THE DEVELOPMENT OF A PURIFICATION PROCEDURE FOR PEPTIDE:*N*-GLYCOSIDASE A FROM *PRUNUS AMYGDALUS*

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ABSTRACT

Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidases cleave the amide bond between N-linked glycans at N-acetylglucosamine and asparagine, liberating intact oligosaccharide chains from glycoproteins. Although PNGase A is commonly used by glycobiologists for removal of N-linked glycans from plant sources, much less is known about it than about PNGase F, an enzyme that is more commonly used for deglycosylating proteins.

New studies on PNGase A have been initiated, with the aim of carrying out complete biochemical and structural studies in order to determine the substrate specificity, isoelectric point, primary, secondary and tertiary structures. Comparisons will then be made with PNGase F, whose three-dimensional structure is known.

The first step in these studies is therefore to obtain some pure protein and amino acid sequence. Although purification protocols have been published previously, it was difficult to produce a homogeneous preparation following these methods and they have hence been modified. The methods used are described in *Chapter 2* and the results of four preparations, using almond meal and almond emulsin as starting materials, are reported in *Chapters 3-6*.

Although PNGase A had not been purified to homogeneity, an active band excised from a native gel and analysed by SDS-PAGE showed four major bands. Which band represents PNGase A remains to be determined.

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LIST OF ABBREVIATIONS

BCA	Bicinchoninic acid
BME or βME	β-mercaptoethanol
BSA	Bovine serum albumin
CM	Carboxymethyl
DEAE	Diethylaminoethyl
DIG	Digoxigenin
EDTA	Ethylenediamine tetra-acetic acid (di-sodium salt)
ENGase	Endo-*N*-acetyl-β-D-glucosaminidase or endoglycosidase
FPLC	Fast protein liquid chromatography
GLC	Gas-Liquid chromatography
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
NaAc	Sodium acetate buffer
NH_4Ac	Ammonium acetate buffer
$(NH_4)_2SO_4$	Ammonium sulphate
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenyl methyl sulfonyl fluoride
PNGase	Peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase A
PVDF	Polyvinylidene difluoride
Q- or QAE	Quaternary amino (hydroxypropyl diethyl aminoethyl)
RPC	Reverse phase chromatography
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TRIS	Tris(hydroxymethyl)aminomethane

Three and one letter code for amino acids

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

Sugar abbreviations

GlcNAc	N-acetylglucosamine	Fuc	fucose
GalNAc	N-acetylgalactosamine		

Linkages are described using conventional carbon ring numbers connected by a slash and anomericity is denoted by α or β. For example: fuc α1-3 linked to GlcNAc.

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CHAPTER I
INTRODUCTION

1.1 Introduction to Carbohydrates

Carbohydrates, one of four classes of biomolecules are aldehyde or ketone compounds with multiple hydroxyl groups. They can exist by themselves and serve as fuels, energy stores and metabolic intermediates. Carbohydrates feature as structural elements in plants and arthropods and are a component of DNA. They are also found covalently linked to proteins and lipids where they have been shown to play important biological roles.

There is greater structural diversity for oligosaccharides compared to nucleic acids and proteins because of the large number of isomers that sugar molecules can form.

Sugar molecules form chains by a condensation reaction between two hydroxyl groups from two different monosaccharides (*figure 1.1*). If two different sugar molecules were linked together, one of four isometric links may be formed between one hydroxyl group of one monosaccharide and the corresponding hydroxyl groups of a second monosaccharide at the C2, C3, C4 or C6 positions. In addition to these 4 possible configurations, each monosaccharide may take up either an α or β configuration (which allows 8 combinations) and exist in a furanose or pyranose form (*figure 1.2*). There are therefore 16 different combinations possible for this disaccharide. For larger oligosaccharides the structural diversity is therefore potentially enormous.

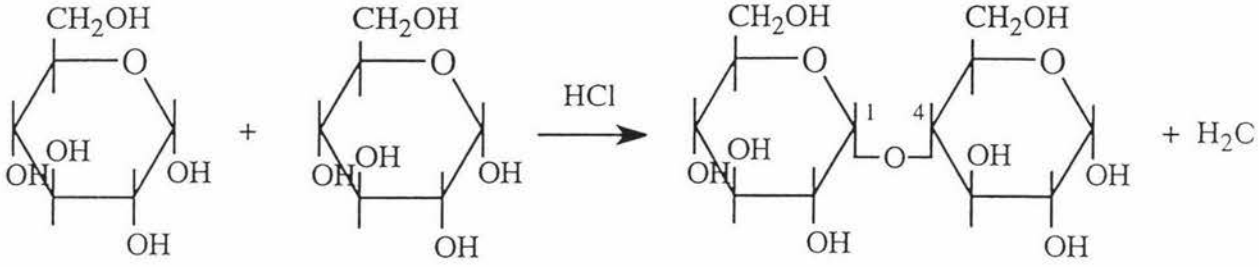


Figure 1.1 Condensation reaction between two monosaccharides.

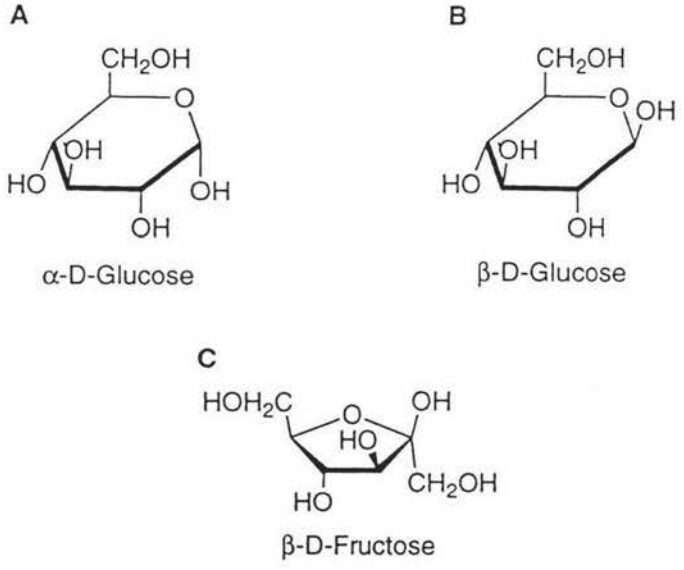


Figure 1.2 Different configurations of monosaccharides. ***A:*** α-D-Glucose, which has an -OH group attached to C1 below the plane of the ring. ***B:*** β-D-Glucose, has an -OH group at C1 above the plane of the ring. Both α-D-Glucose and β-D-Glucose are pyranose forms (aldehyde, C1→C5). ***C:*** β-D-Fructose, in the furanose form (ketone, C2→C5).

1.2 Glycoproteins

Glycoproteins are proteins which possess an oligosaccharide moiety covalently attached to an amino acid side chain via an N-glycosidic bond or an O-glycosidic bond.

1.2.1 N -linked oligosaccharides

Oligosaccharides which are N-linked to glycoproteins have an amide bond between the Asn residue and a GlcNAc moiety at the reducing end of the oligosaccharide, forming a β-aspartylglucosylamine link (*figure 1.3*).

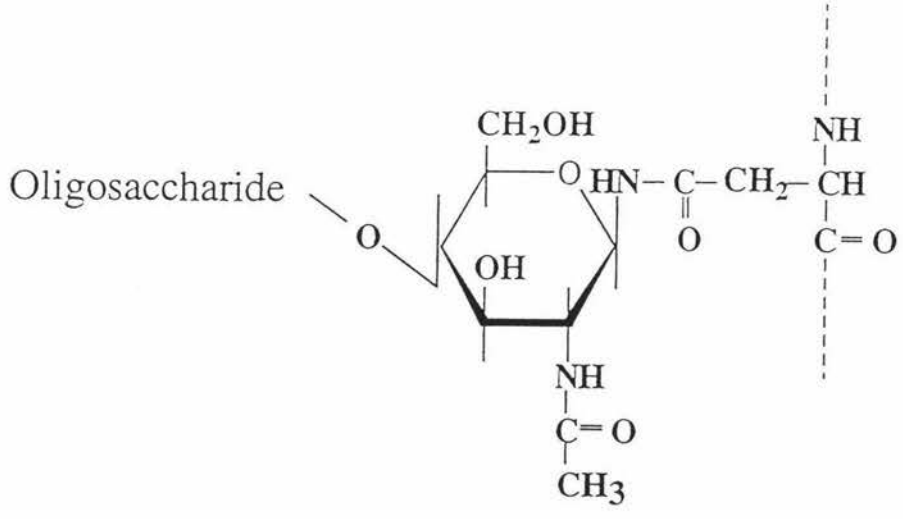


Figure 1.3 An oligosaccharide N-linked to asparagine.

The consensus sequence Asn - X - Ser/Thr (where X is any amino acid except proline or aspartic acid) is necessary for N-glycosylation to occur.

All N-linked glycans have a common trimannosyl core as illustrated in *figure 1.4.* and can be divided into three main categories: high mannose, complex and hybrid.

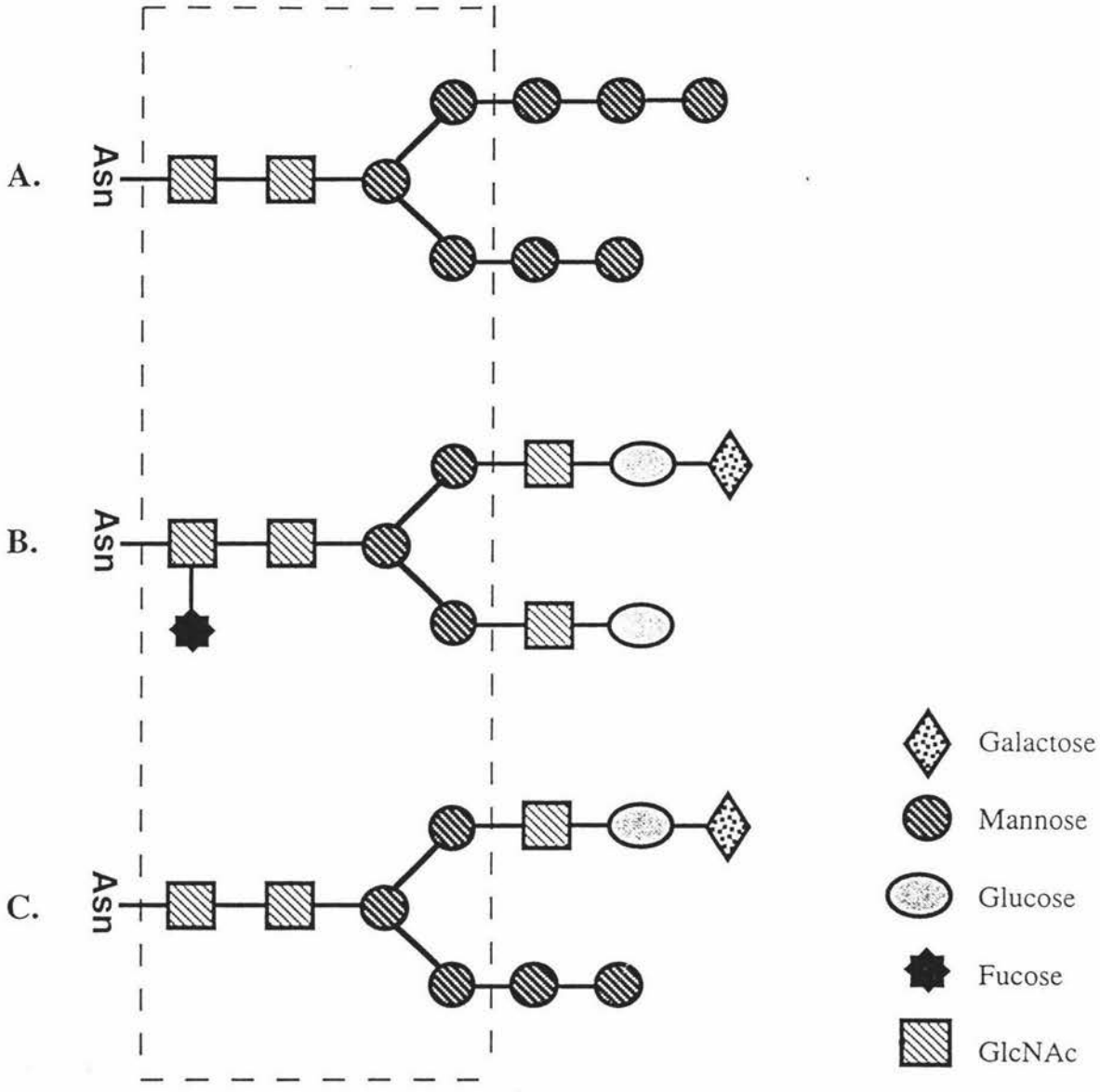


Figure 1.4 The three classes of N-linked glycans. *A:* High mannose. *B:* Complex. *C:* Hybrid. The boxed area denotes the common core.

The high mannose class consists only of mannose residues, outside of the common core. Complex N-linked glycans have no mannose residues other than those within the core and have the greatest structural variation (reviewed by Kobata, 1992). This is due to the formation of one to five chains linked to the trimannosyl core, to give monoantennary, biantennary, triantennary, tetraantennary and pentaantennary branches. The addition of fucose α1-6 or α1-3 linked to the C6 or C3 carbon on the αGlcNAc proximal to Asn adds extra variation. Fucose can also be added to GlcNAc elsewhere in the chain.

Finally, the third class of hybrid N-linked glycans contains a mixture of the previous two classes.

N-glycosylation is a co-translational event which occurs on nascent polypeptide chains in the rough endoplasmic reticulum. Processing and maturation of the N-glycans occurs in the Golgi apparatus (Kornfeld and Kornfeld, 1985).

The primary, secondary and tertiary structure of the protein determines whether the Asn sequon is glycosylated or not. Factors that govern whether or not the protein will be glycosylated include the presence of other glycosylated sites, environmental conditions and cell type. The N-glycosidic link was shown to be rigid and planar and the Asn is preferably in a flexible area within the local environment and sequon, such as on a β bend (Wormald *et al.*, 1991).

1.2.2 O- linked Oligosaccharides

Those attached by an O-link are bound through the side chains of serine, threonine, hydroxyproline and hydroxylysine. They can either be long bulky oligosaccharides or single monosaccharides (usually GalNAc or GlcNAc) and are usually found in exposed areas of the tertiary structure. O-GlcNAc glycosylation is ubiquitous on intracellular proteins and is highly localised to nucleoplasmic and cytoplasmic proteins (Haltiwanger *et al.*, 1991). There appears to be no defined primary sequence necessary for attachment and O-GlcNAc appears to be highly dynamic in a manner not unlike protein phosphorylation (Haltiwanger *et al.*, 1991).

1.2.3 Function of the Oligosaccharide Moiety

Any protein may have one or more glycosylation sites which may or may not be filled. Each glycosylation site may also contain different glycans or oligosaccharide chains. These may vary in both monosaccharide composition and geometric configuration. The population of biomolecules which have the same amino acid sequence but differ in their glycosylation patterns are called glycoforms. This microheterogeneity is due to the lack

of a template for the glycosylation of proteins, disrupted synthesis of glycans and the ability of the monosaccharides to form different links and ways of branching. They may or may not confer different physical and/or biochemical properties on the protein.

The glycan increases the rigidity and stability of the protein (Opdenakker *et al.*, 1993) and may sterically hinder a functionally important site and/or confer resistance against proteases by protecting susceptible sites. An example is the processing of Concanavalin A to the active form by protease cleavage made accessible by glycan removal (Bowles *et al.*, 1986). Glycosylation may confer an orthagonal property, such as a clearance marker on the protein, without affecting other functions of the molecule (Opdenakker *et al.*, 1993 and Cumming, 1991). Examples are the hepaticsialoreceptor which recognises terminal galactose residues and the mannose receptor which mediates the clearance of proteins.

The functions of glycosylation include the following:

- Maintenance of certain physicochemical properties (such as solubility, tertiary conformation, thermal stability and stabilisation of the core protein against proteolysis)
- Cell-cell recognition events
- Signal transduction through the mediation of biological activities
- Intracellular sorting and pinocytic uptake of certain functional glycoproteins
- Proteolytic processing

Cell-cell interactions are mediated by the glycocalyx, which contains all the oligosaccharides bound to both glycoproteins and glycolipids on the outer surface of the cell. The sugar moiety can occupy a large space and subtle changes in the oligosaccharide components may confer particular functions toward the protein and may affect associations between cells. Oligosaccharides are often receptors or target molecules for other proteins such as lectins which play a major role in recognition events, such as the adhesion of leukocytes to endothelial cells during inflammation (Bevilacqua *et al.*, (1987); Kobata, (1992) and Feizi, (1993)).

Oligosaccharides have a role in immunogenicity. They may define an epitope or mask potential antigenic sites. They also play a subtle role in the activity of antibodies, for example in the binding of the F_c region of immunoglobulin G (IgG), which contains more than 30 different N-linked glycans, to the subcomponent C1q of human complement and the F_c receptor (Parekh *et al.*, 1985 and Kobata, 1992).

The glycan primary structure can be an indicator of the homeostatic state of the cell. In a changing cell environment, the flexible nature of the outer sugar moieties means they can change in response to different conditions, according to whether the cell is in a normal, stressed or a pathological state, although the basic core structure remains the same. Changes in glycosylation may lead to malfolded proteins, through disrupted tertiary structure and may ultimately alter the cell phenotype, such as in tumour formation (Cumming, 1991).

From approximately 150 monosaccharides found in nature, less than 20 are found in the oligosaccharide chains (glycans) attached to proteins. These include:

Galactose	N-acetyl glucosamine (GlcNAc)
Glucose	N-acetyl galactosamine (GalNAc)
Mannose	Xylose
Fucose	Arabinose
Sialic acid	Glucuronic acid
Iduronic acid	N-acetyl neuraminic acid (NeuNAc)
	N-glycolyl neuraminic acid (NeuNGc)

1.3 Deglycosylation

1.3.1 Introduction

Now that the importance of glycosylation is known, it has been proposed that this post-translation modification may also be coupled to deglycosylation systems and that the two

are functionally important in all living cells, apart from yeasts where they have not yet been detected (Seko *et al.*,1991; Suzuki *et al.*,1994a and Suzuki *et al.*,1995a).

Whilst the exact *in vivo* biological function of N-deglycosylation has yet to be shown, it is probable that these enzymes are part of degradative pathways and that they may have a function in signalling pathways or enzyme activation pathways, which are reviewed in this section.

Suzuki *et al.*,(1994c) have recently classed the enzymes involved in deglycosylation as 'proximal glycanases' or 'PROXIases'. These enzymes cleave the linkage between the proximal monosaccharide and core protein (or ceramide) or between the two proximal oligosaccharide moieties, releasing free oligosaccharide chains or monosaccharides and apo-glycoconjugates. They can be termed the 'restriction enzymes of the carbohydrate world'. A number of studies are currently underway showing that deglycosylation is crucial for at least certain cellular processes (reviewed by Suzuki *et al.*,1994c).

PROXIases are biochemical tools that enable researchers to analyse the carbohydrate moiety, to study the function of the glycan and to aid in crystallisation of glycoproteins. They can be classified into five main categories: Endo-N-glycanase (ENGases), Peptide-N-glycanase (PNGases), Peptide-*O*-glycanase (POGases), Cytoplasmic β-GlcNAcase (*O*-GlcNAcases) and Endoglycoceramidase (EGCases). Examples of these enzymes and their sources are displayed in *tables 1.1, 1.2* and *1.3.*

Table 1.1 Some ENGases and their source.

Enzyme	Source	Reference
Endo B	Fungi	Bouquelet *et al.*,1980
Endo C	Bacteria	Ito *et al.*, 1975
Endo D	Bacteria	Muramatsu, 1971
Endo F_1 , F_2, F_3	Bacteria	Plummer *et al.*, 1984
Endo H	Bacteria	Tarentino and Maley, 1974
Endo L	Bacteria	Tarentino and Maley, 1974
Endo S	Mould	Freeze and Etchinson, 1984

Table 1.2 PNGases and their source.

Enzyme	Source	Reference
PNGase A	Almonds	Takahashi, 1977 and Taga *et al.*,1984
PNGase F	F. meningosepticum (Bacteria)	Plummer *et al.*, 1984
PNGase L-929	Mouse fibroblast	Suzuki *et al.*, 1994a
PNGase Se	Silene alba (White campion)	Lhernould *et al.*, 1992
PNGase	Oryzias latipes (Medaka fish)	Seko *et al.*, 1991
PNGase J	Jack beans	Sugiyama *et al.*, 1983
PNGase P	Pisum sativum (Pea)	Plummer *et al.*, 1987
PNGase R	Raphanus sativus (Radish)	Berger *et al.*, 1995b
PNGase	Various plant seeds	Plummer *et al.*, 1987
PNGase	Various mouse organs	Kitajima *et al.*,1995
PNGase	Humans, chickens	Suzuki *et al.*,1995a

Table 1.3 Other enzymes involved in deglycosylation. These are outside the scope of this work and shall not be discussed further.

Enzyme	Source	Reference
POGase	Bacteria	Endo and Kobata, 1976
O-GlcNAcase	Rat	Dong and Hart, 1994
Endo-β-Xylase	Rabbit, mollusc	Takagaki *et al.*,1990
EGCase	Animals	Zhou *et al.*,1989
	Bacteria	Ashida *et al.*, 1992

1.3.2 ENGases

Endo-*N*-acetyl-β-D-glucosaminidases (ENGase, EC 3.2.1.96) (endoglycosidases) carry out any hydrolytic cleavage within the Asn-linked oligosaccharide core. These enzymes are present in bacteria, fungi, moulds, plants and animals. Plant ENGases were first detected in a crude fig extract (Ogata-Arakawa *et al*, 1977) and many others have subsequently been found (*table 1.1*). Most of these enzymes have been well characterised and there are two tertiary structures known, of Endo H (Rao *et al.*,1995) and Endo F (Van Roey *et al.*,1994).

The site of cleavage catalysed by these enzymes is shown in *figure 1.5.* The key structural determinants for recognition are a polypeptide chain on either side of the Asn residue and a proximal di-N-acetylchitobiosyl region (two GlcNAc residues) as well as particular structural determinants with respect to the oligosaccharide chain (Maley *et al.*, 1989). Activities and specificities are known for most. While high mannose is the preferred substrate for Endo H, other ENGases have different and restricted substrate specificities.

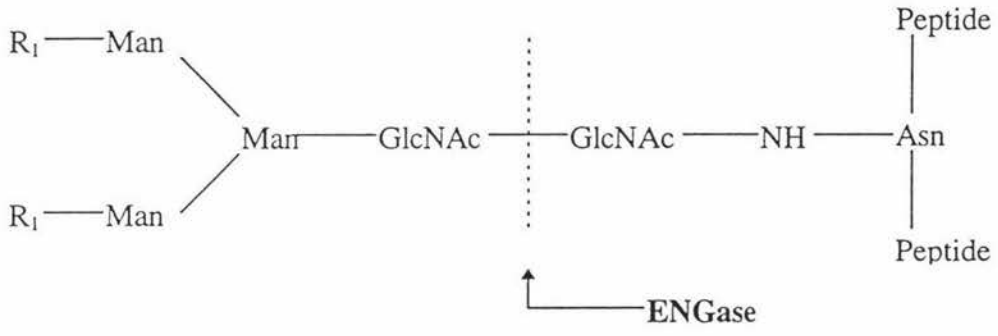


Figure 1.5 Site cleaved by ENGases.

1.3.3 PNGases

Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidases (PNGase, EC 3.5.1.52) cleave N-linked glycopeptides at the β-aspartylglucosylamine bond, between the asparagine residue in peptide linkage and the GlcNAc moiety at the reducing end of the oligosaccharide chain (*figure 1.6).* They are therefore better described as amidases (amidohydrolases) than endoglycosidases, which cleave a glycosidic bond.

The hydrolysis of this bond occurs in two steps, as proven by ^{H}nmr and kinetics studies (Risley and Van Etten, 1985). In the first step in the reaction, the carbohydrate is cleaved from the protein/peptide and the Asn residue is converted to Asp. The intermediate oligosaccharide retains the amino group. This intermediate slowly, non-

enzymatically degrades to an intact oligosaccharide and free ammonia (Risley and Van Etten, 1985) (*figure 1.7*).

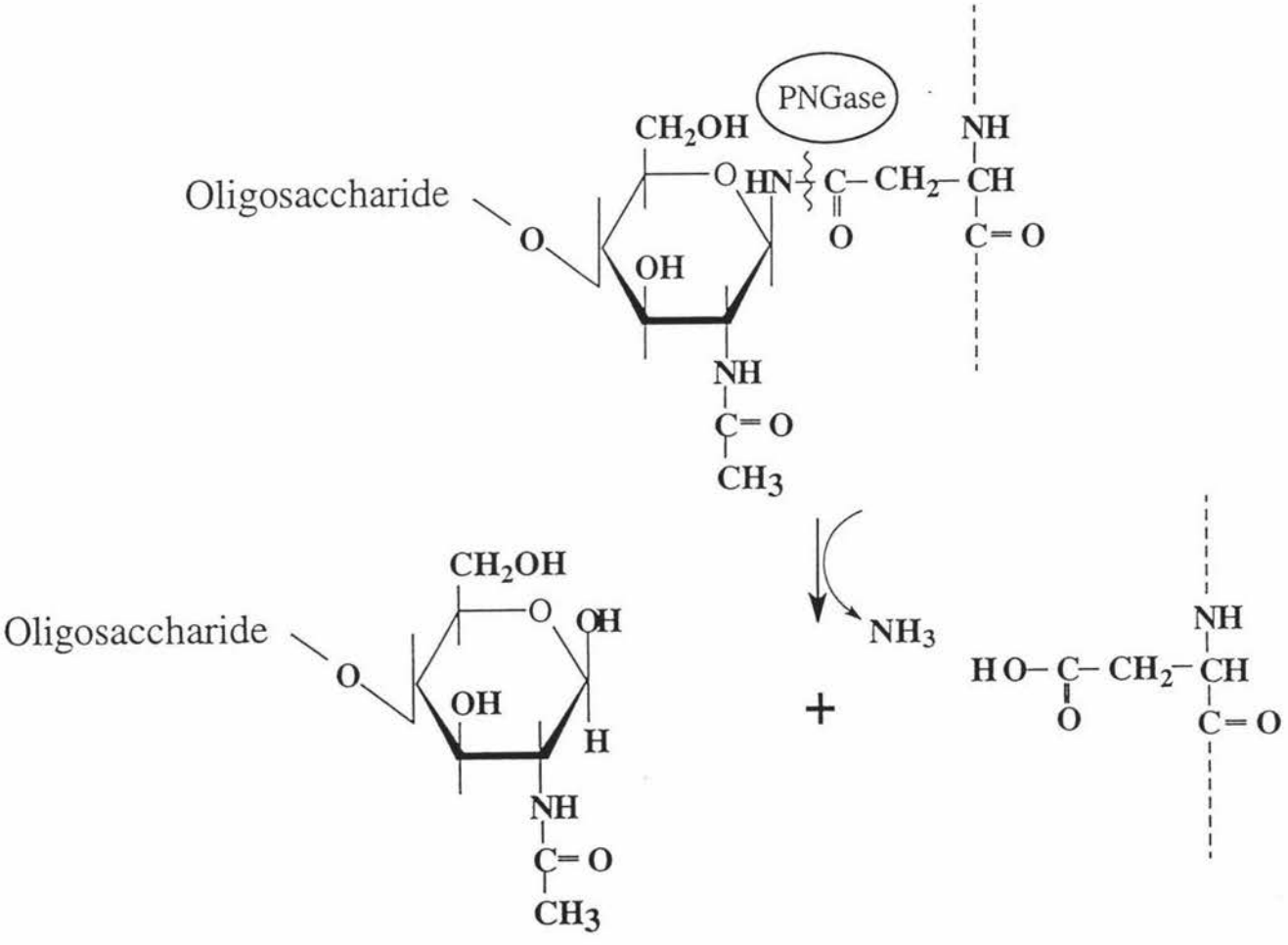


Figure 1.6 The β-aspartylglucosylamine bond cleaved by PNGases.

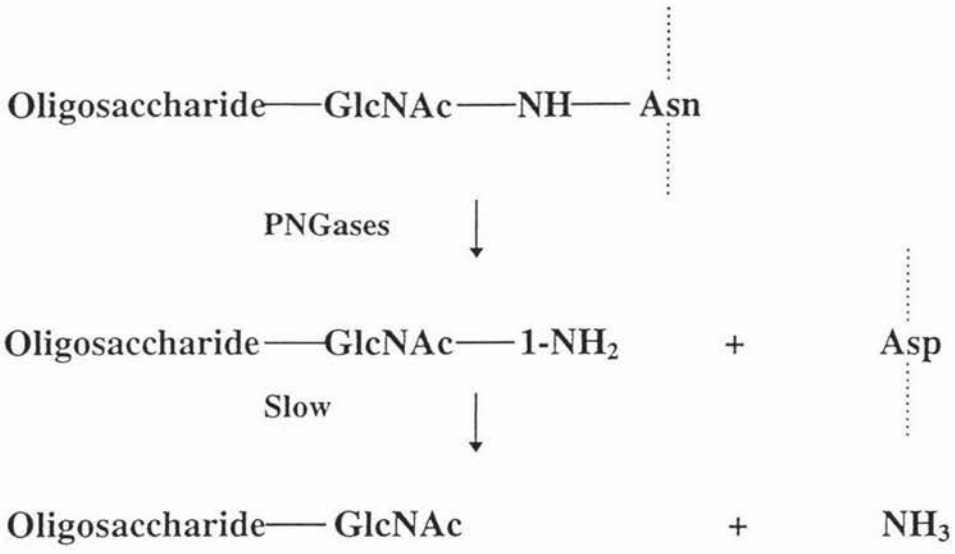


Figure 1.7 The two step reaction catalysed by PNGases which shows the enzyme to be an amidase.

The structural determinants for PNGase activity are the polypeptide chain and the di-N-acetylchitobiosyl region of the glycan chain (Maley *et al.*, 1989). PNGases bind to the polypeptide chain around the glucosylamine junction and the inner GlcNAc core (Chu, 1986).

PNGases do not cleave oligosaccharides on a single Asn residue (glycoasparagine), unlike similar enzymes called aspartylglucosaminidases (NGases) which cleave the same bond (Plummer and Tarentino, 1981 and Suzuki *et al.*, 1994a and 1994c). This has led Suzuki *et al.*, (1994c) and Kitajima *et al.*, (1995) to propose that PNGase activities are a non-lysosomal event (since the pH optimums for PNGase F and PNGase L-929 are 8.6 and 7 respectively). N-linked glycans at the C- or N-termini of the polypeptide chain are unfavourable substrates (Plummer and Tarentino, 1981) and long bulky oligosaccharide chains are favoured.

PNGase F is the best characterised PNGase, however its function *in vivo* is unknown. Purified from *Flavobacterium meningosepticum,* it comprises 314 amino acids, has a

molecular weight of 34 779 Da, has a known tertiary structure (Norris *et al.*, 1994) and an isoelectric point of 8.14. Its optimum activity occurs at pH 8.5 and it is able to cleave a broad range of oligosaccharide chains from both peptides and proteins, including those with a fucose β1-6 linked to the GlcNAc proximal to Asn. However this enzyme is unable to cleave oligosaccharides with a fucose α1-3 linked to the same GlcNAc, a feature common to many plant glycoproteins such as bromelain and horseradish peroxidase (Tretter *et al.*, 1991). PNGase F will not act on an oligosaccharide made up of less than two GlcNAc residues (Chu, 1986).

1.3.4 PNGase A

This enzyme is present in small quantities in almond seeds and was first described by Takahashi in 1977.

The molecular weight of the enzyme was proposed to be 66.8 kDa as analysed by SDS-PAGE, 89 kDa determined by non-reducing conditions, 90 kDa by size exclusion chromatography and 58.896 kDa as determined by amino acid analysis (Taga *et al.*, 1984). However Plummer *et al* (1987), estimated the molecular weight to be 79.5 kDa by HPLC and 80 kDa by size exclusion chromatography (Tarentino and Plummer, 1982). Taga *et al.*,(1984) reported the enzyme to be a glycoprotein, since it tested positive with Schiff's reagent, 18 glucosamine residues were found in the amino acid analysis and the enzyme was retained on Concanavalin A. GLC analysis showed the enzyme to contain GlcNAc, fucose and mannose (Tarentino and Plummer, 1982) with an overall carbohydrate content of 27%. However, no deglycosylation of PNGase A was observed on incubation of PNGase A with PNGase F or Endo F under various conditions (Plummer *et al.*, 1987).

The pH optimum is known to be 4.5 and the enzyme is active between pH 3-6 (Tarentino and Plummer, 1982). After isoelectric focusing, the enzyme appeared as a broad band between 7.0 and 7.5, which may indicate charge heterogeneity indicative of glycoproteins

(Taga *et al.*,1984). Takahashi and Nishibe, (1981) found the isoelectric point for the crude enzyme was between 7.7 and 8.7.

The enzyme is active on glycopeptides, but removal of glycans from proteins is very slow or does not occur (Takahashi and Nishibe, 1981 and Tarentino and Plummer, 1982), with hydrolysis being strongly influenced by the position of the oligosaccharide on the protein (Plummer and Tarentino, 1981). The oligosaccharide chain length and the position of the carbohydrate on the peptide are important structural determinants for activity. PNGase A has a broad substrate specificity. Complex glycans are favoured, with fastest rates obtained with triantennary, then biantennary and tetraantennary structures. Activity increases by 20% with prior removal of sialic acid residues (Plummer and Tarentino, 1981 and Plummer *et al.*,1987). As yet, there is no information about the primary structure of the protein or its glycan component. Circular dichroism results indicate a high α-helical content (Taga *et al.*,1984) in contrast to PNGase F which is made up of only β structure (Norris *et al.*,1994).

Although the substrate specificity is similar to PNGase F, there are some differences. Firstly, PNGase A can cleave fucose α1-3 linked to the GlcNAc proximal to Asn whereas PNGase cannot (Plummer and Tarentino, 1981). Secondly PNGase A is able to cleave a single GlcNAc from an Asn residue in a polypeptide but PNGase F cannot (Tarentino and Plummer, 1982).

