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Glycosylation Of Peanut (arachis Hypogaea L) Peroxidases

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GLYCOSYLATION OF PEANUT (*Arachis hypogaea* L.) PEROXIDASES

by

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ABSTRACT

The cationic (C.PRX) and the anionic (A.PRX) peroxidase isozymes secreted into the medium of cell cultures are both 20% glycosylated proteins. They are stable and occur in high amounts (C.PRX, 5 mg and A.PRX 0.5 mg per litre of spent culture medium), and so provide an ideal system for the study of the effect of glycosylation on the structure and function of glycoproteins.

A novel method to detect glycoproteins and glycopeptides is proposed using periodic-acid Schiff's reagent dot-blotting assay on nitrocellulose membrane. At least 0.1 ug of glycan can be detected within 40 minutes.

The amino acid sequence of C.PRX is confirmed to be the same as the base sequence of cDNA clone prxPNC1 by the amino acid sequencing of the smaller formic acid cleaved peptide fragment. Out of five potential N-glycosylation sites only three, Asn_{60}, Asn_{144} and Asn_{185} are used. Both Asn_{209} and Asn_{275} have a proline residue at the C-terminal of the consensus sequence and are not glycosylated. Computer modelling showed that Asn_{60} and Asn_{185} are located in hydrophillic ß-turns, while Asn_{144} in a hydrophobic ß-sheet. The glycosylated asparagines present a preference for ß-turns.

On the basis of Concanavalin A binding, C.PRX is fractionated into two forms, either binding, (CP+), or not binding, (CP-), to Concanavalin A column. Further investigation showed that CP- and CP+ have the same peptide chain and differ in carbohydrate moiety. All the three glycans of CP- and CP+

probably have the same core structure but different lengths and terminal sugars. This microheterogeneity of glycans is caused by a co-secreted ß-galactosidase, which has two isozymes with mass 60 kd, pI 7.3 and mass 66 kd, pI 7.6 individually.

The antisera against TPCK-tryptic glycopeptides of C.PRX and A.PRX were raised and the antibodies directed to glycans were purified. The epitope mapping showed that the immunogenicity of the three glycans of C.PRX is similar and is mainly directed towards the core structure $[Xyl](Man)_3[Fuc](GlcNAc)_2$. The carbohydrate moiety of many plant proteins may be the basis of often observed antigenic cross reaction among different glycoproteins that are distinct in both function and structure of peptide moiety.

PNGase eliminates C.PRX enzyme activity and this reaction is dose- and time- dependent. Antibodies directed towards glycans of C.PRX or A.PRX do not block enzyme activity, but the antibodies against whole C.PRX or A.PRX inhibit enzyme activity by 60% and 30% respectively. Glycans are probably not directly involved in the enzyme active centre but play a role in maintaining the conformation of the peptide. Removal of glycans may cause a change of the 3-D structure of the peptide and the loss of calcium and heme.

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Abbreviations

Ab	Antibody
Ag	Antigen
anti-GLa	polyclonal antibodies against GLa
anti-GLb	polyclonal antibodies against GLb
anti-GLc	polyclonal antibodies against GLc
anti-GPa	antiserum against GPa
anti-GPb	antiserum against GPb
anti-GPc	antiserum against GPc
A.PRX	Anionic peanut peroxidase
BNG	6-Bromo-2-naphthyl-ß-D-galactopyranoside
BSA	Bovin serum albumin
CcP	Cytochrome c peroxidase
CD	Circular dichroism spectroscopy
CM	Carboxyl methyl
C.PRX	Cationic peanut peroxidase
CPz	Strongly bound fractions on CM column
Con-A	Concanavalin A
DEAE	Diethyl amino ethyl
EDC	N-ethyl-N'-(3-dimethyl-aminopropyl)

	carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
F-C.PRX	PNGase treated C.PRX
Fuc	Fucose
GLa	Asn-glycan fragment of GPa
GLb	Asn-glycan fragment of GPb
GLc	Asn-glycan fragment of GPc
Glc	Glucose
GlcNAc	N-acetyl-glucosamine
GPa	TPCK-tryptic glycopeptide a of C.PRX
GPb	TPCK-tryptic glycopeptide b of C.PRX
GPc	TPCK-tryptic glycopeptide c of C.PRX
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IAA	Indole-3-acetic acid
IEF	Isoelectrofocusing
IgG	Immunoglobulin G
kd	Kilodalton
Man	Mannose
NC	Nitrocellulose membrane
NMR	Nuclear magnetic resonance

PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic-acid Schiff
PBS	Phosphate buffered saline
PNGase	Peptide-N-glycosydase F
RZ	Reinheits Zahl, A_{405}/A_{280}
R-C.PRX	Reconstituted F-C.PRX
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TFMS	Trifluoromethane sulfonic acid
TPCK	l-1-tosylamido-2-phenylethylchloromethyl ketone
Tris	Tris-(hydroxymethyl)-amino methane
Xyl	Xylose

CHAPTER 1

General Review

1.1. Peroxidases: function and structure

Peroxidases (donor: H_2O_2 oxidoreductase; EC 1.11.1.7) were among the first enzymes to be discovered. In fact, the oxidation of guaiacol by hydrogen peroxide was observed by Schonbein as early as 1855 (Saunders et al., 1964). Peroxidases are distributed throughout in living organisms, suggesting that they must be an essential part of all living systems.

Plant peroxidases play important roles in the synthesis and degradation of phytohormones that modulate cell differentiation and growth. Peroxidases degrade auxin, indole-3-acetic acid (IAA) to form either indole-3-acetaldehyde or methylene oxindole through a decarboxylation pathway (Kenten, 1955; Grambow, 1986; Grambow and Langenbeck-Schwich, 1983; Richard and Job, 1974; Brennan and Jacobs, 1983; Sanchez-Bravo et al., 1989; Ernstsen et al., 1987). Peroxidases also catalyze the synthesis of plant hormone ethylene from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Kende, 1989; Apelbaum et al., 1981; Yang and Hoffman, 1984). It is postulated that peroxidases catalyze isodityrosine cross-linking of extensin (Lamport, 1986; Fry, 1987; Zheng and van Huystee, 1991) and diferulate cross-linking of matrix

polysaccharides in the plant cell wall (Fry, 1979, 1986; Shibuya, N. 1984) and thereby control the future shape or size of the cell. Infections of plants by parasitic organisms, e.g. phytopathogenic fungi, bacteria, or viruses generally cause the increase of peroxidase activity. Induced lignification and cross-linking of phenolics or proteins in the plant cell wall by peroxidases provide structural resistance against invading parasites (Gaspar et al., 1982; Biggs and Fry, 1987; Burg, 1962; Imberty et al., 1985). In addition, peroxidases can in themselves be toxic to microorganisms (Chen and Varner, 1985; Epstein and Lamport, 1984; Evans and Himmelsbach, 1991) or produce antimicrobial compounds, such as peroxidative products of phenolic compounds, superoxide anion and hydrogen peroxide (Grambow, 1986). It was reported that peroxidases are involved in various stress responses, such as detoxification of sulphur dioxide and ozone (Pfanz et al., 1993; Alonso et al., 1993), salt tolerance (Dubey, 1993; Valpuesta et al., 1993).

As peroxidases show dramatic colour product formation as a result of their catalytic effect and broad substrate specificity, they are widely used in biotechnology and diagnostic tests (Krell, 1991; Lobarzewski et al., 1988, 1990).

Peroxidases are heme-, calcium- and glyco- proteins. They have similar sizes (from 40 to 50 kd), 2-5 calcium ions per molecule and 20% carbohydrate (Gaspar et al, 1982; Welinder, 1985). The prosthetic group of heme is known to be ferriprotoporphyrin IX. In horseradish peroxidases, peanut peroxidases and other classic peroxidases the heme is linked with the protein envelope

through histidine and catalyses the H_2O_2-dependent one-electron oxidation of the small substrate. Cytochrome C peroxidase CcP has cysteine thiolate as the heme ligands and transfers oxygen from molecular oxygen or alternative oxygen do ors to the large substrate, cytochrome C (Mr = 12,400) (Ortiz de Montellano et al., 1987). The protein envelope plays a key role in the enzyme structure and catalytic mechanism (Ator et al., 1987, Miller et al., 1990; Poulos et al., 1978). Site-directed mutagenesis technique has been used to characterize the role of key amino acids of the protein envelope (Wang et al., 1990; Smulevich et al., 1988a, 1988b; Smith et al., 1990; Fishel et al., 1987; Sivaraja et al., 1989).

Nucleotide sequences for genes encoding peroxidases or their corresponding cDNA have been cloned from horseradish (Fujiyama et al., 1988, 1990; Bartonek-Roxà et al., 1991), peanut (Buffard et al. 1990), Thale cress (Intapruk et al., 1991), barley (Johansson et al., 1992; Rasmussen et al., 1991), cucumber (Morgens et al., 1990), wheat (Rebmann et al., 1991), potato (Roberts et al., 1988; Roberts and Kolattukudy, 1989) and tobacco (Lagrimini et al.,1987). Yeast cytochrome c peroxidase (CcP), horseradish peroxidase E5, their site-directed mutants, their enzyme-substrate and enzyme-inhibitor compounds have been investigated with X-ray crystallography (Edwards et al., 1984, 1987, 1988, 1989; Finzel et al., 1984; Erman et al., 1989; Morita et al., 1991; Poulos, 1988; Poulos et al., 1980). Those data greatly increased the knowledge of the three dimensional structure and enzyme reaction mechanism.

The comparative studies on amino acid sequences, secondary structure predictions and structure modelling based on the crystal structure revealed that plant peroxidases are indeed structurally homologous to yeast CcP. The central core structure of plant peroxidases is the most conserved. The amino acid sequences of substrate binding region, heme binding region, acid/base catalysis region are more conservative. But the surface segments showed little similarity and might pack differently in yeast CcP and plant peroxidases.

The number of glycans of plant peroxidases varies even within the isozymes from the same sources. The carbohydrate sequences of major N-glycans of horseradish (McManus et al, 1988; Ashford et al., 1987) and barley (Johansson et al., 1992) peroxidases have been recently elucidated. Only N-linked glycans were present. However, little precise information on the structure and function of glycans of peroxidases has been known so far. That has led us to the following study.

1.2. Peanut peroxidases: cell culture and peroxidase purification

Peanut cells derived from cotyledon slices (*Arachis hypogaea L.* var Virginia 56R) were routinely cultured in a modified Linsmaier and Skoog medium (Verma and van Huystee, 1969; Linsmaier and Skoog, 1965) with continuous shaking (150 rpm) under constant light at 22^{0}C. The cells were subcultured every two weeks using 25 mL of 14 day-old cell culture and 225 mL fresh medium in 500 mL flasks (Kossatz and van Huystee, 1976) for 14

days (Hu et al., 1987). The spent medium has proved to be an enriched source of peroxidases. The cells secrete around 30 peptides into the medium (van Huystee and Tam, 1988), among which two were identified as peroxidase isozymes, anionic peroxidase (A.PRX) and cationic peroxidase (C.PRX) (Maldonado and van Huystee, 1980). A.PRX and C.PRX account for 2% of the total proteins in the medium and give the yields of 5 mg of C.PRX and 0.5 mg of A.PRX per litre of spent medium (van Huystee et al., 1990). The purification of peroxidases started with filtration of the 14 day-old culture through Whatman No.1 filter paper. The filtrate was brought to 70% acetone and then centrifuged at 9000 X g for 15 min. The pellet was resuspended in 0.02 M Na-acetate buffer, pH 5.0 and centrifuged at 13000 X g for 10 min.. The supernatant was brought to 80% ammonium sulphate and centrifuged at 13000 X g for 10 min.. The pellet was resuspended in 0.02 M Na-acetate buffer and dialysed against water overnight. The dialysate was centrifuged to remove any insoluble matter and the supernatant was ready for CM column. A series of chromatographies were used for the purification and summarized in table 1.1.

Table 1.1 Chromatographies for purification of peanut peroxidases*

Column	CM	Con A	G75	DEAE
Flow rate (ml/min)	1.5	1	1	1
Fraction size (ml)	5	5	5	5
Equilibrate & washing buf.	0.02 M Na-acetate, pH 5.0	TCM-saline	7% isopropanol	0.005 M Tris-HCl, pH 8.4
Bound protein eluted with	gradient of 0.02 M -- 0.10 M Na-acetate, pH 5.0	TCM-saline, o.1 M methyl-α-D-glucopyranoside		gradient of 0.025 M -- 0.15 M NaCl in 0.005 M Tris-HCl, pH 8.4
Column regenerated with	1 M Na-acetate, pH 5.0	0.1 M Na-acetate, 0.5 M HCl, pH 5.0		0.005 M Tris-HCl, 1M NaCl, pH 8.4

*1. Purification scheme for C.. RX: Carboxyl methyl-Sephadex (CM) (bound fraction) ⟶ Concanavalin A-Sepharose 4B (Con A) (flow through) ⟶ Sephadex G-75.

2. Purification scheme for A.PRX: CM (flow through) ⟶ Diethyl amino ethyl (DEAE).

3. Eluents from all columns were detected at 405 nm and 280 nm.

4. TCM-saline buffer (0.01 M Tris-HCl, pH 7.5 containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$).

1.3. Peanut peroxidases: characterization and biosynthesis

As other peroxidases, A.PRX and C.PRX are heme-, calcium, and glycoproteins. A.PRX has a mass of 42 kd, a pI of 4.3-4.6 and 5 calcium ions per molecule. C.PRX has a mass of 40 kd, a pI of 8.9 and 2 calcium ions per molecule. Both have a pH optimum of 5.0 and are glycosylated around 20% by weight (Hu, 1989).

In peanut leaves only 0.2% of protein is peroxidase, but in the culture medium it is 10 fold higher (Chibbar and van Huystee, 1983a; Stephan and van Huystee, 1981). The enhanced synthesis of peroxidases is believed to be due to the greatly enhanced extracellular space (van Huystee and Esnault, 1992). The synthesis of peroxidase in cultured peanut cells has been demonstrated by immunoprecipitation to occur on free polysomes rather than on membrane-bound polysomes (Stephan and van Huystee, 1980. 1981; van Huystee, 1987). The prosthetic group heme is synthesized in the mitochondria as in animals, but the precursor is glutamic acid rather than glycin-succinyl-CoA as in animals (Chibbar and van Huystee, 1983b). The heme group causes the brown colour of peroxidases and contributes an absorbance peak at 405 nm. The Reinheits Zahl (RZ) values of A_{405} / A_{280} are often used for purity estimation of peroxidases (Chibbar and van Huystee, 1984a). When peroxidase is stored at low concentrations the RZ value is readily decreased unless 10 mM Ca^{2+} is present (van Huystee et al., 1990). A similar case occurs for α-amylase. When it is stored in low calcium concentrations the molecule changes conformation

(Bush et al., 1989). So it is possible that when the surrounding calcium concentration is low the tertiary structure of peroxidases uncoils slightly and consequently is apt to lose the heme moiety (Coll, 1987; van Huystee, 1990; van Huystee and Esnault, 1992). The synthesis of glycans is discussed in section 1.5.

