	0.0	0.4	0.0	0.0	1.2	2.2	0.0	0.0	0.0
6	0.0	2.1	1.6	3.0	0.0	0.0	3.1	6.1	0.3	2.9	0.0	0.0	0.5	0.0	0.0	1.1	3.5	0.1	0.0	0.0

AIII.2.7 Sequencing obtained from Lys-C digestion of fragment (e) as seen in Figure 6.4 (Chapter 6). Sequencing was performed by Mr J. Gilroy.

SAMPLE : PAPPA/LYS-C-5/MB
[initiated 10 Nov 1992 8:36am]

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Fmol (raw)	Fmol (-bkgd)	Fmol (+lag)	Pmol Ratio	AAcid ID
1	S or Q	4.90	4.78	6.55	5.62	5.75	21.54	SER
2	Q	5.12	5.12	26.97	19.04	20.19	18.95	GLN
3	A	8.19	8.17	10.93	6.29	5.87	18.53	ALA
4	G	5.70	5.53	23.46	12.47	12.47	17.51	GLY
5	D or P	3.62	3.50	11.99	5.95	6.31	9.06	ASP
6	M or P	13.17	13.13	0.39	0.10		2.00	MET

Handwritten margin notes: beside 5: "Appears to have P in [illegible]"; beside 6: "[illegible] 6 →"

REPETITIVE YIELD ANALYSIS:

Combined AA Repetitive Yield: 90.55 % 0.499

Theoretical Initial Yield: 46.10 pmol (46.10 %)

SAMPLE : PAPPA/LYS-C-5/MB

Sample Amount : 100 pmol
Date/Time of Run : 10 November 1992 8:36am
Cycles Run : 1 STD, 6 AA

Lag Corrected Tabulation ... (in pmols)

Cycle	A ALA	R ARG	N ASN	D ASP	C CYS	E GLU	Q GLN	G GLY	H HIS	I ILE	L LEU	K LYS	M MET	F PHE	P PRO	S SER	T THR	W TRP	Y TYR	V VAL
1	0.0	0.5	0.1	6.2	-1.0	0.4	0.1	5.5	1.0	-1.1	6.0	-0.1	-0.0	0.3	1.2	6.8	0.7	0.3	2.4	-0.2
2	-0.2	-0.4	0.1	0.7	-1.0	2.4	20.2	0.7	-0.5	-1.1	0.3	0.1	-0.0	-0.1	-0.2	0.0	-1.0	-0.1	-1.9	3.4
3	6.7	1.0	-0.4	-0.8	-1.0	-0.0	0.1	-1.3	-0.5	4.7	-0.5	0.3	-0.0	-0.2	-1.5	1.5	9.5	-0.2	9.0	0.3
4	0.8	-0.4	0.1	-0.9	-1.0	-0.9	-1.9	12.5	0.2	4.5	-0.3	-0.1	-0.0	-0.2	2.5	0.0	1.5	-0.4	-2.1	-0.0
5	0.6	-0.4	-0.0	6.3	-1.0	3.3	-0.6	-0.3	0.4	2.0	-0.1	-0.1	-0.0	0.0	6.5	-0.5	0.3	0.1	6.9	-0.2
6	-0.5	0.6	0.1	0.3	-1.0	0.3	1.1	0.5	0.0	-1.4	0.4	-0.1	0.1	0.2	0.3	0.4	-0.1	0.3	1.3	-0.0

AIII.2.8 PAPP-A N-terminal sequence obtained from Glu-C digestion of fragment (a) as seen in Figure 6.5 (Chapter 6). Sequencing was performed by Mr J. Gilroy.

SAMPLE : PAPPA [illegible]
(Initiated 27 Jul 1993 7:03am)

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	E	9.05	9.03	10.17	9.75	12.33	99.00	GLU
2	A	7.83	7.83	20.83	16.25	20.75	99.34	ALA
3	R X	9.05	9.13	1.84	1.98	1.74	9.01	THR
4	G	9.43	9.42	17.33	9.81	7.71	19.99	GLY
5	A	7.82	7.82	14.14	8.55	10.33	29.27	ALA
6	T	9.09	9.13	7.07	3.70	4.99	19.75	THR
7	E	9.07	9.08	6.05	3.02	3.82	17.17	GLU
8	E	9.07	9.03	10.33	4.56	5.84	19.49	GLU
9	P	12.32	12.20	11.10	2.37		9.14	PRO

REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
E: 1, 7, 8	97.02 %	0.732	:GLU
A: 2, 5	90.78 %	1.000	:ALA
T: 3, 6	95.27 %	1.000	:THR

Average AA Repetitive Yield: 94.34 %

Combined AA Repetitive Yield: 98.10 % 0.334

Theoretical Initial Yield: 10.49 pmol 10.49 %

AIII.2.9 Sequencing of iodination grade PAPP-A (Table 6.2, Chapter 6). Sequencing performed by Leeds university central sequencing service.