Furthermore, PNGase A can withstand higher concentrations of chaotropic salts (0.75 M NaSCN for 48 hours at 37°C compared to no more than 0.25 M at 30°C for PNGase F (Tarentino *et al.*,1989). On the other hand, PNGase F is more stable towards ionic detergents such as SDS. At 0.25 M SDS, 90% of PNGase A activity is lost (Tarentino and Plummer, 1982) and at 0.01% SDS (0.0003 M), only 20% of activity is retained (Taga *et al.*,1984). PNGase A is active in non-ionic denaturants such as Triton X-100, Tween 80, Nonidet P-40, BME and 2 M urea (Taga *et al.*,1984).

The main differences between PNGase F (bacterial source) and PNGase A (plant source) are summarised in *table 1.4.*

Table 1.4 Differences between PNGase F and PNGase A.

	PNGase F	PNGase A
Cleave fucose α1-3 linked to GlcNAc next to Asn	No	Yes
Cleave fucose α1-6 linked to GlcNAc next to Asn	Yes	Yes
Needs two GlcNAc residues at reducing end of oligosaccharide	Yes	No
pH optimum	8.6	4.5
SDS tolerance	Higher	Lower
Oligosaccharide removal from proteins and peptides	Able	More active towards peptides
-SH group requirement	No	No
K_m* (mM)	0.525	1.46
Molecular weight (kDa)	35	Proposed to be 66.8

* For fetuin asialoglycopeptide I (Suzuki *et al.*,1994b).

1.4 Functions of PNGases

PNGases are widely used in research for both investigations into the function of the glycan moieties on proteins and in characterisation of the sugar chains themselves. They have also found use in structural studies, where removal of the glycans without compromising the integrity of the protein part, is often necessary to achieve crystallisation. The function of PNGases *in vivo* is not known. Some recent research has highlighted the possible importance of N-deglycosylation in physiological processes.

Inoue *et al.*,(1989) found stage dependent accumulation of free sialooligosaccharides in oocytes of fresh water trout (*Plecoglossus altivelis*) and dace (*Tribolodon hakonensis*), which originated from glycophosphoproteins. These oligosaccharides were complex type N-glycans and terminated with a free di-N-acetylchitobiosyl structure characteristic of N-linked glycoproteins and the products of PNGase cleavage.

These same researchers have also shown that free pentaantennary glycan chains exist in embryos of flounder (*Paralichthys olivaceus*) which were shown to be derived from a cortical alveolus glycoprotein called hyosophorin and another protein, phosvitin. Inoue (1990) proposed that a PNGase catalysed the glycan removal from glycophosphoproteins during oogenesis in fish and that this event was physiologically important in vitellogenesis (the formation of the chief protein constituent in egg yolk). Free N-glycans were also found in Medaka fish (*Oryzias latipes*) by Seko *et al.*,(1991). Since free oligosaccharides were not found in unfertilised eggs, they proposed that PNGases were important in regulation of biochemical events necessary during oogenesis, vitellogenesis and embryogenesis because the free N-glycans were derived from glycoproteins and isolated during these stages of development. These results support the hypothesis that PNGases may have a significant physiological role in living systems that is yet to be elucidated.

Upon finding PNGase activity in the lumen of the endoplasmic reticulum (ER) of rats, Weng and Spiro (1997) proposed its role was to release oligosaccharides from improperly assembled proteins in the ER, where misfolded proteins are degraded.

The presence of free glycans have also been reported in plants cells with ten different free N-glycans being found in mature green tomato pericarp (Priem *et al.*,1993). Arabinosyl, fucosyl and oligomannosyl containing N-glycans were purified and it was proposed that they fulfilled a role in tomato fruit senescence as increasing amounts were found towards ripening. This may be due to an increase in synthesis of the oligosaccharides or an increase in the cleavage of oligosaccharides from N-glycoconjugates by enzymes such as PNGase.

Priem and Gross (1992), previously found that free $Man_5GlcNAc$ was a promoter of tomato fruit ripening when added exogenously. Unconjugated N-glycans are a class of plant oligosaccharins that display biological activity (Albersheim *et al.*,1983 and Priem *et al.*,1994) and are said to play a role in protein regulation (Berger *et al.*,1995a). The release of oligomannose type sugars during seed germination was proposed as the role of an ENGase recently purified from pea (Kimura *et al.*,1996).

Cheung *et al.*,1995 and Wu *et al.*,1995 purified a protein, tobacco transmitting tissue glycoprotein (TSS), which is involved in flower fertilisation. TSS attracts pollen tube growth down the style, from the apical stigma to the basal ovary by forming a polymerised adhesive matrix of itself in varying stages of deglycosylation. It has been postulated that TSS is deglycosylated by enzymes (such as PNGases and ENGases) in the pollen tube, liberating sugars needed for pollen tube growth and forming a TSS gradient (TSS is increasingly glycosylated toward the ovary). They showed the pollen tube interacted with sugar moieties and they proposed that it grew toward those TSS molecules that were more glycosylated (a better nutrient source), sensing it haptotactically (matrix adhesivity driven). It should be noted that sugars forming a gradient on a protein backbone have more stability than free oligosaccharides.

Lhernould *et al.*,(1994) found that carbon starvation increased endoglycosidase activities in white campion (*Silene alba*) with the levels of unconjugated N-glycans increasing in cell suspension cultures. These N-glycans were oligomannosides and xylomannosides which contained α1-3 linked fucose and had previously been shown to confer biological activity on plants (Priem *et al.*,1994). They found that the lower the initial sucrose supply, the faster the increase in activity of both PNGase and ENGase. The highest activity was seen after total sugar depletion and was proposed to be due to glycoprotein breakdown (toward autophagy) related to carbon starvation (cells consume starch, protein and lipid reserves when deprived of sucrose, (Journet *et al.*, 1986)). They named the PNGase from white campion 'PNGase Se' (Lhernould *et al.*, 1992) and have continued to characterise this enzyme (Lhernould *et al.*,1995).

PNGase R and ENGase R activities account for 85-90% of the activity present in cotyledons of radish. This is a major site for the mobilisation of storage macromolecules and it was concluded that both PNGases and ENGases were required for the mobilisation of reserves (Berger *et al.*,1995a, 1995b). They proposed that PNGase was synthesised during seed formation, because high levels of activity were observed during both germination and post-germinative development stages. There was however, a basic level of activity when germination was inhibited (Berger *et al.*,1996). Berger *et al.*,(1996) also proposed that PNGases and ENGases could be induced by different mechanisms and

that they were differentially regulated during plant development. Plummer *et al.*,(1987) proposed that the presence of PNGase is to allow rapid internal hydrolysis of storage proteins during germination. The role of N-deglycosylation by PNGases in plant cells is thought to be complemented by ENGases which are also widespread and which have different substrate specificities.

The role of PNGases in bacteria is also most likely to be for nutrient purposes, to N-deglycosylate foreign material and so make protease access to the protein easier (Berger *et al.*,1995). Studies are continuing on characterising PNGases from different sources, with the aim of obtaining an insight into possible functions. Suzuki *et al.*,(1993a, 1994a) found the occurrence of cytosolic PNGase in mouse-derived L-929 fibroblast cells and BALBc mouse organs (Suzuki *et al.*,1993b). They observed free N-glycans containing 2 x GlcNAc at the reducing end. Searches of databases have found other proteins where the Edman and cDNA sequences differ. These include a honeybee hyaluronidase and bonito and pike eel gonadotrophins (Suzuki *et al.*,1994c) but there is no direct experimental evidence for this. Rather, these discrepancies between the cDNA and Edman sequences are indirect evidence of the hypothesis that N-deglycosylation is a physiological event in most living systems.

An example of N-deglycosylation as a post-translational processing event and regulator of protein activity is the maturation of Concanavalin A, which is synthesised as a glycosylated proprotein and later deglycosylated to the active form (Bowles *et al.*,1986; Sheldon and Bowles, 1992). Ricin A chain has heavy and light forms which are both glycosylated. In the light chain, Asn has been replaced by Asp as confirmed by cDNA analysis (Lamb *et al.*,1985 and reviewed by Suzuki *et al.*,1994a). It has been postulated that PNGases catalysed this site-specific N-deglycosylation.
Suzuki *et al.*,(1994b) proposed that the mammalian PNGase, L-929, has a dual role as both a carbohydrate recognition protein and an enzyme *in vivo*. It is inhibited by oligosaccharides similar to those it releases and binds to immobilised yeast mannan and strongly to $Man_3GlcNAc_2$. They postulated PNGase L-929 to be a lectin-like receptor, because it was able to specifically bind and recognise larger oligosaccharides. This enzyme appears to possess a carbohydrate binding site that is discriminated from the

catalytic site(s) of the enzyme (Suzuki *et al.*, 1995b). With inhibition being observed at high concentrations of free oligosaccharides, PNGase L-929 could thus be regulated (and hence also N-deglycosylation) by feedback inhibition. When the 3D structure of PNGase F was solved (Norris *et al.*, 1994), the two jelly roll domains suggested parallels with lectins and other carbohydrate binding proteins. It is possible that PNGase L-929 may be involved in some intracellular and/or intercellular interactions, similar to other lectins found in mammalian systems. Some glycosidases can act as specific cell-surface receptors for glycan chains on some gametes at fertilisation and embryogenesis (Miller *et al.*, 1993). Interestingly, Seko *et al.*, (1991) and Inoue *et al.*, (1989) found different free N-glycan levels at various developmental stages in oocytes and embryos.

The limited knowledge of PNGases prevents their exact functional role being known. Antibodies against different PNGases will facilitate studies on the cellular localisation and developmental profiles of these enzymes as will the production of PNGase deficient systems. The function of PNGases will become clear as more of these enzymes are characterised.

1.5 Scope of this work

There is a great potential for the study of glycosylation using the complementary enzymes PNGase F and PNGase A. These enzymes have advantages compared to chemical cleavage of oligosaccharides from glycoconjugates, because they are non-destructive and do not modify the oligosaccharide.

Studies of the sugar moieties of glycoproteins are essential for the biotechnology industry. Many recombinant proteins designed for therapeutic use are glycosylated (Cumming, 1991). As glycosylation is species and cell specific and glycosylation is known to play an important role in cell recognition events, knowledge of these structures and their effect on the function of the protein is of vital importance. Techniques for the structural analysis of the oligosaccharide have been established but one of the problems is the lack of pure, well characterised enzymes for their removal from the parent protein.

The PNGases from plants are generally very difficult to purify from their source (Scopes, 1994. Plant cells are highly compartmented and the cytoplasm is only about 2% of the cell volume. The majority of the cell volume is occupied by the vacuole, which contains proteases, alkaloids and polyphenolic compounds which may bind to, degrade and/or inactivate the protein of interest. It is likely that extracts are low in protein. Seeds (such as almonds) are particularly rich in proteases (present for the hydrolysis of storage proteins in germination) making purification difficult (Deutscher, 1990). The ultimate aim of this project was to improve the purification protocol and check the characteristics of the protein (a personal communication from A. Tarentino had indicated that the previous work cited had been carried out on impure protein).

Once the protein has been purified, enough N-terminal and internal sequence will be determined to allow the design of nucleotide probes that will be used to isolate the cDNA coding for the enzyme. The gene will then be cloned and sequenced. The native protein will be completely biochemically characterised. By studying PNGases from different sources, the molecular basis for differences in specificity can be determined.

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

Almond emulsin

β-Glucosidase 2.5 u/mg dry weight. 20 grams total, batch 2204. Worthington Biochemical Corporation.

Almond meal

Batch A-3265, Sigma Chemical Company (U.S.A). 200 grams, BSA defatted.

Buffers and reagents

Analytical or research grade chemicals were used. Solutions for FPLC work were filtered before use through a 0.22 μ filter. Conductivity was measured with a Horiba™ digital conductivity meter (model ES-14) where appropriate. The pH was measured using an Orion digital pH meter, model 410A.

Chromatography

Low, medium and high pressure chromatography was carried out on a Biorad Econosystem, Pharmacia FPLC, Waters 650 FPLC, Pharmacia SMART system FPLC, Philips PU 4100 HPLC and Spectra Physics SP 8800 ternary HPLC.

DIG Glycan/ Protein Double Labelling Kit - Boehringer Mannheim™

The simultaneous differential staining of glycosylated and non-glycosylated proteins was carried out using an immunoassay. Digoxigenin was used as the hapten for labelling sugars and fluorescein for the protein. The staining procedure is described in *Methods, section 2.2.3(e).*

Gel Electrophoresis

Polyacrylamide gels 80 x 100 x 0.75 mm were run using a Mini Protean II (Biorad) system.

Protein Sequencing

Performed on the Applied Biosystems automatic protein sequencer, model 476A.

Ultrafiltration

For very large volumes, an Amicon Diaflo spiral wound membrane ultrafiltration unit was used. For volumes 30 -500 ml an Amicon cell (under pressure) with stirrer was used. Smaller volumes were centrifuged in 15 ml Filtron macrosep centrifugal concentrators (3K or 10K cut-off), 2 ml Amicon centricons (10K), 1 ml Amicon microcons (3K, 10K, 50K) and Filtron 1 ml (30K) concentrators.

The following resins (*table 2.1*) were used.

Table 2.1. Supports used in chromatography.

Chromatographic Method	Trade name (manufacturer)	Matrix base
Ion exchange	CM-Sephadex (Amrad Pharmacia)	Cross-linked dextran
Ion exchange	DEAE Macroprep (Biorad)	Methacrylate
Ion exchange	Q-Sepharose (Pharmacia)	Cross-linked agarose
Ion exchange	Mono-Q 5/5 (Amrad Pharmacia)	Hydrophilic polyether resin
Gel filtration	Superdex 75 HR 10/30; 3.2/30; Hi load 16/60 prep grade	Cross-linked agarose and dextran
Affinity	Con A Sepharose CL-4B (Pharmacia)	Cross-linked agarose
Hydrophobic Interaction	Phenyl sepharose (Amrad Pharmacia)	Cross-linked agarose
Hydrophobic Interaction	Phenyl superose 12 HR 1.6/5; 5/5;	Cross-linked agarose
RPC (HPLC)	Vydac 218 TP C_{18} 4.6 mm x 250 mm (Alltech)	Silica

2.2 Methods

2.2.1 Overview of the Purification of PNGase A

Four main preparations were performed, in an attempt to obtain a pure product of PNGase A. The first preparation was performed using 100g of defatted almond meal, following methods based on those published by Taga *et al.*, 1984 and Plummer *et al.*, 1987. Not only did this procedure fail to give a pure product, the bands on the gel did not match the predicted molecular weight of the enzyme, 66 800 Da (Taga *et al.*, 1984) or 79 500 Da (Plummer *et al.*, 1987). One of the problems when purifying an enzyme of low abundance is to match a band on SDS polyacrylamide gel with an active protein. Extra chromatographic steps were therefore used to try and obtain a pure product and consequently observe a single band on an SDS-PAGE gel. These steps involved hydrophobic interaction chromatography and gel filtration.

The second preparation was performed on a small amount of almond meal (25g) using the methods developed in the first preparation and was used to find the best protocol for scaling up.

The third preparation was a scale up of the previous mini-prep and the fourth preparation was done using a different starting material, almond emulsin, in order to obtain a higher yield. Almond emulsin is said to contain larger quantities of the enzyme (T. Plummer Jr., personal communication).

The main chromatographic methods used were:

(a) Ion exchange chromatography - this method exploits the different net charges on proteins at a given pH. The functional ligands on the matrix interact with proteins mainly by electrostatic attractions. Anion exchange columns have positively charged ligands, such as DEAE and QAE covalently linked to the matrix. Cation exchange columns have negatively

charged ligands, such as carboxymethyl (CM). The charges on these columns are balance by counterions such as Cl^-. The net charge on the protein is the same as the counterion, therefore the protein must displace the counterions and bind to the ligand, hence the term ion exchange. Elution of the proteins is brought about by raising the ionic strength (best method) or changing the pH.

(b) Hydrophobic interaction chromatography - exploits hydrophobic external amino acids on the protein surface which are able to bind to immobilised aliphatic or aromatic residues on an absorbant. Very hydrophobic proteins will bind to hydrophobic groups immobilised on some sort of matrix at low salt concentration. However, because these hydrophobic interactions increase in strength with increasing salt concentration, hydrophobic interaction chromatography (HIC) can be extended to cover all proteins.

Salts commonly used are ammonium sulphate and sodium chloride, the latter being more chaotropic (salting-in). The salts interact with water, removing water molecules from the protein molecule and promoting hydrophobic interactions between hydrophobic patches. Lower pH values strengthen the interaction between the protein and hydrophobic groups on the matrix, while forces between the protein and matrix are weakened by lowering the temperature, increasing the pH and including some detergent, ethylene glycol or organic solvent in the elution buffer, as well as reducing the salt concentration.

(c) Gel filtration (or size exclusion) chromatography- the sample is applied to a column consisting of a matrix of porous beads made up of an insoluble hydrated polymer with pores between 40-120 μm in diameter. Small molecules can enter these beads while larger molecule can only pass around the beads. As a result the larger molecules have a shorter elution time and the different proteins can be sorted on the basis of size since they exit the column at different times (in order of decreasing molecular size). The buffer usually contains a low concentration of salt to minimise interactions between the matrix and protein. The column dimensions are important, a long column with a small diameter operating with a low flow rate gives better resolution of a mixture of proteins compared to a short fat

column. The volume of the sample applied is also important. It should be between 1-5% of the bed volume and for maximum resolution, must be smaller than the separation volume (between peaks).

2.2.2 Details of Procedures Used in the Four Preparations of PNGase A

All buffers used in extraction, dialysis and chromatography contained 1 mM PMSF and 50 mM EDTA to help guard against serine and metalloproteases respectively. All purification procedures were performed at 4°C.

(a) Extraction

For preparations 2 and 3: Almond meal was stirred in the buffer 10 mM NH_4Ac, pH 8.75 overnight to extract the proteins, (25 g and 175 g in 125 ml and 875 ml of buffer respectively). For preparation 1, 100 g of almond meal was extracted in 5 mM Tris/HCl, pH 8.8 and for preparation 4, 20 g of almond emulsin was added to 200 ml of 100 mM Tris/HCl, pH 8.8.

(b) Centrifugation

After extraction, the mixture was centrifuged at 12 000 x g for 40 minutes and the supernatant retained. The pellet was re-extracted in 100 ml of buffer by resuspension before being subjected to centrifugation at 12 000 x g for 40 minutes. The two supernatants were pooled. Assays for PNGase activity showed the supernatant to be active while little activity remained with the pellet.

(c) Salt removal by dialysis

Desalting of the protein solution was done using dialysis.

(d) Anion Exchange Chromatography

DEAE Macroprep was equilibrated in the appropriate buffer according to the manufacturers directions. After ensuring that the pH and conductivity of the protein solution and the resin were equal they were added together and stirred gently overnight at 4°C, using an overhead stirrer. The resin was separated from the protein solution by filtration through a sintered glass funnel and washed with buffer until the filtrate showed no activity.

Q-Sepharose chromatography (column dimensions 2.5 x 20 cm) was carried out in preparation 1. The column was equilibrated in 5 mM Tris/HCl, pH 8.8 and a linear gradient was applied between the equilibration buffer and 5 mM Tris/HCl/0.2 M NaCl, pH 8.8 to elute the bound proteins.

(e) Acidification with 1 M Acetic acid

The active filtrate was made pH 5.5 using 1 M acetic acid with stirring at 4°C. This step was not performed in preparations 1 and 3. A white precipitate formed which was removed by centrifugation (12 000 x g, 40 minutes).

(f) Hydrophobic Interaction Chromatography Using Phenyl Sepharose

For preparations 2 and 4, the supernatant was made 2 M with NaCl and the solution loaded onto a phenyl sepharose column (30 cm x 2 cm diameter) equilibrated in 10 mM NH_4Ac / 2 M NaCl, pH 5.5 (Biorad Econosystem). After loading was complete, the column was washed with the equilibration buffer until the absorbance at 280 nm reached approximately zero. A linear gradient was then applied going from 10 mM NH_4Ac /2 M NaCl, pH 5.5 to 10 mM NH_4Ac, pH 5.5 at a rate of 1 ml/min in 120 minutes. The elution was monitored at 280 nm and 6 ml fractions were collected and assayed. Active fractions were pooled and concentrated by ultrafiltration.

For preparation 3, the active eluent from the DEAE step was made 40% with $(NH_4)_2SO_4$ and the pH was measured to be 6.85. If the conductivity of the solution was greater than that of the loading buffer, it was diluted until it measured the same before being loaded onto a phenyl sepharose column equilibrated with 10 mM NH_4Ac / 40% $(NH_4)_2SO_4$, pH 6.85. The bound proteins were eluted with a linear gradient to 10 mM NH_4Ac, pH 6.85 over 120 minutes at a rate of 1 ml/min.

(g) Ammonium Sulphate Fractionation - Preparation Three Only

SDS-PAGE gels showed the sample was still very impure for preparation 3 at this point. Ammonium sulphate fractionation was therefore trialled. Finely powdered $(NH_4)_2SO_4$ was added in 5% aliquots slowly with stirring to the pooled fractions from the previous step, until the solution became turbid. The solution was let to equilibrate for 2 hours at 4°C before the precipitate was removed by centrifugation. The pellet and supernatant were assayed for activity. The next aliquot of $(NH_4)_2SO_4$ was then added to the supernatant as before until the solution again became cloudy. This procedure was repeated until no activity was detected in the supernatant.

(h) Cation Exchange Chromatography

Ion exchange trials were carried out on active fractions from preparation 3 using batch methods. Quantities of 0.6 ml of CM Sephadex (Pharmacia) were equilibrated in the three buffers 10 mM NH_4Ac, pH 5, 10 mM Phosphate, pH 7 and 10 mM Tris/HCl, pH 8. Three aliquots of 0.4 ml of the protein solution were also equilibrated in these buffers by microconcentration and rinsing, with centrifugation for 30 minutes each rinse. The protein solution was then added to the drained resin in Eppendorf tubes and equilibrated by shaking for an hour (IKA vibromax shaker). The protein solution was removed by centrifugation and tested for both activity and concentration.

The pellet of resin and bound protein, was washed with the appropriate buffer in the Eppendorf 4 x 1 ml for 30 minutes each. Each buffer was then made 1 M with NaCl and was added to the drained resin to elute the bound proteins. After equilibration by gentle shaking for an hour, the protein solution was removed by centrifugation and tested for activity and concentration.

In accordance with the results from the CM Sephadex batch trials (*tables 5.2* and *5.3* in *Results, Chapter V*), CM Sephadex was equilibrated in 10 mM NH_4Ac, pH 5. This chromatographic method was used for preparations 3 and 4. The protein solution was equilibrated by dialysis in the same buffer and loaded onto a column (2 x 20 cm). After loading was complete, the column was washed with buffer until all unbound protein was removed. A linear salt gradient from 10 mM NH_4Ac, pH 5 to 10 mM NH_4Ac/ 1.0 M NaCl, pH 5 was then applied at a rate of 1 ml/min for 120 minutes. Extra 1 M NaCl solution was then used to remove tightly bound protein. The elutions were monitored by the absorbance at 280 nm and fractions having absorbance at this wavelength were assayed for activity. Active fractions were pooled.

(i) Sodium Thiocyanate and β-Mercaptoethanol (BME) Incubation

This step was used in preparations 1, 3 and 4. The pooled active fractions after QAE Sepharose (preparation 1), after phenyl superose (preparation 3) or CM Sephadex (preparation 4) were placed in a beaker and made 0.75 M with sodium thiocyanate (NaSCN) and 0.1 M with β-Mercaptoethanol (BME) and gently stirred for 10 minutes. This solution was incubated at 37°C for 24 hours during which time a thick white precipitate formed. This was removed by centrifugation at 12 000 x g for 40 minutes. The pellet was re-extracted as both supernatants tested positive for PNGase activity. They were pooled and excess salts removed by dialysis.

(j) Gel Filtration Chromatography

Gel filtration was used in preparations 1, 3 and 4. In preparation 1, a 1.0 ml sample was applied to a Superdex 75 column (10/30) equilibrated in the buffer 50 mM Tris/HCl, pH 7.5 then eluted at a flow rate of 0.5 ml/min (Pharmacia FPLC).

The protein pooled from the CM Sephadex column (preparation 3) was concentrated by ultrafiltration before being applied to a preparative Superdex 75 column (Pharmacia high load 16/60) using a Waters 650 FPLC. Best resolution was obtained using a flow rate of 0.5 ml/min with a sample size of 0.5 ml. The buffer used was 10 mM sodium acetate /0.15 M NaCl, pH 5.5. The fractions were tested for activity and the active fractions were pooled and concentrated by ultrafiltration.

For preparation 4, initial trials were carried out using a Superdex 75 column (3.2/30) on the SMART system. 40 µl of protein solution was injected onto the column (3.2 x 300 mm) equilibrated in 10 mM NH_4Ac, pH 5 and eluted at 40 µl/min over a three minute run, collecting 80 µl fractions. Following this, scaled up runs were done using Superdex 75 HR 10/30 (same buffer, with a sample size of 160 µl). Multiple injections of 40 µl were then carried out (on the SMART system), using the same conditions as above.

The remainder of the protein solution from the end of preparation 3 was subjected to gel filtration on the preparative Superdex 75 column (Pharmacia Hi load 16/60, 300 µl sample size) on a Pharmacia FPLC as previously described.

(k) Hydrophobic Interaction Chromatography Using Phenyl Superose

This step was performed with the protein solutions from preparations 2 and 3 using a phenyl superose column (HR 5/5). Trials in preparation 1 were done with phenyl superose HR 1.6/5 (SMART), pre-equilibrated in either 10 mM NaAc/1.6 M $(NH_4)_2SO_4$, pH 5.5 or 10

mM NaAc/2 M NaCl, pH 5.5. With preparation 2, this step followed phenyl sepharose chromatography. Trials were performed with the column equilibrated in 10 mM NH_4Ac/ **2** M NaCl, pH 5 and 10 mM NH_4Ac/ **3** M NaCl, pH 5. Injections of 1.5 ml of the protein solution made 2 M or 3 M (respectively) with NaCl were applied to the column. A linear gradient between the equilibration buffer and 10 mM NH_4Ac, pH 5 was applied over 120 minutes at a flow rate of 0.4 ml/min to elute the bound protein.

The active fractions from FPLC gel filtration in preparation 3 were concentrated by ultrafiltration and rechromatographed using hydrophobic interaction chromatography (HIC) with the column phenyl superose HR 5/5. Four mls of the protein solution made 2 M with NaCl, was injected five times in series every 12 minutes onto the column (5 ml loop) which was equilibrated in 10 mM NH_4Ac/2 M NaCl, pH 5. When all the protein was loaded (49 ml, in two runs), a linear gradient to 10 mM NH_4Ac, pH 5 was applied over 80 minutes with a flow rate of 0.5 ml/min. The peaks recorded at the absorbance 280 nm were assayed for activity and active fractions were pooled.

(l) Mono Q Anion Exchange Chromatography

Mono-Q (5/5) ion exchange chromatography using FPLC (Pharmacia) was carried out in preparation 1 using the buffer 50 mM Tris/HCl, pH 7.5. A linear gradient from 0-0.15 M NaCl (in equilibration buffer) was applied over 60 minutes to elute the proteins. A flow rate of 0.5 ml/min was used, with a sample size of 2 ml.

(m) Affinity Chromatography Using Concanavalin A

Initial trials were carried out using small columns fashioned from Pasteur pipettes. As these appeared successful, a larger column (2 x 15 cm) was equilibrated in the buffer 20 mM NH_4Ac/1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, pH 6.5 according to the manufacturers directions.

The protein was equilibrated in the same buffer by dialysis before it was loaded onto the column. Elution of the proteins was monitored at 280 nm and was achieved by running a gradient between the loading buffer and 0.5 M methyl α-D-mannopyranoside (Sigma) over 120 minutes at a flow rate of 1 ml/min.

2.2.3 General Methods

(a) Bicinchoninic acid (BCA) Total protein determination

Protein was quantitated by the modified Lowry method. Bovine serum albumin was used as the standard. To 50 ml BCA solution, 1 ml 4% $CuSO_4$ was added then 2 ml of this reagent was added to 0.1 ml of standard or sample of unknown concentration. Absorbance at 562 nm was read on a UV/visible spectrophotometer (Hitachi U-1100) after a 30 minute incubation at room temperature. Proteins reduce Cu^{2+} to Cu^{+} and BCA forms a 2:1 complex with Cu^{+}, resulting in a stable, highly coloured chromophore with an absorbance maximum at 562 nm (Smith *et al.*,1985).

(b) PVDF membrane blotting for N-terminal sequencing

A sample of a protein solution containing PNGase A was subjected to electrophoresis (Biorad) on a 12% SDS-PAGE gel but not stained with Coomassie Blue R_{250}. A blotting apparatus was prepared according to the manufacturers instructions in the transfer buffer 10 mM CAPS/ 10% methanol, pH 11. The PVDF membrane was equilibrated in methanol for a few seconds followed by transfer buffer, then placed on the gel in a sandwich made of filter paper (Whatman no.3).

Electrophoresis was for 1¼ hours at 90V (~250 mA). Afterwards the membrane was rinsed in water then stained with 0.1% Coomassie Blue /50% methanol for a few minutes until bands began to appear. It was destained with several changes of 50% methanol/10% acetic acid and finally rinsed in water before air drying. Individual bands were cut out for N-terminal sequencing on the automatic protein sequencer (Applied Biosystems).

(c) Electroelution of PNGase A from non-denaturing gels

A horizontal gel electrophoresis cell (Gibco Horizon) was adapted for the process of electroelution. Slivers of the gel were cut out with a blade in even increments, down from the resolving gel and stack interface. This was performed on one half of the gel, the other half was stained to indicate the positions of the bands. Each sliver of gel was placed into thin width dialysis tubing, which contained about 3 ml of the electroelution buffer 10 mM NH_4Ac, pH 5.

The tubing was tied and numbered then placed in the electrophoresis dish containing the same buffer, which covered the electrodes. This unit was operated in the cold at 4°C and the proteins electroeluted at 50 mA (~130V) overnight. The contents of each tube were concentrated in 3K microconcentrators then assayed for activity and analysed by SDS-PAGE. The original gel was stained with Coomassie Blue R_{250} to ascertain that transfer was complete.

(d) Protocol for Endoglycosidase Assays

Preparation of sample

The substrate used throughout this project was an ovalbumin derived glycopeptide (*figure 2.3*). This contained a biantennary oligosaccharide, which is cleaved by PNGase A to release a free intact oligosaccharide and peptide. The products of this reaction can be assayed for and detected by reverse phase chromatography (RPC) and HPLC.

The substrate was prepared by addition of 1 mg of glycopeptide to 1 ml of 0.25 M sodium acetate buffer, pH 4.5. An aliquot of 50 µl of this substrate is pipetted into Eppendorf tubes and 5 µl of the protein solution (for activity determination) is added. Incubation for dilute samples was overnight at room temperature but as the protein became more pure, at 37°C for 2 hours. The reaction was terminated by heating to 100°C and 5 µl of formic acid was added. The sample was centrifuged for 5 minutes at 14 000 x g before injection to remove any particulate matter. 50 µl was injected on to a Vydac C_{18} column (internal diameter = 4.6 mm, 250 mm in length) and subjected to gradient elution *(table 2.2)*.

Programme for running enzyme assay

Buffer A: Distilled H_2O with 0.1% TFA

Buffer B: Acetonitrile with 0.08% TFA

Table 2.2. PNGase assay gradient programme.

TIME	%A	%B	%C	FLOW
0	80	20	0	1.0
15	60	40	0	1.0
20	80	20	0	1.0
500	80	20	0	0.5

The detector was set at the wavelength 220 nm at a range of 0.5. The chart recorder was set at a speed of 150 mm/min to record the elution of peptides. The chromatograms are displayed in *figure 2.1.*

NOTE: This is a very sensitive assay. Variations in incubation times -carefully monitored- will help distinguish the different degree of activity in otherwise apparently similar active samples. Overnight incubations may not give a precise comparison.

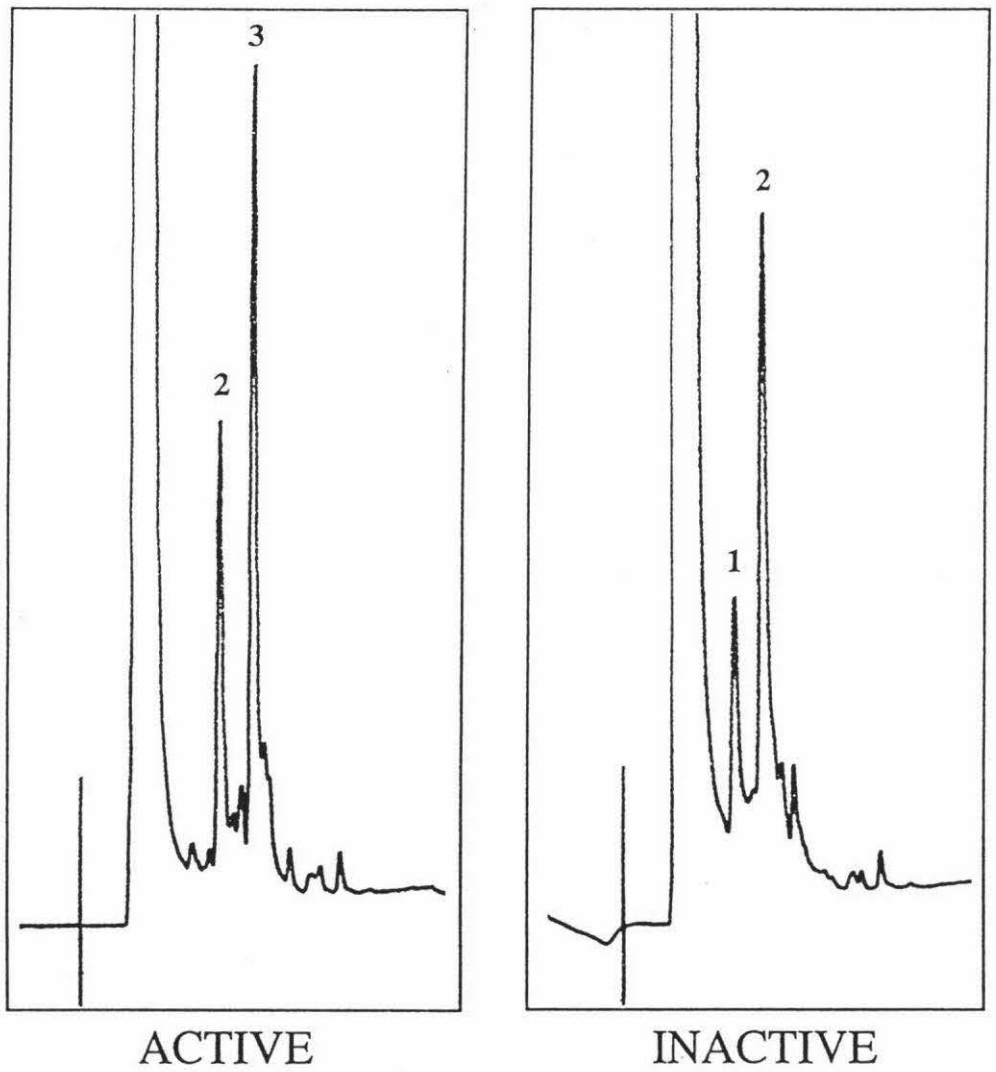


Figure 2.1. The chromatograms from the PNGase assay. ***A:*** An active sample, which shows PNGase activity. ***B:*** An inactive sample, which does not show PNGase activity.