1.4. Glycoproteins: carbohydrate-protein linkages

Glycoproteins are polymers which have one or more carbohydrate chains (oligosaccharides, glycans) attached to a polypeptide. Although at least 200 different monosaccharides are found in nature only 11 are known to occur in glycoproteins (Sharon and Lis, 1981). The oligosaccharides of glycoproteins are classified according to the linkage between sugar and amino acid (Kornfeld and Kornfeld, 1980; Schachter, 1984; Beely, 1985a; Montreuil et al., 1986a). There are mainly two types of primary covalent linkages (N-glycosyl and O-glycosyl) leading to the definition of two classes of glycoproteins (N-glycosylproteins and O-glycosylproteins) and two glycan types of glycoproteins (N-glycans and O-glycans). The only N-glycosidic linkage found in glycoproteins is N-acetyl-glucosaminyl-asparagine. In contrast, the O-glycosidic linkage is much more diverse and is commonly subgrouped into four types (Montreuil et al., 1986a): (1) Mucin type: the alkali-labile linkage between N-acetyl-D-galactosamine and serine or threonine is found mainly in mucins lining the mucous epithelia of the respiratory, genitourinary and gastro-intestinal

systems, and also occurs in immunoglobulins, glycophorin and other molecules (Schachter, 1984). (2) Collagen type: the alkali-stable linkage between D-galactose and 5-hydroxy-D-lysine is found only in the short carbohydrate chains (glucosyl-galactosyl-hydroxylysine and galactosyl-hydroxylysine) of collagens and basement membranes (Schachter, 1984). (3) Proteoglycan type: the alkali-labile linkage between D-xylose and serine is involved in the acidic mucopolysaccharide-protein bond of proteoglycans found predominantly in mammalian connective tissue (Beely, 1985a). (4) Extensin type: the alkali-stable linkage between L-arabinofuranose and 4-hydroxy-L-proline is found in extensin and other plant glycoproteins (Beely, 1985a).

N-glycans are distributed in mammalian and plant glycoproteins more commonly than O-glycans. The structures of a number of N-glycans have been determined and the great majority of these structures seems to fall into three categories which contain the common core pentasaccharide Manα1→3(Manα1→6)Manß1→4GlcNAcß1→4GlcNAcß1-Asn : (1) Simple type or high mannose type: the glycans have a core structure substituted only with mannose residues. (2) Complex type: the glycans have a core structure and both of the α-linked core mannoses are substituted with one or more of the sugars of GlcNAc, Gal, Fuc, sialic acid etc.. Plant complex type glycans often have a fucose and a xylose attached to the core pentasaccharide (Sturm, 1991; Fourent et al., 1987; Hase et al., 1986; Kitagaki-Ogawa et al., 1986). (3) Hybrid type or mixed type: the glycans have a core structure and one α-linked core

mannose has substituents of the "high mannose" type while the other α-linked core mannose has substituents of the "complex" type.

As peanut peroxidases have only N-glycans as shown in the following chapters, I will mainly focus on the N-glycosylation of glycoproteins.

1.5. Glycoproteins: biosynthesis of N-glycans

Even though there is a great variety of structures in N-glycans, all the structures originate from a common precursor and the biosynthesis pathway has been delineated from this precursor through the high mannose type structures to the complex type structures, with branch points to hybrid type structures (Kornfeld and Kornfeld, 1985; Faye et al., 1989; Schachter, 1984; Sharon and Lis, 1981). The pathway is described below in three parts:

1. Synthesis of the dolichol-linked precursor, $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$-ppDol and transfer of the precursor to protein.

Dolichol {CH_3-$C(CH_3)$=CH-CH2-[CH_2-$C(CH_3)$=CH-CH_2-]$_n$-CH_2-$CH(CH_3)$-CH_2-CH_2OH} phosphate serves as a lipid carrier in glycoprotein biosynthesis. The sugar residues of the dolichol-linked precursor are added one at a time by glycosyltransferases from a carrier molecule. The carrier molecule for the first seven monosaccharides is a nucleotide diphosphate sugar (UDP-GlcNAc or GDP-Man), and for the last seven monosaccharides is a derivative of dolichol phosphate (dolichol phosphate mannose or dolichol glucose). The glycosyltransferases are bound to the rough endoplasmic reticulum (RER)

membrane and are highly specific, and thus determine the order of addition of monosaccharides and the specific linkage of one sugar to another. The precursor oligosaccharide $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$ is transferred from the dolichol diphosphate donor to the asparagine residue in the consensus sequence -Asn-Xaa-Thr/Ser- of nascent polypeptide by oligosaccharyltransferase. The transfer of the precursor oligosaccharide in RER occurs usually cotranslationally, although it may occur shortly after translation. Then, the oligosaccharide precursor undergoes a series of processing reactions by exoglycosidases and glycosyltransferases and produces a large variety of structures of oligosaccharides. The processing reactions begin in RER and continue in Golgi apparatus when the glycoprotein traverses the stack of Golgi from the cis through medial to trans cisternae by vesicular transport before it is sorted to the ultimate destinations, such as secretory vesicles, lysosomes and vacuoles.

2. Trimming of the Asn-linked oligosaccharide precursor and the formation of the high mannose type glycans.

The trimming of the precursor begins with the removal of the three glucose residues by glucosidase I and glucosidase II, followed by the cleavage of a single mannose in the middle branch by a mannosidase. The resulting Man_8-$GlcNAc_2$-Asn is transported from RER to Golgi apparatus. There Golgi α-mannosidase I trims three more mannose residues from the precursor. In the absence of further processing, the resulting Man_5-$GlcNAc_2$-Asn is mature and is

found as one of the structure of high mannose type glycans in glycoproteins. In some cases, sulfate, phosphate, fucose and additional mannose may be added to this structure to form other high mannose type structures.

3. Formation of complex type and hybrid type glycans

In the Golgi apparatus, the first step in the conversion of the high mannose structure to complex or hybrid structure is the addition of a GlcNAc to the α1,3-linked core mannose by GlcNAc-transferase I. A complex glycan will be formed if the next reaction is the removal of the other two mannoses by Golgi α-mannosidase II, followed by the addition of another GlcNAc to the α1,6-linked core mannose by GlcNAc-transferase II. If the two mannoses are not cleaved, or cleaved but no GlcNAc is added to the α1,6-linked core mannose because of the absence of GlcNAc-transferase II, the oligosaccharide will have mannose on one arm of the core and GlcNAc initiated antennae on the other, and will be a hybrid glycan. Other GlcNAc-transferases may also add GlcNAc to any of the three core mannoses and initiate as many as 5 antennae (Takasaki et al., 1980). In complex or hybrid glycans, each of these GlcNAc initiated branches from the core is termed an antenna (the term "antenna" is not used to describe high mannose structures, even if they are branched). Each of the antenna initiated by a GlcNAc may be completed by the addition of other sugars, such as galactose, fucose, sialic acid and etc.. It is GlcNAc-transferases that control the biosynthesis of complex and hybrid glycans and cause the diversification of antennae. So far at least 7 GlcNAc-transferases with

different specificities have been characterized (Schachter, 1984, 1986; Brockhausen, 1989).

1.6. Glycoproteins: function of carbohydrate moiety

The function of glycans of glycoproteins is one of the central problems in contemporary cell biology and has attracted the interest of many researchers. Three approches are frequently used to study this problem: 1. deglycosylation of glycoproteins using chemicals, such as TFMS, periodate, or glycosidases, such as PNGase; 2. using inhibitors such as tunicamycin to inhibit glycosylation during the synthesis of glycoproteins; 3. using site-directed mutagenesis to inhibit glycosylation of glycoproteins. A general pattern of the function of glycans has emerged from the recent surge of interest in this study. Although many aspects are still unknown, the carbohydrate moiety of glycoproteins is no longer an ignored component.

1. Protection of peptide chain against proteolysis

The concept that the carbohydrate moiety protects the peptide chain of glycoprotein against proteolytic attack is generally agreed on by almost all researchers. Enzymatically or chemically deglycosylated peanut peroxidase (Hu and van Huystee, 1989a), DNAase, RNAase B and carboxypeptidase Y (Olden et al., 1982), human chorionic gonadotropin (Matzuk and Boime, 1988); and tunicamycin treated acetylcholine receptor, myoblast fusion protein (Olden et al., 1982), variant surface glycoprotein (Reinwald, 1985), membrane

glycoprotein (Polonoff et al., 1982), cell surface glycoprotein (Olden et al., 1978) are more sensitive to protease. A mutant form of the contact site A protein of *Dictyostelium discoideum* lacking carbohydrate is more unstable than the wild type (Hohmann et al., 1987).

As glycans are often added to the hydrophillic ß-turns of peptide chains (Beely,1977; Anbert et al., 1976), it is proposed that glycans would mask the surface of glycoproteins and prevent access of proteases (Beely, 1976, 1977). For example, the carbohydrate moiety of haemagglutinin of the influenza virus covers 17-20% of the available surface of the protein including many of the potential trypsin and chymotrypsin cleavage sites (Wilson et al., 1981). Arginine and lysine are largely localized on hydrophillic sites of proteins (Janin et al., 1988).

2. Secretion or sorting

Whether glycans are essential for intracellular targeting or secretion is still controversial, and some reports show that glycans are not essential for the secretion or sorting of glycoproteins (Lafayette and Travis, 1988; Voelker et al., 1989). But there are also many references suggesting that glycans are required for secretion and intracellular targeting. The secretion of ß-fructosidase synthesized in suspension-cultured carrot was apparently inhibited by tunicamycin. But pulse-chase experiments showed that the unglycosylated ß-fructosidase did not remain in the cells and seemed to be secreted in the same way as glycosylated ß-fructosidase. It was suggested that the apparent

inhibition of the secretion of ß-fructosidase may be explained by the breakdown of the unglycosylated protein during secretion (Faye and Chrispeels, 1989). In contrast, when both N-glycosylation sites of vesicular stomatitis virus G protein were eliminated using site-directed mutagenesis the unglycosylated G protein could not be transported to the cell surface but remained in the cells (Machamer et al., 1985).

3. Induction and maintenance of biological activity

In some cases, glycans are nessesary for the expression of biological activity of glycoproteins. Tunicamycin treatment completely inhibited the secretion of active acid phosphatase synthesized by yeast protoplasts but had no effect on protein synthesis. The nonglycosylated acid phosphatase was accumulated inside the protoplasts and was enzymatically inactive (Mizunaga and Noguchi, 1982). Thyrotropin (TSH), a dimeric glycoprotein hormone, has two complex type N-glycans on the α-subunit and one on the ß-subunit. The removal of the glycans by PNGase did not affect their receptor binding activity, but greatly decreased the hormone stimulation action (Thotakura et al., 1992). The catalase inhibitor from maize scutella has 4 galactose residues per subunit. Removal of the carbohydrate with ß-galactosidase or blockage with a galactose-specific lectin abolished the biological activity of the inhibitor (Tsaftaris et al., 1980). Probably human tissue plasminogen activator (tPA) is the first example in which the unique biological properties of a glycoprotein have been shown to be dependent on the structural features of the oligosaccharides at

distinct positions within the peptide. When the glycans at glycosylation sites 184 and 448 were altered either to high mannose type with the treatment of deoxymannojirimycin (dMM) or to neutral, unsialated forms with the treatment of neuraminidase, dMM treatment was found to increase both lysine affinity and the fibrin-stimulated catalytic activity of tPA. Neuraminidase treatment increases the catalytic activity, but was without effect on affinity for lysine. A further study showed that the modification of glycans at glycosylation sites 184 and 448 has different effects on the catalytic activity and lysine affinity (Howard et al., 1991). Why are the biological activities of glycoproteins related to carbohydrate moiety? Probably the change of the glycan structure or the removal of glycans directly or indirectly induces a conformational alteration in the protein structure. Antibodies against nonglycosylated envelope proteins of Semliki Forest virus do not react with their native glycosylated counterparts, but do react with the high mannose type precursor of the glycoproteins. This suggests that maturation of the carbohydrate chain confers conformational features on the mature glycoprotein not present in the nonglycosylated species (Schwarz et al., 1979). This can not be resolved definitively until the tertiary structure of glycosylated versus nonglycosylated (or modified) forms of glycoproteins are compared (Olden et al., 1985).

4. Recognition

Sugar chains of glycoproteins and glycolipids are predominant features of the surface of cells. Thus it is not surprising that they serve as cell surface

ligand for blood group and tumor associated antibodies, viruses, cell attachment proteins of some bacteria (Feizi et al., 1984; Paulson, 1989; Sharon and Lis, 1981).

5. Immunogenicity

Glycans of glycoproteins are important immunogenic components. This will be discussed in chapter 4.

1.7. Glycoproteins: glycosylation engineering

The investigation of structure and function relationships of carbohydrate has flourished in recent years and a new field of glycosylation engineering is emerging. The engineering of the peptide moiety of glycoproteins is straightforward, but altering the covalently attached carbohydrates is more complicated because they are the products of a series of transferases and glycosidases encoded in the cellular genome (Stanley, 1992).

The advantages of glycosylation engineering include: (i) to alter the glycan structure to obtain optimally active biomolecules, for example the sequential treatments of glucocerebrosidase purified from placenta with sialidase, ß-galactosidase, and ß-hexosaminidase produce a more effective form of the enzyme to treat patients with Gaucher's disease (Barton et al., 1991); (ii) to reduce or eliminate the heterogeneity inherent in carbohydrates of mature glycoproteins in order to aid in purification (Stanley, 1989); (iii) to take advantage of carbohydrates in purifying a glycoprotein by lectin affinity; (iv) to

reduce the carbohydrate complement of glycoprotein to a minimum to aid in crystallization; (v) to reduce the carbohydrate complement of a glycoprotein to lower the immune response.

The methods used in glycosylation engineering may include: (i) production of glycoproteins in specific host cells expressing different glycosyltransferases and glycosidases to alter glycan structure (Kukuruzinska et al., 1987; Lee et al., 1989 (ii) mutation of cloned glycoproteins to remove or add glycosylation sites (Moonen et al., 1987); (iii) production of proteins in the presence of glycosylation inhibitors; (iv) treatment of a purified glycoprotein with glycosidases or glycosyltransferases (Stanley, 1992)

1.8. Thesis objectives

Glycosylation is a major modification of proteins and glycans perform important biological functions. The research described in this thesis focuses on the structure and function of glycans of peanut peroxidases to increase our understanding of both peroxidases and glycoproteins.

1. Location of glycosylation sites and the relationship between glycosylation and peptide chain structure

Peanut peroxidase is 20% glycosylated based on the loss of molecular weight caused by treatment with TFMS and determined on SDS-gel (Hu and van Huystee 1989a). Horseradish peroxidases have at least 9 isozymes and the number of glycans varies from 1 to 9 among their isozymes (Welinder 1985;

Roberts et al 1988). Two cDNAs for peanut peroxidases were cloned (Buffard et al., 1990). C.PRX and A.PRX were digested and their amino acid sequences were determined and compared with the predicted amino acid sequences by the base sequences of cDNA. As soon as the cDNA sequences are matched with the amino acid sequences of the isozymes, the genetic information of the base sequences will greatly promote the research on the enzymes. C.PRX and A.PRX were digested with trypsin and the tryptic glycopeptides were purified. The amino acid sequences of the glycopeptides were determined to locate glycosylation sites on the peptide chain. To investigate the relationship between glycosylation and peptide structure, a computer modelling program was used to predict the secondary structure of the peptide around glycosylation sites.

2. The heterogeneous glycosylation of C.PRX

There are two different forms of cationic peroxidase, CP- and CP+ based on their Con A binding. The investigation on the enzyme activity, peptide mass, and the Soret band absorption did not reveal any differences between these two forms and they were suggested to be two heterogeneously glycosylated forms of an enzyme. To elucidate their differences the structures of the glycans of CP- and CP+ were studied using lectin technique, size exclusion chromatography, electrophoresis and HPLC. To interpret what causes the heterogeneity of glycans the glycosidase activities in the culture medium were determined and the role of ß-galactosidase in the conversion of

CP- to CP+ was studied.

3. The immunogenicity of glycans

C.PRX and A.PRX showed some immuno- cross-reaction, which is suggested on the basis of their glycosidic side chains (Hu and van Huystee 1989b). To understand the distribution of epitopes on glycans and what caused the cross reaction between C.PRX and A.PRX, glycopeptides were coupled to a carrier protein, antisera directed to individual glycopeptide were raised, and the antibodies directed to individual glycan were purified. Epitope mapping and investigations of the cross reaction were completed using ELISA.