1	D 305407						
2	Q 233398	L 212150	V 151089			M 80247	
3	E 537985	N 205455	W 9241			R 104403	
4	L 132629	G 85863	P 61198			F 25356	
5	N ~ 130000	A 37134	V 39651			I 22160	
6	D 302697	K 103652	L 122542			R 54965	
7	K ~ 140000	V 87325	F 83389	A 70327		E 50189	I 23390
8	E 146323	R 80349				T 6786	
9	F 63570	I 66705					
10	E 129956	K 57378	T 17441	G 54771		A 32566	Y 23587
11	E ~ 250000	M 127154	A ~ 70000	P 22209			
12	N 82359	G 36509	P ~ 35000	S 7792		H 10705	
13	F 70746	D 20123	R 2240				
14	W 46383	L 53281	N ~ 85000				
15	V 64726	Q 60915	F 47309	A 36694		G 21137	I 17747
16	V ~ 75000	D 50641	L 41281				

(Note, 10,000 equivalent to 1 pmol)

AIII.2.10 Sequence obtained for design of cPAPP-3 primer from fragment (b) from Glu-C digestion as seen in Figure 6.5 (Chapter 6). Sequencing was performed by Mr J. Gilroy.

SAMPLE : PAPPA/K-2/MB
(Initiated 27 Jul 1992 2:00pm)

Sample Amount: 100 pmol

AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	H	7.55	7.50	11.43	9.71	11.81	35.87	HIS
2	F	19.82	19.83	3.87	3.92	3.07	9.52	PHE
3	N	3.93	3.89	12.35	10.71	12.89	32.78	ASN
4	A	7.92	7.83	14.36	9.82	11.18	17.22	ALA
5	D	3.60	3.55	7.08	3.03	3.89	10.24	ASP
6	G	5.50	5.45	11.83	7.12	8.14	12.65	GLY
7	G	5.48	5.47	11.57	6.90	8.65	8.20	GLY
8	L	18.62	18.50	12.44	2.91	3.69	4.56	LEU
9	S	4.62	4.55	3.44	0.43		1.38	SER

REPETITIVE YIELD ANALYSIS:

		Rep.Yield	Variance	
G:	6, 7	95.91 %	1.001	:GLY
Average AA Repetitive Yield:		95.91 %		
Combined AA Repetitive Yield:		78.45 %	0.430	

Theoretical Initial Yield: 15.09 pmol (15.09 %)

SAMPLE : PAPPA/K-3/MB

Sample Amount : 100 pmol
Date/Time of Run : 24 August 1992 8:54am
Cycles Run : 1 STD, 9 AA

Lag Corrected Tabulation ... (in pmols)