Mechanism of Assay

The ovalbumin glycopeptide is glycosylated at the Asn residue. When the glycan is cleaved by PNGase A, Asn is converted to Asp and the hydrophobicity of the peptide is increased, due to the removal of many hydrophillic -OH groups on the glycan (*figure 2.2*). The product is therefore retained longer on the column. This peak is recorded further away from the formic acid reference peak. The peptide will always have a positive charge due to Lys (K) but the total charge remains the same since there is no charge on the protonated Glu (E) or Asp (D) at this low pH.

Note: (ϒ) represents the glycan.

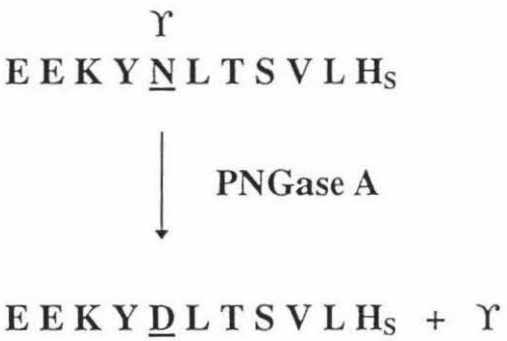
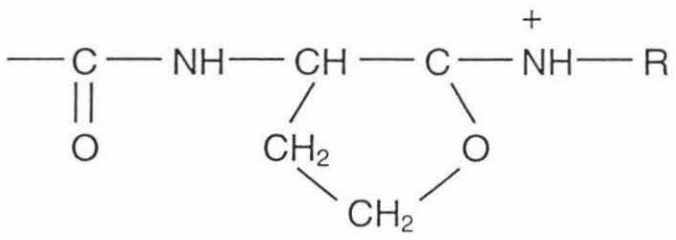


Figure 2.2 Mechanism of assay: Cleavage of the glycan from the ovalbumin glycopeptide, by PNGase A.

The molecular weight for the product is 1278 in the lactone form. Homoserine at the carboxyl terminus of the peptide is acidified to homoserine lactone as shown in *figure 2.3*.

Homoserine



⇓ Acidification

Homoserine lactone

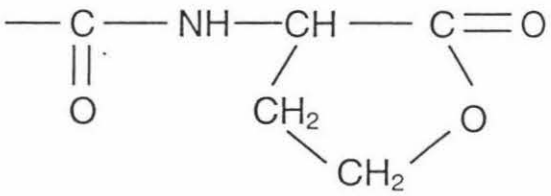


Figure 2.3. The homoserine lactone ring formation.

Specific Activity of PNGase A During the Purification

In order to perform specific activity assays, tritiated substrates were required. These were not obtained due to their expense, which exceeded the budget of this investigation. Plummer and Tarentino (1981), used [^{3}H]-labelled 5-dimethylaminonaphthalene-1-sulphonyl octaglycopeptide substrate, derived from ovalbumin. From a 30% yield of PNGase A, they obtained a specific activity value of 438 nmol of [^{3}H]-dns-octaglycopeptide formed min/mg of protein. Fetuin-derived [^{3}H]-dns-pentaglycopeptides were used in later quantifications (Plummer *et al.*, 1987) and by many other researchers.

(e) DIG Glycan / Protein Double Labelling

This method used a kit supplied by Boehringer Mannheim® and can be used to differentiate between glycoproteins and non-glycosylated proteins which appear as different coloured bands on the same blot.

The sugar and protein component are each labelled with a different hapten and detected with the corresponding antibody. With the sugar component, periodate oxidises the -OH groups to aldehydes, which couples it with digoxigenin. An anti-digoxigenin-POD (antibody coupled to an enzyme, peroxidase) is added and upon substrate addition the colour reaction can be seen. The hapten for proteins (labelling of $-NH_2$ groups) is fluorescein and the antibody conjugate has the enzyme alkaline phosphatase.

Method for labelling of proteins and glycoproteins already immobilised on a filter

All filters are incubated by gentle agitation at room temperature except for the staining reaction, which is not shaken.

Materials

The vial contents are listed as follows:

1. Sodium metaperiodate. Dissolved 21.4 mg in 10 ml 100 mM sodium acetate pH 5.5.
2. DIG-hydrazide (Digoxigenin-3-O-succinyl-ε-aminocaproic acid-hydrazide hydrochloride)
3. FLUOS (5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester)
4. Anti-digoxigenin-POD
5. Anti-fluorescein-AP
6. X-phosphate (5-bromo-4-chloro-3-indolyl phosphate)
7. INT (2-(-4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride)
8. TETON (4-Triethylenetrioxy-1-naphthol)
9. Blocking reagent. Dissolved 0.5 g in 100 ml TBS, pH 7.5 by heating at 50-60°C for 1 hour.

The solutions made are listed as follows:

A. 0.1 M Sodium acetate buffer, pH 5.5

B. TBS (Tris buffered saline) pH 7.5 (0.05 M Tris/HCl, 0.15 M NaCl)

C. Tris buffer pH 8.0 (0.1 M Tris/HCl: 0.05 M $MgCl_2$: 0.1 M NaCl)

D. Nonidet®P40

E. PBS (phosphate buffered saline) pH 6.5 (0.05 M K_3PO_4: 0.15 M NaCl)

F. 30% (w/v) H_2O_2

Procedure

The membrane that had been blotted was washed in 50 ml PBS, pH 6.5 then incubated in 10 ml 0.1 M sodium acetate buffer, pH 5.5 with 21.4 mg sodium metaperiodate for 20 minutes. Following this the filter was washed three times, for five minutes each in 50 ml PBS.

Next it was incubated with 2 µl DIG hydrazide in 10 ml of 0.1 M sodium acetate buffer, pH 5.5 for 1 hour at room temperature, followed by incubation in a mixture of 5 µl FLUOS in 10 ml of 0.05 M potassium phosphate buffer, pH 8.5 and 0.01% Nonidet P40 for 1 hour. This was followed by washing three times for five minutes each in 50 ml TBS.

Incubation for 30 minutes in the blocking solution followed. The membrane was washed three times for five minutes each in 50 ml TBS then incubated for 1 hour with anti-digoxigenin-POD and anti-fluorescein-AP (20 µl added of each of the two conjugate solutions to 10 ml TBS).

Finally the filter was washed three times for five minutes each in 50 ml TBS and the staining reaction was prepared including the following:

10 ml Tris buffer, pH 8.0

50 µl INT

50 µl X-phosphate

50 µl TETON

6 µl H_2O_2

The filter was incubated without shaking and the colour development occurred within 20 seconds. The reaction was terminated by immersing the filter in water and rinsing continued through several changes then the filter was left to air dry.

Non-glycosylated proteins are identified by a reddish brown colour while glycoproteins are coloured blue.

(f) Mass Spectroscopy

Samples were sent to the University of Waikato for analysis. Samples were made up to 200 µl with 1:1 MeCN/H_2O for injection (plenty of sensitivity).

(g) Urea Gel Electrophoresis

A non-denaturing 12% gel was made with a 2 M urea solution replacing the water. The stacking gel did not contain urea.

(h) Molecular Weight Curve of SDS Markers for Protein Molecular Weight Determination

With every gel run, a lane which contained low range SDS-PAGE molecular weight standards (Biorad), was loaded alongside the protein samples. The bands seen after staining with Coomassie Blue R_{250}, were measured from the dye front and a curve was drawn using these known distances and molecular weights of the markers (manufacturers directions). The unknown molecular weights of the protein samples were determined by reading the values from a plot similar to that shown in *figure 2.4*. A new curve was plotted for every gel run, based on the migration of the standards in that particular gel, to allow for precise comparisons to be made between gels.

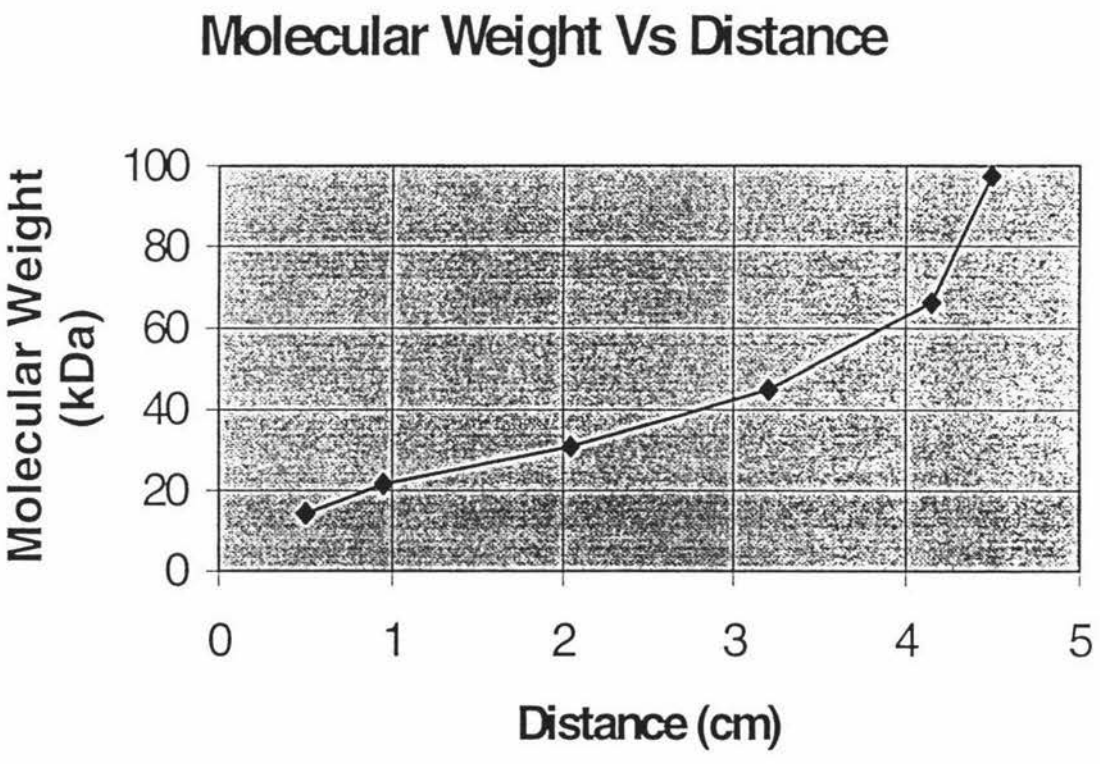


Figure 2.4 Curve generated by plotting the molecular weight of the low range molecular weight standards run on a 12% SDS polyacrylamide gel vs. the distance migrated from the dye front, in centimetres.

2.2.4 Summary of purification steps

PREPARATION 1

1) Extraction
2) Centrifugation
3) Dialysis
4) Q-Sepharose
5) NaSCN/BME incubation
6) Gel filtration using FPLC
7) Mono Q using FPLC
8) Gel filtration using FPLC
9) NaSCN/BME incubation
10) Phenyl superose (FPLC)

PREPARATION 2

1) Extraction
2) Centrifugation
3) Dialysis
4) DEAE Macroprep
5) Acidification to pH 5
6) Phenyl sepharose
7) Phenyl superose (FPLC)

PREPARATION 3

1) Extraction
2) Centrifugation
3) Dialysis
4) DEAE Macroprep
5) Phenyl sepharose
6) Ammonium sulphate fractionation
7) CM Sephadex
8) Gel filtration using FPLC
9) Phenyl superose (FPLC)
10) Con A trials
11) Gel filtration using FPLC

PREPARATION 4

1) Extraction
2) Centrifugation
3) Dialysis
4) DEAE Macroprep
5) Acidification to pH 5
6) CM Sephadex
7) Phenyl sepharose
8) Gel filtration using FPLC
9) Gel filtration using SMART

CHAPTER III

RESULTS OF PREPARATION 1

3.1 Purification Using Almond Meal

(a) Ion Exchange Chromatography

It was found that cellulose based resins were not suitable for use because of cellulase activity. Q-Sepharose was found to be suitable. The PNGase appeared to bind to the resin and the elution (outlined in *Methods, section 2.2.2(d)*) was monitored by measuring the absorbance at 280 nm (*figure 3.1*).

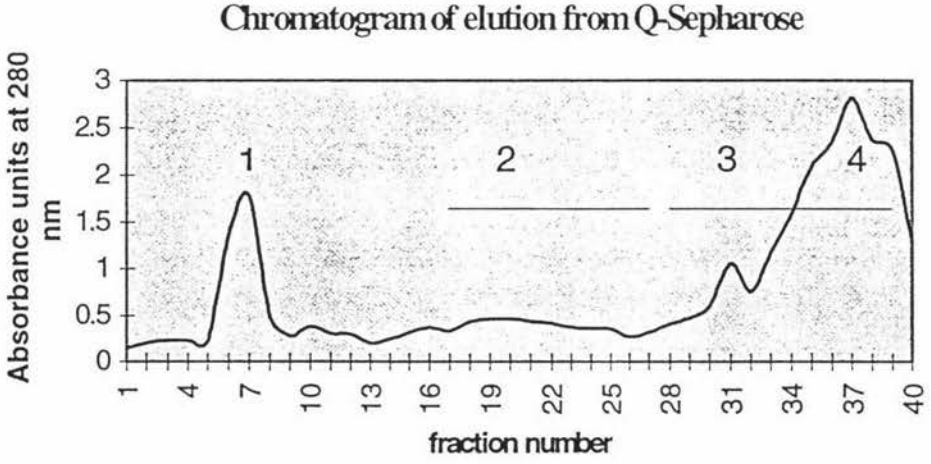


Figure 3.1 Chromatogram of elution from Q-Sepharose. The peaks monitored are numbered. The active fractions were pooled as indicated by the *bars*.

Peaks two (fractions 17-27), three and four (fractions 28-39) contained PNGase activity. Before pooling, the fractions within each peak were analysed by SDS-PAGE (*figure 3.2*).

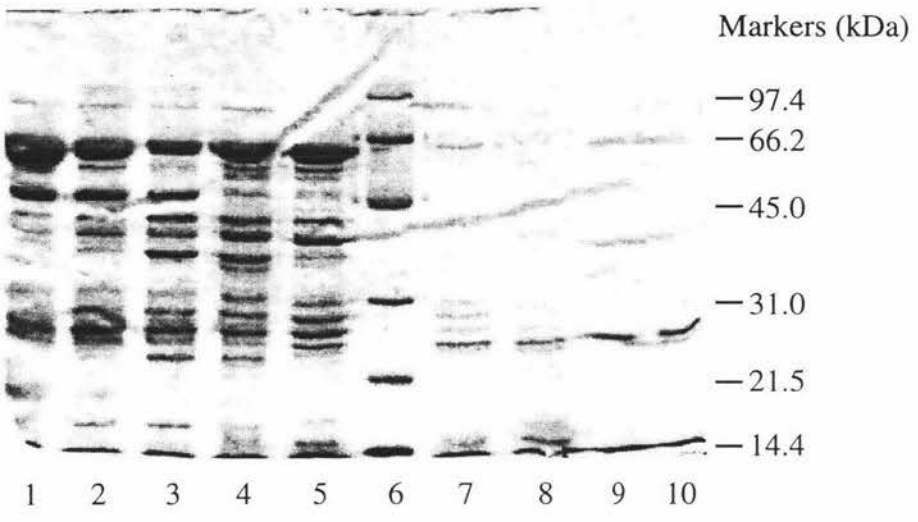


Figure 3.2 SDS gel of fractions across the two peaks eluted from Q-Sepharose which contained PNGase activity. *Lanes 1-5:* Fractions in the third and fourth peak (pool 2): 40, 38, 36, 34, 32 (all active). *Lane 6:* Marker. *Lanes 7-10:* Fractions in the second peak (pool 1): 28, 24, 20, 18 (all active).

Many bands were seen for each of these active samples. The third and fourth peak (*lanes 1-5*) which was pool 2, showed bands mainly at approximately 91, 66, 58 (darkest) and 27 kDa (faint). The second peak (pool 1) in *lanes 7-10* showed faint bands at approximately 66, 27, 25 and 16 kDa.

Fractions 17-27 (pool 1) and 28-39 (pool 2) were combined and concentrated by ultrafiltration (Amicon) using a PM-30 membrane. The filtrate tested inactive.

(b) NaSCN and βME Incubation

This step removes many contaminating proteases and glycosidases (outlined in *Methods, section 2.2.2(i)*). After incubation, the precipitate was removed by centrifugation and assayed. No PNGase activity was detected in the pellet yet the supernatant was active.

The thiocyanate salts were removed using extensive dialysis, then the samples were concentrated using ultrafiltration.

Pool 1: 18 ml

Pool 2: 25 ml

Each pool was then made 70% in ammonium sulphate to precipitate the protein. As the protein is said to lose activity on freezing (Taga *et al.*, 1984 and Takahashi, 1977), an ammonium sulphate precipitate is the most appropriate storage method. The pellet was removed by centrifugation (40 minutes at 12 000 x g) then resuspended in the minimum of buffer.

Each of the pools from this experiment were treated differently. The pellet from pool 2, containing the most impure fractions, was resuspended in a minimum of buffer and divided into 2 fractions, one of which was subjected to gel filtration, the other to further ion exchange chromatography in order to determine an appropriate method for further purifying the protein.

(c) Gel Filtration Using FPLC

The sample (1 ml) was subjected to gel filtration on a Superdex 75 (HR 10/30) column as described in *Methods, section 2.2.2(j)*. The elution (*figure 3.3*) was monitored by measuring the absorbance at 280 nm and fractions were analysed by both SDS-PAGE (*figure 3.4*) and assays for PNGase activity. The only active fraction was 8 (*lane 2*), which contained bands corresponding to molecular weights of approximately 66, 61, 46, 27 and 25 kDa (diffuse). The inactive fractions (in the second peak) also had bands corresponding to molecular weights of 66, 61 and 46, as well as extra bands corresponding to 40, 37 and 31 kDa. Thus the bands at 66, 61 and 46 kDa are probably not associated with PNGase activity.

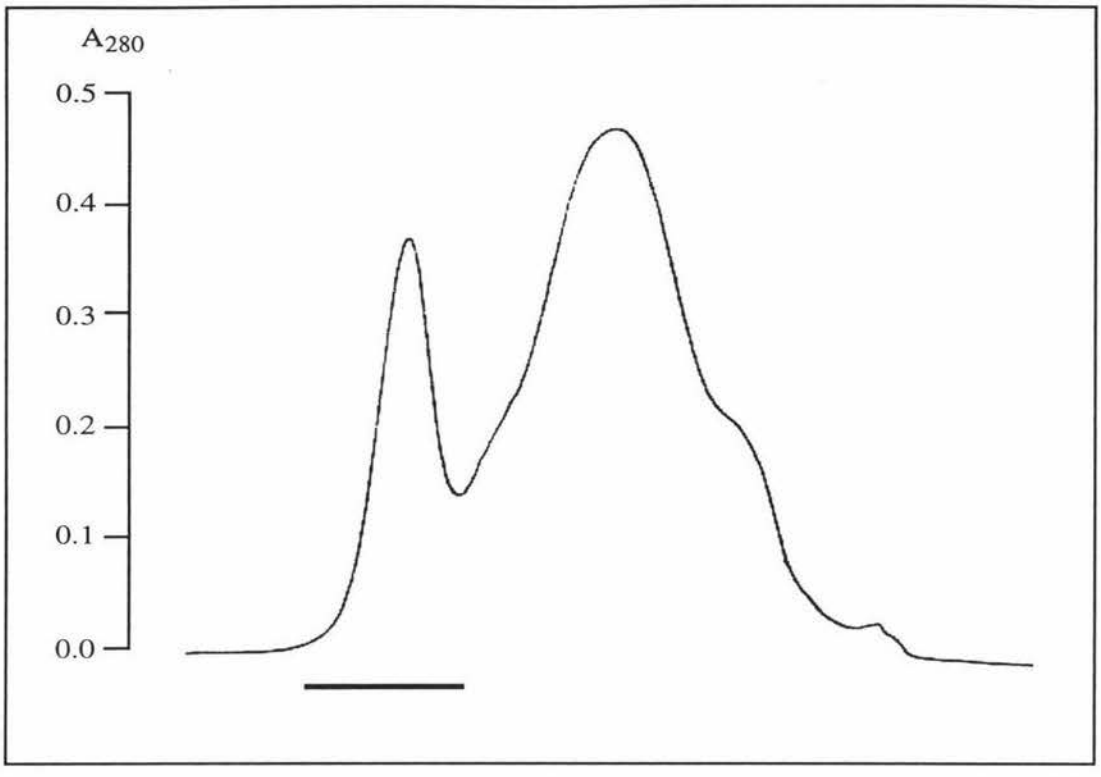


Figure 3.3 Chromatogram of pool 2 subjected to gel filtration, using FPLC. The active fractions were pooled as indicated by the *bar*.

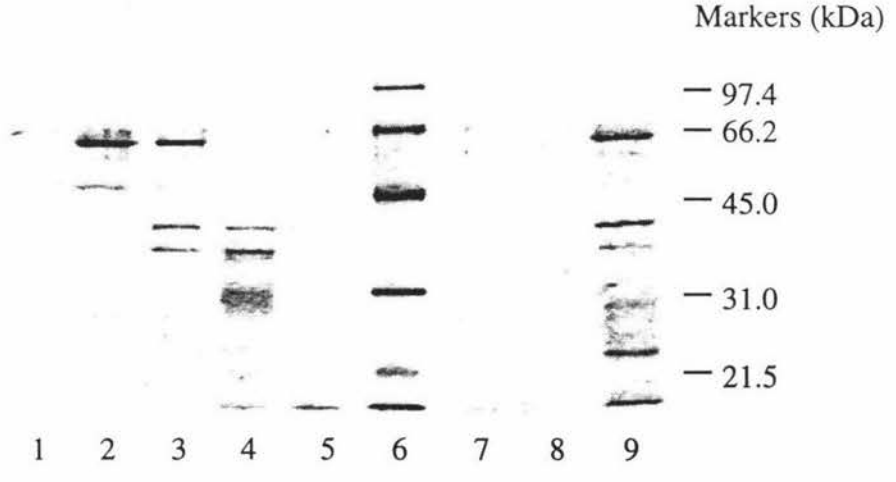


Figure 3.4 SDS gel of gel filtration fractions which contained PNGase activity. From pool 2, which was subjected to gel filtration. *Lane 1:* Fraction 7 (small peak, inactive). *Lane 2:* Fraction 8 (small peak, active). *Lanes 3-5:* Fractions 9-11 (large peak, inactive). *Lane 6:* Marker. *Lanes 7 and 8:* Fractions 12 and 13 (inactive). *Lane 9:* Active sample before gel filtration (for comparison).

(d) Mono-Q Ion Exchange Chromatography

The rest of the sample was equilibrated in buffer by dialysis before being loaded onto a Mono-Q column as described in *Methods, section 2.2.2(l).* Although anion exchange had been used earlier, the column used was prepacked with beads of a smaller, more even size, which should result in a better separation of the different proteins in the sample.

The elution was monitored by absorbance at 280 nm. Active fractions eluted first at a concentration of about 0.025 M NaCl, in a series of low absorbance peaks. The chromatogram is shown in *figure 3.5.* The active fractions were pooled, concentrated by ultrafiltration then analysed by SDS-PAGE (*figure 3.6*).

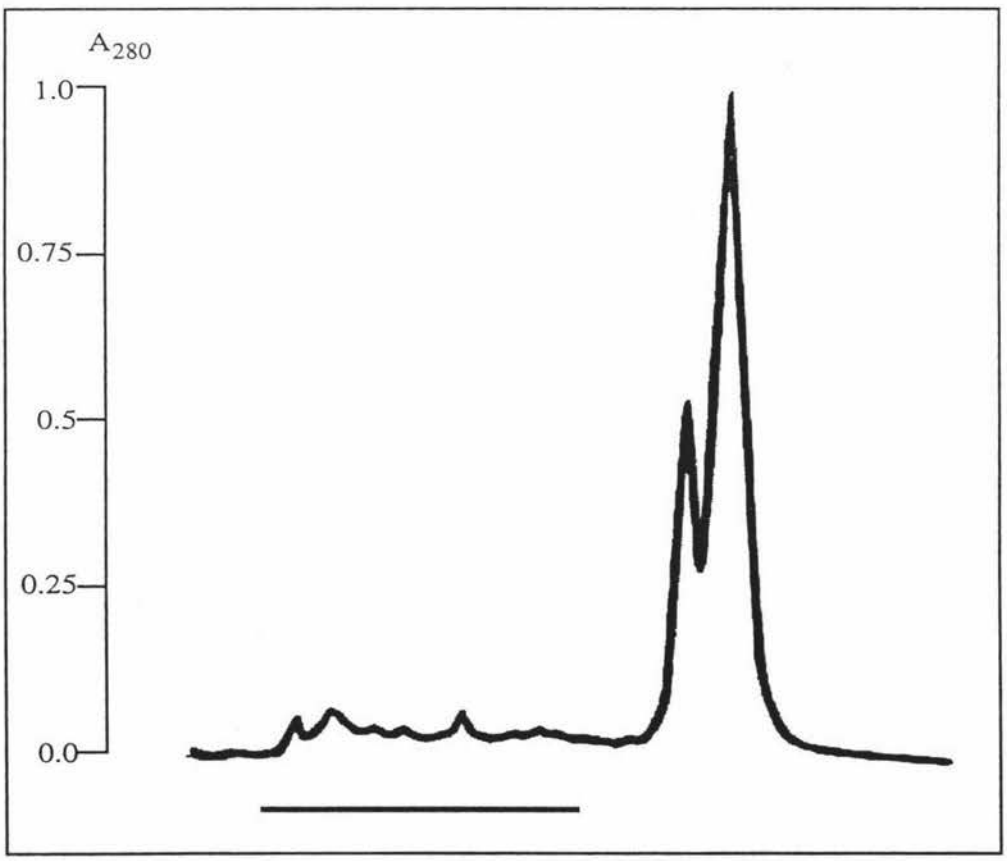


Figure 3.5 Chromatogram of the elution from Mono-Q ion exchange chromatography. The active fractions were pooled as indicated by the *bar.*

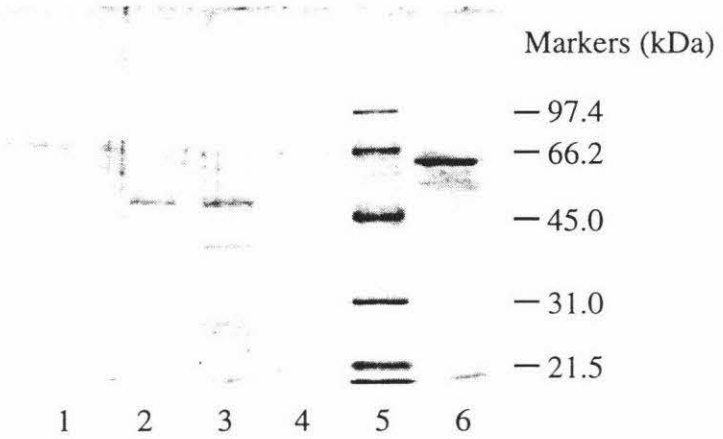


Figure 3.6 SDS gel of active fractions from Mono-Q elution. *Lane 1:* Inactive fraction 4. *Lanes 2-4:* Active fractions 5, 7 and 8. *Lane 5:* Marker. *Lane 6:* Inactive fraction 9 (peak of highest absorbance).

Active fractions (*lanes 2, 3* and *4*) showed bands at ~66, 51, 39, 27 and 25 kDa. Bands in the inactive sample of high absorbance (*lane 6*) contained a vivid band at ~60 kDa and a faint band at ~54 kDa.

A comparison with the results obtained from gel filtration show that the only bands in common for the active fractions were those at ~66, 27 and 25 kDa. There appears to have been a shift in molecular weight for the 61 and 46 kDa bands from the previous gel (*figure 3.4*) to 51 and 39 kDa. However, as the inactive fractions also had a band at ~66 kDa, it is possible that this is not associated with active protein or there were two proteins with molecular weights of approximately 66 kDa in the previous gel.

Figure 3.5 showed this step was very successful in the elimination of a large amount of contaminating proteins.

(e) Gel Filtration Using FPLC

Pool 1 (from part *(a)*) was also subjected to gel filtration under the same conditions as used above. The chromatogram was similar to that of the previous runs. The fractions were assayed for activity and analysed by SDS-PAGE (*figure 3.7*).

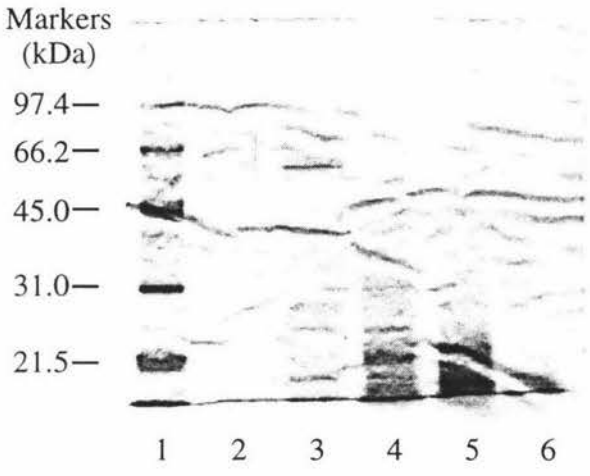


Figure 3.7 SDS gel of pool 1: Fractions spanning the peaks eluted by gel filtration. *Lane 1:* Marker. *Lanes 2-4:* Active fractions 38-40. *Lanes 5 and 6:* Inactive fractions 41 and 42.

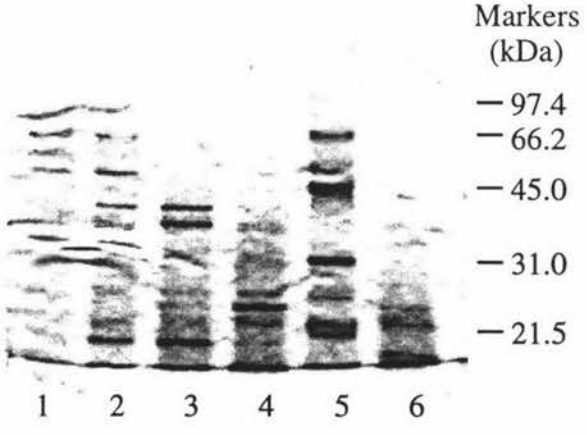


Figure 3.8 SDS gel of pool 2: Fractions spanning the peaks eluted by gel filtration. *Lanes 1-3:* Active fractions 9-11. *Lane 4:* Inactive fraction 12. *Lane 5:* Marker. *Lane 6:* Inactive fraction 13.

The most distinct band in the active peak was at ~56 kDa and faint bands also appeared at ~27 and 25 kDa. The inactive peaks showed bands at approximately 21, 18 and 15 kDa, showing that this step was successful in the elimination of lower molecular weight proteins. However, as these bands had not been observed earlier, it seemed degradation must have occurred and that the band at ~66 kDa previously seen (*figure 3.2*) had been replaced by a band at ~56 kDa. Bands at ~27 and 25 kDa were observed to be still present in the active fractions.

A small amount of pool 2 was re-chromatographed by gel filtration after Mono Q ion exchange to check possible degradation of the proteins. The chromatogram was similar to that seen for the original gel filtration, before Mono Q (chromatogram not shown), which was not expected. The fractions were assayed for activity and analysed by SDS-PAGE (*figure 3.8*).

Activity was detected in fractions 9, 10 and 11. Bands in these active fractions ran at approximate molecular weights of 66, 51, 42, 39, 27 and 18 kDa. The band at 25 kDa, previously present in the active peaks, was most distinct in an inactive sample (large, overlapping peak). Although the bands at approximate molecular weights of 66, 51, 39 and 27 kDa were still present, the appearance of bands at ~42 and 18 kDa indicated some proteolysis was occurring.

Pool 1 was further divided into two pools A_1 and B_1 , where 'A' represents the active peak and 'B' represents the tail of this peak (also active) which overlapped with the following inactive peak. For the same reasons, pool 2 was divided into A_2 and B_2.

(f) NaSCN and βME Incubation

As most proteins are inactivated in the presence of such a chaotropic salt, this step was performed a second time to remove proteolytic activity because degradation of protein was shown to occur by SDS gel analyses (shifts in molecular weights). The two pools of fractions, A_2 and B_2 from gel filtration, were incubated in 0.75 M NaSCN and 0.1 M

βME and the pellet removed by centrifugation as described in *Methods, section 2.2.2(i).* The pellet tested inactive and the activity resided in the supernatant.

(g) Hydrophobic Interaction Chromatography (HIC)

As there were still several bands in the SDS gels, the protein samples from each pool (1 and 2) were subjected to HIC chromatography because this had proved very successful for PNGase F and it had not been done before. It had been observed (SDS-PAGE) in previous purification steps that aggregation occurred between proteins and gel filtration did not separate some low molecular weight proteins.

Trials were carried out using SMART, which enables a better separation of different proteins due to the smaller, more even bead size and small sample load. The protein sample containing PNGase A was chromatographed on phenyl superose 12 (HR 1.6/5) pre-equilibrated in 10 mM NaAc/1.6 M ammonium sulphate, pH 5.5, since high salt was required for binding (see *Methods, section 2.2.1* for background information). The fractions containing activity eluted last in fractions 18, 19, 20 and 21 (*figure 3.9*).