4. The relationship between the enzyme activity and the carbohydrate moiety

It was established that the glycans of peanut peroxidases greatly stabilize the enzymes. To investigate whether glycans are essential for the expression of enzyme activity, the enzyme was enzymatically deglycosylated by PNGase, and then the enzyme activity, the absorption spectra, CD spectra, mass and calcium content of the deglycosylated form were studied.

CHAPTER 2

Improved Determination of Glycoproteins and Glycopeptides by Periodic-acid Schiff's Reagent Dot-blotting Assay on Nitrocellulose Membrane

2.1. Introduction

In recent years, research interest in glycosylation of glycoproteins has intensified because continued investigation of glycoproteins has indicated that the carbohydrate moiety of glycoproteins confers important biological functions (see section 1.3). A sensitive and rapid determination of glycans is desired for the purification and identification of glycoproteins and glycopeptides. In the past few years, several dot blot assay methods to detect glycoproteins have been reported (Faye and Chrispeels 1985; Weitzhandler and Hardy 1990; Hsi et al., 1991; Buee et al., 1991). These methods use mainly lectins to probe glycans and, in some cases, are selective because of the sugar binding specificity of lectin. When we studied the structure and function of glycans of peanut peroxidases, we distinguished glycopeptides by directly dot blotting samples onto nitrocellulose membrane and staining the membrane with periodic acid-Schiff's (PAS) reagent using an improved staining method. We demonstrate here that it is possible to eliminate the time-consuming fixation

step and shorten staining and washing steps. At least 0.1 ug of glycan can be detected within 40 minutes.

2.2. Materials and methods

2.2.1. Cell culture and C.PRX purification

As described in section 1.2.

2.2.2. TPCK-tryptic digestion of C.PRX

To remove heme and denature the protein, C.PRX dissolved in water was treated with 10 volumes of -20^{0}C acidic acetone (0.5% HCl v/v) at ambient temperature for 15 min., followed by centrifugation (Chibbar et al., 1984). The pellet was resuspended in water and lyophilized. The apoprotein was incubated in 0.1 M NH_4HCO_3, pH 8.0 with l-1-tosylamido-2-phenylethylchloromethyl ketone treated trypsin (TPCK-trypsin, Worthington Biochemical Corporation) at 37^{0}C for 40 min. The ratio of C.PRX:TPCK-trypsin is 30:1(w/w). The trypsin was inactivated by boiling for 3 min.

2.2.3. Gel filtration chromatography

The TPCK-trypsin digested peroxidase was applied to Biogel P6 column(1.5 cm x 90 cm, 200-400 mesh, molecular weight range 1,000-6,000 daltons, Bio-Rad laboratories). The column was equilibrated and eluted with

0.1 M ammonium acetate, pH 7, containing 0.02% NaN_3 at room temperature at flow rate 8 ml/h. Fractions of 2 ml were collected . Glycopeptide fractions were identified by dot-blotting assay on NC membranes (section 2.2.5) or by the phenol-sulphuric acid method (section 2.2.6).

2.2.4. Schiff reagent preparation

The Schiff reagent was prepared by the method of Segrest and Jackson (1972) with slight modifications. Five grams of basic fuchsin was dissolved in 1 L of boiling distilled water . After cooling 100 ml of 1 N HCL and 5 g of sodium metabisulfite were added. The solution was left in the dark overnight and then twice vacuum-filtered through 4 layers of Whatman No.1 filter paper after adding 10 g of activated charcoal. The filtrate was clear and colourless.

2.2.5. PAS staining of glycoproteins or glycopeptides on nitrocellulose (NC) membrane

The procedure is based on modified published methods (Segrest and Jackson, 1972; Konat and Mellah, 1984). NC membranes (Schleicher and Schuell, BA 85, 0.45 μm or PH 79, 0.1 μm) were wetted with distilled water, followed by removal of excess water with filter paper. Then, 5 ul of sample solution was placed on the membrane using a Justor 1100 DG digital pipette (Nichiryo Co. LTD.). After air drying, the membrane was soaked sequentially in 0.2% aqueous periodic acid, 0.2% sodium metabisulfite, Schiff's reagent, and

0.2% sodium metabisulfite in 40% ethanol and 5% acetic acid for 10 min, or varying times as indicated for each, at room temperature. The membrane was washed with one change of 10% acetic acid.

2.2.6. Phenol-sulphuric acid color reaction

Sugar detection was done according to a modified method (Dubois et al.,1956). Briefly, 0.25 ml of sample was mixed with 0.3 ml 5% phenol and then 2 ml of concentrated sulphuric acid was added quickly. The absorbance was measured at 490 nm on Shimadzhu UV-160 spectrophotometer after 30 min.

2.3. Results

PAS reagent is widely used to stain glycoproteins. In order to estimate the staining time and sensitivity of dot-blotting assay using this reagent, two well-characterized glycoproteins were employed. Cationic peroxidase has a mass of 40 kd with 20% carbohydrate, and fetuin, around 60 kd, has 6 glycans per molecule, which account for one third of the whole molecular weight (Nilsson et al., 1979; Rupar and Cook, 1982). The reaction product of PAS and glycoproteins or glycopeptides on NC membrane presents magenta spots. Fig. 2.1 shows that there is no significant difference of staining intensity amongst 5, 10, 20, and 40 minute treatments. But, there is a clear difference of staining intensity between treatments with and without sodium metabisulfite.

Sodium metabisulfite appears to enhance staining (Konat and Mellah, 1984). Glycoprotein can be detected within 40 minutes at least. Fig. 2.2 shows that 0.5 ug of cationic peroxidase, equal to 0.1 ug carbohydrate, is the lower level that may be detected. In contrast, 40 ug of cationic peroxidase, equal to 8 ug of carbohydrate, gave only an absorbance of 0.05 at 490 nm with the phenol-sulphuric acid method (Fig. 2.3), another popular sugar-detecting technique. Compared with the phenol-sulphuric method, the dot blotting assay of glycoprotein is more sensitive and less sample-consuming. Fig. 2.4 shows the identification of glycopeptide fractions of TPCK-tryptic cationic peroxidase eluted from P6 column. Both the PAS dot-blotting assay and the phenol-sulphuric acid method revealed that fractions 28-43 contained glycopeptides. The presence of glycosylation of peanut peroxidases can be detected more readily with this approach.

2.4. Discussion

PAS reagent has been employed to stain glycoproteins and other macromolecules with carbohydrate content, such as in the staining of glycoproteins in polyacrylamide gels (Segrest and Jackson, 1972). As the gel impedes the contact of staining reagent with glycoproteins in the gel, a staining procedure takes more time and requires a protein fixation step before staining. NC membranes have a strong binding capacity with proteins and staining chemicals can reach blotted glycoprotein easily. So, it is possible by protein

blotting to eliminate the fixation step and greatly decrease the staining period. Because PAS reagent does not require any specific terminal sugars, it is suitable for staining a variety of glycoproteins. Dot blotting assays for glycopeptides are slightly more complex than for glycoproteins. This is due to the weaker binding of glycopeptides onto the membrane, especially when the glycopeptide has a short peptide and a large glycan. The second issue is that glycopeptides often have a small mass, which allows glycopeptides to travel readily across membranes with larger pores. Therefore, it is important to select the proper membrane. The mass of TPCK-tryptic glycopeptides of cationic peanut peroxidase is around 3000-5000 daltons based on peptide lengths and masses of glycans. The NC membrane with 0.1 um pore is the best-suited membrane for the dot blotting assay according to my experiences. The PVDF (polyvinylidene fluoride) membrane is another choice for PAS staining. Although Nylon membranes have a high binding capacity for proteins, they have a high background when stained with PAS reagent.

As various detergents and buffer reagents such as SDS, Tris, phosphate or acetate do not prevent the binding of proteins onto NC membranes (data not shown), dot blotting assay following chromatography or Western blotting after electrophoresis is a useful tool to detect glycoprotein or glycopeptide fractions. The procedure described here supplies a simple, rapid and sensitive determination of glycoproteins and glycopeptides and will be used extensively for the remainder of this research.

Fig. 2.1. Comparison of varying staining time of dot-blotted glycoproteins on NC membrane with PAS. Five ul of sample (0.4 ug glycoprotein/ul) was dot-blotted on a BA 85 NC membrane. Upper row, cationic peroxidase (C.PRX); lower row, fetuin. The staining time of each step was lane 1: 5 min; lane 2: 10 min; lane 3: 20 min; lane 4: 40 min; and lane 5: 20 min, but without the last treatment of sodium metabisulfite.


C.PRX
Fetuin
1
2
3
4
5

Fig. 2.2. The sensitivity of PAS staining of dot-blotted glycoproteins on NC membrane. Five ul of serially diluted samples containing 5 ug, 3 ug, 2 ug, 1 ug, 0.5 ug of C.PRX or fetuin, respectively, were dot-blotted on a BA 85 NC membrane. Upper row, fetuin; lower row, C.PRX. Each staining step lasted 10 min..

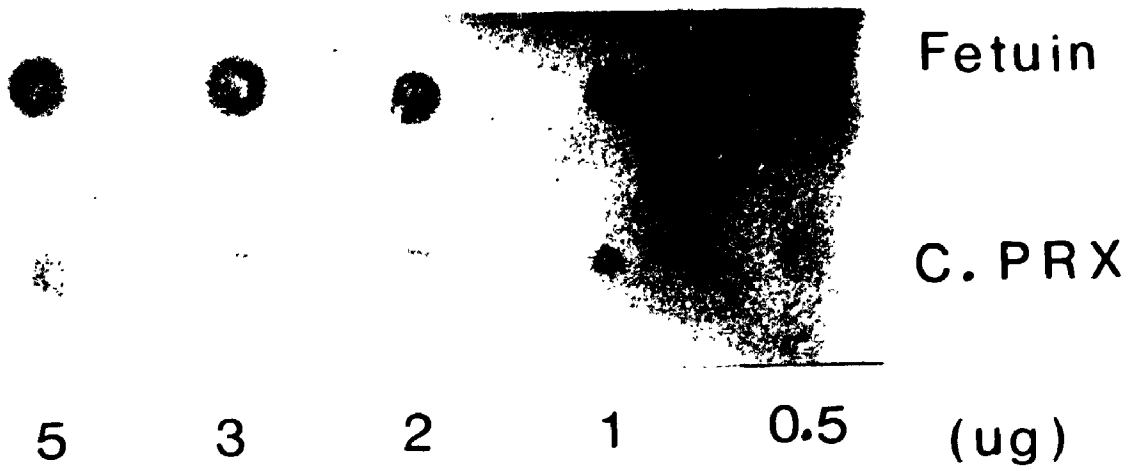

Fetuin
C. PRX
5
3
2
1
0.5
(ug)

Fig. 2.3. **The sensitivity of phenol- sulphuric acid color reaction.** A sample of 0.25 ml containing different amount of C.PRX was mixed with 0.3 ml 5% phenol. After addition of 2 ml concentrated sulphuric acid, the absorbance was measured at 490 nm.

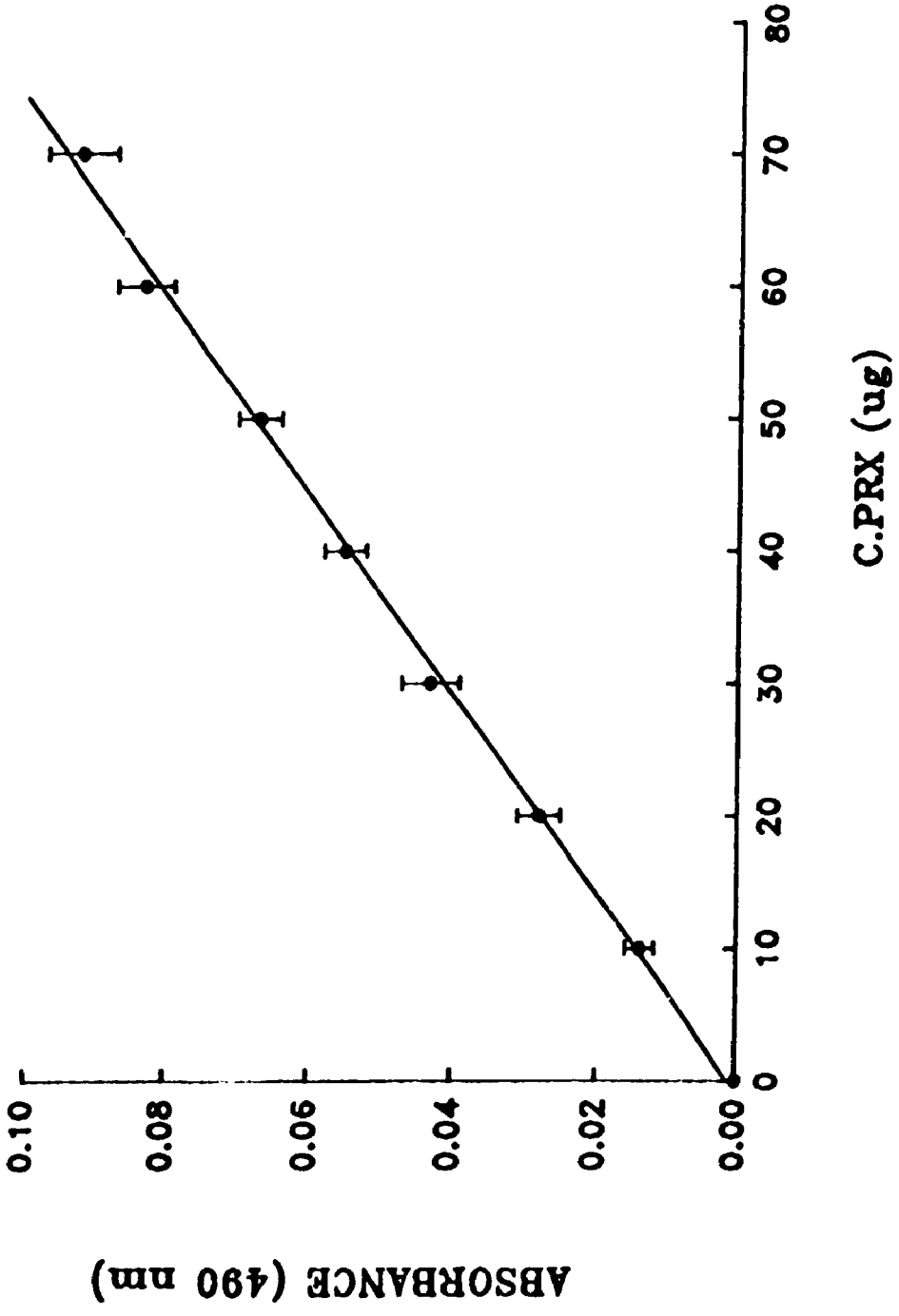
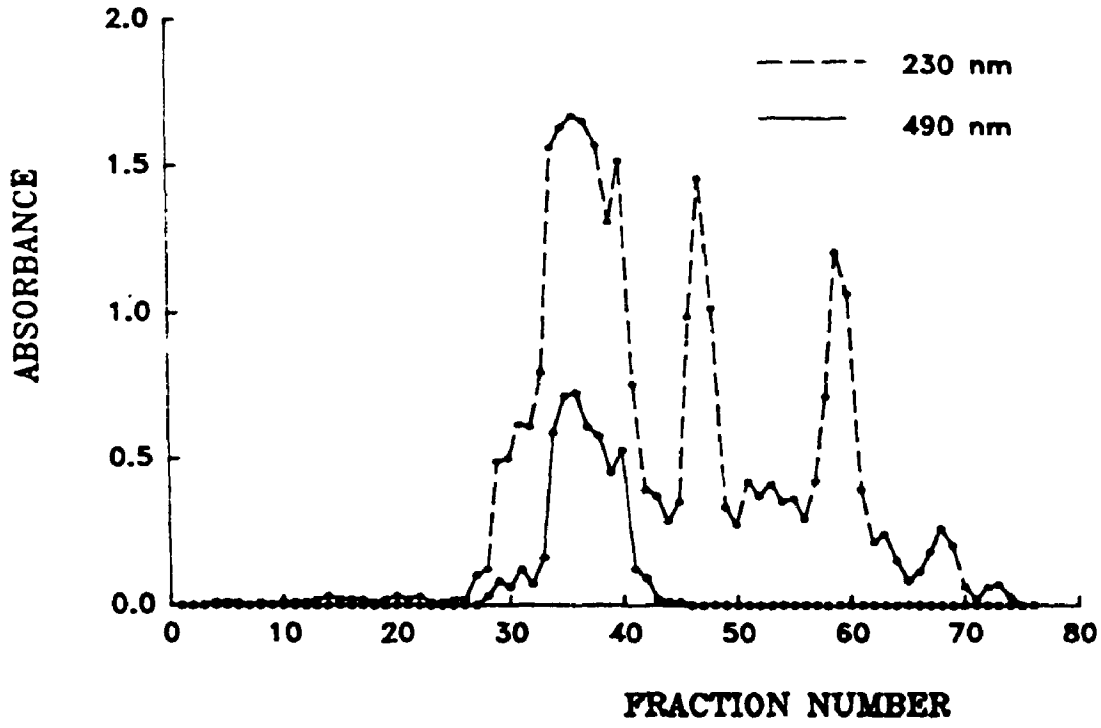
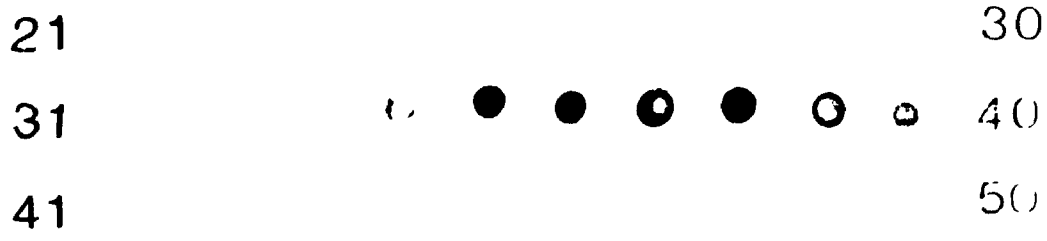