Cycle	A ALA	R ARG	N ASN	D ASP	C CYS	E GLU	Q GLN	G GLY	H HIS	I ILE	L LEU	K LYS	M MET	F PHE	P PRO	S SER	T THR	W TRP	Y TYR	V VAL
1	-0.7	1.2	0.2	0.8	-1.0	-1.0	0.0	11.5	0.4	24.0	-0.4	-0.5	0.1	10.4	-0.0	19.0	10.9	4.2	-0.3	0.5
2	1.1	-0.4	-0.5	37.5	-1.0	0.3	-0.2	-0.6	0.1	0.0	0.5	12.9	-0.0	0.0	10.9	0.0	2.3	0.0	0.5	-1.6
3	32.1	0.7	0.0	9.2	-1.0	0.5	-0.2	0.0	0.0	0.0	-0.5	0.0	-0.3	-0.1	3.4	0.0	0.0	0.0	-0.5	3.0
4	15.7	0.1	0.4	0.0	-1.0	2.1	-0.2	0.0	-0.0	-0.4	17.9	-0.2	-0.2	0.2	0.0	-0.0	0.0	-0.4	-0.2	1.6
5	0.0	1.9	0.0	-0.1	-1.0	0.0	1.0	5.9	-0.3	-0.4	0.0	0.3	7.0	-0.4	0.9	-0.5	-0.4	-0.4	-0.3	0.9
6	0.0	-0.3	0.1	-0.8	-1.0	5.0	2.3	1.0	-0.5	-0.3	12.8	0.3	0.0	-0.3	-0.2	-0.3	-0.5	-0.3	1.3	0.1
7	0.2	-0.3	-0.0	-0.5	-1.0	2.0	1.2	1.4	-0.6	0.4	2.9	1.0	-0.2	1.5	0.4	-0.4	1.5	-0.0	0.4	2.5
8	-0.9	9.9	-0.1	0.3	-1.0	0.4	0.0	0.0	0.9	0.1	1.5	1.9	0.0	0.0	-0.1	0.7	-0.2	0.1	0.1	0.8
9	0.2	7.1	-0.1	0.7	-1.0	-0.3	-0.5	-0.3	0.2	0.0	-1.1	-0.4	-0.2	-0.3	-0.1	0.0	0.4	0.4	-0.5	-0.9

(Chapter 6) from Glu-C digestion . Sequencing was performed by Mr J. Gilroy.

SAMPLE : PAPPA/K-3/MS
[Initiated 24 Aug 1992 8:55am]

Sample Amount: 100 pmol

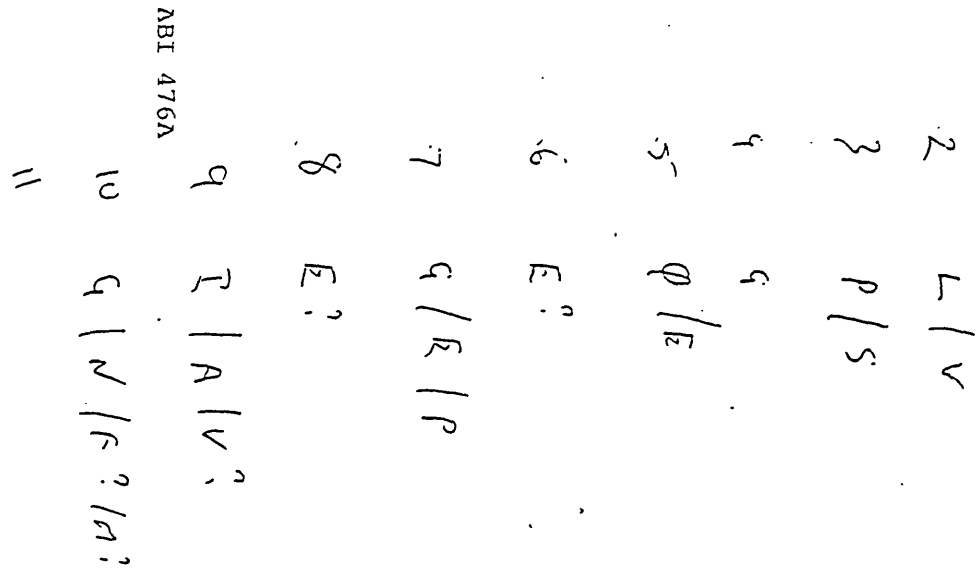
AAcid #	AAcid ID	R.Time (min)	C.Time (min)	Pmol (raw)	Pmol (-bkgd)	Pmol (+lag)	Pmol Ratio	AAcid ID
1	I	16.03	15.87	25.65	20.26	23.99	73.16	ILE
2	D	3.58	3.57	38.35	25.61	37.45	77.62	ASP
3	A	7.90	7.85	35.71	22.22	32.14	70.65	ALA
4	L	16.53	16.55	20.30	13.01	17.99	18.84	LEU
5	M	12.92	12.83	8.93	6.26	7.00	25.29	MET
6	L	16.80	16.55	16.65	10.27	12.83	12.08	LEU
7	E	6.03	6.00	11.07	3.31	2.82	6.50	GLU
8	R	11.77	11.77	4.52	7.42	9.26	37.15	ARG
9	R	11.77	11.77	4.80	9.52		28.87	ARG

REPETITIVE YIELD ANALYSIS:

	Rep.Yield	Variance	
L: 4, 6	98.84 %	1.000	:LEU
R: 8, 9	109.73 %	1.000	:ARG
Average AA Repetitive Yield:	104.29 %		
Combined AA Repetitive Yield:	83.51 %	0.542	

Theoretical Initial Yield: 28.82 pmol (28.82 %)

AIII.2.12 Sequence obtained from Group-X contaminants from illustrated blot in Figure 5.4 (Chapter 5). Sequencing was performed by Dr A. Moir.