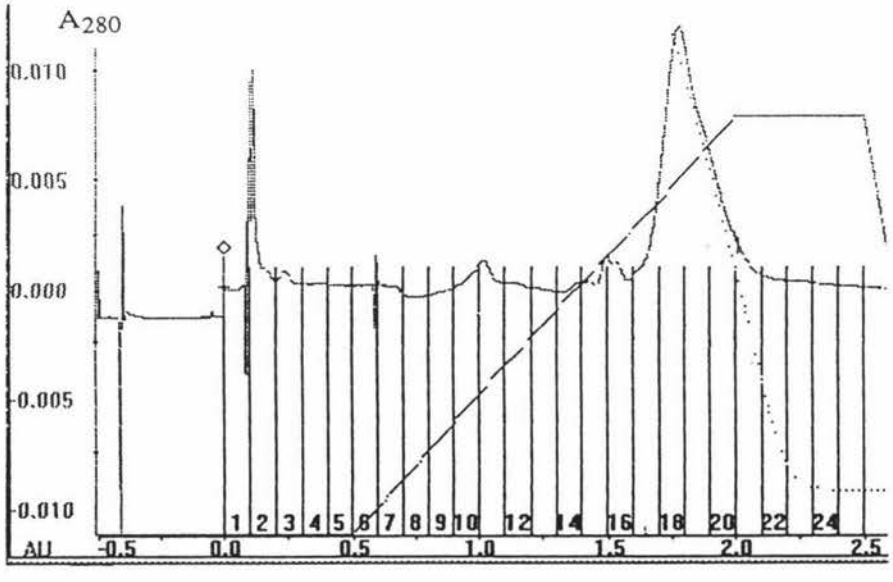


Figure 3.9 Chromatogram from phenyl superose elution, equilibrated with ammonium sulphate. The bound proteins were eluted using a linear gradient from 10 mM NaAc/1.6 M $(NH_4)_2SO_4$, pH 5.5 to 10 mM NaAc, pH 5.5. Active fractions were pooled as indicated by the *bar.*

Since the activity was contained in fractions within a broad peak, separation of different proteins may not have been achieved. A more chaotropic salt, NaCl, was used in the next trial because it facilitates weaker hydrophobic interactions (is more 'salting-in'), compared to SO_4^{2-}. With the same column equilibrated in 10 mM NaAc/2 M NaCl, pH 5.5, the active fractions eluted last in fractions 16, 17, 18, 19 and 20 (*figure 3.10*). The active fractions eluted at 58% of 2 M NaCl (0.84 M NaCl). No activity was recovered from a trial which involved the column equilibrated at 4 M NaCl.

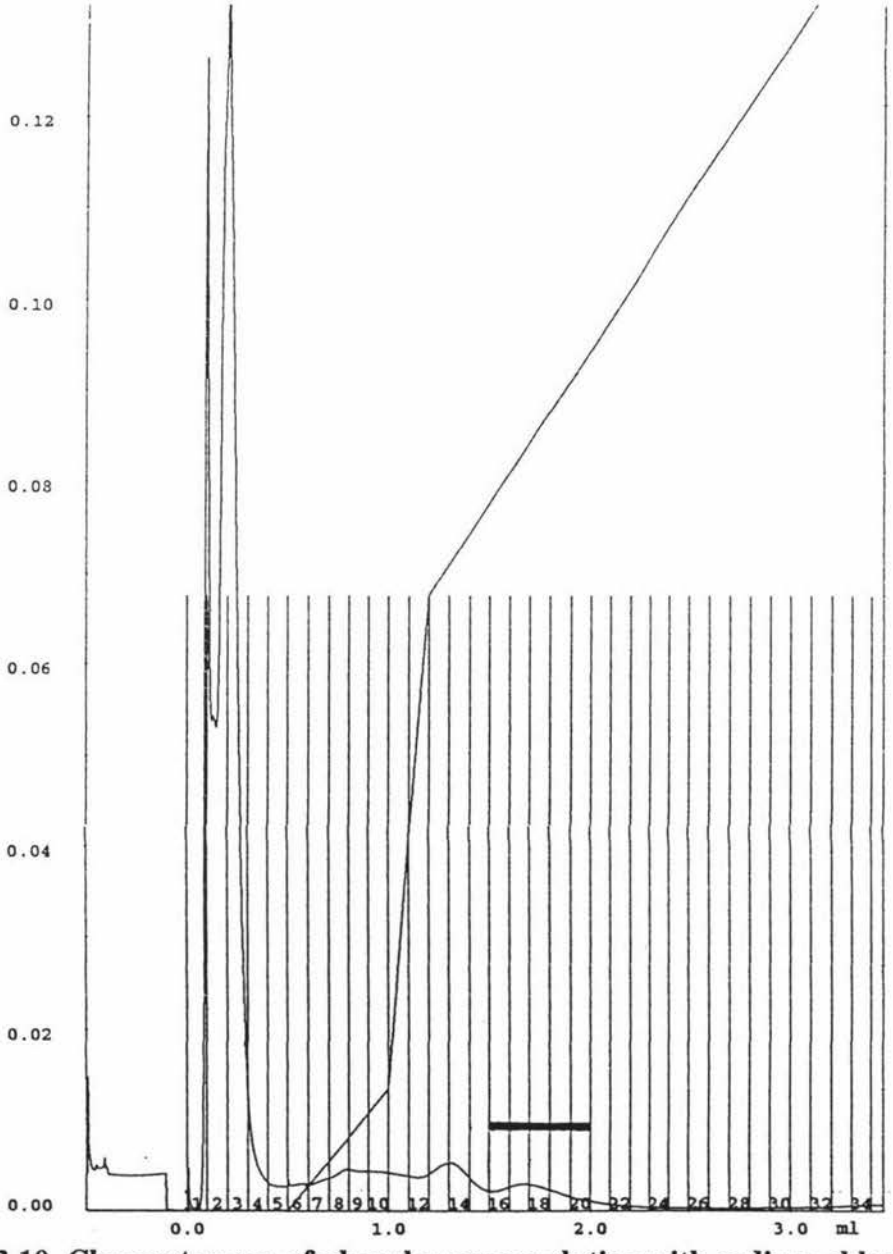


Figure 3.10 Chromatogram of phenyl superose elution with sodium chloride. The bound proteins were eluted using a linear gradient from 10 mM NaAc/2 M NaCl, pH 5.5 to 10 mM NaAc, pH 5.5. The active fractions were pooled as indicated by the *bar.*

Optimum separation was obtained using 2 M NaCl and as it seemed to eliminate a relatively large amount of contaminants, this method was scaled up. FPLC was used (Pharmacia) under the same conditions as the latter trial and two runs were carried out, combining B_1 with B_2 and A_1 with A_2. Elution of active fractions occurred at 66 % 10 mM NH_4Ac, pH 5.5 (0.68 M NaCl). This was seen as a peak of low absorbance, compared to the contaminants which eluted at the start (chromatogram not shown). The active fractions were analysed by SDS-PAGE and silver stained (*figure 3.11*).

The A_1 + A_2 sample revealed many bands were still present, corresponding to the approximate molecular weights of 66 (a faint band), 62, 54, 45, 42, 38, 27 and 25 kDa. Although a faint band could be seen at ~66 kDa (66.8 kDa is the molecular weight of PNGase A proposed by Taga *et al.,* 1984), no band could be seen at 79.5 kDa (the molecular weight proposed by Plummer *et al.,* 1987). The only bands that could be seen on a SDS gel stained using Coomassie Blue R_{250}, were those corresponding to proteins of molecular weights 54, 27 and 25 kDa (gel not shown).

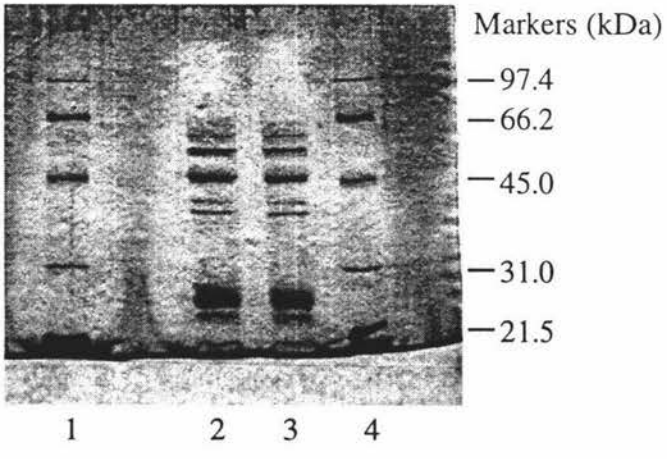


Figure 3.11 SDS silver stained gel of sample containing PNGase A, after phenyl superose chromatography. *Lane 1:* Marker. *Lanes 2* and *3:* Sample containing PNGase A. *Lane 4:* Marker.

To ensure it was PNGase A which was purified and traced with the assays, the eluted peaks from the assay using HPLC, were collected for analysis by mass spectroscopy. The peaks were labelled as shown in *figure 2.1* (*Methods, section 2.2.3(d)*).

The second peak (substrate peak) gave three positive ion $[M+H]^{2+}$ peaks at 1248.5, 1329.5 and 1451.5, which corresponded to M values of 2495, 2657 and 2901 respectively. These assignments were confirmed by negative ion mode. These three molecular weights represent the different glycoforms of the glycan on the substrate peptide.

The third peak, which appears only in active samples (product peak, after the glycan is removed) gave a positive ion $[M+H]^{+}$ peak at 1280 and a $[M+2H]^{2+}$ peak at 640.5. Therefore M=1279. This was confirmed by negative ion spectra. This was an unambiguous clean sample (personal communication). Peak 3 therefore complied with the weight of the peptide in the lactone form, with the glycan removed and Asn converted to Asp.

E E K Y N L T S V L H_S (MW= 2495, 2657, 2901)

↓

E E K Y D L T S V L H_S (MW= 1279)

CHAPTER IV
RESULTS OF PREPARATION 2

4.1 Preliminary Study Using Almond Meal

(a) DEAE Ion Exchange Chromatography

Protein not bound to the resin, was removed by washing with successive aliquots of buffer until fractions showed no activity (900 ml). The optical density at 280 nm was 0.211, indicating there was about 190 mg of protein in the eluent. More proteins were eluted with 1 M NaCl but there was no PNGase activity detected in this solution. Samples up to this stage were analysed by SDS-PAGE (*figure 4.1*).

This chromatographic step was quick and useful in the elimination of contaminating proteins which bound to the support. UV spectroscopy can be used to measure the optical density of a solution at 280 nm and provide an estimation of the concentration in mg/ml. The absorbance at 280 nm gives an approximate estimation of protein concentration. It is due entirely to the absorbance by aromatic groups and therefore is dependent on the number of Tyr, Trp and to a lesser extent Phe residues in the polypeptide chain. For most proteins, the values lie between 0.4 and 1.5 for a 1 mg/ml solution but there are exceptions.

The SDS gel shows many bands are still present in the protein solution at this point. No band is visible at 54 kDa in the present preparation at this stage, compared to the band seen at this place in the sample from preparation 1 (*lanes 8, 9* and *10*). However a band they both have in common is at 27 kDa. All samples except those in *lanes 6* and *7* exhibit

PNGase activity. A band at about 58 kDa in *lane 3* is not present in *lane 4* after removal of the precipitate formed when the pH was lowered.

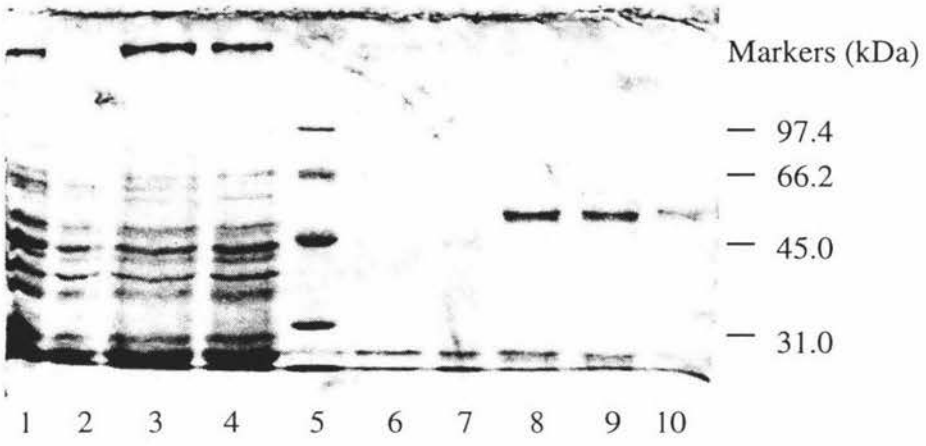


Figure 4.1 SDS gel of current protein samples. *Lane 1:* Supernatant after first centrifugation. *Lane 2:* Supernatant after second centrifugation. *Lane 3:* Active eluent from DEAE at pH 5.5. *Lane 4:* Supernatant after removal of precipitate. *Lane 5:* Marker. *Lane 6:* Column eluent during sample loading onto phenyl sepharose (very faint). *Lane 7:* Precipitate from pH lowering to 5.5 (very faint). *Lane 8, 9 and 10:* PNGase A sample (proven by mass spectroscopy) from the end of preparation 1, for comparison to this current preparation.

(b) Phenyl Sepharose Chromatography

When the pH was lowered to 5.5, a precipitate formed. This was removed by centrifugation. The pellet resolubilised and was tested for PNGase activity, which was absent.

Hence by lowering the pH, impurities were removed. The supernatant was loaded onto a phenyl sepharose column under the conditions outlined in *Methods, section 2.2.2(f).* The eluent from the loading and rinse of the column (*figure 4.2*) did not contain PNGase activity. The elution was monitored by absorbance at 280 nm (*figure 4.3*) and fractions containing PNGase activity were detected spanning several peaks. The active fractions were pooled as indicated by the *bar.*

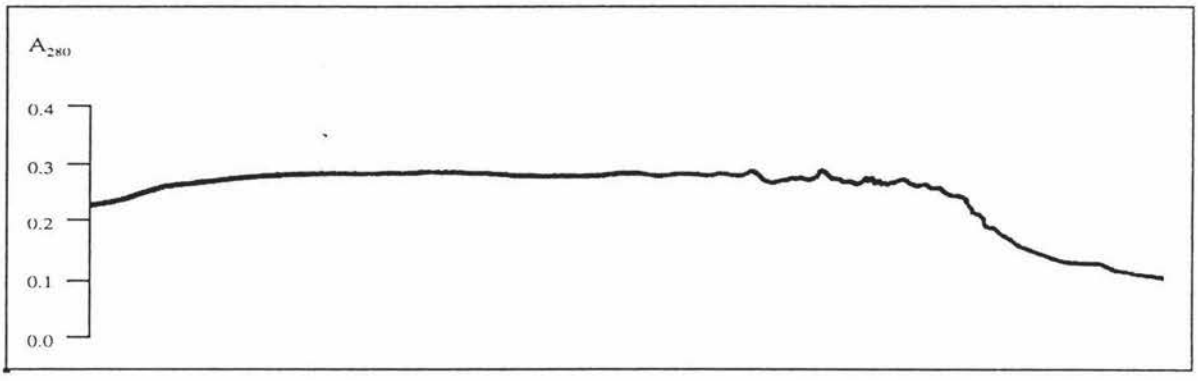


Figure 4.2 Phenyl sepharose sample load and rinse with 10 mM NH_4Ac/2 M NaCl, pH 5.5. There is no PNGase activity contained in this eluent.

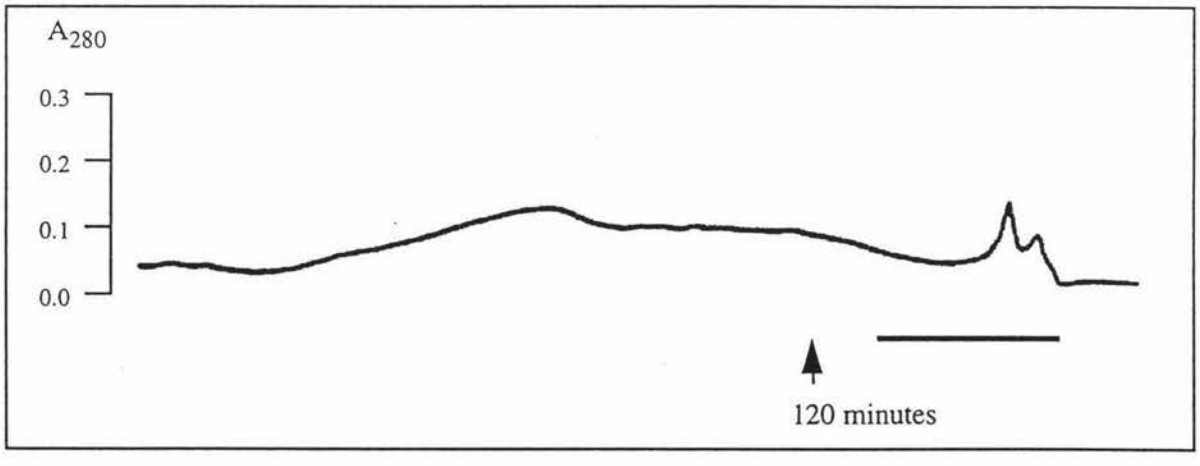


Figure 4.3 Phenyl sepharose elution of bound proteins with a gradient from 10 mM NH_4Ac/2 M NaCl, pH 5.5 to 10 mM NH_4Ac, pH 5.5. PNGase activity was detected at the end of the gradient and was pooled as indicated by the *bar.*

PNGase A was only eluted when the end of the gradient was attained. Fractions 23-29 were pooled and concentrated. The samples were analysed by SDS-PAGE (*figure 4.6*). Note: The samples from after DEAE and after phenyl sepharose are labelled as pre-sepharose and pre-superose respectively.

As can be seen in the chromatogram of the sample load and rinse from phenyl sepharose (*figure 4.2*), more proteins were present in the breakthrough eluent during loading than in the following elution of bound proteins (containing PNGase A). These results show phenyl sepharose chromatography to be a useful step.

(c) FPLC Phenyl Superose Chromatography (HR 5/5)

A sample of 1.5 ml was found to produce good resolution. A large peak that did not contain PNGase activity eluted immediately after each injection. PNGase A eluted after approximately 98 minutes from injection and was seen as a peak of extremely low absorbance. Fractions 23-29 were pooled as indicated by the *bar* to give a volume of about 11 ml (*figure 4.4*). To test whether these were the optimum conditions, the sample was equilibrated in 10 mM NH_4Ac / 3 M NaCl, pH 5.5 and the sample made 3 M with NaCl. Under these conditions, PNGase was not completely separated from other proteins (*figure 4.5*) and was eluted in fractions 17-20 (6.5 ml). The best conditions for hydrophobic interaction chromatography therefore involve using phenyl superose with 10 mM NH_4Ac/2 M NaCl, pH 5.5.

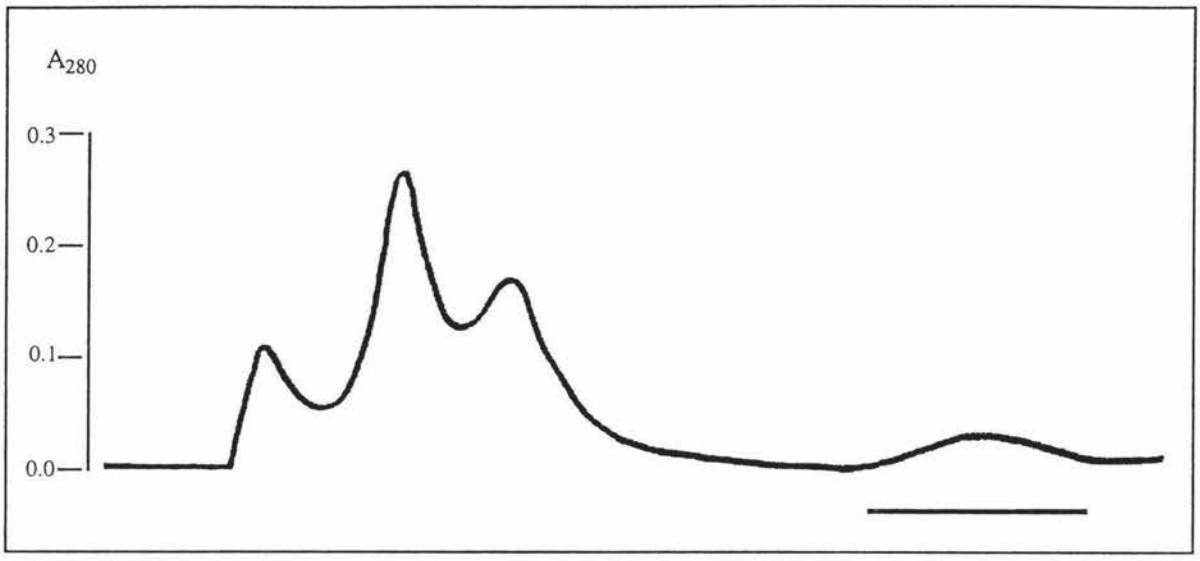


Figure 4.4 Run 1. Phenyl superose chromatogram showing the elution of PNGase A with a gradient from 10 mM NH_4Ac/2 M NaCl, pH 5.5 to 10 mM NH_4Ac, pH 5.5. Active fractions were pooled as indicated by the *bar*.

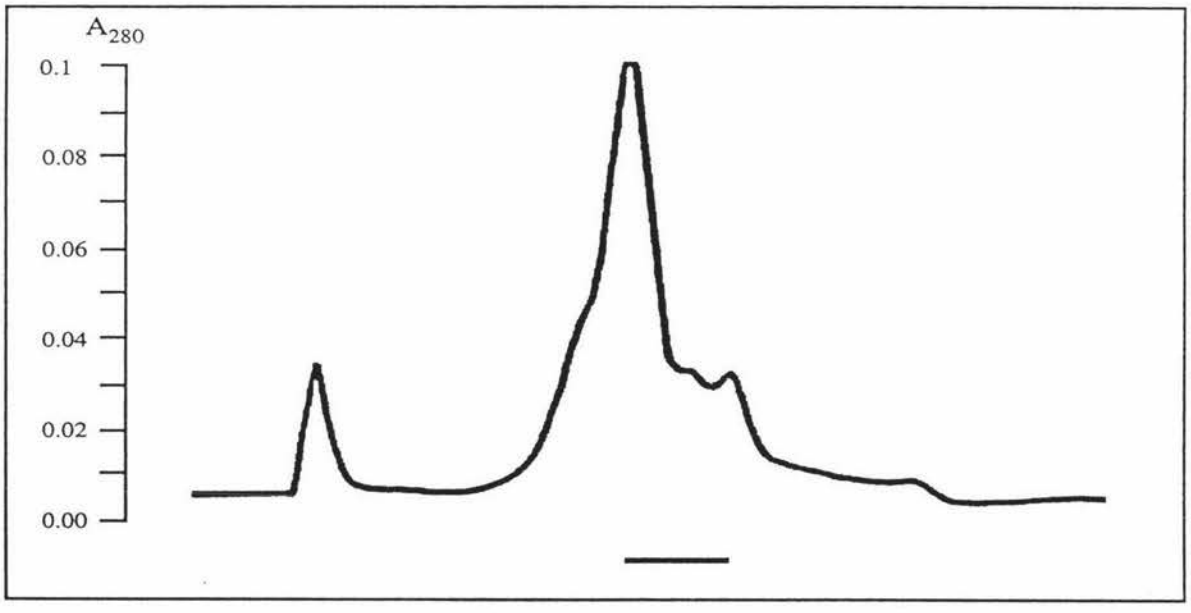


Figure 4.5 Run 2. Phenyl superose chromatogram showing the elution of PNGase A with a gradient from 10 mM NH_4Ac/3 M NaCl, pH 5.5 to 10 mM NH_4Ac, pH 5.5. Active fractions were pooled as indicated by the *bar*.

The SDS gel on the results of the phenyl sepharose and phenyl superose step, show a band at 66 kDa for all the samples (*figure 4.6*). However a band at 62 kDa is most intense in the pre-superose samples (*lanes 3* and *4*), a result from the phenyl sepharose step. The samples from post phenyl superose (at 2 M NaCl) which were loaded in *lanes 5, 7, 8* and *9* are only clearly seen in *lane 8*. A single band at 66 kDa was seen, which agreed with the molecular weight proposed by *Taga et al.*,(1984), which was 66 800 using SDS-PAGE.

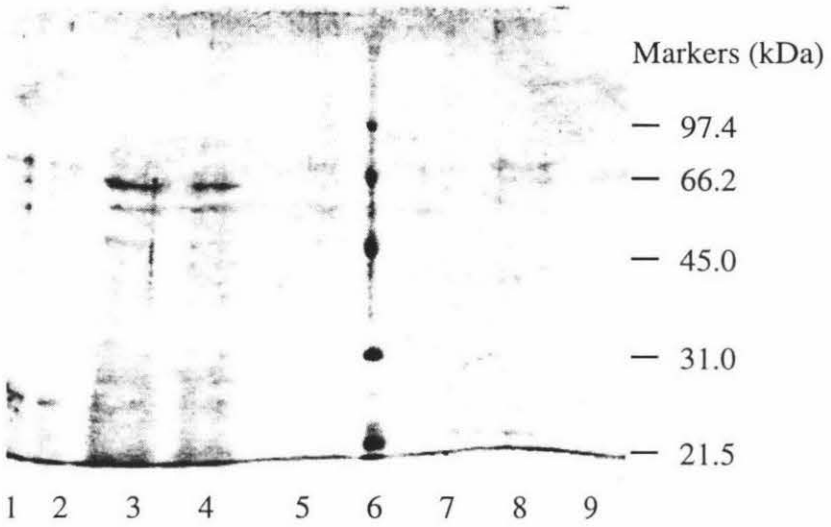


Figure 4.6 SDS gel of samples from phenyl sepharose and phenyl superose chromatography. *Lanes 1 and 2:* Pre-sepharose samples (after DEAE). *Lanes 3 and 4:* Pre-superose samples (After phenyl sepharose). *Lane 6:* Marker. *Lanes 5, 7, 8 and 9:* Post-superose samples (2 M NaCl).

These results indicate a purification has been achieved with hydrophobic interaction chromatography, especially with FPLC as a final step (phenyl superose).

To concentrate the protein, pooled samples from the two phenyl superose runs were made 70% with ammonium sulphate and equilibrated at 4°C overnight before being centrifuged at 12 000 x g for 30 minutes.

The pellet (from run 1) was redissolved in a minimum of buffer to test for activity (1a). However it was discovered that a sticky residue remained in the bottom of the tube. When more buffer was added to this it dissolved and was labelled (1b). PNGase activity resided in the pellets 1a and 1b but the supernatants did not contain activity. There was no activity in either the pellet or the supernatant from the 3 M NaCl run (samples 2a and 2b). These samples were analysed by SDS-PAGE together with the sample from preparation 1 (*figure 4.7*).

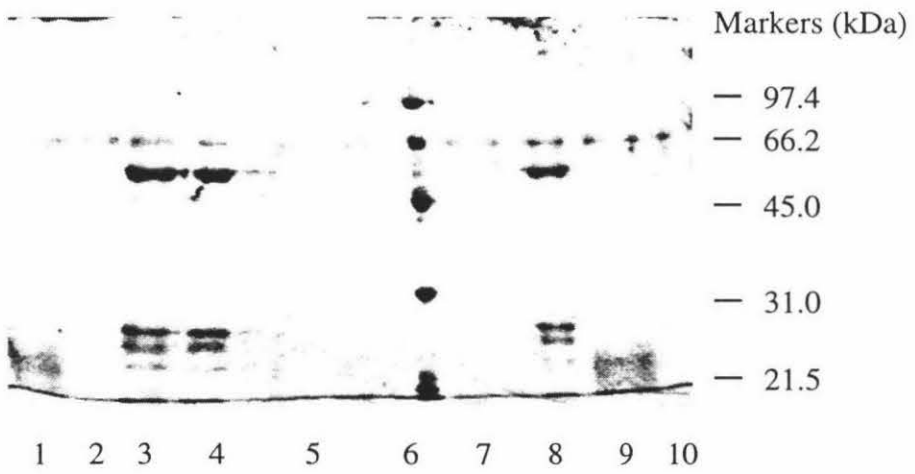


Figure 4.7 SDS gel of final protein products. *Lanes 1 and 9:* Pellet 1b, active. *Lanes 2 and 7:* Pellet 2b, inactive. *Lanes 3, 4 and 8:* Sample from preparation 1. *Lane 5:* Pellet 1a, active. *Lane 6:* Marker. *Lane 10:* -.

A band at 66 kDa is common throughout which may indicate it is an artefact. A faint band at 54 kDa can be seen in *lane 9* from sample 1b, which was also present in the sample from preparation 1 where this was most distinct. Samples 1b (*lanes 1 and 9*) had diffuse bands at about 23 kDa, not seen in the preparation 1 sample. The 27 and 25 kDa bands are still only seen in the samples from preparation 1. There was no 23 kDa band seen in pellet 1a (before the re-extraction of the supernatant) or in pellet 2b (inactive sample). The active pellet 1a (*lane 5*) and inactive pellet 2b (*lane 7*) look identical, both having a single band at 66 kDa. The band corresponding to a molecular weight of about 66 kDa could be an artefact of the gel. The bands at molecular weights of approximately 23 kDa and 54 kDa seen in the present preparation could be breakdown products since a single band was previously seen after phenyl superose (*lane 8, figure 4.6).*

A scale-up preparation was expected to yield better results. In the first instance, hydrophobic interaction chromatography (HIC) worked well to remove the contaminating proteins since there was a great reduction of bands seen on gels after these steps, compared to at the beginning (*figure 4.1*). The next preparation was based on the success of HIC used in this preparation.

CHAPTER V

RESULTS OF PREPARATION 3

5.1 Purification Using Almond Meal

(a) DEAE Ion Exchange Chromatography

The results in preparation 2 showed that although PNGase did not bind to the resin, other contaminating proteins did. It was therefore thought to be a valuable step. After loading, the resin was washed with equilibration buffer until no more protein eluted (the total volume was 5.5 L).

(b) Phenyl Sepharose Chromatography

The protein solution containing PNGase A was made 40% (~1.8 M) using ammonium sulphate ($(NH_4)_2SO_4$), in 10 mM NH_4Ac, pH 6.85 and applied to a pre-equilibrated column of phenyl sepharose as described in *Methods, section 2.2.2(f).* The breakthrough elution during sample loading contained protein which was not active (chromatogram not shown) similar to the results observed in preparation 2. The removal of protein from the matrix was accomplished by gradient elution and was monitored by measuring the absorbance at 280 nm. Fractions were tested for PNGase activity and active fractions were all contained within a large single broad peak (*figure 5.1*).

PNGase activity was first detected at 44% 10 mM NH_4Ac, pH 6.85. Elution therefore starts when the buffer is ~18% $(NH_4)_2SO_4$ (0.8 M $(NH_4)_2SO_4$). A second loading and elution from phenyl sepharose yielded similar results (chromatogram not shown).

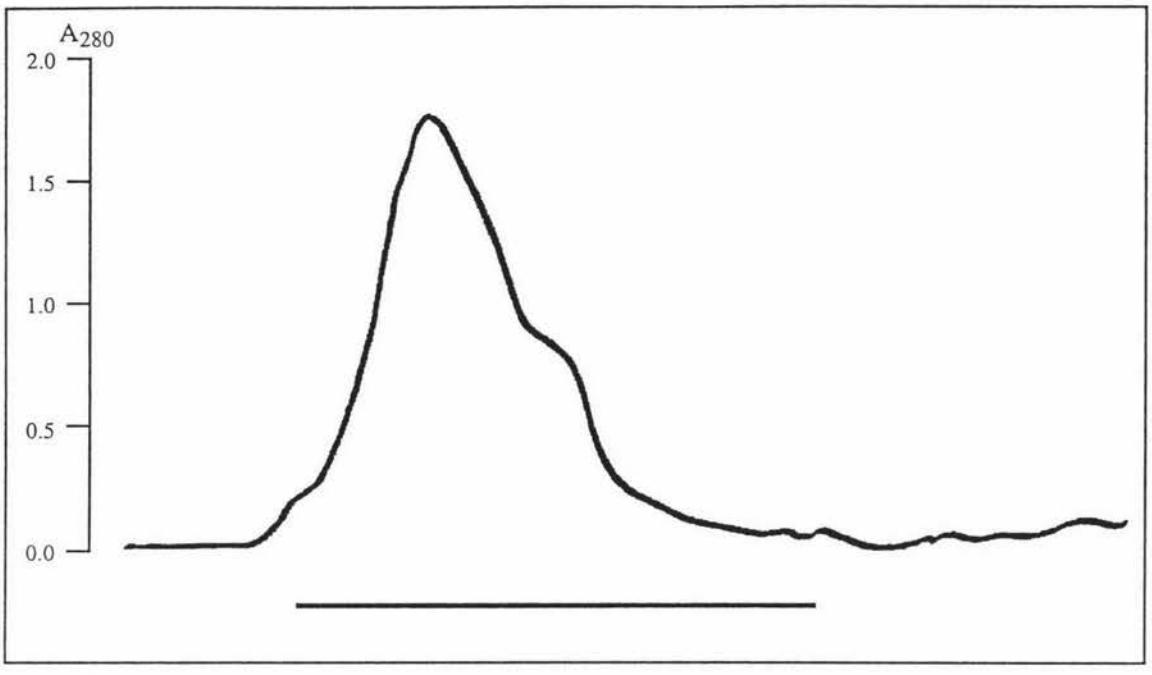


Figure 5.1 Chromatogram of phenyl sepharose elution. Proteins eluted during a 180 minute linear gradient from 10 mM NH_4Ac/ 40% $(NH_4)_2SO_4$, pH 6.85 to 10 mM NH_4Ac, pH 6.85 at a flow rate of 0.5 ml/min. Active fractions were 18-61 contained within the single peak as indicated by the *bar.*

(c) Ammonium Sulphate Fractionation

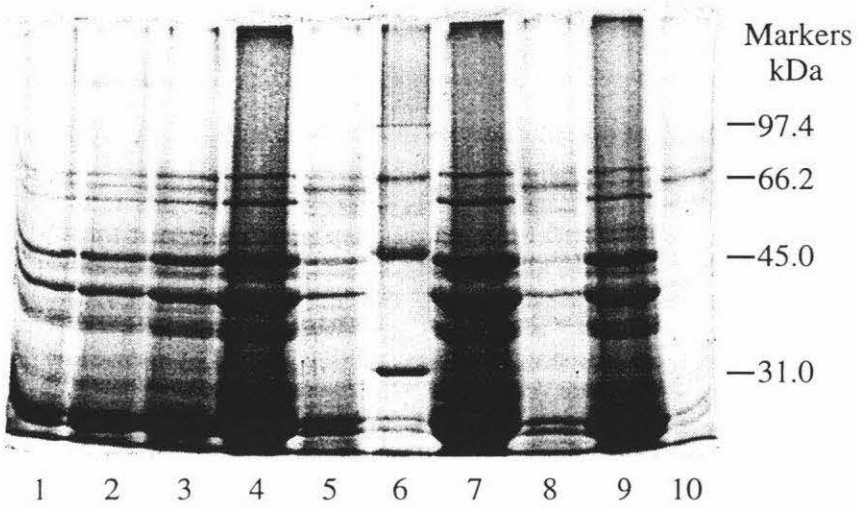
The protein sample was concentrated to a volume of 53 ml by ultrafiltration. Optical density measurements by absorbance at 280 nm gave an approximate concentration result of 106 mg/ml. A method of fractionation of PNGase A from the other contaminating proteins was needed. Ammonium sulphate fractionation was used in a series of graduated steps (*Methods, section 2.2.2(g)*). The protein solution was diluted with 10 mM NH_4Ac, pH 7.0 to a volume of 125 ml. The results are displayed in *table 5.1.*

Table 5.1 Ammonium sulphate fractionation trials.