ABSORBANCE (490 nm)
0.00
0.02
0.04
0.06
0.08
0.10
0
10
20
30
40
50
60
70
80
C.PRX (ug)

Fig. 2.4. Detection of glycopeptide fractions of TPCK-tryptic cationic peroxidase eluted from a Biogel P-6 column. Fifty mg of acitic acetone treated cationic peroxidase was incubated with TPCK-trypsin at 37^{0}C for 40 min. The column was eluted at 8 ml/h.. Fractions of 2 ml were collected. A. the absorbance at 230 nm was used to detect peptide fractions and 0.25 ml of each fractions was used for phenol-sulphuric acid method to identify glycopeptide fractions. B. Five ul of fractions 21-50 was dot blotted on a PH 79 NC membrane. Each staining step lasted 10 min.

A


2.0
1.5
1.0
0.5
0.0
ABSORBANCE
230 nm
490 nm
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

B


21
31
41
30
40
50

CHAPTER 3

Location and Secondary Structure of Glycosylation Sites of Cationic Peanut Peroxidase

3.1. Introduction

Nucleotide sequences for genes encoding peroxidases or their corresponding cDNA have been cloned from tobacco (Lagrimini et al.,1987), potato (Roberts et al., 1988) and horseradish (Fujiyama et al., 1988). Recently two genes, prxPNC1 and prxPNC2, encoding two peanut peroxidases have been cloned (Buffard et al. 1990). But there has not been any proof of sequence identity between cDNA and protein so far. In this chapter I shall show that the amino acid sequence of C.PRX corresponds to the base sequence of cDNA clone prxPNC1. Also, the glycosylation sites on the peptide chain will be located and the relationship between glycosylation and secondary structure of peptide moiety will be investigated.

3.2. Materials and methods

3.2.1. Cell suspension culture and peroxidase purification

As described in section 1.2.

3.2.2. TPCK-tryptic digestion of C.PRX

As described in section 2.2.2.

3.2.3. Formic acid treatment

Heme depleted peroxidase was treated with 70% formic acid at 37^{0}C for 96 hours at a concentration of 0.5 mg of peroxidase/ml of 70% formic acid. Subsequently, the mixture was diluted with 2-3 volumes of water and lyophilized.

3.2.4. Electrophoresis, Western blotting and staining

The fragments of formic acid cleaved peroxidase were separated on 15% polyacrylamide-SDS gel (Laemmli 1970). In order to minimize N-terminal blockage or side chain modification of the peptide, the gel was pre-run in 50 mM Tris-HCl, pH 8.0, 0.1% SDS and 0.1 mM sodium thioglycollate for 30 min. at 100 V (Dr. S. Dunn, personal communication). After electrophoresis the fragment bands were blotted to an Immobilon-P transfer membrane (0.45 μM, Millipore) at 250 mA for 1 hour in a blotting buffer of 10 mM $NaHCO_3$, 3 mM Na_2CO_3 and 20% methanol. For detection of blotted protein, the membrane was stained with 0.1% amido black 10B, 45% methanol and 5% acetic acid for 10 seconds and destained with 90% methanol and 2% acetic acid. For carbohydrate staining on the membranes, the improved procedures described in section 2.2.5. were employed. Glycopeptides of TPCK-tryptic C.PRX were

separated on a mini gel (Bio-Rad) employing Swank and Munkres's (1971) SDS-polyacrylamide gel system (T 13.8%, C 9.1%, 8 M urea). The progress of electrophoresis was recorded with rainbow protein molecular weight markers (Amersham) and the glycans were visualized by staining with PAS reagent. The slab gel was fixed in 7.5% aqueous acetic acid for 45 min first and then the PAS staining procedure described in section 2.2.5 was followed, except that each step lasted 45 min rather than 10 min.

3.2.5. Gel filtration chromatography

The glycopeptides of TPCK-trypsin digested C.PRX were separated on a Biogel P6 column as described in section 2.2.3.

3.2.6. HPLC (high performance liquid chromatography) analysis

TPCK-tryptic glycopeptides of C.PRX from Biogel P6 column were subsequently purified on a reverse-phase HPLC using a u-Bondapak^{TM} C_{18} column (3.9 x 300 mm, Waters). HPLC was carried out with Beckman Gold system at 1.2 ml/min. The mobile phase was 0.1% TFA in water and the mobile phase modifier was acetonitrile containing 0.1% TFA. The eluent was monitored at 230 nm.

3.2.7. Amino acid sequencing

The glycopeptide peaks purified by reverse-phase HPLC were collected

separately. Acetonitrile was removed first under nitrogen. After lyophilization, the glycopeptides were subjected to amino acid sequence analysis. Amino acid sequencing employed the Edman reaction to degrade sequentially N-terminal amino acid of the protein and convert it to a phenylthiolhydantoin derivative for amino acid identification. Sequencing was performed with a Porton gas-phase sequencer. The amino acid sequencing was done by Mr. W. Chuang of the Department of Biochemistry, UWO.

3.2.8. Prediction of the secondary structure of peptides around potential glycosylation sites and hydropathy measure of C.PRX

The conformation of peptide chains adjacent to the potential glycan attachment sites was predicted with the computer program PepPlot written by Gribskov and Devereux (Gribskov et al., 1986) according to the rules of Chou and Fasman (Chou and Fasman, 1978). The relative probability that a tetrapeptide forms a reverse ß-turn was given by Lewis et al (1971): $p_t = (f_i)(f_{i+1})(f_{i+2})(f_{i+3})$, where f_i, f_{i+1}, f_{i+2} and f_{i+3} are the frequency of occurrence of a certain residue at the first, second, third and forth position of the ß-turn, respectively, and were evaluated by Chou and Fasman based on the X-ray coordinates of 29 proteins (Chou and Fasman 1978). If $p_t > 0.75 \times 10^{-4}$ (3/2 the average probability of 408 ß-turns observed in 29 proteins) and $<P_\alpha> < <P_t> > <P_\beta>$, where $<P_t>$, $<P_\alpha>$, $<P_\beta>$ are, respectively, the average conformational potential for the tetrapeptide to be in the ß-turn, α-helix and ß-

sheet conformation, a tetrapeptide ß-turn is predicted. The prediction of α-helices and ß-sheets is based on $<P_\alpha>$ and $<P_\beta>$ values over a window of four adjacent residues and the rules established by Chou and Fasman (Chou and Fasman,1974, 1978). The hydropathy indices of C.PRX was plotted with the computer program PepPlot according to Kyte and Doolittle (1982). The curve is the average of residue specific hydrophobicity indeces over a window of nine residues. A positive hydropathy indicates hydrophobicity and a negative value, hydrophilicity.

3.3. Results

The Asp-Pro bond can be cleaved specifically by treatment with formic acid (Jauregui-Adell and Marti, 1975). Cleavage of C.PRX by 70% formic acid resulted in two unequal fragments (approximately 26 and 14 kd) and some minor bands as shown in Fig. 3.1. As cDNA clone prxPNC1 (Buffard et al. 1990) has only one Asp-Pro bond and the result of amino acid sequencing of the smaller fragment of 14 kd is the same as predicted by the sequence of the cDNA clone prxPNC1 (Fig. 3.2), it was confirmed that the amino acid sequencing of C.PRX corresponds to the sequence of cDNA clone prxPNC1. As shown in Fig. 3.2, there are five sites of consensus sequence -Asn-Xaa-Thr/Ser- for N-glycosylation in cDNA clone prxPNC1. To investigate whether all five sites are glycosylated, C.PRX was digested with TPCK-trypsin and the fragments were separated on SDS-polyacrylamide gel. Fig. 3.3 shows that

digestion of C.PRX with a low ratio of 1/600 to 1/300 (TPCK-trypsin/C.PRX, w/w) at 37^{0}C for 30 min was not completed, but within a wide range from 1/200 to 1/20, C.PRX was equally well digested and presented only three glycopeptide bands after staining with PAS reagent. Compared to protein molecular weight markers, glycopeptides migrated much slower on SDS-polyacrylamide gel. The lagging of glycopeptides results from their associated oligosaccharides, which prevent the proper binding of SDS to peptides and cause drag on the peptides, and thus may cause overestimation of the mass of glycopeptide on SDS-polyacrylamide gel. According to the sequence of the cDNA clone prxPNC1, the larger fragment produced by cleavage with 70% formic acid has three potential glycosylation sites (Asn_{60}, Asn_{144} and Asn_{185}) and the smaller one has two (Asn_{209} and Asn_{275}). The fact that the smaller fragment does not stain with PAS reagent (Fig. 3.4) implies that Asn_{209} and Asn_{275} are not used for glycosylation. Furthermore, when TPCK-trypsin digested C.PRX was passed through a Bio-gel P6 column to separate glycopeptide fractions (Fig. 3.5) and the total of the PAS positive peaks were identified on a reverse-phase HPLC, only 3 glycopeptide peaks were detected (Fig. 3.6). N-terminal amino acid sequencing of the 3 TPCK-tryptic glycopeptides of C.PRX (Table 3.1) indicated that TPCK-trypsin catalyses precisely the hydrolysis of lysyl and arginyl peptide bonds. A comparison of the amino acid sequencing results with that of the cDNA clone prxPNC1 verified again that the protein sequence is the same as cDNA and that GPa contains glycosylation site Asn_{185}; GPb, Asn_{60} and GPc,

Asn_{144}. Asn_{185} of GPa can not be detected on gas chromatography, because glycosylated residues tend to interfere with sequencing via Edman degradation (Clark and Shannon, 1976), and was assigned as X. No glycopeptides corresponding to either Asn_{209} or Asn_{275} could be detected by HPLC separation.

To investigate the relationship between glycosylation and protein conformation the secondary structure of peptides around glycosylation sites was computer analyzed. The computed ß-turn profile for C.PRX is shown in Fig. 3.7 with the probable ß-turn around 5 potential glycosylation sites listed in Table 3.2. There are 9 tetrapeptides above the cutoff point, $p_t = 0.75 \times 10^{-4}$ (Table 3.2). It can be seen that tetrapeptide 140-143 is not predicted as ß-turns because its $<P_t>$ value is lower than both $<P_\alpha>$ and $<P_\beta>$ values. Neither are tetrapeptides 58-61, 144-147, 273-276 predicted as ß-turns because adjacent tetrapeptides have higher ß-turn probability p_t (Chou and Fasman, 1978, 1979). Five predicted ß-turns are indicated in both Fig. 3.7 and Table 3.2. Fig. 3.8 displayed the α-helix and ß-sheet propensities for C.PRX. At position of 142 there is a peak for ß-sheet propagation ($<P_\alpha> = 1.24$), which is higher then both ß-sheet threshold ($<P_\beta> = 1.03$) and the potential for α-helix propagation at this position ($<P_\alpha> = 1.04$). Also, the tetrapeptide 142-145 has three ß-formers (Phe, Phe and Leu) and so satisfies the criteria for the initiation of a ß-sheet (Chou and Fasman,1974,1978). As the adjacent tetrapeptides 141-144 and 143-146 are tetrapeptide breakers (both have two ß-formers, one ß-

indifferent and one ß-breaker), the ß-sheet segment 142-145 do not extend in both directions. Leu_{145} at the C-terminal of ß-sheet also participates in the following ß-turn. So, both Asn_{60} and Asn_{185} are located in ß-turns and Asn_{144} is in a ß-sheet near a ß-turn. The hydropathy profile of C.PRX (Fig. 3.9) showed that glycosylated Asn_{60} and Asn_{185} are in hydrophillic (surface) regions, while Asn_{144} is in the hydrophobic (interior) region.

3.4. Discussion

3.4.1. The location of glycosylation sites of C.PRX

As the amino acid sequence of C.PRX is first confirmed to be the same as predicted by the base sequence of the cDNA clone prxPNC1 by amino acid sequencing of the smaller fragment cleaved by 70% formic acid and three TPCK-tryptic glycopeptides of C.PRX, the nucleotide sequence of prxPNC1 supplies useful information to study the structure and function of C.PRX. It is showed that out of five potential N-glycosylation sites, only three of Asn_{60}, Asn_{144} and Asn_{185} are used. As not all the consensus sequences -Asn-Xaa-Thr/Ser- are glycosylated, it was concluded that the primary structure of the tripeptide is necessary, but not sufficient, for glycosylation. The constraints which govern whether a particular potential glycosylation site is used are unclear, although the conformation of the protein in the region of the glycosylation site is probably important (Kornfeld and Kornfeld, 1985).

3.4.2. Prediction of the secondary structure of proteins

To understand the mechanism of folding and the biological function of proteins, it is necessary to know their spatial conformation. X-ray diffraction is successful in elucidating the three dimensional structure of proteins. However, this analysis is quite laborious, expensive, and time-consuming. Furthermore, for some proteins it is difficult to obtain suitable crystals, so that other approaches may be explored to give structural information. The classical experiments of Anfinsen et al (Anfinsen, 1973; Anfinsen et al.,1961) showed that ribonuclease could be denatured and refolded without loss of enzyme activity. This implied that the amino acid sequences contained sufficient information to define the three-dimensional structure of a protein in a particular environment. Many efforts have been made to predict protein structure on the basis of the sequence data. Today there are around 15-20 proposals for the prediction of the secondary structure of protein conformation from the amino acid sequence, among which the proposals of Chou and Fasman, Garnier, Osguthorpe et al. (Lim, 1974a, 1974b) are the most widely used methods. The first two methods use parameters obtained from the analysis of known sequences and structures, and the third method is based on stereochemical criteria.