AIII.3 Oligonucleotides Synthesised

AIII.3.1 N-PAPP-1 primer designed from initial PAPP-A N-terminus (see AIII.2.2).

Sequence name : N-PAPP1
Sequence length : 21
Date last edited : 25/02/91
Author name :
Description :

Base X : Inosine

Base Y :

```
                10          20
5' -AAAGCXAGAG  GXGCXACXGA  A- 3'
     G   C G
```

Date : 25/02/1991
Sequence : N-PAPP1
Synthesis : N-PAPP1
Scale : 0.2 micromole
Sequence Length : 21
Column : 1
Final Detritylation : Yes
Coupling Efficiency Threshold : 90 %

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
21	A	0.40	0.58	470	52.86	-
20	AG	0.38	0.69	766	88.95	-
19	G	0.37	0.57	715	76.12	-
18	xC	0.39	0.66	783	84.91	-
17	C	0.41	0.70	715	90.50	-
16	Ax	0.40	0.67	804	93.20	101.2
15	xC	0.40	0.65	807	87.22	-
14	CG	0.42	0.74	766	96.31	102.1
13	Gx	0.39	0.55	702	77.62	100.3
12	xG	0.41	0.69	776	91.37	-
11	G	0.41	0.63	738	79.68	101.3
10	G	0.41	0.66	742	78.86	99.0
9	AG	0.42	0.68	731	84.90	-
8	G	0.42	0.65	674	79.23	100.2
7	AC	0.44	0.72	760	92.60	-
6	xC	0.44	0.70	780	87.94	-
5	CG	0.45	0.72	778	92.24	99.6
4	G	0.44	0.66	613	75.44	98.8
3	AG	0.45	0.68	615	79.09	-
2	A	0.45	0.71	678	83.89	99.3

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
1	A	0.45	0.75	763	89.63	106.8

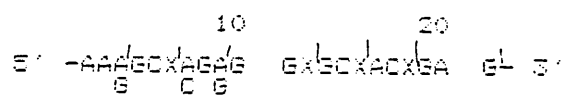
Total synthesis yield from start = 100.0 %

AIII.3.2 N-PAPP-2 primer designed from initial PAPP-A N-terminus (see AIII.2.2).

Sequence name : N-PAPP2
Sequence length : 21
Date last edited : 26/02/91
Author name :
Description :

Base X :

Base Y :



Date : 26/02/1991
Sequence : N-PAPP2
Synthesis : N-PAPP2
Scale : 0.2 micromole
Sequence Length : 21
Column : 2
Final Detritylation : Yes
Coupling Efficiency Threshold : 90 %

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
21	G	0.39	0.56	367	41.68	-
20	AG	0.37	0.49	916	100.79	-
19	G	0.36	0.63	877	88.05	-
18	xx	0.37	0.66	922	94.05	-
17	C	0.39	0.71	924	102.12	-
16	A	0.39	0.66	906	95.26	98.6
15	xx	0.39	0.68	925	99.05	-
14	C	0.40	0.73	1083	118.37	105.0
13	G	0.38	0.67	816	86.96	99.8
12	xx	0.39	0.70	889	104.84	-
11	G	0.40	0.64	714	81.30	96.7
10	G	0.39	0.67	779	83.92	103.2
9	AG	0.40	0.66	798	86.23	-
8	G	0.40	0.64	732	78.82	96.9
7	AC	0.42	0.74	826	94.89	-
6	xx	0.42	0.74	806	90.71	-
5	C	0.43	0.77	776	94.96	97.6
4	G	0.42	0.73	642	69.26	96.8
3	AG	0.43	0.79	726	79.10	-
2	A	0.43	0.89	766	85.00	99.2

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
1	A	0.44	0.92	773	84.33	99.2

Total synthesis yield from start = 87.0 %

AIII.3.3 cPAPP-3 internal primer designed from sequence obtained from Glu-c digestion (see AIII.2.11).