% $(NH_4)_2 SO_4$	Total amount added to supernatant (g)	Result of activity test on pellet	Result of activity test on supernatant	Appearance of pellet	Appearance of supernatant
20	14.8	slightly active	very active	yellow	yellow turbid
25	18.0	slightly active	very active	yellow	yellow turbid
30	22.0	slightly active	very active	yellow	yellow turbid
35	26.0	slightly active	very active	yellow viscous	yellow clear
40	30.4	slightly active	very active	yellow viscous	yellow clear
45	38.1	active	very active	yellow viscous	yellow clear
50	50.9	active	very active	white viscous	dark yellow clear
55	57.1	active	very active	white	colourless
60	66.3	very active	inactive	white	colourless

The samples were analysed by SDS-PAGE (*figure 5.2*).

A.



B.

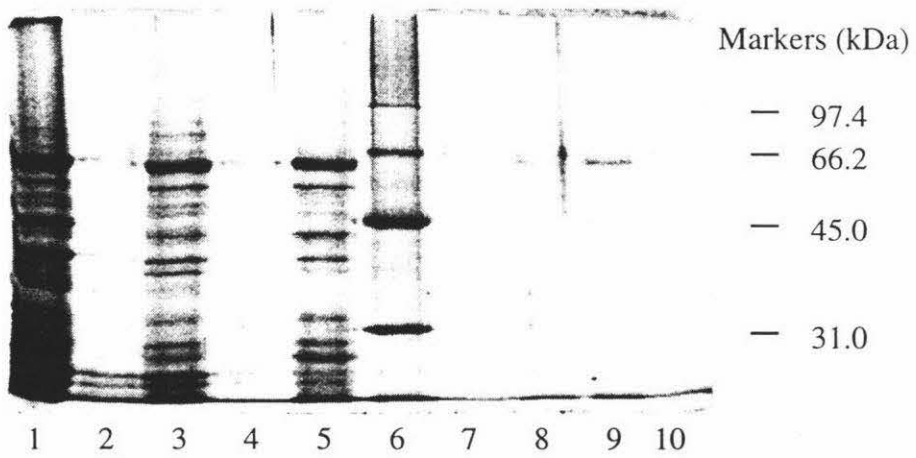


Figure 5.2 SDS gel of ammonium sulphate fractionations. The supernatants and pellets of each fraction are loaded alternatively.

A: *Lane 1:* 25% pellet. *Lane 2:* 30% pellet. *Lane 3:* 30% supernatant. *Lane 4:* 35% pellet. *Lane 5:* 35% supernatant. *Lane 6:* Marker. *Lane 7:* 40% pellet. *Lane 8:* 40% supernatant. *Lane 9:* 45% pellet. *Lane 10:* 45% supernatant. ***B:*** *Lane 1:* 50% pellet. *Lane 2:* 50% supernatant. *Lane 3:* 55% pellet. *Lane 4:* 55% supernatant. *Lane 5:* 60% pellet. *Lane 6:* Marker. *Lane 7:* 60% supernatant. *Lane 8:* 65% pellet. *Lane 9:* 70% pellet. *Lane 10:* 80% pellet.

Most of the pellets had some PNGase activity, however the most activity resided in the pellets between 50 and 60% $(NH_4)_2SO_4$. The supernatant at 60% ammonium sulphate contained no PNGase activity and the pellet at 60% is most active, which meant that this was the percentage of $(NH_4)_2SO_4$ needed to precipitate the majority of PNGase A.

Increasing salt concentration will have the effect of promoting the interaction of PNGase A molecules with other protein molecules, accounting for the salting out of the protein at lower concentrations of $(NH_4)_2SO_4$. However, as most of the activity is precipitated between 50 and 60% $(NH_4)_2SO_4$, many impurities can be removed by making the solution 40% in $(NH_4)_2SO_4$ and the supernatant will contain the majority of PNGase A.

(d) Cation Exchange Chromatography

CM -Sephadex cation exchange resin (C-50) is negatively charged and binds proteins in their positive ionic state (below their isoelectric point). Batch trials which determined which pH facilitated best binding of PNGase A to the resin were outlined in *Methods, section 2.2.2(h).* The results are displayed in *tables 5.2* and *5.3.*

Table 5.2 CM -Sephadex trials using a batch method to determine the relative binding abilities of PNGase A to resins equilibrated at different pH values.

pH of solution	Dilution factor	Absorbance at 280 nm	Concentration of supernatant (non-binding proteins) (mg/ml)	PNGase assay result (supernatant)
no resin	1/20	0.875	17.5	active
5	1/10	0.141	1.4	least active
6	1/10	0.235	2.4	least active
7	1/20	0.605	12.1	most active
8	1/10	0.301	3.0	active

Table 5.3 Elution of bound proteins from the CM- Sephadex resin, with 1 M NaCl.

pH of solution	Concentration of supernatant (bound proteins) (mg/ml)	PNGase assay result (supernatant)
5	0.490	active
6	0.157	active
7	0.031	inactive
8	0.098	inactive

These results (*tables 5.2* and *5.3*) show that PNGase binds best to the resin at pH 5, but along with almost all the proteins.

At pH 7 and above, protein binds, but what binds is inactive (*table 5.3*). Although the following chromatographic step using CM-Sephadex, was performed at a pH of 5 where bound PNGase was later removed by gradient elution, perhaps a better strategy to try in future would have been to batch, at pH 8, all proteins that did not bind which included PNGase A. The results in *table 5.2* showed that few protein is present in the supernatant at pH 8 and that it was active. *Table 5.3* showed no PNGase bound to the resin at pH 8.

Table 5.3 indicated that PNGase A could possess an isoelectric point between the pH values of 6 and 7. It bound to the resin at a pH of 5 and 6, which meant it was positively charged and since it did not bind at a pH of 7 and above (due to its net negative form), the isoelectric point is perhaps not between pH 7.7 and 8.7 as stated by Takahashi and Nishibe (1981).

The protein samples were analysed by SDS-PAGE and the results are shown in *figures 5.3* and *5.4*.

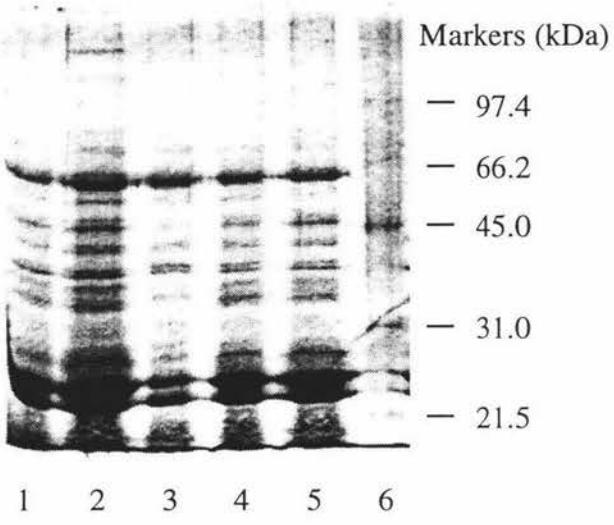


Figure 5.3 SDS gel of the supernatants at different pH values, which contained proteins not bound to CM-Sephadex. *Lane 1:* Original sample before resin addition. *Lane 2:* pH 7. *Lane 3:* pH 5. *Lane 4:* pH 6. *Lane 5:* pH 8. *Lane 6:* Marker.

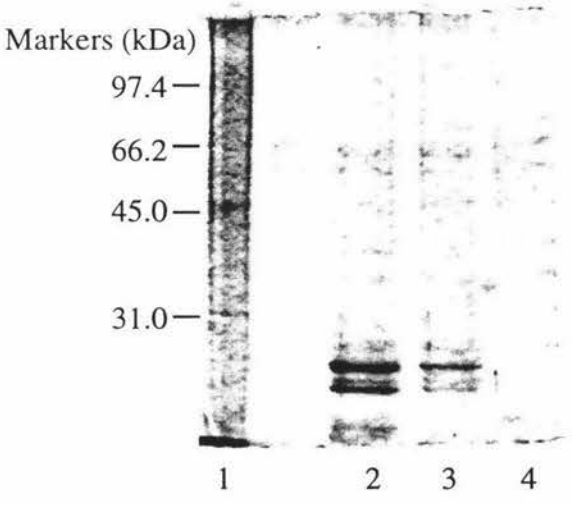


Figure 5.4 SDS gel of the supernatants at different pH values, which contained proteins that bound to the CM-Sephadex resin. *Lane 1:* Marker. *Lane 2:* pH 5. *Lane 3:* pH 6. *Lane 4:* pH 7.

SDS-PAGE analysis showed that at pH 7, few proteins bound to the resin compared to pH 5, 6 and 8 (gel not shown for pH 8 binding result). Interestingly, the patterns of protein binding (*figure 5.3* and *table 5.2*) were similar at both low and high pH which was unexpected. Bands at molecular weights of 66 kDa, 27 and 25 kDa were prominent. When bound protein was removed by increasing the ionic strength, the only bands common to pH 5 and 6 (the active fractions) were three bands at molecular weights of approximately 25, 26 and 27 kDa.

On the basis of these findings, cation exchange chromatography was carried out on the protein solution, according to the procedure outlined in *Methods, section 2.2.2(h).* *Figures 5.5* and *5.6* show the chromatograms from the loading/wash and the gradient elution respectively.

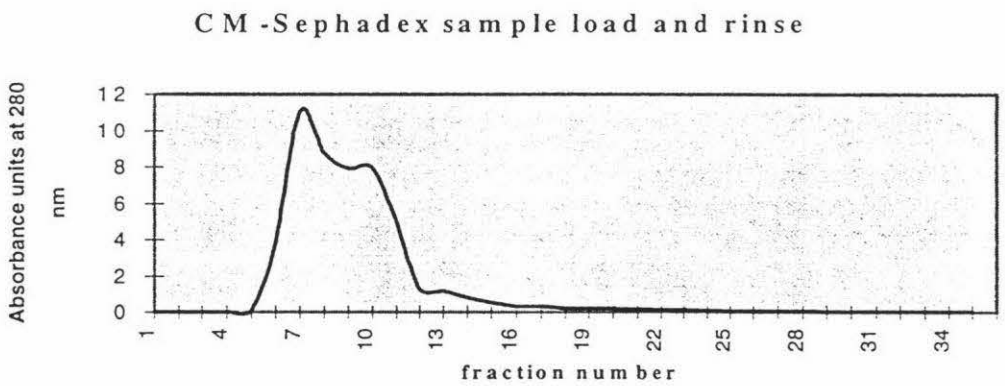


Figure 5.5 Chromatogram of CM-Sephadex loading of protein solution and wash with 10 mM NH_4Ac, pH 5. These fractions tested negative for PNGase activity.

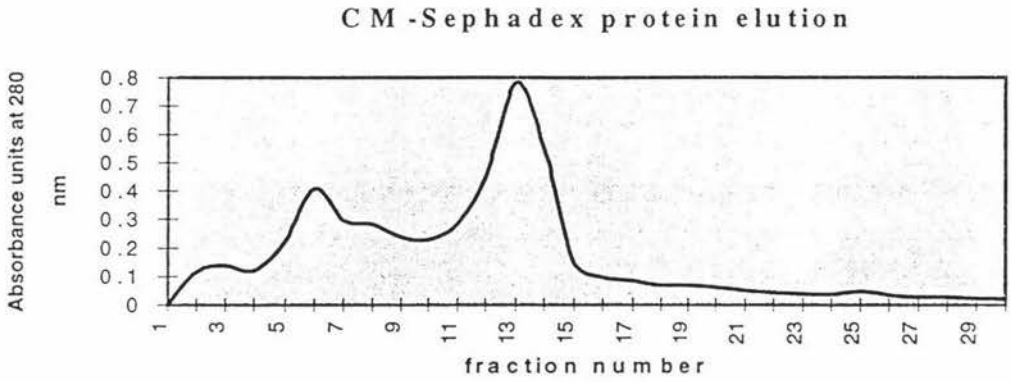


Figure 5.6 Chromatogram of CM-Sephadex protein elution by gradient application from equilibration buffer to 10 mM NH_4Ac /0.15 M NaCl, pH 5. Fractions 6-20 contained PNGase activity.

The equilibration buffer was run through the column until UV absorption in the eluent returned to the baseline (*figure 5.5*) and bound proteins were eluted with an increasing linear salt gradient from 0-1 M NaCl in 10 mM NH_4Ac, pH 5 (*figure 5.6*). The gradient was held at 0.15 M NaCl, pH 5 until all the fractions containing PNGase activity were eluted. More proteins were eluted when the solution was made 2 M with NaCl (*figure 5.7*). There was no PNGase activity detected in these fractions, likewise from the loading and rinse of the column.

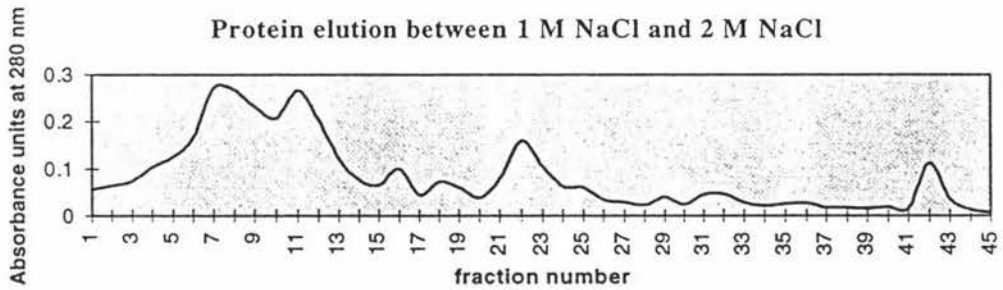


Figure 5.7 CM-Sephadex protein elution at high salt. A linear gradient was applied between 1-2 M NaCl in 10 mM NH_4Ac, pH 5 after the active fractions were eluted.

The results of the CM-Sephadex step were analysed by SDS-PAGE (*figure 5.8*).

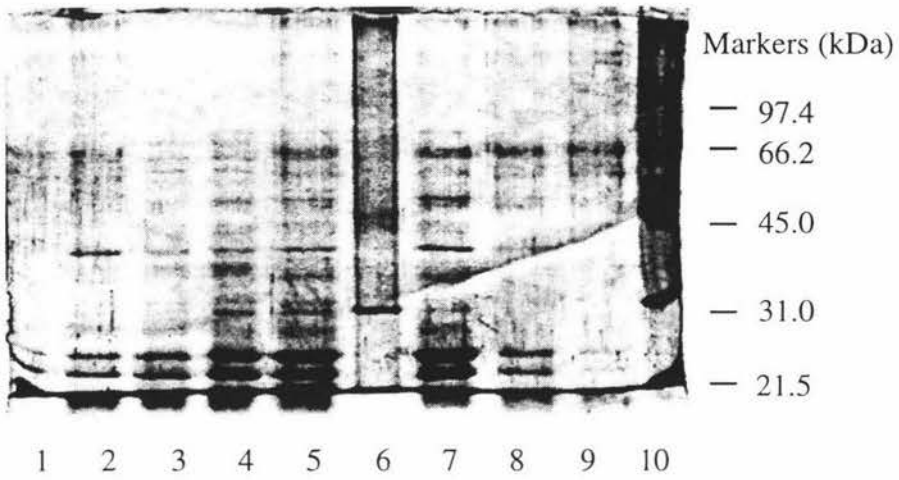


Figure 5.8 SDS gel of fractions across the active peak eluted from CM-Sephadex. *Lane 1:* Fraction 5. *Lane 2:* Fraction 7. *Lane 3:* Fraction 9. *Lane 4:* Fraction 11. *Lane 5:* Fraction 13. *Lane 6:* Marker. *Lane 7:* Fraction 14. *Lane 8:* Fraction 15. *Lane 9:* Fraction 17. *Lane 10:* Marker.

PNGase assay results indicated that fraction #13 (*lane 5, figure 5.8*) was most active and this sample also had the highest optical density (seen on the chromatogram, *figure 5.6*). The darkest bands were those at 27 kDa, 25 kDa and 66 kDa. Other bands present were at 54 kDa, 49 kDa, 42 kDa and 31 kDa. However fraction #17 (*lane 9*) was still very active and the obvious bands were those at 66 kDa and 54 kDa. The bands at 27 kDa and 25 kDa were scarcely visible although the 66 kDa and 54 kDa bands were at the same intensity as for the most active fraction (#13).

The breakthrough from the loading of the column and the 2 M NaCl wash were analysed by SDS-PAGE (gel not shown). The major contaminating proteins from the sample loading had a high optical density (11.04 mg/ml by absorbance at 280 nm, as seen in *figure 5.5*) due to a slight precipitation, which was inactive when assayed. Main bands present on the gel were at 58 kDa, 54 kDa, 42 kDa, 38 kDa, 34 kDa, 25 kDa and 18 kDa.

These fractions showed very slight activity but were classed as inactive and were not worth pooling for further work.

The column wash after sample loading displayed the same band patterns except the band at 54 kDa was of a greater concentration. The fractions from the elution to 2 M NaCl after the active fractions had been eluted, did not contain any activity but showed a 66 kDa band and many faint bands including those at 27 kDa, 25 and 19 kDa.

A conclusion that could be drawn from these results is that the active and inactive samples have bands in common. These bands are at 66, 54, 27 and 25 kDa. Bands representing proteins responsible for activity may be those at 49, 42 and 31 kDa.

(e) Gel Filtration Using FPLC

The elution was monitored by absorbance at 280 nm and fractions were assayed for activity. Active fractions were pooled and concentrated by ultrafiltration. The chromatogram is shown in *figure 5.9.*

Despite the appearance of the chromatogram which indicated that several impurities should have been removed, SDS-PAGE of these fractions (*figure 5.10*) showed that this technique had not been successful (*lanes 1* and *2; 4* and *6*). An inactive fraction (*lane 3*), showed an identical pattern of bands to the active fractions in the surrounding lanes. This would suggest that PNGase activity is associated with a band so weak that it cannot be visualised with Coomassie R_{250}. Some of these samples (*lanes 1, 3* and *6*) have been concentrated and therefore appear darker and may contain some bands not seen in preceding samples.

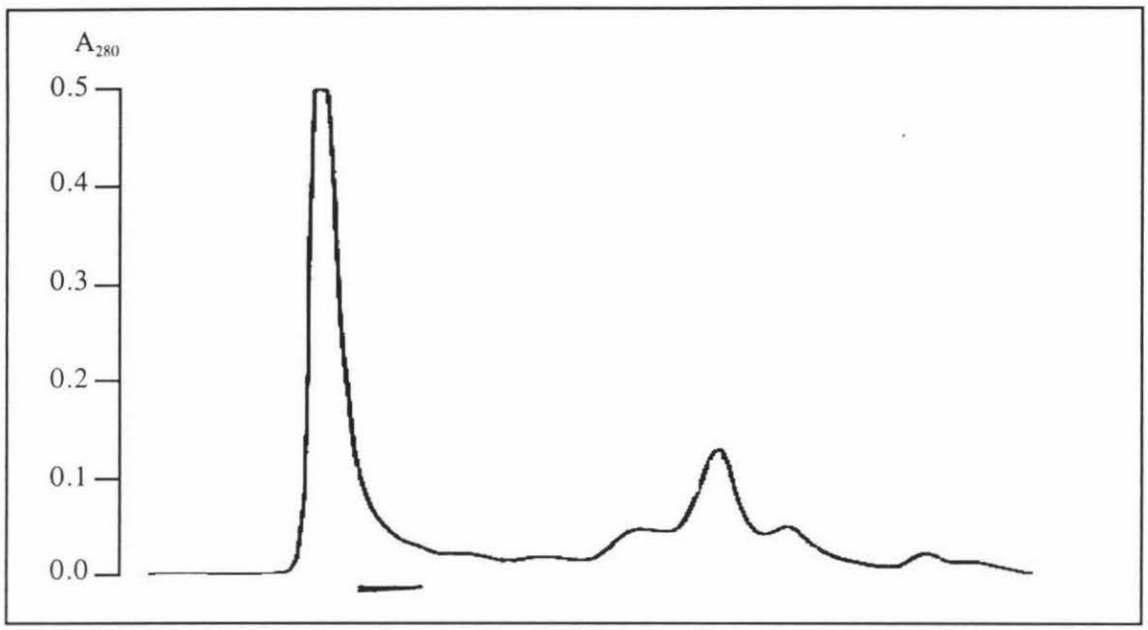


Figure 5.9 Chromatogram from gel filtration, using FPLC. Superdex-75 (Hi load 16/60) was equilibrated in 10 mM NH_4Ac/0.15 M NaCl, pH 5.5. The active fractions were pooled as indicated by the *bar*.

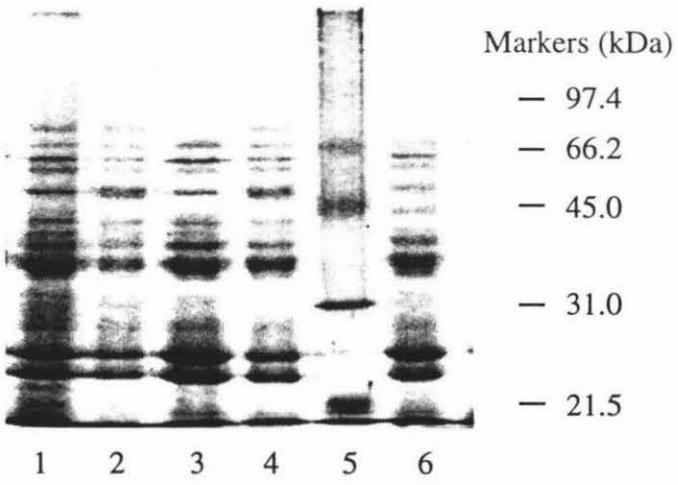


Figure 5.10 SDS gel of the pooled active fractions from gel filtration.

Lane 1: Protein sample before gel filtration (active). *Lane 2:* Pooled fractions after gel filtration (active). *Lane 3:* First peak from gel filtration (inactive). *Lane 4:* Another active sample, before gel filtration. *Lane 5:* Marker. *Lane 6:* After gel filtration.

(f) NaSCN and β-ME Incubations

This step was done to inactivate proteases, because they can not tolerate these conditions. Plummer *et al.*,(1987) stated that more than 99% of the contaminating exoglycosidases are irreversibly denatured by this treatment, but PNGase A remains fully active. As expected, the supernatant contained a high degree of PNGase activity and the white precipitate which was removed by centrifugation, tested negative for PNGase activity.

(g) Phenyl superose FPLC

The first fraction containing PNGase activity eluted at 49% 10 mM NH_4Ac, pH 5. The chromatogram is shown in *figure 5.11*. The fractions were monitored by absorbance at 280 nm and were assayed for activity. The active fractions were pooled, concentrated (ultrafiltration) and analysed by SDS-PAGE (*figure 5.12*). This revealed six bands: 62, 54, 49, 42, 27 and 25 kDa. The most intense band was at 54 kDa.

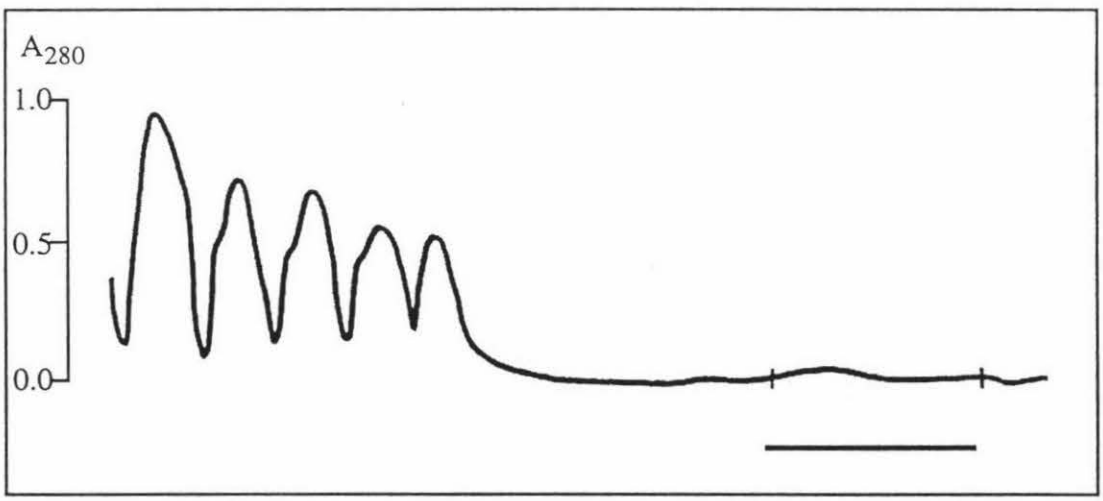


Figure 5.11 Chromatogram of phenyl superose chromatography. Active fractions were pooled as indicated by the *bar*.

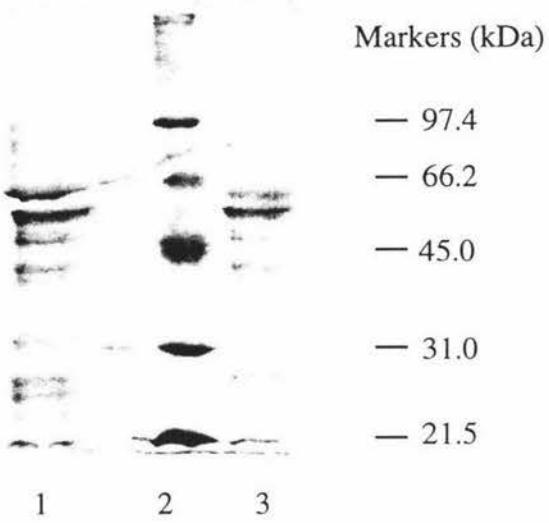


Figure 5.12 SDS gel of pooled and concentrated fractions containing PNGase activity, after phenyl superose chromatography.

Lane 1: 10 µl loading. *Lane 2:* Marker. *Lane 3:* 10 µl loading.

This looked to be successful, since many proteins previously present after gel filtration (*figure 5.10*) were now eliminated after this step, especially those represented by the prominent bands between 45 and 31 kDa and the bands at 27 and 25 kDa.

(h) N-Terminal Sequencing

A protein sample which contained PNGase of high purity (after phenyl superose chromatography) was electroblotted from an SDS gel onto a PVDF membrane as outlined in *Methods section 2.2.3(b).*

Eight bands were present: 62, 54, 49, 42, 32, 27, 25 and 15 kDa. The 54 kDa band was cut out and sequenced from the N-terminal on the automatic sequencer. The results are displayed in *table 5.4.*

Table 5.4 Partial N-terminal sequence of the 54 kDa band. Numbers across the table denote the amino acid number from the N-terminus (each cycle). The first row contains the first choice (human interpretation) of amino acids present. The second row contains secondary (lesser concentrations of) amino acids present.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	**L**	**A**	**S**	**G**	**Y**	**H**	**S**	**-**	**A**	**D**	**L**	**I**	**L**	**P**	**I**
2	**R**	**G**	**Y**				**L**		**L**						

The first choice of sequence was very clear and strong. The results here showed an aliphatic N-terminal.

(i) DIG Glycan and Protein Double Labelling

The same sample of PNGase, used in the above experiment, was electroblotted onto a PVDF membrane which was stored in 50 ml PBS pH 6.5 overnight (see *Methods, section 2.2.3(e)* for buffers and procedures used in DIG staining). The results are shown in *figure 5.13.*

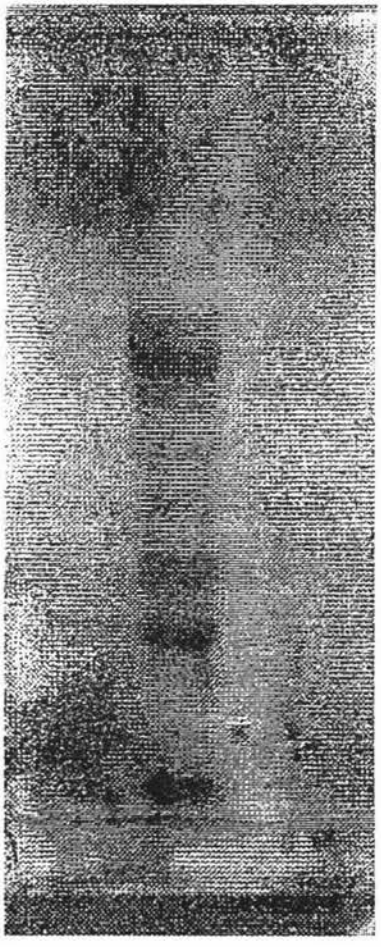


Figure 5.13 The DIG glycan and protein labelled PVDF blot.

Reddish brown bands represented non-glycosylated proteins and blue bands depicted glycosylated proteins. The results here indicated that the 54 kDa protein was glycosylated and was the only one as such. It had been stated by Plummer *et al.*,(1987) and Taga *et al.*, (1984) that PNGase A is glycosylated and has a weight of 66.8 kDa (Taga *et al*) or 79.5 kDa (Plummer *et al*). These results do not agree with either the molecular weight proposed or a match of the proposed weight to glycosylation.

(j) Affinity Chromatography Using Concanavalin A

Taga *et al.*,(1984) reported that PNGase A was a glycosylated protein and furthermore, contained a high proportion of mannose, along with other hexoses and hexosamines. Goldstein *et al.*,(1965) determined that at least 2 mannosyl residues were necessary for binding to the column and Ogata *et al.*,(1975) found that the common backbone of Manα1-6(Manα1-3)Manβ1-4GlcNAc was the main determinant for binding. Concanavalin A (Con A) resin binds glycans, especially those rich in mannose.

The DIG glycan stain results suggested that one of the proteins (or parts thereof) present in the PNGase active sample was glycosylated. If PNGase A is glycosylated, it should bind to the resin under appropriate conditions. Affinity chromatography using Con A should separate this protein from others in the mixture.

From the batch trials using the miniature columns fashioned from Pasteur pipettes, it was found that fractions containing PNGase activity were eluted with the stepwise addition of 0.1 M α-methyl mannopyranoside. More proteins were eluted with the application of 0.5 M α-methyl mannopyranoside.

The eluent from the loading of the protein solution and rinse of the column did not contain PNGase activity (*figure 5.14*). The first fraction eluted, which was positive for PNGase activity, eluted at 0.05 M α-methyl mannopyranoside (*figure 5.15*).

A single weak peak eluted with a large tail. The fractions containing PNGase activity were pooled, concentrated and analysed by SDS-PAGE (*figure 5.16*).

The SDS gel showed many bands were still present. It is likely that some non-glycosylated proteins interacted with the glycosylated protein which bound to the column. This chromatographic method did not seem to be useful in the elimination of proteins.

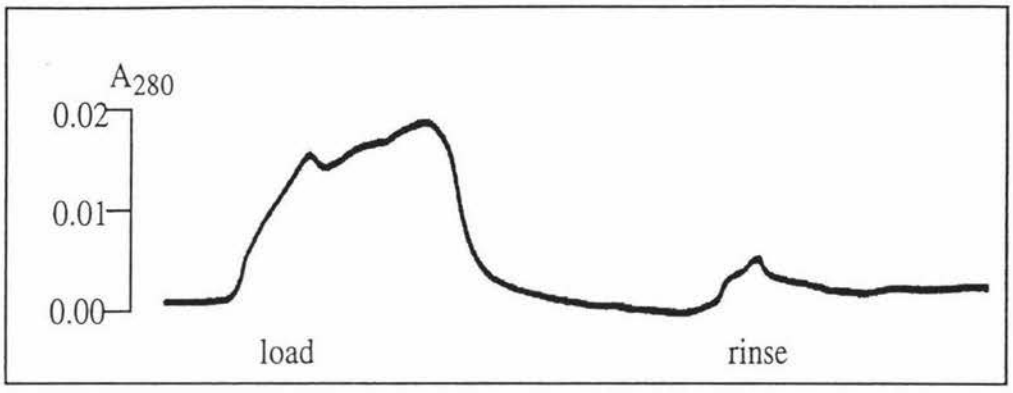


Figure 5.14 Chromatogram of Concanavalin A sample application and rinse.

The column was equilibrated with 20 mM NaAc/ Mg^{2+}, Ca^{2+}, Mn^{2+}, pH 6.5.