Chou and Fasman made a statistical survey of 29 proteins based on X-ray diffraction data (Chou and Fasman 1973). The frequency of all 20 amino acids in the α-helical (f_α), β sheet (f_β), coil (f_c), and β-turn (f_t) regions can be obtained when their occurrence in each conformational state is divided by their

total occurrence in the 29 proteins. The average frequency of all residues in the α-helical (<f_α>), ß-sheet (<f_β>), coil (f_c>), and ß-turn (f_t>) regions are, respectively, 38%, 20%, 42% and 30%. When the frequency of each amino acid in the α-helical, ß-sheet, coil, and ß-turn regions is divided by the respective average frequency, the normalized conformational parameters are obtained: $P_\alpha=f\alpha/<f_\alpha>$, $P_\beta=f_\beta/<f_\beta>$, $P_c=f_c/<f_c>$, and $P_t=f_t/<f_t>$. For example, for Ala: $f_\alpha=234/434=0.539$, $f_\beta=71/434=0.164$, $f_t=85/434=0.196$, $P_\alpha=0.539/0.38=1.42$, $P_\beta=0.164/0.20=0.82$, $P_t=0.196/0.30=0.66$. The conformational parameters P_α, P_β and P_t for the 20 amino acids are arranged in hierarchical order, and their assignments as formers, indifferent, and breakers of α and ß regions are also given. Using the protein conformational parameters P_α, P_β, P_t and a set of rules, the secondary structure of soluble or globular proteins can be predicted. The method of Chou and Fasman was computerized by Drs. Gribskov and Devereux of the Genetics Computer Group. This protein secondary structure analysis program, namely PEPPLOT, was first described in Nucl.Acids Res. 14(1): 327-334 (1986).

3.4.3. The secondary structure of peptides around glycosylation sites

With the computer program, both Asn_{60} and Asn_{185} are predicted to reside in ß-turns and Asn_{144} in a ß-sheet near a ß-turn. The ß-turn is a structural feature of protein conformation involving four consecutive residues, thus enabling a polypeptide chain to reverse itself by nearly 180^0. The ß-turn has

has also been called ß-bend, hairpin loop, and reverse turn. In some cases, the ß-turn is not specified and is assumed to be part of a random coil or irregular region (Lewis et al., 1971; Chou and Fasman 1978).

The preference of glycosylated asparagines for ß-turns has been reported in other glycoproteins (Beely, 1977, Aubert et al. 1976, Mononen and Karjalainen, 1984). Under these considerations, 19 out of 28 glycosylated asparagine (Aubert et al. 1976), 22 out of 31 (Beeley, 1977), and 98 out of 139 (Mononen and Karjalainen, 1984) were reported in ß-turns. The glycosylated asparagines in ß-turns accounted for 69%, 70% and 71% of the total glycosylated asparagines, respectively. The statistical parameters of 29 proteins based on X-ray data (Chou and Fasman, 1978) demonstrated that in a total of 4741 residues 1400 were found in ß-turns, with a percentage 1400/4741=30% and that in a total of 230 asparagines 106 were found in ß-turns, with a percentage 106/230= 46%. So, asparagines occur frequently in ß-turns. But comparing 46% with 70%, glycosylated asparagines present an even higher preference for ß-turns. The hydropathy profile of C.PRX (Fig. 3.9) showed glycosylated Asn_{60} and Asn_{185} are in hydrophillic (surface) regions, while Asn_{144} is in a hydrophobic (interior) region. As ß-turns are often correlated with the termination of hydrophobic α-helices or ß-sheets and exposed at the surface, this type of conformation may contribute to appropriate accessibility of oligosaccharyltransferase and favours the glycosylation of asparagines. Proposing a space-filling model, Bause and Legler (Bause, 1983;

Bause and Legler, 1981) suggested that ß-turns represent a spatial arrangement of the peptide chain that favours the hydrogen-bond contact between the ß-amide of asparagine and the hydroxy group of the hydroxy amino acid (Ser or Thr). As a result, the nucleophilicity of the ß-amide electron pair is increased, thus giving rise to a higher reactivity toward the glycosyl donor. Both Asn_{209} and Asn_{275} have a proline residue at the C-terminal of the consensus sequence and the asparagines are not glycosylated. The same results that C-terminal proline of the tripeptide inhibits the glycosylation of asparagine were also observed in other in vivo or in vitro experiments (Bause, 1983; Bause and Legler, 1981; Roitsch and Lehle, 1989). Probably proline handicaps the formation of favourable conformation of glycan acceptor peptide because of its pyrrolidine side chain (Bause, 1983).

Fig. 3.1. **Cleavage pattern of C.PRX with 70% formic acid.** After electrophoresis the fragments were blotted to Immobilon-P transfer membranes (0.45 μM) and stained with 0.1% amido black 10B. Lane 1, formic acid cleaved C.PRX, 40 μg. The arrowheads show the two major cleaved fragments. Lane 2, C.PRX, 20 μg. Lane 3, 10 μg of standard protein molecular weight marks (Sigma): bovine albumin, 66 kd; egg albumin, 45 kd; glyceraldehyde-3-phosphate dehydrogenase, 36 kd; carbonic anhydrase, 29 kd; trypsin inhibitor, 20.1 kd and α-lactalbumin, 14.4 kd. The smaller fragment was cut off and subjected to amino acid sequencing.

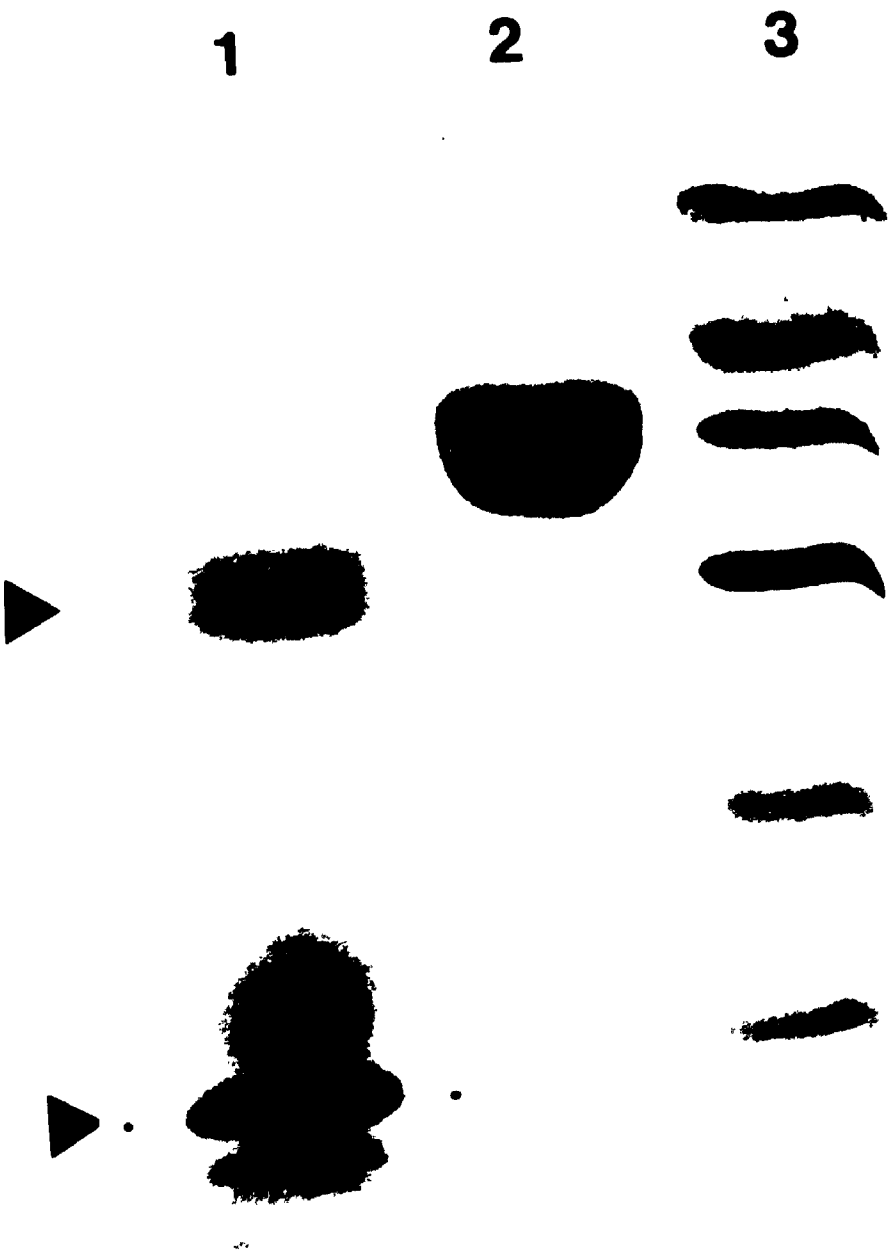
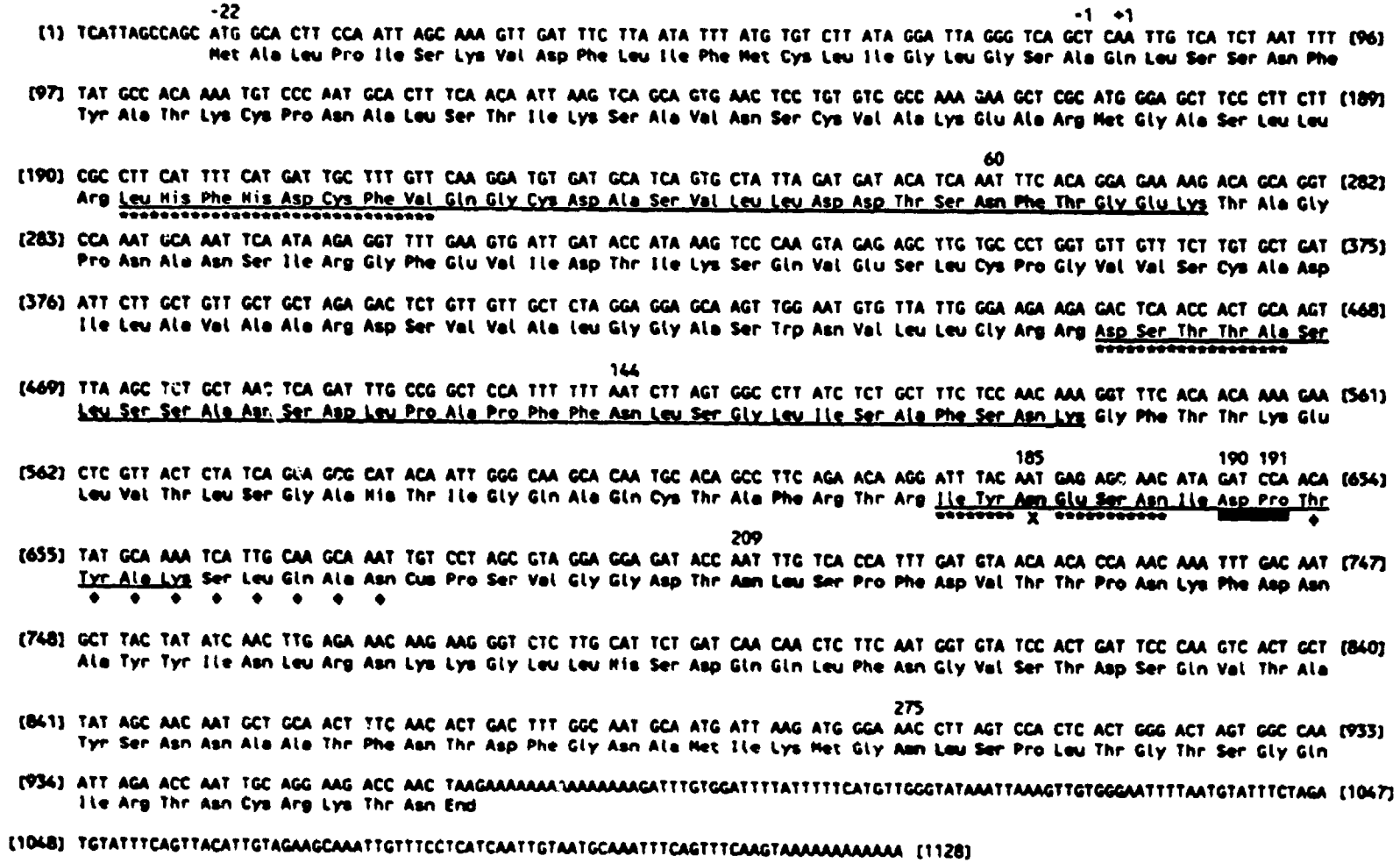

1
2
3

Fig. 3.2. Nucleotide and deduced amino acid sequence of the cDNA clone prxPNC1. The mature protein starts at position +1. The sole Asp-Pro bond is shown with ▬ and the partial N-terminal amino acid sequence of the cleaved smaller fragment is indicated with sign ♦. The positions of potential glycosylation sites are labelled and the consensus sequence -Asn-Xaa-Thr/Ser- for N-glycosylation is given in bold letters. The TPCK-tryptic glycopeptides are underlined and the results of partial N-terminal amino acid sequencing of glycopeptides are indicated with sign *. Asn_{185} of GPa can not be detected on gas chromatography, because glycosylated residues tend to interfere with sequencing via Edman degradation, and was assigned as X.



[1] TCATTAGCCAGC ATG GCA CTT CCA ATT AGC AAA GTT GAT TTC TTA ATA TTT ATG TGT CTT ATA GGA TTA GGG TCA GCT CAA TTG TCA TCT AAT TTT [96]

[97] TAT GCC ACA AAA TGT CCC AAT GCA CTT TCA ACA ATT AAG TCA GCA GTG AAC TCC TGT GTC GCC AAA GAA GCT CGC ATG GGA GCT TCC CTT CTT [189]

[190] CGC CTT CAT TTT CAT GAT TGC TTT GTT CAA GGA TGT GAT GCA TCA GTG CTA TTA GAT GAT ACA TCA AAT TTC ACA GGA GAA AAG ACA GCA GGT [282]

[283] CCA AAT GCA AAT TCA ATA AGA GGT TTT GAA GTG ATT GAT ACC ATA AAG TCC CAA GTA GAG AGC TTG TGC CCT GGT GTT GTT TCT TGT GCT GAT [375]

[376] ATT CTT GCT GTT GCT GCT AGA GAC TCT GTT GTT GCT CTA GGA GGA GCA AGT TGG AAT GTG TTA TTG GGA AGA AGA GAC TCA ACC ACT GCA AGT [468]

[469] TTA AGC TCT GCT AAC TCA GAT TTG CCG GCT CCA TTT TTT AAT CTT AGT GGC CTT ATC TCT GCT TTC TCC AAC AAA GGT TTC ACA ACA AAA GAA [561]

[562] CTC GTT ACT CTA TCA GGA GGG CAT ACA ATT GGG CAA GCA CAA TGC ACA GCC TTC AGA ACA AGG ATT TAC AAT GAG AGC AAC ATA GAT CCA ACA [654]

[655] TAT GCA AAA TCA TTG CAA GCA AAT TGT CCT AGC GTA GGA GGA GAT ACC AAT TTG TCA CCA TTT GAT GTA ACA ACA CCA AAC AAA TTT GAC AAT [747]

[748] GCT TAC TAT ATC AAC TTG AGA AAC AAG AAG GGT CTC TTG CAT TCT GAT CAA CAA CTC TTC AAT GGT GTA TCC ACT GAT TCC CAA GTC ACT GCT [840]

[841] TAT AGC AAC AAT GCT GCA ACT TTC AAC ACT GAC TTT GGC AAT GCA ATG ATT AAG ATG GGA AAC CTT AGT CCA CTC ACT GGG ACT AGT GGC CAA [933]

[934] ATT AGA ACC AAT TGC AGG AAG ACC AAC TAAGAAAAAAAA\AAAAAAAACATTTGTGGATTTTATTTTTCATGTTGGGTATAAATTAAAGTTGTGGGAATTTTAATGTATTTCTAGA [1047]

[1048] TGTATTTCAGTTACATTGTAGAAGCAAATTGTTTCCTCATCAATTGTAATGCAAATTTCAGTTTCAAGTAAAAAAAAAAAA [1128]

Fig. 3.3. SDS-polyacrylamide gel electrophoresis of TPCK-tryptic C.PRX (T 13.8%, C 9.1%, 8 M urea). Lane 1-8 were individually applied with 200 μg of C.PRX digested with TPCK-trypsin at 37^{0}C for 30 min in different ratio of C.PRX to TPCK-trypsin (w/w): 1. 1/600, 2. 1/300, 3. 1/200, 4. 1/150, 5. 1/100, 6. 1/60, 7. 1/30, 8. 1/20. Lane 9, 5 μg of C.PRX. The gel was electrophorezed at 20 V for 0.5 h. and subsequently at 40 V for 18 h. at room temperature. The progress of electrophoresis was indicated with rainbow protein molecular weight markers: ovalbumin, 46 kd; carbonic anhydrase, 30 kd; trypsin inhibitor, 21.5 kd and lysozyme, 14.3 kd. The gel was stained with PAS reagent to reveal glycopeptide bands as described.