Sequence length : 21
Date last edited : 1/01/84
Author name :
Description :

cPAPP-3

Base X :

Base Y :

10 20
5'-XCCXCCGTCX GCATTCAAAGT G- 3' (Complementary Strand)
A G G A

Date : 1/01/1984
Sequence : PAPP-3
Synthesis : PAPP-3
Scale : 0.2 micromole
Sequence Length : 21
Column : 1
Final Detritylation : Yes
Coupling Efficiency Threshold : 90 %

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
21	G	0.42	0.63	515	61.00	-
Monitor failed to autozero for base 21 (reading=3942)						
20	T	0.44	0.73	717	98.09	-
19	GA	0.41	0.66	779	94.08	-
18	A	0.42	0.64	801	92.89	-
17	A	0.42	0.64	800	93.97	101.2
16	AG	0.41	0.62	796	91.42	-
15	T	0.45	0.69	758	98.55	100.1
14	T	0.46	0.71	764	99.66	101.1
13	AG	0.42	0.67	734	90.61	-
12	C	0.44	0.68	758	96.01	-
11	G	0.26	0.13	141	4.24	-
Monitor failed to autozero for base 11 (reading=114)						
10	X	0.42	0.62	785	87.61	-
Monitor failed to autozero for base 10 (reading=114)						
9	C	0.45	0.68	741	92.84	98.9
Monitor failed to autozero for base 9 (reading=114)						
8	T	0.46	0.72	748	91.79	98.6
7	GA	0.44	0.69	702	86.15	-
6	C	0.45	0.67	713	89.06	98.6
5	C	0.46	0.68	699	87.68	98.4
4	XC	0.45	0.68	699	87.02	-
3	C	0.46	0.69	683	88.04	100.2
2	C	0.46	0.69	662	86.39	98.1

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
1	X	0.46	0.68	671	83.16	-

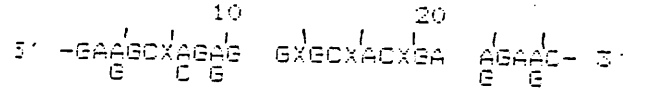
Total synthesis yield from start = -

AIII.3.4 PAPP-4 primer designed from consensus PAPP-A N-terminus (see AIII.2.3).

Sequence name : PAPP-4
Sequence length : 25
Date last edited : 1/01/84
Author name :
Description :

Base X :

Base Y :



Date : 1/01/1984
Sequence : PAPP-4
Synthesis : PAPP-4
Scale : 0.2 micromole
Sequence Length : 25
Column : 2
Final Detritylation : Yes
Coupling Efficiency Threshold : 90 %

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
25	C	0.40	0.45	691	55.12	-
24	AG	0.35	0.47	1142	89.90	-
23	AG	0.36	0.47	1192	74.76	-
22	GG	0.36	0.43	1059	84.33	-
21	AG	0.40	0.58	757	74.28	-
20	AG	0.40	0.60	780	79.61	102.1
19	GxC	0.40	0.61	719	69.26	102.5
18	AxC	0.41	0.54	760	76.52	-
17	CA	0.42	0.61	793	78.97	-
16	AxC	0.42	0.57	770	75.83	98.1
15	CA	0.42	0.58	761	73.95	-
14	CG	0.44	0.59	773	76.71	99.0
13	GxG	0.43	0.56	660	62.57	98.3
12	GxG	0.43	0.58	689	70.10	-
11	GG	0.43	0.57	649	63.59	92.5
10	G	0.44	0.55	596	58.97	110.0
9	AG	0.45	0.59	571	62.36	-
8	G	0.45	0.57	588	58.56	99.7
7	AC	0.45	0.63	624	64.27	-
6	x	0.45	0.58	621	60.52	-

Pos	Base	Retention mins	Duration mins	Peak ht %FS	Acc Area %min	Last eff %
5	C	0.46	0.61	614	64.39	98.1
4	G	0.45	0.57	525	53.78	97.4
3	AG	0.47	0.52	472	45.40	-
2	A	0.46	0.59	559	57.16	98.2
1	G	0.46	0.53	495	48.23	97.0

Total synthesis yield from start = 87.0 %