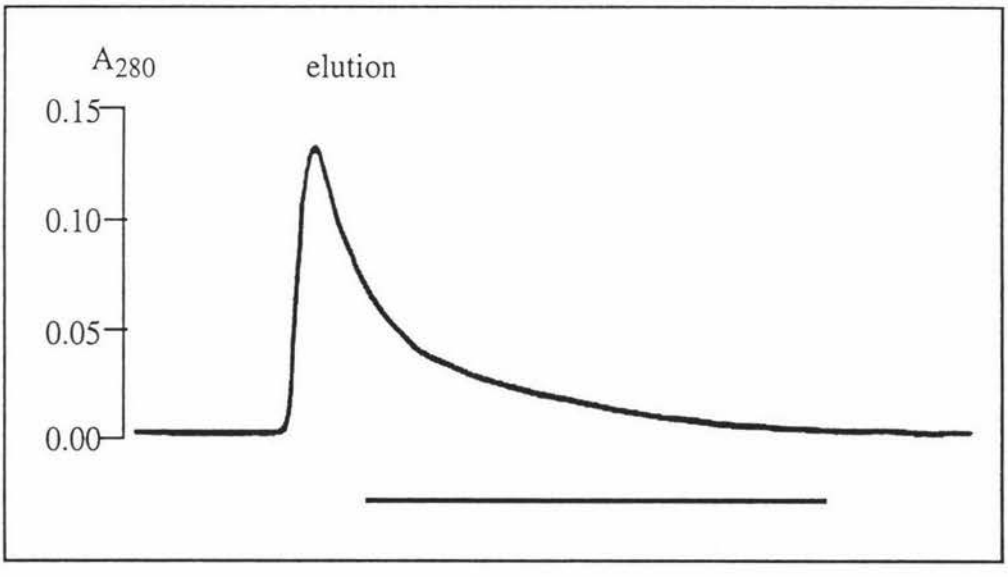


Figure 5.15 Chromatogram of Concanavalin A elution.

A gradient was applied from 20 mM NaAc/ Mg^{2+}, Ca^{2+}, Mn^{2+}, pH 6.5 to 20 mM NaAc/ Mg^{2+}, Ca^{2+}, Mn^{2+}/0.5 M α-methyl mannopyranoside, pH 6.5 for a duration of 120 minutes. PNGase activity was detected within this peak.

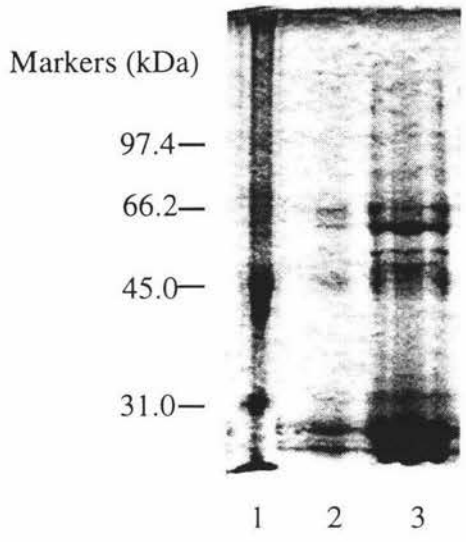


Figure 5.16 SDS gel of active fractions pooled and concentrated from Concanavalin A chromatography. *Lane 1:* Marker. *Lane 2:* Sample after Con A chromatography (10 µl load). *Lane 3:* Same sample (20 µl load).

(k) Native gel trials

The protein sample containing PNGase was loaded onto each half of a gel and after electrophoresis, one half was soaked in 0.05% SDS for half an hour then stained with Coomassie. Two bands were seen after destaining, one at the top of the resolving gel and one 12 mm from this (gel not shown). Four slivers were cut from the other half of the gel from the top of the resolving gel, covering the area of these bands (four lanes wide) and each placed in an Amicon Nebuliser. These were assayed after incubation and a 2 mm area about 5 mm from the top of the gel was active. This did not correspond to any visible band.

(l) Gel Filtration Chromatography

The PNGase samples were subjected to FPLC using Superdex 75 (HR 10/30), to see if there was a change in the chromatogram now that a lot of protein was eliminated. Five slightly different traces resulted (monitored by absorbance at 280 nm), however they were still similar to the previous chromatogram (*figure 5.9*). Active fractions were pooled and concentrated by ultrafiltration, according to the sample batch and similarity of chromatogram (*figure 5.17*). In all cases, active fractions eluted 18 minutes from the time of injection, with an elution volume of 7.2 ml.

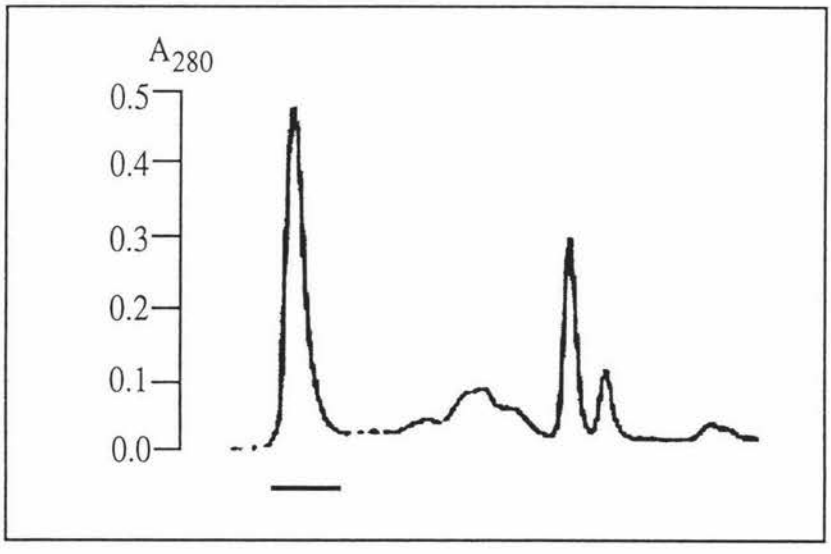


Figure 5.17 Chromatogram representative of the five pools of active PNGase fractions after gel filtration using FPLC. The active fractions were pooled as indicated by the *bar*.

One of these active pools was concentrated to a higher degree then rechromatographed on a Superdex 75 Hi Load Prep Grade 16/60 column (conditions outlined in *Methods*) using FPLC (Waters). The fractions positive for PNGase A eluted at the tail of a single peak, which was similar to the results seen in previous gel filtration runs in this preparation (*figure 5.9*) except no lower molecular weight products were seen. The fractions spanning the peak were analysed by SDS-PAGE (*figure 5.18*).

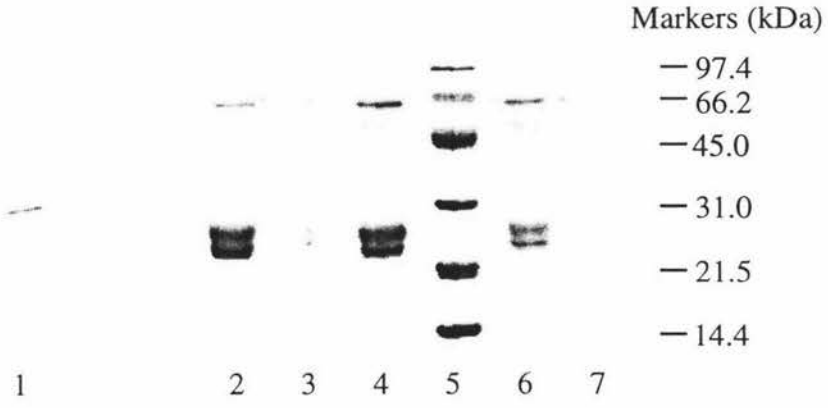


Figure 5.18 SDS gel of fractions spanning the peak eluted by gel filtration.

Note: *Lanes 1, 2, 4* and *6* contain concentrated samples.

Lane 1: Fraction 14, inactive. *Lane 2:* Fraction 17, inactive. *Lane 3:* Fraction 18, active. *Lane 4:* Fraction 19, active. *Lane 5:* Marker. *Lane 6:* Fraction 20, active. *Lane 7:* Fraction 21, inactive.

Activity was detected in fractions 18, 19 and 20 (slight), seen in *lanes 3, 4* and *6.* For these active samples, bands corresponded to the molecular weights of 58, 27 and 25 kDa. This band pattern seems the same as that of the inactive sample in *lane 2*, (although a few other faint bands at 45, 42, 15 and 12 kDa were seen in the latter sample). Fraction 14 contained a small 'shoulder' preceding the peak (*lane 1*) and bands at 31, 17 and 8 kDa were seen. These add to make a 58 kDa protein, as observed in the other lanes, however this fraction was inactive.

The active fractions were pooled, concentrated and used in the following experiments:

- urea gel analysis
- electroelution from non-denaturing gels with the aim to recover activity and match this to a particular band seen on the gel
- DIG staining

(m) Urea Gel Analysis

Urea gels were prepared as described in *Methods, section 2.2.3(g).* PNGase A is active in 2 M urea (Taga *et al.*, 1984). This was confirmed by assaying. The protein sample from gel filtration (Superdex 75, 16/60) was analysed by urea gel electrophoresis (*figure 5.19*). Several lanes were loaded but only one was stained. One clear band was seen 8 mm from the top of the gel (stack/resolving gel interface). Another faint band was seen 4 mm above this. These bands were cut out and electroeluted. The former band (at 8 mm) was very active when assayed. When the same protein was retrieved by elution from an Amicon Nebuliser, a positive result for activity was also seen. The electroeluted proteins were concentrated and analysed by SDS-PAGE (*figure 5.20*).

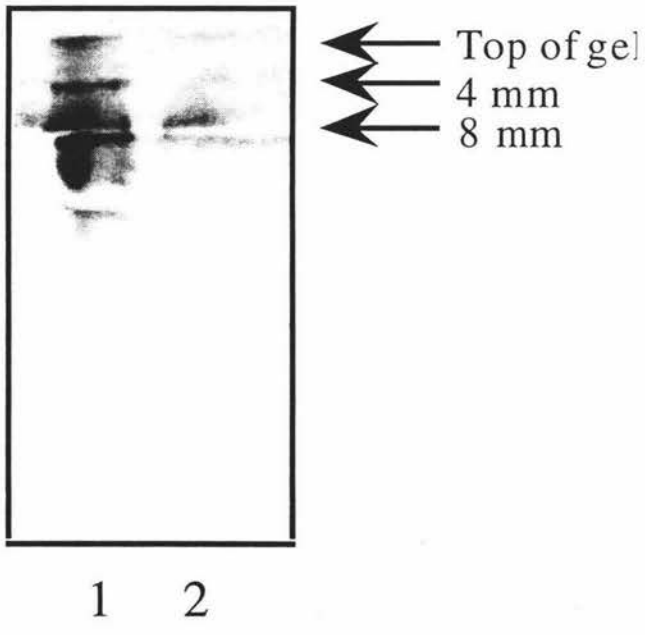


Figure 5.19 Urea gel of a protein sample containing PNGase A.

Lane 1: 20 µl sample load. *Lane 2:* 5 µl sample load.

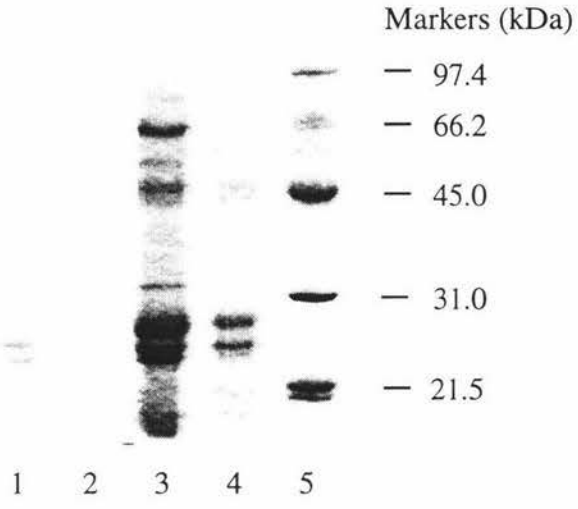


Figure 5.20 SDS gel of protein electroeluted from the urea gel.

Lane 1: First gel slice, 4 mm from top, inactive. *Lane 2:* Gel slice 6 mm from top, inactive. *Lane 3:* Gel slice 8 mm from top, active. *Lane 4:* Gel slice 10 mm from top, inactive. *Lane 5:* Marker.

The results shown in *lane 3, figure 5.20*, indicated bands at positions 62, 54, 45, 33, 27 and 25 kDa. The inactive band 4 mm from the top gave rise to bands at 27 and 25 kDa. These results indicate that a single band from a non-denaturing gel gave rise to multiple bands with SDS-PAGE and the band pattern is the same as that observed with previous SDS gels. These results also suggest that the 27 and 25 kDa bands always present in active samples, may not be responsible for activity separately or together.

To further assess whether the single bands in native gels were aggregates of different proteins, contaminants or tightly aggregating breakdown products of the same protein, the active protein electroeluted from the urea gel was subjected to gel filtration using FPLC (Superdex 75 3.2/30, SMART). The chromatogram which resulted is displayed in *figure 5.21.*

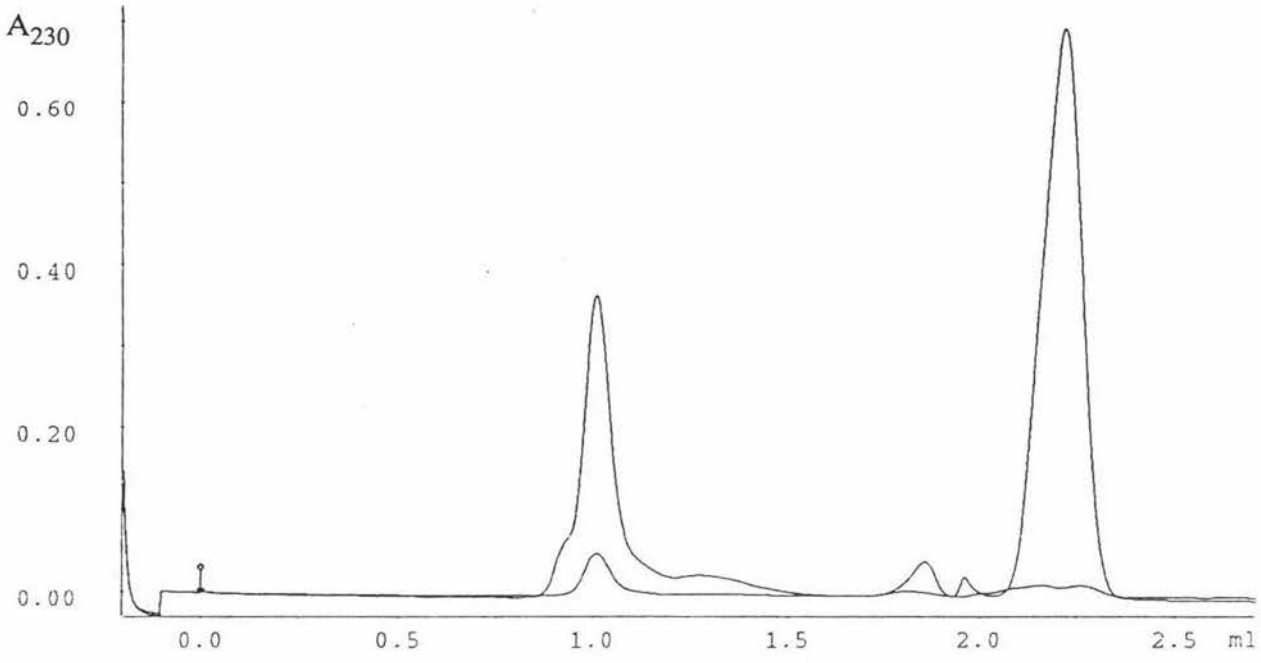


Figure 5.21 Chromatogram of PNGase active sample subjected to gel filtration, following urea gel electroelution. Activity was detected in the first peak which eluted. The elution was monitored by measuring the absorbance at 230 nm and 280 nm.

The elution was best monitored by measuring the absorbance at 230 nm (larger peaks) and PNGase activity was detected in the first peak, including the 'shoulder' which preceded it. The elution volume was 1 ml, for the active fraction. The second main peak was inactive and of high absorbance and three small peaks were present between the two (230 nm). Interestingly, concerning the first active peak, assays performed with the 'shoulder' and main peak separately did not show activity (likewise with the second run) but when the two samples were combined from both runs and concentrated further, activity was detected.

The fractions were pooled into four groups and concentrated by ultrafiltration. The active fractions were in pool 1 and the final large peak in pool 4 (and the smaller peaks were in pools 2 and 3). These pools were analysed by SDS-PAGE (*figure 5.22*).

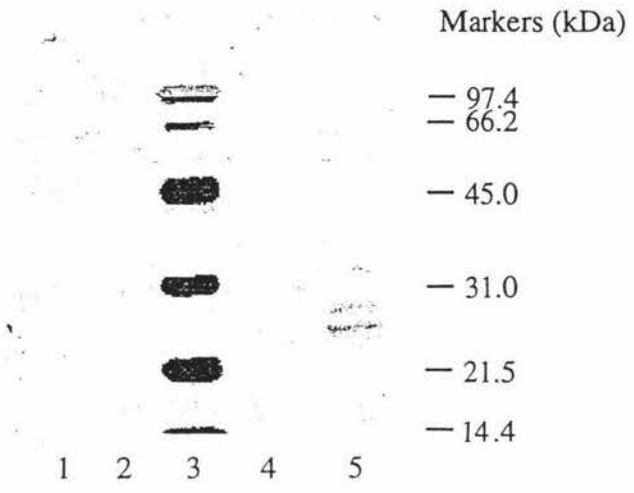


Figure 5.22 SDS-PAGE of fractions after gel filtration (SMART) which followed electroelution from a urea gel. *Lane 1:* Fourth pool, inactive. *Lane 2:* Third pool, inactive. *Lane 3:* Marker. *Lane 4:* Second pool, inactive. *Lane 5:* First pool, active.

Lane 5, which contained the active peak, revealed bands at 62, 54, 50, 38, 27 and 25 kDa. *Lane 4* had bands at 62, 38, 27 and 25 kDa which were very faint. More faint were the bands at 66, 54 and 38 in *lanes 1* and 2. Another gel run on these same samples (not shown) revealed a band at 66 kDa was common for all samples, although only pool 1 was active, which suggests that this band may be an artefact. In this latter gel, present in the active sample but absent in the other three inactive samples, were bands at 50, 40, 25 and 23 kDa. These results suggest that the 50 or 40 kDa proteins may be responsible for activity.

It is likely there was some type of interaction (most likely hydrophobic, since salt was used in gel filtration) between the proteins in the sample. From observing the SDS gels, the low molecular weight proteins (27 and 25 kDa) were not expected to appear with the high molecular weight proteins (62, 54 kDa) in the first peak from gel filtration and from a single active band in the urea gel. These 27 and 25 kDa proteins do not seem to be readily separated from the active sample and the same pattern of bands was observed before and after gel filtration (*figure 5.20* and *figure 5.22*).

(n) Second DIG Stain Analysis

The active protein after gel filtration (Superdex 75,16/60) was analysed by DIG staining (as outlined in *Methods, section 2.2.3(e)*) and is shown in *figure 5.23.*

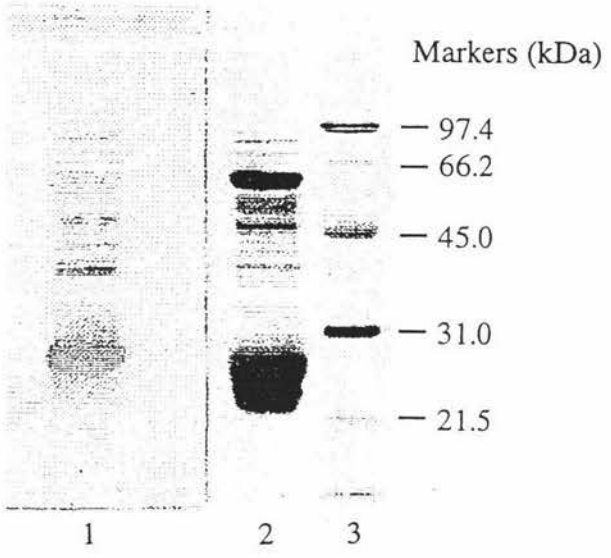


Figure 5.23 DIG stain showing the proteins and glycosylated proteins contained in a sample with PNGase activity. *Lane 1:* DIG stained (PVDF blot). *Lane 2:* Coomassie Blue R_{250} stained (PVDF blot). *Lane 3:* Markers (PVDF blot).

Many bands showed up from this sample, however it was clear that the band at 38 kDa stained blue, which indicated it was glycosylated. It is likely that many of these bands represented breakdown products, since a sample from this same preparation was previously DIG stained and fewer bands were seen, when this sample was less pure (*figure 5.13*). On the contrary, the previous DIG stain had the band at 54 kDa stained blue. The 54 kDa glycosylated protein may have broken down such that the glycosylated part now belongs to a smaller protein.

(o) Total Protein Estimates

The total protein estimations were done by measuring the absorbance at 280 nm and the results are displayed in *table 5.5.* The Biuret assay is inaccurate for the determination of total protein in this preparation due to the presence of ammonium salts in phenyl

sepharose chromatography and in all buffers. The presence of EDTA in concentrations of at least 10 mM in all the buffers, also affects the Biuret assay by chelating the Cu^{2+} (Smith *et al.*, 1985). The BCA solution was unavailable at this time.

Table 5.5 Total protein estimates (by absorbance at 280 nm).

Step	Sample	Volume (ml)	Dilution	A_{280}	Concentration (mg/ml)	Total protein (mg)
DEAE Chromato -graphy	Super-natant	5500	-	0.535	0.535	2940 *
Phenyl Sepharose	Active fractions	1. 53 2. 330	1/100 1/5	1.095 0.642	102.9 3.21	5450 1060
CM-Sephadex	Active fractions	1. 31 2. 500	1/20 -	0.120 0.328	2.4 0.328	75 164
Gel filtration	Active fractions	1. 70 2. 36.5 3. 225	- - -	0.169 0.235 0.321	0.169 0.235 0.321	12 9 72

* Only 1 aliquot was measured, further batches which were less concentrated had been unaccounted for.

Taga *et al.*, (1984) started with 70 g of almond meal and obtained a final amount of 3 mg of active protein at the end of their preparation. This was a 45% yield and the purification factor was 9000-fold. Approximately 10 mg of protein was left at the end of this preparation.

CHAPTER VI

RESULTS OF PREPARATION 4

6.1 Purification Using Almond Emulsin

Because of the very small amounts of protein obtained from the almond meal and the difficulties in obtaining a pure product, it was decided to carry out a new preparation using almond emulsin as a starting material. It was used because it is said to contain a higher amount of PNGase A than almond meal (T. Plummer Jr., personal communication).

(a) Anion Exchange Chromatography

Protein not bound to the DEAE resin was removed by successive washing with buffer until no further PNGase activity was detected. Bound proteins were eluted with 1 M NaCl which tested negative for PNGase activity.

(b) Cation Exchange Chromatography

Lowering the pH to 5 resulted in the formation of a white precipitate. The supernatant was divided in half and applied in two aliquots to a pre-equilibrated CM-Sephadex column, under the conditions outlined in *Methods, section 2.2.2(h).* Under these conditions, PNGase binds to the column, while other contaminating proteins pass straight through (chromatogram not shown). After washing the column with loading buffer to remove all contaminating protein, bound proteins were eluted by application of a linear salt gradient, the elution being monitored by measuring the absorbance at 280 nm. Fractions were assayed and those showing activity (represented by the *bar* in *figure 6.1*) were pooled.

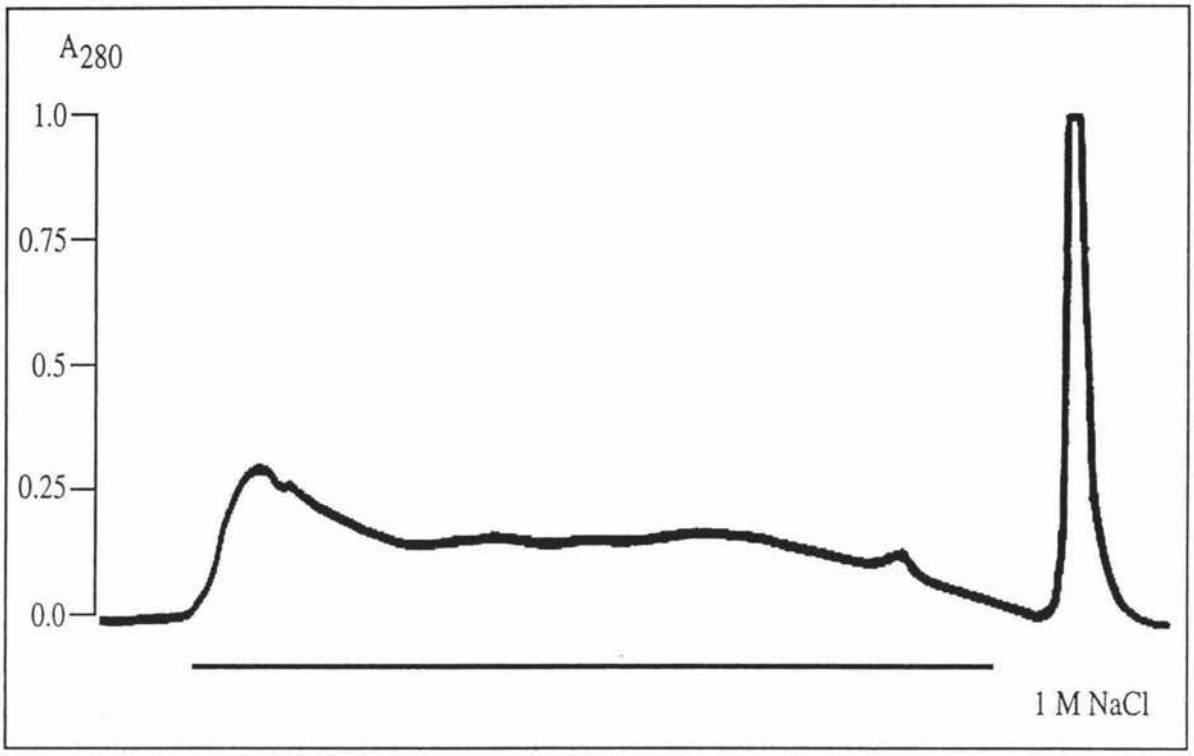


Figure 6.1 CM-Sephadex protein elution. The linear gradient was applied from 10 mM NH_4Ac, pH 5 to 10 mM NH_4Ac /1 M NaCl, pH 5 in 120 minutes. Active fractions were pooled as indicated by the horizontal *bar*.

Ion exchange chromatography is thus a good step to perform at the beginning of the purification, as a large number of contaminating proteins are removed, firstly by lowering the pH, then by the chromatography itself.

(c) Sodium Thiocyanate and β-Mercaptoethanol Incubation

Most proteins would be denatured under these conditions with the exception for PNGase A, which remains fully active. This step should therefore get rid of most protease activity. The pellet was removed by centrifugation at 12 000 x g for 40 minutes and tested negative for PNGase activity.

(d) Phenyl Sepharose Hydrophobic Interaction Chromatography

The supernatant was subjected to chromatography on a phenyl sepharose column (2 x 30 cm) as described under *Methods, section 2.2.2(f)*. The breakthrough from the column during sample loading and rinsing tested negative for PNGase activity which agreed with previous runs (chromatogram not shown). The elution of bound protein yielded two peaks and the active fractions eluted at zero salt concentration. The active fractions eluted as a very broad dilute peak (*figure 6.2*). The fractions containing PNGase activity (fraction # 30 onwards) were concentrated to 4.5 ml by ultrafiltration (Amicon), using a YM-5 membrane then 10 K centricons.

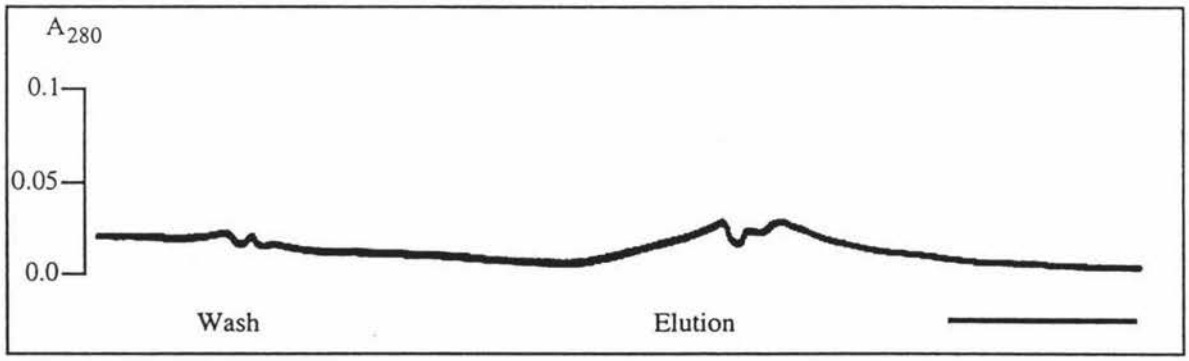


Figure 6.2 Phenyl sepharose column wash and elution. Active fractions eluted after a linear gradient from 10 mM NH_4Ac/2 M NaCl, pH 5 to 10 mM NH_4Ac, pH 5 in 120 minutes, at a very low absorbance (indicated by the *bar*).

The results of each purification stage to date were analysed by SDS-PAGE and are shown in *figures 6.3* and *6.4*.

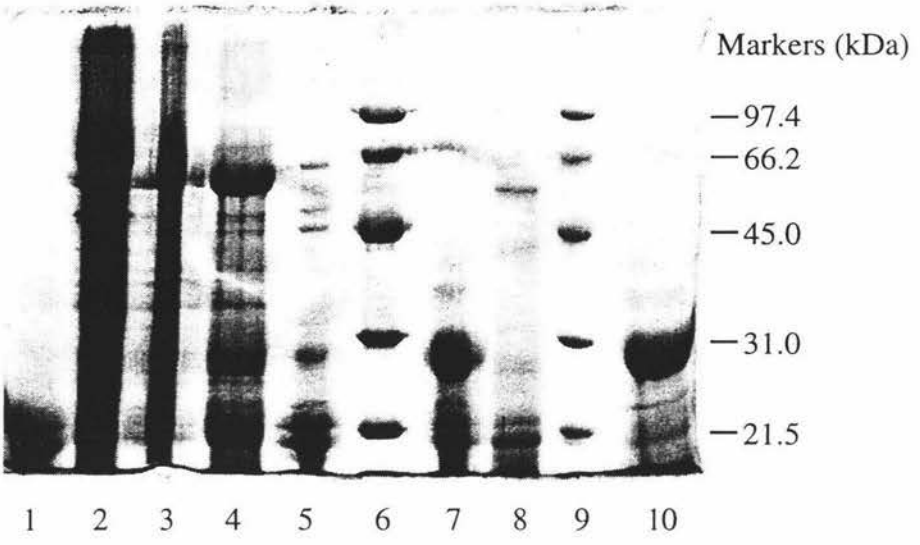


Figure 6.3 SDS gel of fractions which contain PNGase activity, from each stage of the purification to date. The lanes are labelled on the facing page.

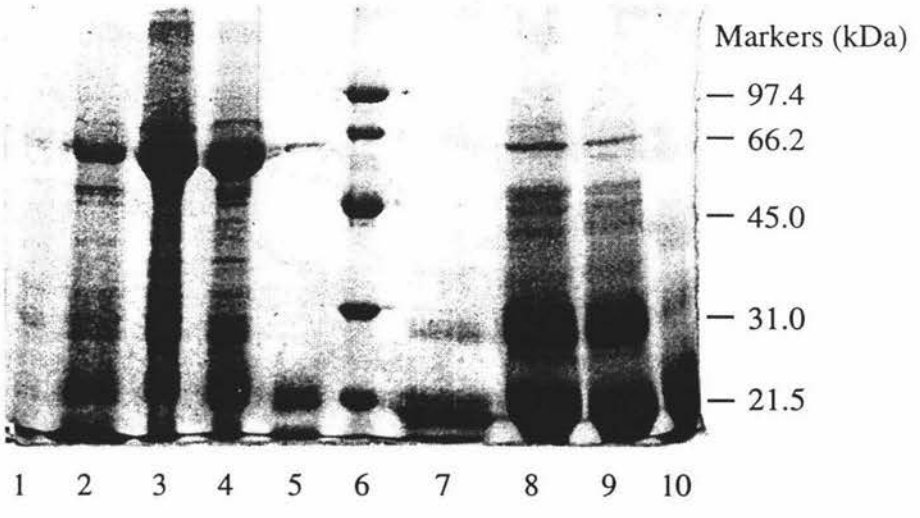


Figure 6.4 SDS gel of protein solutions which do not contain PNGase activity, from each stage of the purification to date. The lanes are labelled on the facing page.

Figure 6.3 SDS gel of fractions which contain PNGase activity, from each stage of the purification to date. *Lanes 1 and 10 are inactive samples. Lane 1:* Pellet from NaSCN/BME incubation (inactive). *Lane 2:* Supernatant after centrifugation of almond emulsin. *Lane 3:* Breakthrough from DEAE. *Lane 4:* Supernatant after removal of pellet from lowering pH to 5. *Lane 5:* After CM-Sephadex. *Lane 6:* Marker. *Lane 7:* Supernatant after removal of pellet from NaSCN/BME incubation. *Lane 8:* After phenyl sepharose. *Lane 9:* Marker. *Lane 10:* Phenyl sepharose breakthrough (non binding, inactive).

Figure 6.4 SDS gel of protein solutions which do not contain PNGase activity, from each stage of the purification to date. See also *lanes 1* and *10* in *figure 6.3*. *Lane 1:* DEAE salt eluent (1 M NaCl). *Lane 2:* Pellet from lowering pH to 5. *Lane 3:* CM-Sephadex loading breakthrough (run 1). *Lane 4:* CM-Sephadex loading breakthrough (run 2). *Lane 5:* CM-Sephadex wash breakthrough (run 1). *Lane 6:* Marker. *Lane 7:* CM-Sephadex wash breakthrough. *Lane 8:* CM-Sephadex 1 M NaCl elution, run 1 (after actives were eluted). *Lane 9:* CM-Sephadex 1 M NaCl elution, run 2. *Lane 10:* Phenyl sepharose loading breakthrough.