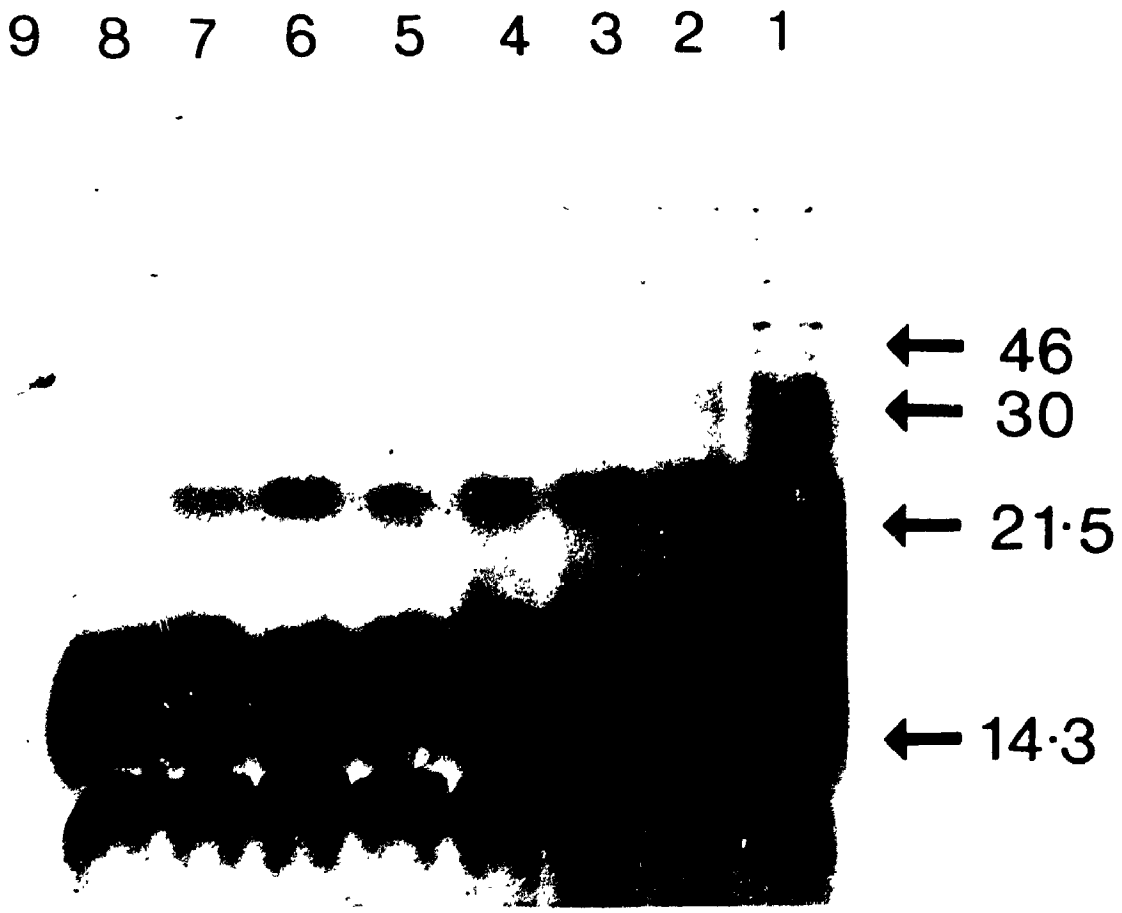

9 8 7 6 5 4 3 2 1
46
30
21·5
14·3

Fig. 3.4. Glycopeptide mapping of 70% formic acid cleaved C.PRX.

After electrophoresis the cleaved fragments were blotted to Immobilon-P transfer membranes (0.45 μM). Lane 1-4 were stained with 0.1% amido black 10B, lane 5-7 were stained with PAS reagent twice. Lane 1, 20 μg of standard protein molecular weight marks as in lane 3 of Fig. 3.1.; lane 2, C.PRX, 20 μg; lane 3, formic acid cleaved C.PRX, 80 μg; lane 4, formic acid cleaved C.PRX, 40 μg; lane 5, formic acid cleaved C.PRX, 40 μg; lane 6, formic acid cleaved C.PRX, 80 μg; lane 7, C.PRX, 20 μg.


7
6
5
4
3
2
1

Fig. 3.5. Separation of glycopeptides of cationic peroxidase on Biogel P6 column. Fifteen milligrams of heme depleted C.PRX were digested with TPCK-trypsin at 37^{0}C for 30 min in a ratio of 1/100 (TPCK-trypsin/C.PRX, w/w). The mixture was applied to a Biogel P6 column (1.5 cm x 90 cm) and eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% NaN_3 at flow rate 8 ml/h. The eluent was monitored at 280 nm using a UV-2 monitor (Pharmacia) and fractions of 2 ml were collected. Glycopeptide fractions were identified (section 2.2.5), pooled for further analysis and cross-hatched.

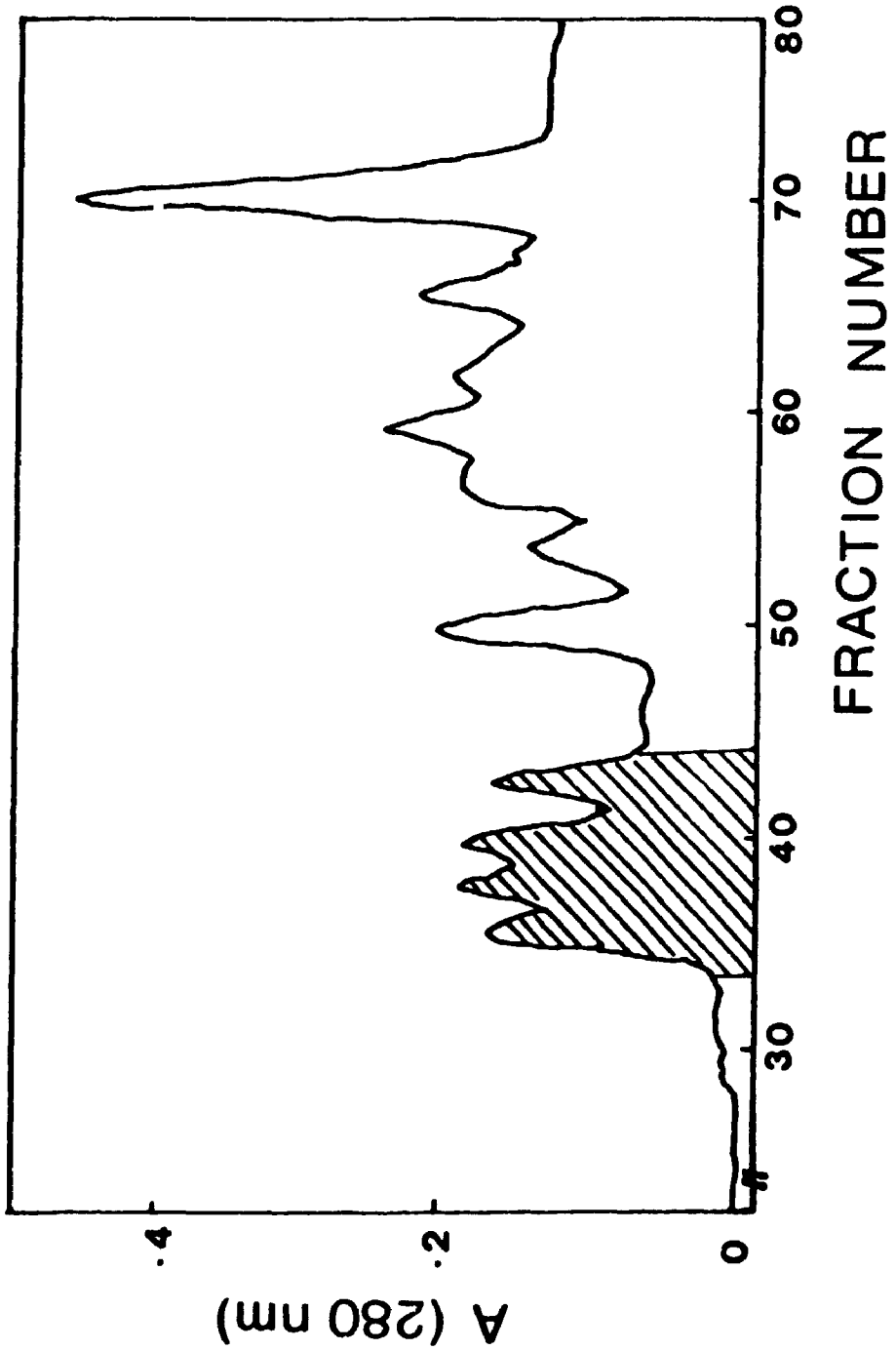

A (280 nm)
.4
.2
0
#
30
40
50
60
70
80
FRACTION NUMBER

Fig. 3.6. Purification of glycopeptides of C.PRX with reverse-phase HPLC. Glycopeptide fractions of 2 mg of TPCK-tryptic C.PRX separated on Biogel P6 column were applied to a Waters u-Bondapak™ C_{18} column (3.9 x 300 mm) and eluted with an acetonitrile gradient at 1.2 ml/min. The gradient was programmed with Beckman Gold system and shown as dash line. The elution was monitored by UV absorption at 230 nm. The glycopeptide peaks were identified with phenol-sulphuric acid technique, and were designated as GPa, GPb, GPc, respectively.

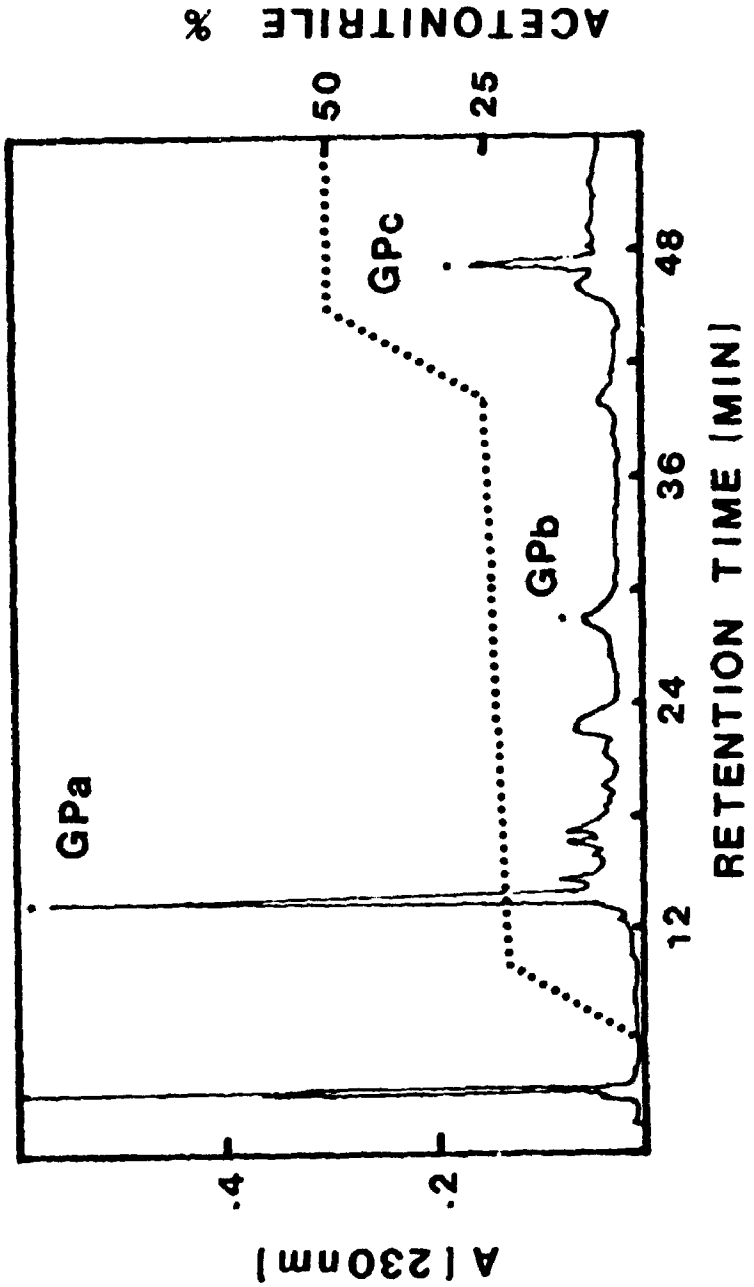

ACETONITRILE %
50
25
GPc
GPb
GPa
48
36
24
12
RETENTION TIME (MIN)
.4
.2
A (230nm)

Table 3.1. Partial N-terminal amino acid sequences of TPCK-tryptic glycopeptides of C.PRX. Sequencing was performed with a Porton gas-phase sequencer. The third amino acid residue of GPa could not be detected and was designed as X.

Glycopeptides	N-terminal amino acid sequence
GPa	Ile-Tyr-X-Glu-Ser-Asn
GPb	Leu-His-Phe-His-Asp-Cys-Phe-Val
GPc	Asp-Ser-Thr-Thr-Ala

Fig. 3.7. Probability of tetrapeptide ß-turns (p_t) for C.PRX. The dashed line corresponds to cutoff values of $p_t = 0.75 \times 10^{-4}$ (3/2 the average ß-turn probability). The numbered peaks indicate ß-turns predicted at position i using the computer predictive algorithm around potential glycosylation sites.