Figure 6.3 revealed a definite elimination of bands as the purification progressed. *Lane 4* showed many bands were present at the start and major bands appeared at about 58-61 kDa (overloaded), 49, 45, 35-38, 26, 24, 21 and 19 kDa. CM-Sephadex was an efficient step as many proteins were eliminated, leaving representative bands at 61, 49, 45, 35, 27, 23 and 19 kDa (*lane 5).* After the NaSCN/BME incubation, there were faint bands at ~61, 54, 49, 40 and 38 kDa and very dark bands representing a much higher concentration, present at 25-27, 23 and 19 kDa. The results from the next lane after phenyl sepharose showed bands of a darker relative intensity at 54 and 19 kDa. Other bands present were at 42 kDa (diffuse), 31, 27 and 23 kDa.

These bands, which corresponded to approximate molecular weights, did not seem to match exactly from one step to the next. A protease may have been present, which could explain the slight shifts in molecular weights, although the general band pattern looked similar. Dark bands, predominant in later purification steps, were at positions representing lower molecular weights, further evidence of protease activity despite the protease inhibitors EDTA and PMSF being added to the buffers used in each purification step. PMSF inhibits serine proteases and some thiol proteases, is very unstable in aqueous solutions and its inhibition effect may be reversed in high salt (such as during the phenyl sepharose step). At 4°C, 0.1 mM PMSF is inactivated in 30 hours at pH 7.6 and the inactivation time shortens with increases in temperature and pH. However, since protease activity still occurred, there may be acid proteases (active at low pH) and/or other thiol proteases present. The inclusion of pepstatin and leupeptin would have inhibited these proteases respectively.

Figure 6.4 showed a wide range of eliminated proteins. Proteins which had bound to the DEAE resin seemed to be at 61, 49, 33, 27 and 21 kDa. A similar pattern of bands was present for the pellet removed at pH 5 and the darkest band was at 61 kDa (*lane 2).* Many bands were present in the breakthrough from CM-Sephadex (*lanes 3* and *4*), which correlated well with the disappearance of many bands in the active pooled fractions after CM-Sephadex (*lane 5, figure 6.3*). The dark overloaded band at 61 kDa was eliminated

during the loading of the protein solution and the rinsing of the column showed the removal of proteins at 45, 38, 27, 23, 19 and 15 kDa (mainly 19 and 23 kDa). However a large number of proteins with molecular weights 27, 23 and 19 kDa were still present in the active fractions (*lane 7, figure 6.3).* The proteins which had bound very tightly to CM-Sephadex and which were subsequently removed by 1 M NaCl were mainly of molecular weights 61, 49, 45, 42, 27 and 19 kDa (the latter two being very overloaded). The pellet from the NaSCN/BME incubation contained a lot of protein at 23 and 19 kDa (*lane 1, figure 6.3)* and the protein at 27 kDa remained in the active supernatant (*lane 7, figure 6.3).*

Proteins that did not bind to phenyl sepharose had molecular weights of 19 kDa and a small amount of 27 kDa. The wash contained more of the 27 kDa protein and a lesser amount of 19 kDa (*lane 10, figure 6.4 and lane 10, figure 6.3* respectively), indicating that this protein was somewhat retarded by the matrix but did not bind.

A summary of the total protein amounts for the fractions containing PNGase activity is displayed in *table 6.1.*

Table 6.1 Total protein amounts for active samples

Step	Sample	Volume (ml)	Activity	Total protein (mg)	Appearance
Extraction	Emulsin	200	✓	✧ 8 600	Brown, viscous
DEAE chromatography	Supernatant	1130	✓	10 622	Yellow, clear
pH lowering to 5	Supernatant	1130	✓	6 893	Yellow, clear
CM-Sephadex	Active fractions	424	✓	× 806	Colourless, clear
NaSCN/BME	Supernatant	990	✓	* 5 940	Colourless, clear
Phenyl Sepharose	Active fractions	187.5	✓	× 45	Colourless, clear

✧ This value is inaccurate since this is the starting material and it may contain interfering compounds.

× At least this amount, because only the first of two active pools were accounted for.

* This value is inaccurate due to the presence of BME (a reducing agent) and high salt (NaSCN), which both increase the readings markedly (Smith *et al.*, 1985).

All of these values are lower than they should be due to the presence of 50 mM EDTA in all the buffers used. Smith *et al.*,(1985) reported that this concentration of EDTA affected the results with BCA such that the apparent amount of protein was nearly half that of the actual amount present. This is because EDTA is a copper chelating reagent and BCA is a sensitive, stable and highly specific reagent for Cu^{+}. Proteins reduce Cu^{2+} to Cu^{+} in an alkaline environment so less Cu^{2+} are available to proteins in the presence of EDTA. Worse results were shown to occur with the Lowry assay, for the same reasons (Smith *et al.*, 1985).

(e) Gel Filtration Chromatography

A Superdex 75, 3.2/30 column was used for preliminary trials on the SMART system. The column was pre-equilibrated in 10 mM NH_4Ac, pH 5 and the duration of the run was 80 minutes. Three main peaks monitored by absorbance at 280 nm were eluted. PNGase

activity was found in the first peak (fractions 8-11). PNGase A eluted after an elution volume of 1 ml (*figure 6.5*).

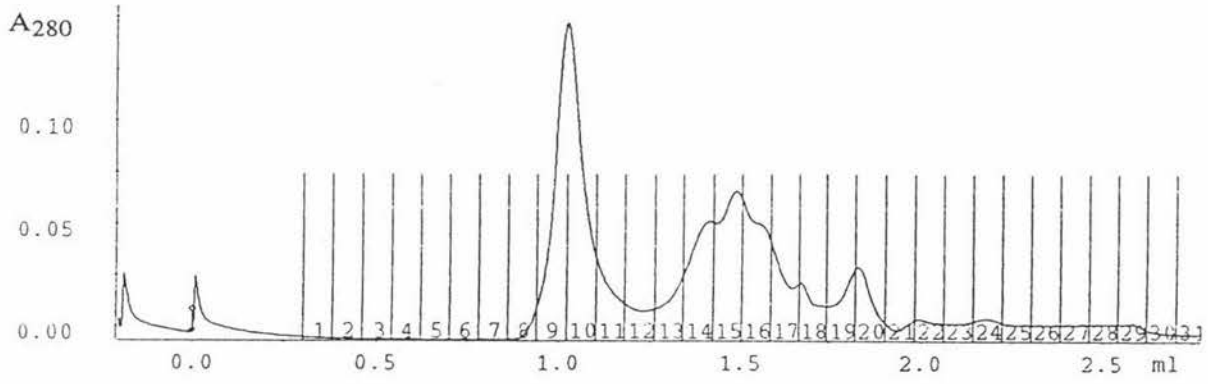


Figure 6.5 Gel filtration on the SMART system using Superdex-75 (3.2/30). PNGase activity was detected in fractions 8, 9, 10 and 11 and peaks were monitored by absorbance at 280 nm.

In order to determine the molecular weight of the active fraction, a mixture of marker proteins were loaded onto a column equilibrated in 20 mM HEPES/0.2 M NaCl, pH 7.5 (hexokinase 104 kDa, bovine serum albumin 66 kDa, ovalbumin 45 kDa and chymotrypsinogen A 25 kDa). They were eluted using identical conditions to the PNGase elution. Superimposing the two elution diagrams, PNGase A seemed to slightly precede the elution of the 104 kDa marker protein and appeared at approximately 108 kDa. The next peak eluted at the positions of 27 kDa and 25 kDa (second shoulder in line with 25 kDa marker). These elution diagrams are shown in *figure 6.6*.

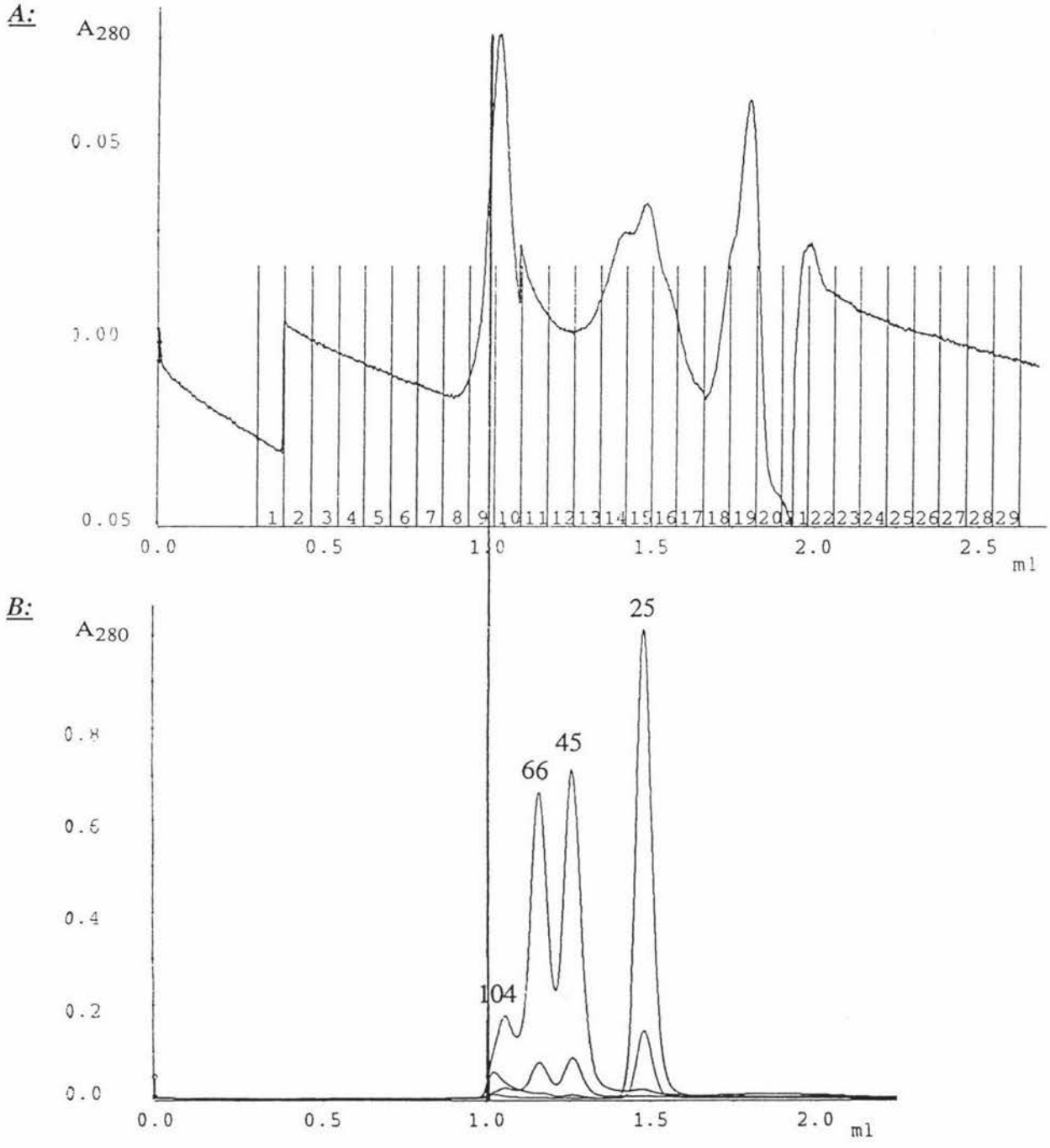


Figure 6.6 A: Superdex-75 gel filtration on the SMART System of the protein solution containing PNGase A. The column was equilibrated in 20 mM HEPES/0.2 M NaCl, pH 7.5. Active fractions were 9, 10 and 11. ***B***: Markers (104, 66, 45 and 25 kDa) loaded under the same conditions. Note that PNGase A eluted before the 104 kDa marker.

In another trial, a different set of markers and buffers were used (β-galactosidase 116 kDa, albumin 66 kDa and carbonic anhydrase 29 kDa). In this case, the 66 kDa marker protein eluted at the same volume as the PNGase active peak (*figure 6.7, B*). This suggested that PNGase A had a molecular weight of about 66 kDa.

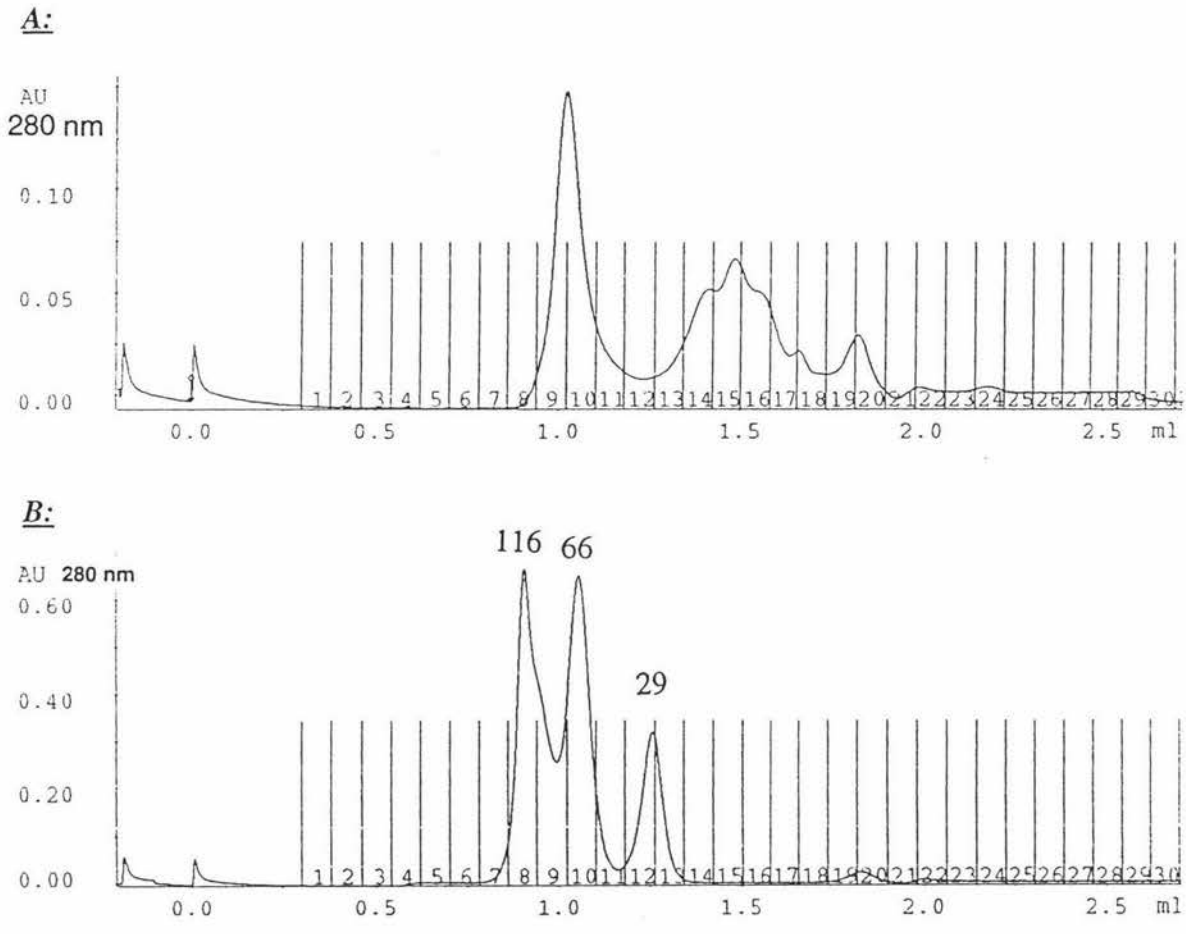


Figure 6.7 Superdex-75 gel filtration on PNGase A solution and marker proteins. The SMART system was used and the column was equilibrated in the buffer 10 mM NH_4Ac/0.15 M NaCl, pH 5. ***A***: PNGase A containing sample. ***B***: Marker proteins (116, 66, 29 kDa).

However, the run using the column equilibrated with HEPES buffer pH 7.5, at the higher salt concentration of 0.2 M salt, suggested that PNGase A had a molecular weight of approximately 108 kDa. This suggests that some form of interaction between proteins is occurring. This may be a result of either pH or salt concentration, which will both have an effect on the interactions between protein molecules. It may be that the PNGase is forming homodimers with itself or it may represent the interaction of PNGase with another protein.

Hydrophobic interactions would have increased with the higher salt concentration used, compared to the second run, but may have been counteracted by the higher pH, which decreases hydrophobic interactions. Since the isoelectric point of PNGase A is not known for sure, the effect of pH on ionic interactions is difficult to assess. Results from preparation 3 indicated however, that PNGase A was negatively charged at pH 5 and positively charged at a pH of 7 in CM-Sephadex binding trials (Chapter 5, *section 5.1 (d)*). This is in conflict with the fact that the protein appeared not to bind to DEAE at pH 8.8.

The pH and ionic composition of buffer used in gel filtration can have an effect on the net charge of proteins. Conformational changes and dissociation of subunits or cofactors may occur. In this case, PNGase A is inactive at pH 7.5, which may be due to the fact that it forms dimers at this pH (PNGase A is most active at pH 4.5). Possible examples of proteins (seen on SDS gels) associating to make 108 kDa are : 54 + 54 kDa; 66 + 42 kDa and 54 + 27 + 27 kDa but there is no direct evidence for this.

Electrostatic or ionic interactions may have increased in the second run, since a lower pH and lower salt concentration were used. The lower pH would have increased hydrophobic interactions, whereas the lower salt would have decreased these interactions. Together with ionic interactions (PNGase may be of a different net charge under these conditions) a different chromatogram from the HEPES trial resulted, as seen in *figure 6.7.*

Referring back to *figures 6.5* and *6.7*, the peaks represent the following proteins at 66 (fraction 10), 27, 25, 23 (fractions 14-16), 19 (fractions 17 and 18) and 15 kDa (fraction 19). These agree with bands seen in SDS gels such as in *figures 6.3* and *6.4*. Fractions 8, 9, 10 and 11 which contained PNGase activity were pooled, concentrated then re-chromatographed using the lower pH, low ionic strength buffer (*figure 6.8*).

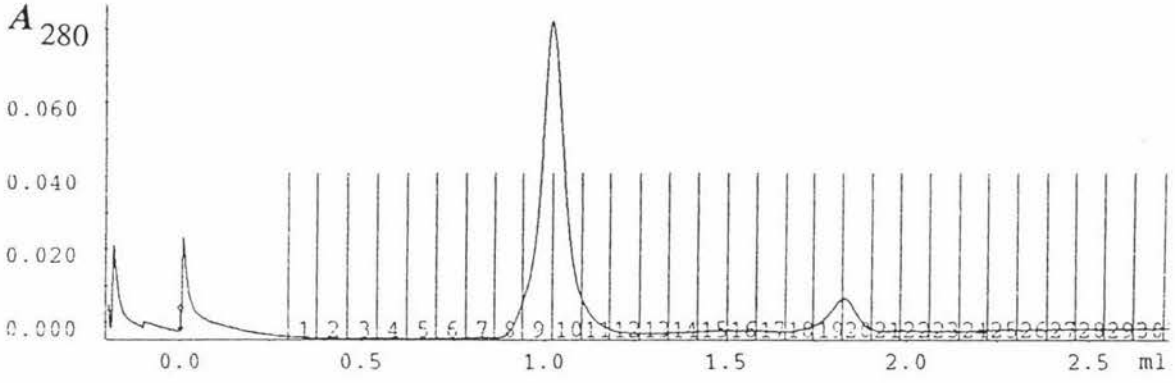


Figure 6.8 Active fractions of PNGase A re-chromatographed by gel filtration on Superdex 75 using the SMART system. Two peaks were seen at 66 and 15 kDa.

This second chromatogram showed the same peak at the same elution volume for PNGase A. However a small second peak at 1.8 ml (fractions 19 and 20) was also observed, which was the same as that seen in the previous run. The smaller peak, estimated to be of a 15 kDa protein, did not possess PNGase activity. This suggests that a small protein was strongly attached to PNGase in the active fractions, the interaction being hydrophobic in nature. While this could also be due to proteolysis of the PNGase, all the chromatography was done at 8°C and the two runs were carried out within 2 hours of each other.

Fractions containing PNGase activity (*figure 6.8*), were pooled, concentrated by ultrafiltration and analysed by SDS-PAGE (*figure 6.9*).

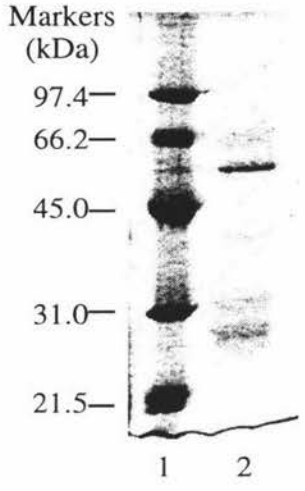


Figure 6.9 SDS gel of rechromatographed active fractions from SMART.
Lane 1: Markers. *Lane 2:* First peak from SMART (fractions 8, 9, 10 and 11 pooled and concentrated) rechromatographed then pooled and concentrated again for this gel.

Three bands were clearly visible. The most obvious and intense band was at 53 kDa. Other bands present were at 35 and 27 kDa. Plummer *et al.,* (1987) proposed the molecular weight to be 79.5 kDa and Taga *et al.,*(1984) proposed the molecular weight to be 66.8 kDa. Three things are possible from these results:

1. The enzyme has been hydrolysed by proteolytic activity and there is an active fragment left in the mixture.
2. The enzyme bears some post translational modification which causes it to run anomalously.
3. One of the bands represents PNGase A. The rest are impurities which closely stick to it, under the conditions of chromatography.

(f) Gel Filtration Using FPLC (Scaled Up)

Gel filtration using FPLC (Superdex 75 HR 10/30) was used with the remainder of the protein solution from phenyl sepharose chromatography. Good resolution was optimised with sample applications of 160 µl. The elution was monitored by absorbance at 280 nm and PNGase activity was detected in the first peak which had an elution volume of 7.2 ml, 18 minutes from the time of injection. The elution diagram is shown in *figure 6.10.*

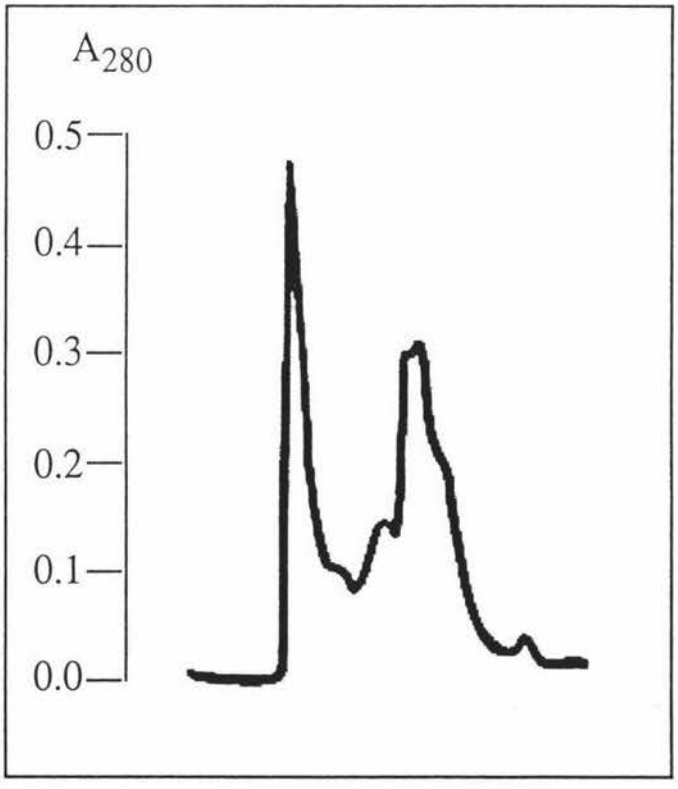


Figure 6.10 Gel filtration of protein solution containing PNGase A, using FPLC. PNGase activity was detected in the first peak.

Fractions comprising the first peaks were pooled after they were assayed and then analysed by SDS-PAGE (*figure 6.11*).

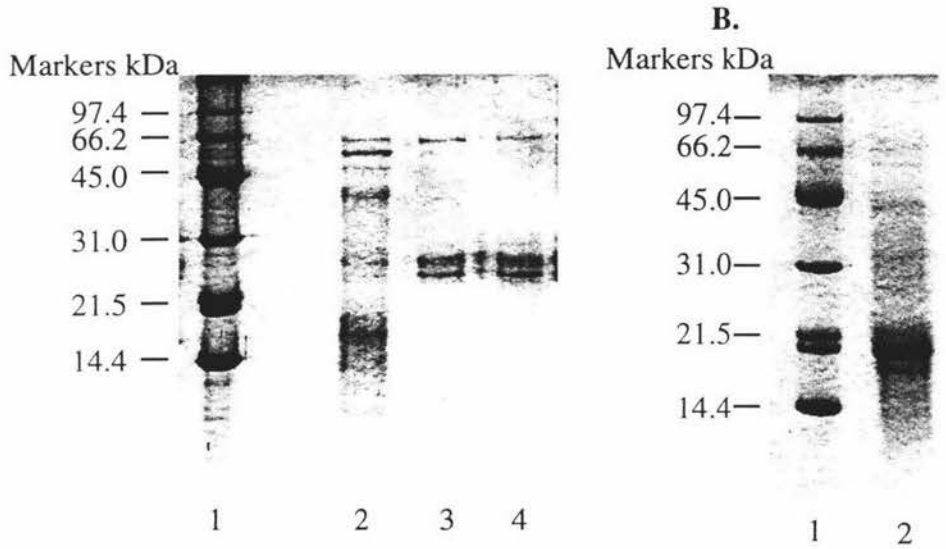


Figure 6.11 SDS gel of active fractions after gel filtration. The Superdex 75 column (HR 10/30) was equilibrated in 10 mM NH_4Ac/0.2 M NaCl, pH 5. ***A:*** *Lane 1:* Marker. *Lane 2:* Peak 1, which contained PNGase activity. *Lanes 3 and 4:* Preparation 3 samples which contained PNGase activity, after gel filtration under the same conditions. ***B:*** *Lane 1:* Marker. *Lane 2:* Fraction which contained the first peak.

Compared with the samples from preparation three (*lanes 3* and *4, figure 6.11A),* the emulsin sample revealed a different pattern of bands. Bands in common were at 62, 27 and 12 kDa. There was a band of high intensity at ~17 kDa in the emulsin sample (*lane 2, gel A*). Before pooling, fractions had been analysed by SDS-PAGE (*figure 6.11 B*). The most intense band (compared to the other bands) was at 17 kDa (*lane* 2, *gel B*) and was very diffuse.

(g) Gel Filtration Using the SMART System

Multiple runs with the concentrated protein sample (after FPLC) were performed using 40 µl sample volumes, as a final high resolution separation step. Resolution is better using SMART because the beads are smaller and more evenly sized. The elution was monitored by absorbance at 280 nm and active fractions were 8, 9, 10 and 11 (similar to

previous runs). After chromatography was complete, the same fractions from each run were pooled and concentrated, then each pool assayed with only a 15 minute incubation. It was found that fraction 10 was most active, then fractions 9, 11 and 8. Fractions 7 and 12 did not contain PNGase activity. The four pools were analysed by SDS-PAGE (*figure 6.12*).

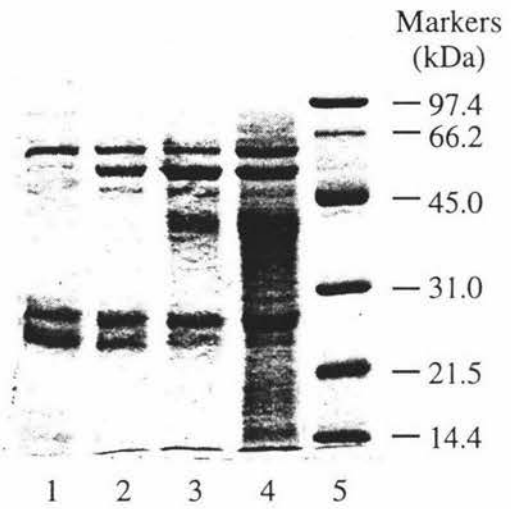


Figure 6.12 SDS gel of fractions across the active peak of about 66 kDa. These samples are very concentrated compared to *figure 6.10. Lane 1:* Fraction 8 (concentrated). *Lane 2:* Fraction 9. *Lane 3:* Fraction 10. *Lane 4:* Fraction 11 (concentrated). *Lane 5:* Marker.

The results shown in the SDS gel of the active peak (*figure 6.12*) indicate there were bands in common for all samples. Fractions 8 (*lane 1*) and 11 (*lane 4*) had to be concentrated with a 3 K microcon in order to be visible with Coomassie stain. From looking at relative intensities within the same lane, the most active sample (*lane 3*) had strongest bands at 62, 54 and 27 kDa. No band was seen at 66 kDa across the peak. A diffuse band at 42 kDa showed up at a high intensity in fraction 11 (*lane 4*). The spread of this diffuse band indicated it could possibly be a glycosylated protein. Although fraction 11 was not very active (before concentration) the relative intensities of the bands were the same as in fraction 10 except for the 42 kDa band which was more prominent in fraction 11.

Analysis of the inactive peaks by SDS-PAGE (gel not shown) showed mainly a diffuse 19 kDa band present from fractions 12 to 16. Very faint bands were also visible across this peak at 66 kDa and 54 kDa. Therefore, these bands may not correlate to activity in these samples.

(h) Glycan Determination by DIG Staining

The contents of fraction 10, the most active emulsin sample were analysed by DIG staining (*figure 6.13*) as outlined in *Methods, section 2.2.3(e).*

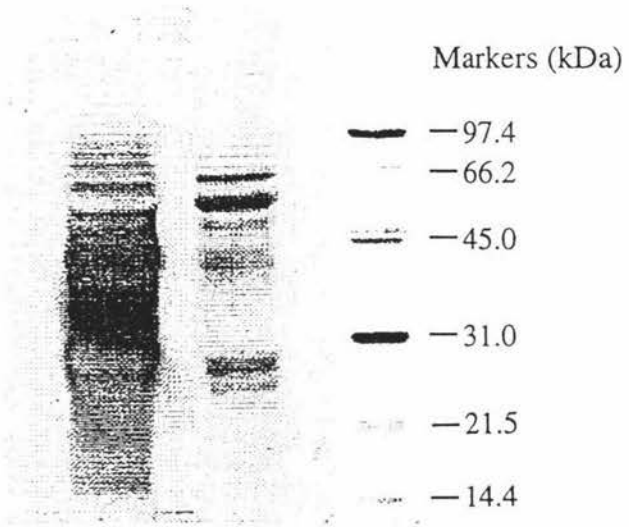


Figure 6.13 DIG glycan and protein double labelled stain of the most active PNGase sample, from a PVDF blot. *Lane 1:* DIG stain. *Lane 2:* Coomassie Blue R_{250} stain (PVDF blot), for comparison. *Lane 3:* Markers.

The results showed one blue band, which represented a glycosylated protein with a molecular weight of approximately 42 kDa. All other bands were reddish brown. Taga *et al.*, (1984) proposed that PNGase A was glycosylated and had a molecular weight of 66.8 kDa. These results show there is no glycoprotein with a molecular weight of 66-67 kDa. Furthermore, in preparation 3 (almond meal), DIG staining showed the band representing a molecular weight of 54 kDa, was glycosylated (stained blue) and the

protein with a molecular weight of 42 kDa was reddish brown (*figure 5.13).* It is possible that the 42 kDa protein may be a breakdown product cleaved at a different place. N-terminal sequencing of all the bands in the fraction may indicate that cleavage has occurred if two identical sequences are found.

(i) Affinity Chromatography with Concanavalin A Sepharose

Affinity chromatography was performed because it was known from preparation 3 that PNGase A bound to a Concanavalin A column. The glycosylated protein with a molecular weight of 42 kDa (determined from DIG staining) was expected to bind to this column and hence separate from the other proteins present in the sample. If one band is seen on an SDS gel, which belongs to an active fraction, PNGase activity could then be matched to this protein.

Fractions 17-29 from phenyl sepharose chromatography were active but contained within two small peaks and therefore were not as pure as the following active fractions of low absorbance (*figure 6.2*), so these fractions were pooled and subjected to Concanavalin A chromatography. The column eluent collected during sample loading and washing, tested negative for PNGase activity.

Fractions 10-22 exhibited PNGase activity and eluted at a concentration of approximately 0.05 M methyl-α-D mannopyranoside. The pooled fractions were analysed by SDS-PAGE (*figure 6.14*).

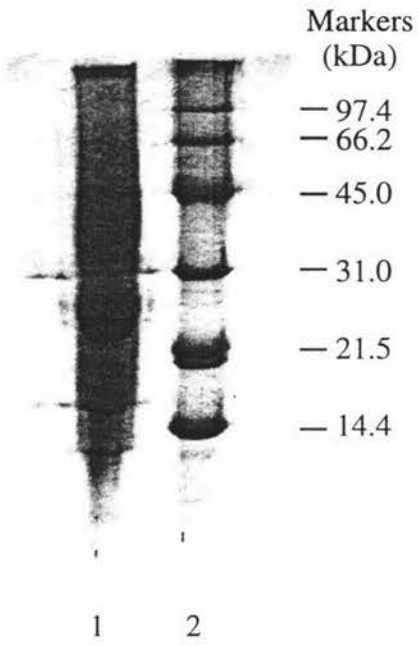


Figure 6.14 SDS gel of the results from Concanavalin A chromatography.
Lane 1: Pooled fractions 10-22 containing PNGase activity. *Lane 2:* Marker.

Seven main bands were revealed, corresponding to proteins with molecular weights of approximately 59, 41, 31, 27, 25, 17 and 12 kDa. Although this sample had been concentrated, these bands were different to what had been seen in the protein sample analysed after phenyl sepharose chromatography (*figure 6.3, lane 8*). While some of these bands matched those seen in the SDS gel shown in *figure 6.3*, others were very different. For example the 59 kDa band seen in *figure 6.14*, was 54 kDa in *figure 6.3* and likewise the 17 kDa band in *figure 6.14*, was previously 19 kDa. A band at 12 kDa appeared here, which was not in the previous gel. This indicates some breakdown of protein may be occurring.

(j) Ultrafiltration Studies

Protein samples containing PNGase activity were subjected to ultrafiltration using 50 K membranes (Amicon). If PNGase A was more than 50 K in molecular weight (such as 66.8 kDa) then it would be expected to be retained by this membrane. This would separate PNGase A from the other low molecular weight proteins seen in SDS gels and

would be a purification step. One of the buffers used contained salt and was at a low pH, to increase hydrophobic interactions between the protein molecules. This was to see which proteins (especially of low molecular weight) aggregate and stay in the retentate, when they are otherwise expected to be in the filtrate. The results are displayed in *table 6.2.*

Table 6.2 Assays on filtrates from ultrafiltration using a 50 K membrane.