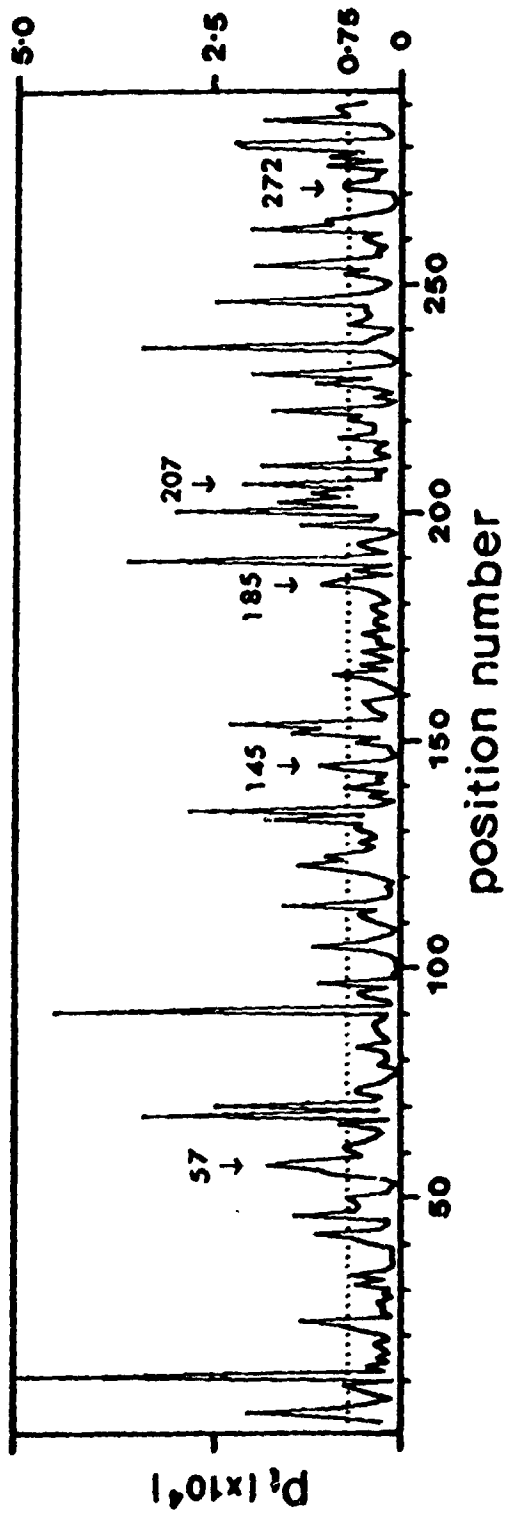

5·0
2·5
0·75
0
272
207
185
145
57
50
100
150
200
250
position number
p_i (x10⁴)

Table 3.2. Predictions of tetrapeptide ß-turns around potential glycosylation sites of cationic peanut peroxidase[a]

Position	Tetrapeptide	p_t (x10^4)	$<P_t>$	$<P_\alpha>$	$<P_\beta>$
57-60*	Asp-Thr-Ser-Asn_{60}	1.81	1.35	0.82	0.84
58-61	Thr-Ser-Asn-Phe	1.48	1.14	0.85	1.05
140-143	Ala-Pro-Phe-Phe	0.76	0.85	1.06	1.04
144-147	Asn_{144}-Leu-Ser-Gly	0.76	1.29	0.81	0.92
145-148*	Leu-Ser-Gly-Leu	1.1[illegible]	1.04	0.94	1.03
185-188*	Asn_{185}-Glu-Ser-Asn	1.10	1.32	0.91	0.73
207-210*	Asp-Thr-Asn_{209}-Leu	2.12	1.14	0.93	0.98
272-275*	Lys-Met-Gly-Asn_{275}	0.78	1.18	0.96	0.86
273-276	Met-Gly-Asn_{275}-Leu	0.77	1.08	0.98	1.00

[a] $p_t = (f_i)(f_{i+1})(f_{i+2})(f_{i+3})$ is the probability of tetrapeptide ß-turn occurrence. $<P_t>$, $<P_\alpha>$, $<P_\beta>$ are, respectively, the average conformational potential for the tetrapeptide to be in the ß-turn, α-helix and ß-sheet conformation. Tetrapeptides around potential glycosylation sites with $p_t > 0.75 \times 10^{-4}$ were listed as potential ß-turns. The predicted ß-turns (denoted with *) all have $<P_\alpha> < <P_t> > <P_\beta>$.

* Predicted ß-turns.

Fig. 3.8. Probability of α-helices (<p_α>) and ß-sheets (<p_β>) for C.PRX. The curves are the average conformational potential for the tetrapeptide to be in α-helix (dashed line) and ß-sheet (solid line) conformation. The thresholds (<P_α> = 1.03, <P_β> = 1.05) shown in broken or solid horizontal lines indicate the minimum levels for predicting alpha and beta structures. Positions below the thresholds indicate possible tetrapeptide breakers.

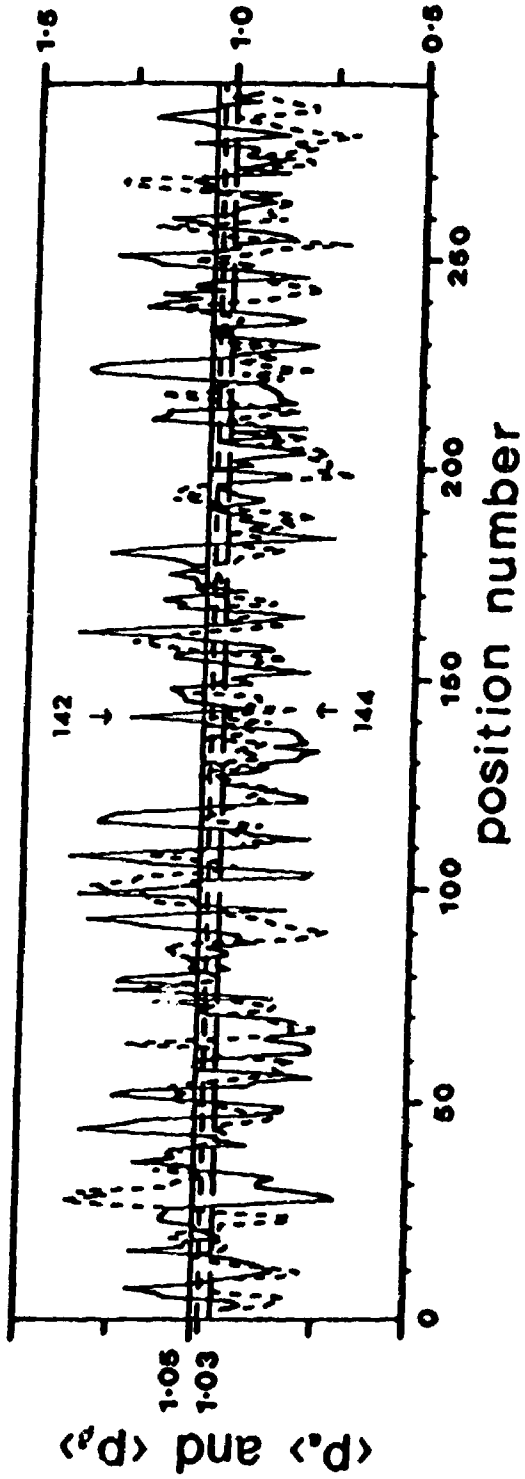
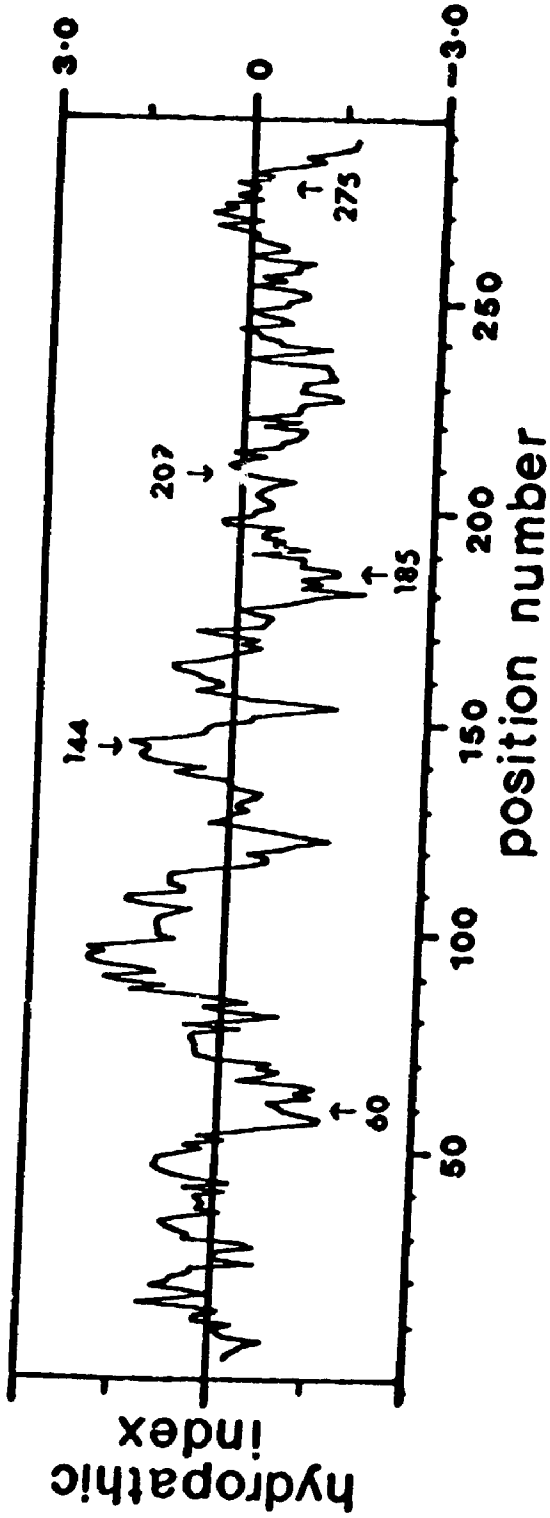

1·5
1·0
0·5
250
200
150
100
50
0
position number
142
144
1·05
1·03
⟨ρ_s⟩ and ⟨ρ_φ⟩

Fig. 3.9. **Hydropathy profile of C.PRX.** The plot is a running average over nine residues of the hydropathy scale of Kyte and Doolittle. Peaks indicate hydrophobic (interior) regions, and valleys hydrophillic (surface) regions. The hydropathic indices of 5 potential glycosylation sites are: Asn_{60}, -0.94; Asn_{144}, 1.24; Asn_{185}, -1.54; Asn_{209},0.00; Asn_{275}, -0.10.


hydropathic index
3·0
0
-3·0
50
100
150
200
250
position number
60
144
202
185
275

CHAPTER 4

Immunogenicity of the N-glycans of Peanut Peroxidase

4.1. Introduction

Polyclonal and monoclonal antibodies against A.PRX and C.PRX have been raised (Hu and van Huystee, 1989b; Xu and van Huystee, 1990). It was observed that there is immuno-cross reaction between these two isozymes and the antibodies directed to peanut peroxidases even recognize other plant glycoproteins of different sources and functions (Xu et al., 1990; Xu and Huystee,1991). Such cross reaction was also found with antibodies directed to other glycoproteins (McManus et al, 1988) and is suggested to be due to the carbohydrate moiety. As antibodies are widely used as molecular probes to identify antigens, in some cases cross reaction may cause problems. To investigate the immunogenicity of carbohydrate moiety of glycoproteins, we raised polyclonal antisera against tryptic glycopeptides of peanut peroxidases, isolated antibodies directed towards the N-glycans, and determined the distribution of epitopes on glycans.

4.2. Materials and methods

4.2.1. Purification of TPCK-tryptic glycopeptides of C.PRX, A.PRX and fetuin

The digestion of C.PRX with TPCK-trypsin and the purification of the three glycopeptides, GPa, GPb and GPc on Biogel P6 column and HPLC are described in section 3.2.2 and section 3.2.6).

A.PRX and fetuin were individually digested with TPCK-trypsin and then applied to P10 column (1.5 cm x 90 cm, 200-400 mesh, molecular weight range 1500-20,000 daltons, Bio-Rad) using the same procedure as for a Biogel P6 column. The glycopeptide fractions of fetuin were pooled together, dialysed (Spectro/Por membrane tubing, MWCO, 1000, Fisher), lyophilized and directly used for coupling trials. The glycopeptide fractions of A.PRX from Biogel P10 column were subsequently purified on HPLC as described in section 3.2.6. The sole glycopeptide was identified with phenol-sulphuric acid technique (Dubois et al., 1956) and named GP.

4.2.2. Coupling of glycopeptides to bovine serum albumin (BSA)

To raise polyclonal antisera against individual tryptic glycopeptides of C.PRX and A.PRX, a modified coupling procedure (Harlow and Lane, 1988) was employed. For one injection, each glycopeptide from either 20 mg (0.5 μM) C.PRX (around 2 mg glycopeptide) or 8 mg (0.2 μM) A.PRX (around 2 mg glycopeptide) and 3 mg bovine serum albumin (BSA, Sigma) was dissolved in 1 mL 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride, and vortexed for 5 min.. Then 0.1 mL 1% (v/v) glutaraldehyde

(Eastman Kodak Company) was added dropwise with constant agitation. The mixture was incubated with shaking at 4^0C overnight. The coupling reaction was stopped with addition of 250 μL of 1 M glycine. After incubation with glycine and continuous shaking for 3 hours at room temperature, the glycopeptide-BSA conjugate was separated from excess glutaraldehyde by dialysis against 0.01 M sodium phosphate buffer, pH 7.2 (MWCO, 1000) overnight and lyophilized. In order to optimize the coupling condition the glycopeptides of TPCK-tryptic fetuin were used for a trial assay.

4.2.3. Immunization

Lyophilized glycopeptide-BSA conjugate was dissolved in 0.6 mL double distilled water and homogenized in 0.6 mL complete (for initial injection) or incomplete (for subsequent boosting) Freund's adjuvant using a Kontes Pellet Pestle Motor (Baxter Healthcare Corp.). The emulsion was injected subcutaneously into New Zealand rabbits. The rabbits were boosted every three weeks until the titre was sufficiently high.

4.2.4. Affinity purification of antibodies specific to carbohydrate moiety of C.PRX

Each of the TPCK-tryptic glycopeptides, GPa, GPb and GPc of 40 mg C.PRX isolated on HPLC was extensively digested with 0.5 mg pronase (Boehringer Mannheim) in 1 mL 50 mM Tris-HCl buffer, pH 8.0 containing 20

mM calcium chloride and 0.02% sodium azide at 37^{0}C for 72 hours with additions of 0.2 mg pronase every 24 hours. To eliminate any possible impurity of endoglycosidase activity , pronase was dissolved in Tris-HCl buffer and preincubated at 37^{0}C for 30 min (Beely, 1985b). The produced glycan-Asn fragments, named GLa, GLb and GLc accordingly, were separated from amino acids on Bio-Gel P4 column (1.5 x 90 cm, Bio-Rad) and coupled to ECH Sepharose 4B (Pharmacia) according to the manufacturer's instruction. Briefly, GLa, GLb, or GLc of 40 mg C.PRX was dissolved in 1 mL double distilled water and mixed with 2 mL ECH Sepharose 4B gel suspension. One mL of 0.4 M N-ethyl-N'-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC, Sigma) was added dropwise to the mixture with agitation. After 1 h., the pH was adjusted to 5.0 using 0.2 M sodium hydroxide and left to agitate at 4^{0}C overnight. To block the remaining carboxyl groups, 200 μL ethanolamine (Sigma) was added and incubated at room temperature for 5 h.. The gel was packed (5 X 40 mm) and washed alternatively three times with acetate buffer (0.1 M, pH 4) and Tris-HCl buffer (0.1 M, pH 8.0) each containing 0.5 M sodium chloride to remove any uncoupled ligand. The columns were equilibrated with 10 mM Tris-HCl, pH 7.5. Under the above conditions, the coupling efficiency is around 20% based on the measurement of the amount of uncoupled ligand recovered in the washings. Antiserum against GPa, GPb or GPc was mixed with equal volume of saturated ammonium sulphate and left at 4^{0}C overnight. After centrifugation, the precipitated IgGs were dissolved in and dialysed against 10 mM Tris-HCl,

pH 7.5 at 4^{0}C overnight. Then, the IgGs were passed twice through a correspondingly conjugated ECH Sepharose 4B affinity column at a flow rate of 6 mL/h. After washing the column with 10 mM Tris-HCl, pH 7.5 until A_{280} returned to the baseline, bound antibodies against individual glycans, designated anti-GLa, anti-GLb and anti-GLc respectively, were eluted with 3 M magnesium chloride and dialysed against water to remove salt (Chibbar and van Huystee, 1984b). To regenerate the columns, they were washed immediately with 10 mM Tris-HCl, pH 7.5 .