	10 mM NH_4Ac/0.2 M NaCl, pH 5	**10 mM NH_4Ac, pH 5**
First spin	Inactive	Inactive
Second spin	Active	Inactive

The same procedure was repeated with 30 K membranes (Filtron) using the same buffers. This was to see whether the protein responsible for activity was retained by a membrane this size and whether salt affects the outcome. The results are displayed in *table 6.3.*

Table 6.3 Assays on filtrates from ultrafiltration using a 30 K membrane.

	10 mM NH_4Ac/0.2 M NaCl, pH 5	**10 mM NH_4Ac, pH 5**
First spin	Active	Active

Using the 30 K cut-off membrane, the filtrate is active both in the presence and absence of NaCl. This experiment was repeated in the absence of salt, since these results did not agree with that seen for the 50 K cut-off membrane (filtrate inactive). The active emulsin sample (from fraction 10, SMART) was washed three times with 1 ml of the same buffer and the resulting filtrate was concentrated in 3 K microcons (filtrates inactive). The filtrates and retentates were assayed for activity (*table 6.4*) and analysed, together with the 50 K ultrafiltration filtrates, using SDS-PAGE (*figure 6.15*).

Table 6.4 Repeated assays on retentate and filtrate from 30 K ultrafiltration.

Sample	Retentate	Filtrate
1	Very active	active
2	Very active	active

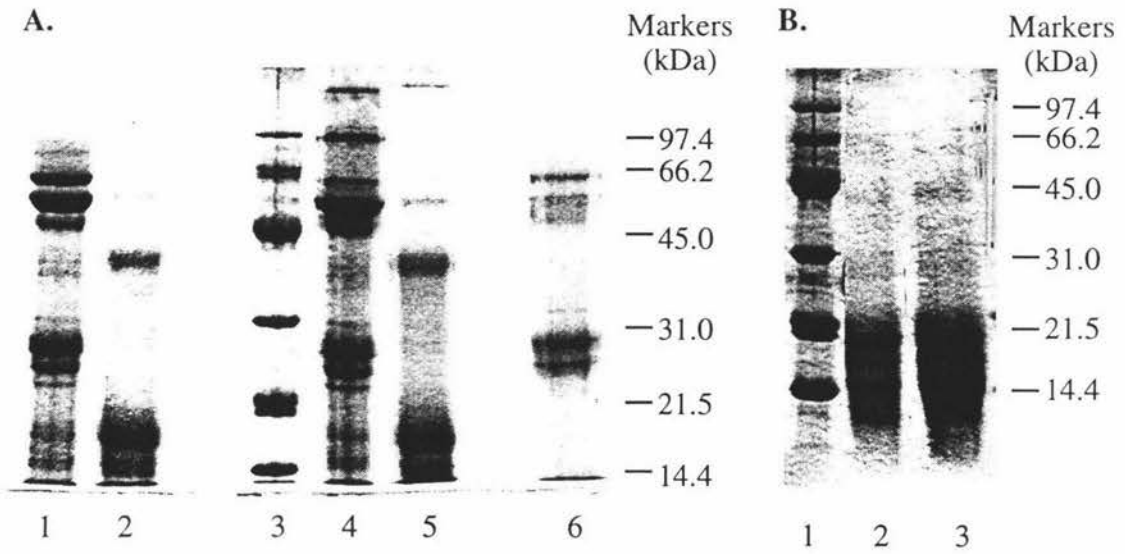


Figure 6.15 SDS gel of retentates and filtrates from ultrafiltration.

Gel A: **30 K membrane samples, without salt:** *Lane 1:* Retentate. *Lane 2:* Filtrate. *Lane 3:* Marker. *Lane 4:* Retentate without boiling sample before gel loading. *Lane 5:* Filtrate without boiling sample. *Lane 6:* Preparation 3 almond meal sample, after gel filtration (Superdex 75, 16/60) for comparison.

Gel B: **50 K membrane filtrates:** *Lane 1:* Marker. *Lane 2:* Filtrate without salt. *Lane 3:* Filtrate with salt.

The filtrate from the 50 K cut-off membrane (*figure 6.15, Gel B*) revealed a few low molecular weight bands of high intensity, which were more concentrated than the other higher bands within the lane. Bands were present at the higher molecular weights of 66, 61 and 42 kDa, despite the membrane having a nominal cut-off of 50 kDa. The increasing intensity of the lower molecular weight bands would seem to suggest that

some breakdown of the larger proteins is occurring, especially as these are very different from what was observed in previous gels run on these protein samples.

Lane 2, (Gel B) which contained the filtrate without salt had an identical band pattern to *lane 3*, which contained 0.2 M NaCl. However, a band at about 61 kDa present in *lane 2* had disappeared in *lane 3* and instead a new band appeared here at 31 kDa. This may be due to the presence of salt and this protein may be associated with the activity of the filtrate.

Analysis of *Gel A* showed the following proteins were present in the retentate: 66, 56, 49, 41 (faint), 27, 25, 19 and 15 kDa. The filtrate contained proteins of the molecular weights 66 (faint), 56, 41, 18 and 15 kDa. It should be noted that the filtrate may have been more concentrated than the retentate because some bands in the filtrate were relatively darker than in the original sample.

The samples which were not boiled agreed with these molecular weights but contained an extra two bands in the retentate, which were at about 71 and 130 kDa. The 130 kDa band was also present in the filtrate. The band at about 56 kDa was lighter in intensity, in the retentate of the non-boiled sample, which may partly account for these extra bands.

The manufacturers stated the 30 K Filtron Nanosep best retains proteins 90-180 kDa. Therefore if the protein of interest is more than 60 kDa, then some would be expected in the filtrate. However, they also stated that the 30 K cut-off membrane will retain 90-100% of albumin (67 kDa) and 80-89% of α-chymotrypsinogen (24.5 kDa). Therefore both the retentate and filtrate were expected to be active, because even a 1-2% leakage may be enough for the sensitive assay to detect activity. A few particular proteins however, seemed to pass through the membrane easier than others and some seemed to be tightly associated.

The 56 and 42 kDa proteins appeared early in the filtrate but were expected to be largely retained. The 25 and 27 kDa proteins were probably tightly associated with a larger

protein (or were part of a parent protein) because they were present in the retentate (same intensity) but absent in the filtrate, where they were expected.

(k) Urea Gel Trials

Taga *et al.*,(1984) showed that PNGase A was active in 2 M urea. In preparation 3, incubation trials performed which included 2 M urea in the substrate, showed activity was still retained. Slight activity was seen after incubating the enzyme in 5 M concentrations for an hour.

Urea gel trials were performed in an attempt to match a single band seen on the gel to activity. The protein sample after gel filtration using FPLC (before using SMART), was analysed by urea gel electrophoresis (*figure 6.16 gel A*). This was also compared to the sample from preparation 3 (*figure 6.16 gel B*).

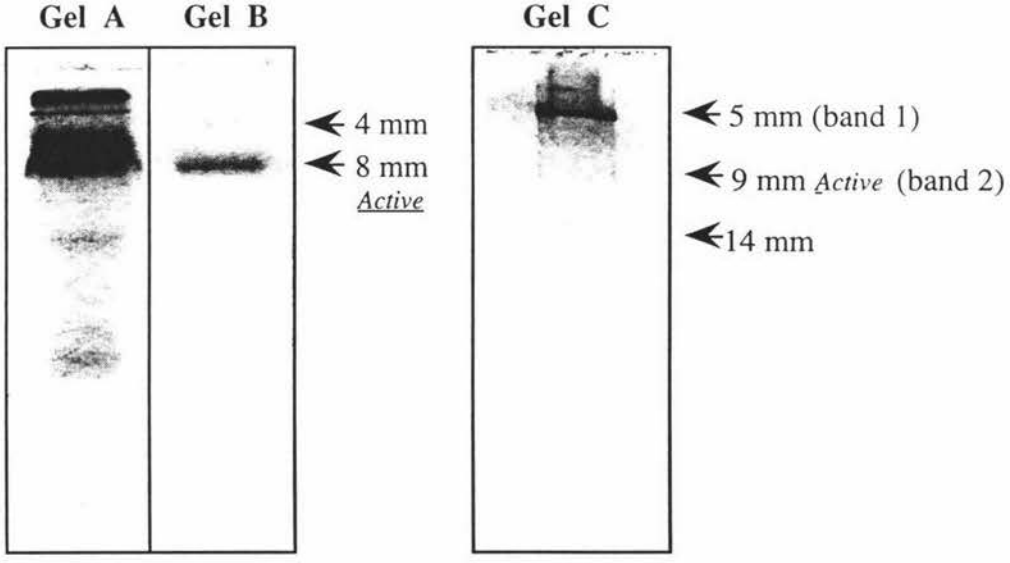


Figure 6.16 Urea gel of samples containing PNGase activity.

Gel A: Sample from almond emulsin, before gel filtration on SMART. ***Gel B:*** Sample from preparation three after gel filtration (Superdex 75,16/60 with FPLC) for comparison (from *figure 5.19*). ***Gel C:*** Sample from almond emulsin after gel filtration on the SMART system (Superdex 75, 3.2/30).

As can be seen in *Gel A, figure 6.16*, there are many bands still present in the emulsin sample compared to the previous almond meal preparation (*Gel B*). After gel filtration on the SMART system (Superdex 75, 3.2/30), the sample was analysed again using a gel made 2 M with urea (*Gel C*). One half was stained with Coomassie Blue and the other half (four lanes) was sliced into 1 mm strips from the top of the resolving gel and electroeluted as described in *Methods, section 2.2.3(c)*).

A slice 9 mm from the top of the resolving gel exhibited PNGase activity, which was seen as a faint band (band 2), (*Gel C, figure 6.16*). The slice relating to the band clearly seen 5 mm from the top, tested negative for PNGase activity (band 1). The active band 9 mm from the top agreed well with the results obtained with the almond meal sample from preparation three (*figure 5.19*) where the active band resided 8 mm from the top of the gel.

The protein samples which were electroeluted from bands 1 and 2 were concentrated and analysed by SDS-PAGE (*figure 6.17*).

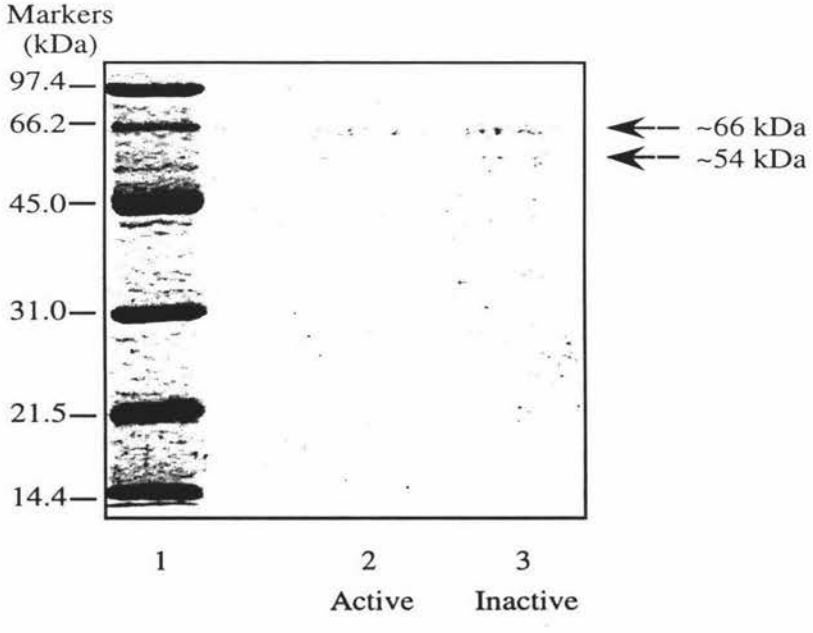


Figure 6.17 SDS gel of protein electroeluted from the previous urea gel.
Lane 1: Marker. *Lane 2:* Band 2, at 9 mm, positive for PNGase activity. *Lane 3:* Band 1, at 5 mm, negative for PNGase activity.

These bands were very faint. However, from close scrutiny of the actual dried gel, the active protein in *lane 2* revealed a band at about 66 kDa. The inactive protein in *lane 3* revealed two bands at 66 and 54 kDa. There were possibly some proteins of a lower molecular weight, such as at 27 kDa but they were difficult to see.

Fractions across the active peak from SMART were run on a urea gel in duplicate and blotted onto PVDF and stained. The blot was cut in half and one half was stained with Coomassie Blue R_{250} and showed a dark band 4 mm from the top of the resolving gel, such as that seen in *figure 6.16C* but no band 9 mm from the top. Fraction 11 (not as active) showed many bands and a smeared lane was observed under the band at 4 mm for fraction 10 (blot not shown).

The other half of this blot was DIG stained. Red bands 4 mm from the top were revealed (unglycosylated proteins) and a weak broad blue band (of length 5 mm), was present below it. This represented a glycosylated protein, which travelled up to 9 mm from the top of the resolving gel (data not shown).

The active band seen in the urea gel may therefore be glycosylated, although no discrete band was determined from the DIG stain to match.

(l) Native Gel Experiments

The protein solution was analysed by native gel electrophoresis at pH 8.8. This was performed in duplicate using electroelution and Amicon Nebulisers to extract the protein from the gel. Both electroelution and nebulisation gave the same results and showed PNGase activity to reside in a visible band 15 mm from the top of the resolving gel.

A second gel was run and blotted onto a PVDF membrane. On staining with Coomassie Blue R_{250}, four bands were visible, at the distances 4, 15, 24 and 33 mm from the top of the resolving gel. The active band at 15 mm was cut out and sequenced from the N-terminus (results to follow).

The active samples from the native and urea gels were then analysed by SDS-PAGE (*figure 6.18*) to relate the activity to molecular weight.

Although the bands were faint, it can be seen in *lane 3* that the single band from the native gel separated into four main bands which compared favourably to the bands seen in the most active sample in *lane 1* (fraction 10 from SMART). The bands were placed at 64, 58, 27 and 25 kDa. However the bands which *lanes 4, 5* and *6* have in common, were at 66 and 59 kDa and came from the second native gel and the urea gels.

The experiments using non-denaturing gels have shown that a single band cut from these gels separates into at least two bands on SDS gels. About four bands could sometimes be seen, at either 66 and 59 kDa or 64 and 58 kDa and then at 27 and 25 kDa.

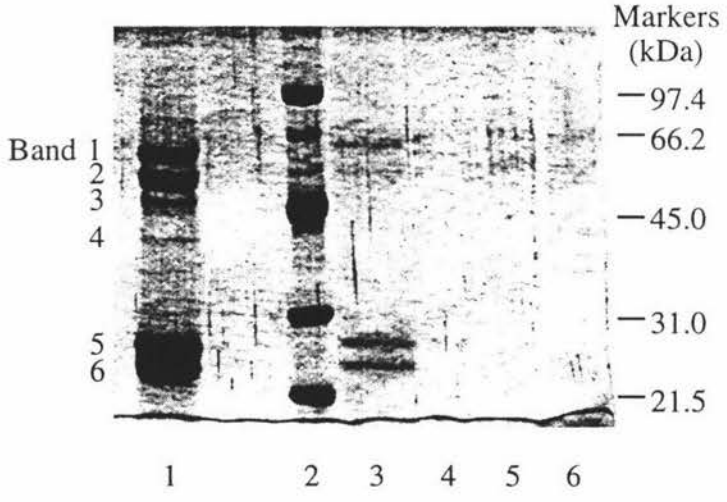


Figure 6.18 SDS gel of protein electroeluted from native and urea gels.
Lane 1: Most active emulsin sample (fraction 10). *Lane 2:* Marker. *Lane 3:* Native gel band at 15 mm, active. *Lane 4:* Another native gel, band at 15 mm, active. *Lanes 5 and 6:* Active band from urea gel.

Evidence of breakdown (sugar or protein) may be signified by the slightly different band pairs of high molecular weight, although the band patterns looked the same. The difference between the top two bands was about 6-7 kDa, in both cases.

Another urea gel trial showed activity to reside 2 mm form the top of the gel (data not shown) although a similar band pattern to that in *figure 6.16C* was observed. Limited proteolysis and denaturation may therefore result in PNGase activities in native and urea gels correlating to different bands seen in these gels. This is summarised in *table 6.5*.

Table 6.5 Summary of band locations in non-denaturing gels.

Gel type	Assay results for activity	Distance from top of resolving gel for emulsin sample (mm)	Distance from top of resolving gel for almond meal sample (mm)
Native	Present in both samples	15 14	-
Urea	Present in all samples	9 4 2	8 7

(m) N-Terminal Sequencing

The most active PNGase sample (fraction 10) was blotted onto PVDF from an SDS gel. The six bands present were at 62, 54, 49, 42, 27 and 25 kDa. These bands were cut out and sequenced. The results are displayed in *table 6.6.*

Table 6.6 N-terminal sequencing of bands seen in an SDS gel, an active peak after gel filtration and a native gel blot. Amino acid numbers are at the top from 1-14 and molecular weights from the SDS gel are labelled in bold under **M.** The numbers under **C** represent the call order. **SM** is peak one from gel filtration on the SMART, **NA** is the blot from a native gel.

M	C	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	1	R	G	T	P		L	N	V	A	Y				
62	2	L	A	S	G				D	A	G				
	3			L	E	X	N		A	S	G				
	1	L	A	S	G	Y	H	S	E	A	D				
54	2	R	G	L	E	X	L	N	V		-				
	3				-	D	E		Q		L				
49	1	K	N	L	E	-	-	-							
	2	R	G	-	G	E	X	X							
	1	G	Q	-	-	G	G	Q	G	G	-				
42	2	-	E	-	-	E	Q	N	R	R	-				
	3	T	P	-	Y	-	K	X	X	P					
	4	S					V								
	1	E	P	T	P	L	H	N	A						
27	2	A	G	L	Y	D	K	D	T						
	3	K	D	K	H	-	-	G	V						
	4	X			G			S							
25	1	E	G	P	E	T	L	N							
	2	R	Y	F	K	-		F							
SM	1	T	P	K	Y	D	K	Y	A	F	D	A	P	L	
	2			D					K						
NA	1	T	K	K	Y	D	K	G	A	F	D	A	P	L	L
	2		D	D					K	G					R
	3	S	Q			E	V			P					

Each row within the molecular weight category has up to four results (under **C** for call). The first is human interpretation of which amino acids are present. The second to fourth calls are mixtures of human interpretation and machine interpretation on which amino acids are at that position.

N-terminal sequencing was performed to see if the bands present in the latest SDS gel (*lane 1, figure 6.18*) represented proteins with the same N-terminal, meaning breakdown products. If a high molecular weight protein is breaking down, another band with the same N-terminal sequence would be expected to be seen further down.

The gel filtration SMART sample and the native gel blot sample gave clear results, which closely agreed. However, the sequences present for the SDS gel blot were less obvious. The only very clear sequence for the SDS gel blot is that of call one for the 54 kDa band. This agreed with the clear sequence obtained previously for preparation 3 (*table 5.4*) and so is the most reliable. From looking at the first calls, no particular pattern emerged. When these first calls were entered into the Blitz Database, no match of any N-terminal was found but some short internal sequences were close.

However, some patterns clearly emerged from a mixture of the possible calls on amino acid sequences, for the SDS gel blot. These are shaded in *table 6.6*. The most probable sequences are listed first.

Firstly, band 1 at 62 kDa contained the following possible sequences:

1. **R G L E - L N V S I**
2. L A S G - - - - A -
3. R G L G L N

Band 2 at 54 kDa contained the following sequences:

1. **L A S G Y H S E A D** (very clear sequence, same as preparation 3)
2. R G L - E L N V - I

The third band at 49 kDa contained the following sequences:

1. R G L E E -

2. R G L G - N

The fourth band at 42 kDa (glycosylated in DIG stain) contained:

1. T P - Y - K - - G -

2. S Q - - E V - - P

The fifth band at 27 kDa contained the following N-termini:

1. X P K Y D K G A

2. R G L G L -

3. R G L - - - N V

Finally the sixth band at 25 kDa showed the sequence:

1. R G L E - L N

Although some of these amino acids were not the most probable interpretation, they all appeared to be present in that particular sample or band.

The sequence beginning with RGL--- seemed to be in all the samples except band 4, therefore this was suspected of being a contaminant. Possible proteins responsible for the sequences RGLGEL and RGLGLN were searched for through the Blitz Database system. It was found that a few 100% and 93.5% matches (respectively) were made to common human proteins. Seeing different proteins present for each band was not expected from a blot. More than one protein may be expected to be contained in a peak from gel filtration chromatography, but this is less likely on an SDS gel, although it is possible for two proteins of identical molecular weight. Furthermore, proteins with sequences beginning with R are more common as protease products, than those beginning with T.

Since more than one peptide with an N-terminal was deduced to be present in each band, signals from within each peptide may have had different intensities. This meant that by looking at the first call only (row 1) for each band, none of these peptides had any amino acid sequence in common.

The SMART sample originated from preparation 3, after gel filtration on Superdex 75, Hi load 16/60 (*section 5.1(l)*). The protein from that step was run on a urea gel, the active band excised, electroeluted then subjected to further gel filtration chromatography on the SMART (Superdex 75, 3.2/30 column, *section 5.1(m)*). The first peak eluted was active. This was vacuum-dried then sequenced.

The native gel sample was the band cut out from the blot (15 mm from the top of the resolving gel) described in this chapter, *section 6.1(l)*.

The resulting sequences of these two samples show nearly an exact match. The mismatches are underlined. There were also a few matches seen in band 4 (42 kDa) and band 5 (27 kDa) from the SDS gel sequenced earlier.

SMART:	**T P K Y D K Y A F D A P L**	66 kDa
NATIVE:	**T D K Y D K G A F D A P L**	66 kDa
BAND 4:	**T P - Y - K - - G**	42 kDa
BAND 5:	**X P K Y D K G A** **D**	27 kDa

The band corresponding to 27 kDa from SDS gels, most closely matched the band from the native gel blot. The 27 kDa band is present in most active samples. P or D was found in this sample at position 2, which meant it could match either the SMART peak or the native blot. Mismatches occurred at positions 2 (P and D) and 7 (Y and G). Although F was present at residue 9 in both the SMART and native gel samples, this residue was G in band 4, which corresponds to the protein of molecular weight 42 kDa, from the SDS gel. Band 4 (of 42 kDa), had been previously shown to be

glycosylated (*figure 6.13*). It must also be noted that cysteine residues were not detected by the sequencer.

In summary, there were three main N-terminals deduced between all the bands seen by SDS-PAGE analysis. One starting with the sequence RGLE..., the next with LASGY... and the third by TDKYD... . Three proteins shared the first sequence (61, 49 and 25 kDa) and one clearly exhibited the second (54 kDa). The 42 and 27 kDa proteins shared the third sequence together with the SMART and native samples. The SMART and native gel samples did not possess any other peptide sequence such as RGL..., suggesting that this latter sequence is characteristic of SDS gels.

Some of these proteins may be either contaminants or breakdown products from a larger precursor or a tight aggregation of subunits. This was apparent from the SDS-PAGE blot where any one band contained a mixture of N-termini also found in common with other bands.

When the sequences TPKYDKYAFDAPL and TDKYDKYAFDAPL were searched for using the Blitz Database, no match with internal sequences of a variety of non-human proteins was found. This suggests that these sequences from almond meal and almond emulsin respectively, perhaps belong to PNGase A, since activity has been linked to both.

CHAPTER VII

DISCUSSION

At the end of preparations 3 and 4, a single active band of high concentration had still not been isolated by SDS-PAGE. Not surprisingly, there appeared to be changes in mobility of some bands in the SDS gels, during the progression of the purifications which were all very long, taking months rather than weeks. This could be due to a number of reasons:

1. The enzyme may have been hydrolysed by proteases but a fragment possessing activity remained in the mixture, tightly associating with other proteins or fragments of the same or different proteins.

2. One of the bands represents PNGase A and the rest are impurities which strongly associate with the active enzyme.

3. All visible bands are impurities and the PNGase is present in such low concentrations it is completely masked or not visible with Coomassie Blue R_{250}.

The bands representing the approximate molecular weights of proteins present in each sample after a particular chromatographic method are summarised in *table 7.1*.

Table 7.1 Approximate molecular weights of proteins present in samples after each chromatographic method.

Prepara -tion	Chromato-graphic step	Bands present in active samples (kDa)	Bands present in inactive samples (kDa)
1 Pool 2	Q-Sepharose	66 58 27	
	Gel filtration	66 61 46 27 25	66 61 46 4037 31
	Mono Q	66 51 39 27 25	60 54
	Gel filtration	66 51 4239 27 18	25
Pool 1	QAE	66 27 25 16	
	Gel filtration	54 27 25	211815
	Silver stained	66 62 54 45 4238 27 25	
2	Phenyl seph	66 62	
	P-superose	66	
	A/Sulphate	66 54 23	
3	CM-Sephadex	66 54 49 42 31 27 25	58 54 4238 34 25 18
			66 27 25 18
	P-superose	62 54 49 42 27 25	41 -31 27 25
	N-term blot	62 54 49 42 32 27 25 15	
	Gel filtration	58 27 25	58 45 42 27 25 1512
	Urea gel	62 54 45 33 27 25	27 25
	G.F (SMART)	62 54 50 38 27 25	66 62 54 38 27 25
4	CM-Sephadex	61 4945 35 27 23 19	61 45 38 27 231915
		61 54 49 4038 27 2523 19	61 4945 42 27 19
	BME/NaSCN	54 42 31 27 23 19	2319
	P-Sepharose	66 27-23 1915	27 19
	Gel filtration	53 35 27	
	G.F rechromat	62 54 49 42 27 25 12	
		62 54 42 27	
	G.F (SMART)	59 41 31 27 25 1712	66 54 19
	Con A	66 56 49 41 27 25 1915	
	Ultrafiltr 30 K	66	
	Urea to SDS	64 58 27 25	66 54
	Native to SDS	66 59	
		62 54 49 42 27 25	
	SDS blot		

Note: Slight differences in molecular weight (1-2 kDa) may be due to non-uniformities of the gel. The molecular weights are grouped accounting for small differences between different gels, which are not meaningful. The groups are:

66-64	62-58	59-57	54-51	50-45
42-38	35-31	27-23	19-12	

A comparison of the groups of bands from both active and inactive samples, it shows that both samples have the same bands in common. Therefore, no band could be unambiguously labelled as that representing the protein responsible for activity. As it is possible to have two bands of the same molecular weight, representing two different proteins, future work could involve sequencing the bands seen in SDS blots of the inactive samples and comparing these to the sequences of the same molecular weight in the active samples. This way, bands of particular molecular weight and sequence can be characterised and accurately identified as being inactive. Due to the number of inactive samples in each preparation, this seemed to be beyond the budget of this investigation.

Table 7.1 also showed bands of low molecular weight appearing at later stages of each preparation as well as shifts in the molecular weights of proteins. These results indicate evidence of proteolytic degradation. It should be noted that many combinations of these bands either add up to other bands seen in the same protein sample, or to the proposed molecular weights of PNGase A (66 kDa by Taga *et al.*, 1984 and 79.5 kDa by Plummer *et al.*, 1987) as well as other proposed molecular weights mentioned in *section 1.3.4.* However, there is no direct evidence that these proteins may associate as such to make up these combinations.

Further evidence for the occurrence of proteolysis came from the observation of degradation of a glycosylated protein during the preparation, as monitored by DIG staining. *Figure 5.13* showed a 54 kDa protein (almond meal) to be glycosylated. A later DIG stain on the almond meal sample showed the molecular weight of the glycosylated protein had dropped to 38 kDa (*figure 5.23*).

Native gel trials have also shown differences in mobility of the proteins showing activity (*table 6.5*), a further indication of the occurrence of slow proteolysis. Breakdown would have affected both the net charge on the protein and the molecular weight, making identification of breakdown products difficult.

Proteolytic degradation could have been prevented by including a 'cocktail' of proteolytic inhibitors (such as those suggested by Deutscher,1990). Although 1 mM

PMSF was included in the earlier procedures to inhibit serine proteases, this has a short half life, measured in minutes in aqueous solutions of high pH. Deutscher (1990) suggested the use of PMSF with 3 other competitive inhibitors, such as benzamide and ε-amino-n-caproic acid. Pepstatin could have been included to inhibit the activity of aspartic proteases, which are most active at low pH, which was predominantly used throughout these preparations. Cysteine (thiol) proteases would be inhibited by the inclusion of sodium p-hydroxymercuribenzoate (PHMB), the competitive inhibitors leupeptin and antipain or the covalent inhibitor iodoacetate. EDTA was used throughout the preparation but this does not chelate Mg^{2+} ions, which are often involved in activation of metalloproteases. EGTA, which does chelate Mg^{2+} ions, could be used in the future.

The sequenced 42 kDa protein had an N-terminus that matched that of an active protein of 66 kDa, polished by high resolution gel filtration chromatography (SMART) and derived from almond meal (*section 5.1(m)*). Both N-termini also matched that from an almond emulsin protein of 66 kDa which was electroeluted from a native gel (*section 6.1(l)*) and had the correct activity (previously shown by mass spectroscopy, *section 3.1(g)*).

Strong interactions between proteins were evident throughout the purification and were quite likely to be hydrophobic in action, since salt was used in nearly all chromatographic procedures. Proteins which carry out the same function often have similar structural features. For example, the structure of lactoferrin has many features in common with other iron-binding proteins (transferrins) and bacterial periplasmic binding proteins specific for L-arabinose, D-galactose, leucine/isoleucine/valine and sulphate (Anderson *et al.*, 1987 and Baker *et al.*, 1987). Each of these proteins is similar in size to a single lobe of lactoferrin and they possess similar domain structures, substrate binding and conformational changes. It would therefore not be unreasonable to assume that there would be similar structural features between PNGase F and A. PNGase F and A are both carbohydrate-binding proteins and this category of proteins and enzymes are known to possess similar structural features. PNGase F possesses two domains of all β-structure (β jelly roll configuration) and has a large cleft in the interdomain region which contains many hydrophobic residues and is a putative substrate binding site (Norris *et*

al.,1994). This structure imparts a hydrophobic nature to PNGase F that is exploited in its purification. While PNGase A is thought to be much larger than PNGase F, a similar domain may impart similar hydrophobic properties which have been exploited in the hydrophobic interaction chromatography. However, this also means that these proteins bind to other proteins through hydrophobic interactions, particularly at high salt. Chromatography trials without salt or the presence (in low concentrations) of a non-ionic detergent are suggested for future preparations.

Although PNGase activity appeared to bind to a Concanavalin A column, the glycosylated protein was not separated from the other proteins. Separation was expected to occur even though a band matched to activity in a urea gel did not discretely stain positive for carbohydrate presence, using DIG (the stain was diffuse in the general area of the active band, *section 6.1(k)*). Other proteins seemed to associate with this protein, even though the solution was of low ionic strength and the pH was only slightly acidic (pH 6.5). The proteins of molecular weights 27 and 25 kDa, were present in every SDS gel, in both active and inactive fractions, throughout the purification and were not removed (together with a few other low molecular weight proteins) by ultrafiltration (30 and 50 K) or gel filtration chromatography.

The presence of contaminants should not be ruled out. The N-terminal sequencing of bands in the SDS gel blot did not give clear sequences (*section 6.1(m)*). In other words, a number of different interpretations of the data could be made. However, the N-terminal sequences for the protein sample from gel filtration (SMART) as well as that from the blot from the native gel were unambiguous. The blot from the native gel had fewer bands compared to the blot from the SDS gel and since the sequence of the former was unambiguous, the SDS sample buffer and heating may be causing breakdown of the proteins. The urea gel trials (*section 6.1(k)*) showed that activity could be matched to a band, from both an emulsin and almond meal source and that these both showed very close sequence homology (*section 6.1(m)*).

There were however, many conflicting results. Taga *et al.*,(1984) and Takahashi and Nishibe (1981) proposed the isoelectric point of PNGase A to lie within the pH range

7.7-8.7. It was found that PNGase A (in the net positive form) bound to CM-Sephadex at both pH 5 and 6 but did not bind at pH 7 and above, which indicated an isoelectric point less than pH 7.0. In contrast, PNGase A did not bind to the anion exchanger DEAE, at pH 8.8, which meant that it was not negatively charged at this pH, indicating its isoelectric point was greater than pH 8.8. A possible explanation is the hydrophobic association between the active protein and other proteins which bind to the resins under different conditions. The isoelectric points would seem to differ. Throughout these preparations it had been observed that some proteins (the 27 and 25 kDa proteins for example) associate strongly and were not eliminated by size exclusion, hydrophobic interaction or ion exchange chromatography. Therefore different experimental conditions could deliver results based on the properties of proteins other than PNGase A, which remains tightly associated throughout different purification steps.

Trials with cation exchange showed that PNGase A binds to CM-Sephadex at pH 6.0 below. However, so do many other proteins. In contrast, although PNGase A does not bind at pH 8.0, more total protein in the mixture does, indicating this may be a useful step. A first step involving batch ion exchange at pH 5, could be used to get rid of a large amount of contaminating proteins which do not bind (as seen in *figure 5.5*). This could be followed by column chromatography using CM-Sephadex pre-equilibrated at pH 8.0.

The buffers should initially contain the protease inhibitors aforementioned and the NaSCN/BME incubation stage should also be performed early, since most proteins will not tolerate long exposure to this chaotropic salt. Polyphenols are present in the vacuoles of plant cells and can bind to and may inactivate enzymes during extraction. Inactivators such as 1.5% insoluble poly(vinylpyrrolidone)borate could be used in the extraction buffer (Deutscher, 1990).

It has been found throughout the course of these preparations, that PNGase A retains its activity in the presence of many impurities, some of which are likely to be proteases, for at least 18 months. This indicates the enzyme or active fragment is very stable and that the active domain of the protein is very well protected.

While this project had not succeeded in purifying the protein, it has laid the foundation for future successful purifications. Using refinements to the method noted above, it should be possible (despite the difficulties in purifying this protein), which will enable a full investigation of its biochemical properties to be undertaken.

CHAPTER VII

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