4.2.5. Enzyme-linked immunosorbent assay (ELISA)

The modified procedures for ELISA (Xu et al., 1990) was employed. Briefly, each well of a round bottom polystyrene plate (Corning) was coated with 50 μL of HPLC purified TPCK-tryptic glycopeptide, GPa, GPb, GPc or GP, in 0.05 M sodium carbonate buffer, pH 9.6 containing 0.02% sodium azide at 37^{0}C for 1 h. and then at 4^{0}C overnight. The amount of coated glycopeptides was given either as indicated in the case of titre determination of antisera or optimised as GPa, 1 μg of equivalent C.PRX/well, GPb and GPc, 0.5 μg of equivalent C.PRX/well in competitive ELISA. The unbound glycopeptides were washed off with washing buffer (0.01 M phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 0.1% gelatin, 0.02% sodium azide and 0.05% Tween-20). Then, 50 μL of antibodies appropriately diluted with washing buffer was added to each well. After incubation at 37^{0}C for 1 h., the plate was washed

with washing buffer and incubated with 50 μL of alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, 1:5000 diluted in washing buffer) at 37^{0}C for 1 h. Following washing, the plate was incubated with 50 μL p-nitrophenylphosphate (Sigma, 0.5 mg/mL in 9.7% diethanolamine, pH 9.8) at 37^{0}C for 1 h.. The reaction was stopped by addition of 50 μL of 3 M sodium hydroxide. The absorbance was read at 405 nm on an EIA ELISA reader (Biorad).

For competitive ELISA, the purified antibodies directed towards the individual glycans of C.PRX were preincubated with various competitors at 37^{0}C for 1 h. and then at 4^{0}C overnight. Fifty μL of the mixture was transferred to each well coated with antigen, and ELISA was processed as above. The concentration of antibodies employed was: anti-GLa, 5 ng/μL; anti-GLb, 0.3 ng/μL and anti-GLc, 2.5 ng/μL prepared in washing buffer. The stock concentration of the competitors was: glycopeptides (GPa, GPb, GPc), 10 μg/μL; glycoproteins (bromelain from pineapple stem, Sigma; fetuin; A.PRX; C.PRX; horseradish peroxidase, HRP, Sigma), 4 μg/μL and monosaccharides (mannose, galactose, fucose, xylose, N-acetyl glucosamine, all purchased from Sigma), 1 M. Each competitor tested was diluted from the initial concentration as either five-fold for monosaccharides or two-fold for glycoproteins and glycopeptides series.

4.2.6. SDS-electrophoresis

In order to probe the suitable amount of glutaraldehyde needed, TPCK-tryptic glycopeptides of 5 mg fetuin isolated on Bio-gel P10 column were coupled to 3 mg BSA with increasing amounts of glutaraldehyde and then separated on 12% SDS-gel (Laemmli, 1970). After electrophoresis the gel was stained with either PAS reagent as in section 3.2.4 to show carbohydrate or Coomassie blue R to show protein.

4.2.7. Protein determination

The technique of Bradford (Bradford, 1976) was employed for protein determination and BSA used as standard.

4.3. Results

4.3.1. Raising of Abs against TPCK-tryptic glycopeptides

TPCK treatment eliminates the impurity of chymotrypsin activity in trypsin (Schoellmann and Shaw, 1962) so that TPCK-trypsin cleaves peptide bonds precisely at the carboxyl end of lysine and arginine as confirmed by amino acid sequence analysis (see section 3.3). All three TPCK-tryptic glycopeptides of C.PRX, GPa, GPb and GPc have one free amino group at the N-terminal and one at the C-terminal lysine (see Fig. 3.2). These can be linked to a carrier protein by a bifunctional coupling reagent such as glutaraldehyde. BSA with mass around 66 kd is not a glycoprotein and has 59 lysine residues (30-35 are

available for coupling) (Harlow and Lane, 1988). Therefore BSA is a good carrier for raising antibodies against glycopeptides as haptens. In order to establish the optimal coupling condition, TPCK-tryptic glycopeptides of fetuin were used to probe the amount of glutaraldehyde needed. Fetuin, with a mass of around 60 kd, has 3 N-linkage and 3 O-linkage glycans (Nilsson et al., 1979). Fig. 4.1 shows a clear separation of its TPCK-tryptic glycopeptides from other peptides on Bio-gel P10 column . Approximately 0.5 μM glycopeptides (from 5 mg fetuin) was used for coupling with 3 mg BSA using different amounts of glutaraldehyde and then separated on gel (Fig. 4.2). Lane 5 shows the least uncoupled BSA and lane 9 is strongly stained with PAS reagent, which demonstrates that 10 μM glutaraldehyde is a sufficient amount for the coupling of 0.5 μM glycopeptides to 3 mg BSA.

TPCK-trypsin digested A.PRX shows a sole glycopeptide on Bio-gel P10 column and HPLC (Fig. 4.3 and Fig. 4.4), which is consistent with the data obtained by purification on ion-exchange columns (Xu and van Huystee 1991).

Antisera were collected after 4 immunizations and ELISA readings of the titre determination are summarized in Table 4.1 Antisera to GPa and GP showed titres of around 100 times lower, compared with antiserum to GPb and GPc. Since GPa has the largest glycan and the shortest peptide chain (13 amino acid residues) among the three glycopeptides of C.PRX, and GP has a very large glycan of 8000 daltons (Xu and van Huystee 1991), the lower titres are probably caused by the poor binding of glycopeptides to the ELISA plate.

There was no significant difference of ELISA readings for antiserum against GPa with coated antigen of 2.5 μg/well to 0.63 μg/well (Table 4.1). Also, little difference in ELISA readings for antisera against GPb and GPc was observed with coated antigen amounts from 2.5 μg/well to 0.16 μg/well, 1.25 μg/well to 0.32 μg/well, respectively (Table 4.1). GP coated at higher amounts presented even lower readings (Table 4.1). This may be caused by overlapping of glycopeptides on the plate so that absorbed antigen molecules became detached during the test and reduced the sensitivity. The optimal amount of antigen coated on ELISA plate was chosen as: GPa, 1 μg of equivalent C.PRX/well; GPb and GPc, 0.5 μg of equivalent C.PRX/well and GP, 2 μg of equivalent A.PRX/well.

4.3.2. Competitive ELISA

In order to investigate the distribution of epitopes on individual glycans, the antibodies specific to the carbohydrate domain were purified by passing 50% saturated ammonium sulphate precipitated IgGs through a correspondingly conjugated ECH Sepharose 4B affinity column. The bound antibodies are thus specific to carbohydrate and are named anti-GLa, anti-GLb and anti-GLc, respectively. Fig. 4.5 shows the sigmoidal response curves of isolated antibodies specific to the carbohydrate moiety. The dilutions that gave a linear response (A_{405} was about 1) were used as working concentrations for competitive ELISA (anti-GLa, 5 ng/μL; anti-GLb, 0.3 ng/μL and anti-GLc, 2.5

ng/μL). Sugar composition and linkage analyses demonstrated that the glycans of C.PRX are composed of N-acetyl glucosamine, mannose, galactose, xylose and fucose and that all the glycans share a common core structure [Xyl](Man)$_3$[Fuc](GlcNAc)$_2$-Asn (van Huystee et al., 1992). Competitive ELISA showed that the reactivity of all three categories of antibodies, anti-GLa, anti-GLb and anti-GLc, is inhibited around 25% to 40% by 1 M fucose (Fig. 4.6). As fucose occurs only in the core structure of glycans (van Huystee et al., 1992), this suggests that all the glycans may have a fucose-directed epitope in the core structure. N-acetyl glucosamine and xylose slightly inhibit the reactivity of all the three categories of antibodies, but mannose and galactose do not inhibit the reactivity of anti-GLa in contrast with a slight inhibition of anti-GLb and anti-GLc.

GPa and GPc almost completely inhibit the reactivity of anti-GLb, while anti-GLa and anti-GLc are less inhibited by the other two rival glycopeptides (Fig. 4.7). This cross reactivity reflects the similarity of the three glycans in core structure and sugar composition, and the diversity in peripheral structure. When any of the three glycan-specific categories of antibodies was passed through the other two conjugated ECH Sepharose 4B affinity columns, no flow-through antibodies were found (Table 4.2). This indicated that none of the three classes of antibodies has any specific antibody that is not recognized by the other two rival glycans. All three categories of glycan-specific antibodies recognize epitopes on both A.PRX and HRP (Fig. 4.8). Also, their reactivity is

inhibited more than 70% by bromelain at 4 $\mu g/\mu L$. In contrast, at the same concentration, fetuin does not compete in the case of anti-GLa and anti-GLc and only slight inhibition in the case of anti-GLb (Fig. 4.8). The detailed structures of glycans of bromelain, horseradish peroxidase and fetuin are well established. Bromelain has a short N-linkage glycan $[Xyl](Man)_2[Fuc](GlcNAc)_2$-Asn (Ishihara et al., 1979; Bouwstra et al., 1990), which is only one mannose less than the core structure of glycans of C.PRX. The major N-linked glycan of HRP has the same core structure $[Xyl](Man)_3[Fuc](GlcNAc)_2$-Asn (McManus et al, 1988; Ashford et al., 1987). Fetuin has three O-linkage and three N-linkage glycans, whose structure is much different from the structure of glycans of C.PRX. There is neither fucose nor xylose attached to the core structure of N-glycans, and the peripheral structure of all the six glycans has terminal N-acetylneuraminic acid (Nilsson et al., 1979). The potent inhibition by bromelain proved that anti-GLa, anti-GLb and anti-GLc are strongly directed toward the common core structure. In the case of anti-GLa and anti-GLc, bromelain presented an even stronger inhibition than C.PRX (Fig. 4.8), probably because of the easier access of shorter glycan of bromelain to the antibodies. The specificity of antibodies toward the common core structure were recognized by horseradish peroxidase but not by fetuin. Although the sugar composition of the glycan of A.PRX is known (van Huystee et al., 1992), the detailed structure is difficult to establish because of its large size. According to the competition test, the glycan of A.PRX has probably

the same core structure.

4.4. Discussion

In the last decade antibodies against varieties of glycoproteins have been raised and antigenic analyses showed that oligosaccharides are major antigens of glycoproteins (Feizi and Childs, 1987; Vautherot et al., 1992; Nakamura et al., 1991; Judelson et al., 1987; Masutani et al., 1990; Hu and van Huystee, 1988; Holmes and Greene, 1991; Wycoff et al., 1987; McManus et al., 1988; Chen and Bahl, 1992; Xu and van Huystee, 1991). The present study directly demonstrated that glycans of glycoproteins are important immunogenic components. Antibodies with carbohydrate specificity are easy to raise and isolate using the methods employed here.

Monosaccharides presented weaker competition for the binding of antibodies to antigen at much higher concentration compared with glycopeptides or glycoproteins. For example, anti-GLb is inhibited 42% by 1 M fucose (Fig. 4.6B), in contrast with 95% inhibition by GPa at a concentration of 10 μg/μL (equal to 250 μM, Fig. 4.7B) and 92% inhibition by HRP at 4 μg/μL (equal to 100 μM, Fig. 4.8B). This is due to the small size of monosaccharides and that monosaccharides may be only a part of the overall epitope recognized by antibody directed towards carbohydrate. Despite the disadvantage, monosaccharides are still useful competitors for epitope mapping of carbohydrate (Masutani et al, 1990), especially because of the difficulty to

obtain specific oligosaccharides.

The three categories of polyclonal antibodies directed towards the three individual N-glycans of C.PRX exhibit high hor: ogeneity as proved by the competitive ELISA. Obviously, the similar immunogenicity of the three N-glycans of C.PRX is due to the identical nature of sugar composition and core structure. It was reported that antiserum against C.PRX recognizes A.PRX (Hu and van Huystee, 1989b) and that monoclonal antibodies directed to A.PRX recognize C.PRX (Xu and van Huystee, 1990). The data shown here reveal that the antigenic cross reaction between C.PRX and A.PRX is caused by the carbohydrate moiety.

Although all the carbohydrate sequences of glycoproteins are potential antigens, the antigenic epitopes characterized so far involve mainly the core backbone and peripheral sugar residues (Feizi et al., 1984; Holmes and Greene, 1991; Masutani et al., 1990; Ohbayashi et al.,1989). The reactivity of the three categories of antibodies, anti-GLa, anti-GLb and anti-GLc is inhibited 25% to 40% by fucose, which suggested that the antibodies are mainly directed to the fucose-containing core backbone. This is further supported by the inhibition test with bromelain. Pineapple stem bromelain is a thiol protease and contains one N-linkage glycan per molecule (Ishihara et al., 1979). It was the first N-linkage glycan in higher plants whose primary structure was well established (Ishihara et al., 1979; Bouwstra et al., 1990). As bromelain inhibits by more than 70% the reactivity of the three classes of antibodies directed to

glycans of C.PRX, the core structure [Xyl](Man)$_3$[Fuc](GlcNAc)$_2$ is confirmed to be the major antigenic determinant. This fucose-containing core structure is widely distributed in complex-type N-glycans of plant glycoproteins, such as Ricin D from castor plant seeds (Kimura et al., 1988), phaseolin from bean (Sturm et al., 1987), *Sophora japonica* lectin (Fourent et al., 1987), protease inhibitor from barbados pride seeds (Hase et al., 1986), *Clerodendron trichotomum* lectin (Kitagaki-Ogawa et al., 1986), *Erythrina cristagalli* lectin (Ashford et al., 1987), S-glycoproteins from *B. campestris* (Takayama et al., 1987), 52/54-kDa medium protein and ß-fructosidase from carrot (Sturm, 1991), laccase from sycamore cells (Takahashi and Hotta, 1986), HRP (McManus et al, 1988; Ashford et al., 1987). The homogeneity of carbohydrate may be the basis of often observed antigenic cross reactions among different glycoproteins that are distinct in both function and structure of peptide moiety (McManus et al, 1988; Xu et al., 1990; Xu and Huystee, 1991).

Antibodies, including monoclonal antibodies and polyclonal antisera to glycoproteins are increasingly used as probes in biochemical and biological studies, such as investigating embryonic development, cell differentiation and oncogenesis, and monitoring the biosynthesis and subcellular localization of specific glycoproteins of enzymes or receptors (Feizi and Childs, 1987; Jacob,1979; Childs et al., 1983; Feizi et al.,1984). Critical for the proper application of antibodies in such studies is an awareness of a potential cross reaction based on carbohydrate moiety, especially the core structure of

oligosaccharide chains. In order to determine whether antibodies have carbohydrate specificity or to eliminate the immunogenicity of carbohydrate moiety of glycoproteins, various methodologies have been developed. Deglycosylation of antigens of glycoproteins is widely used. For example, enzymatic deglycosylation by exo- or endo-glycosidases, chemical deglycosylation with trifluoromethane sulfonic acid (TFMS), and biosynthesis of proteins in the presence of glycosylation inhibitor, such as tunicamycin may be used. But complete deglycosylation with either glycosidases or chemical treatment may be difficult to achieve and chemical deglycosylation may modify the peptide moiety (Tang and Feizi, 1987; Edge et al., 1981). Tunicamycin treatment may decrease the secretion of proteins (Ravi et al., 1986; Hu and van Huystee, 1989a) and cause degradation of proteins before they are sorted (Faye and Chrispeels,1989).

We supplied an effective way to determine or rule out the carbohydrate specificities of antibodies. The antigen of glycoprotein is digested with pronase and then the produced glycan-Asn fragments (in some cases two or more amino acid residues may remain attached with the glycan depending on the accessibility of pronase to the peptide) are coupled to ECH Sepharose 4B. This glycan-conjugated affinity column could be valuable for determining or isolating antibodies with carbohydrate specificity. Conversely, the antibodies with affinity to a well-defined glycan may be used as a probe to elucidate structures of other carbohydrate chains.

Fig. 4.1. **Separation of glycopeptides of fetuin on a Bio-gel P10 column.** Thirty milligrams of fetuin were digested with TPCK-trypsin in a ratio of 1/30 (TPCK-trypsin/fetuin, w/w) at 37^{0}C for 15 hours. The mixture was applied to a Bio-gel P10 column (1.5 cm x 90 cm) and eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% sodium azide at a flow rate 7.2 mL/h. The eluent was monitored at 280 nm using a UV-2 monitor (Pharmacia) and fractions of 1.8 mL were collected. Glycopeptide fractions were identified and cross-hatched.

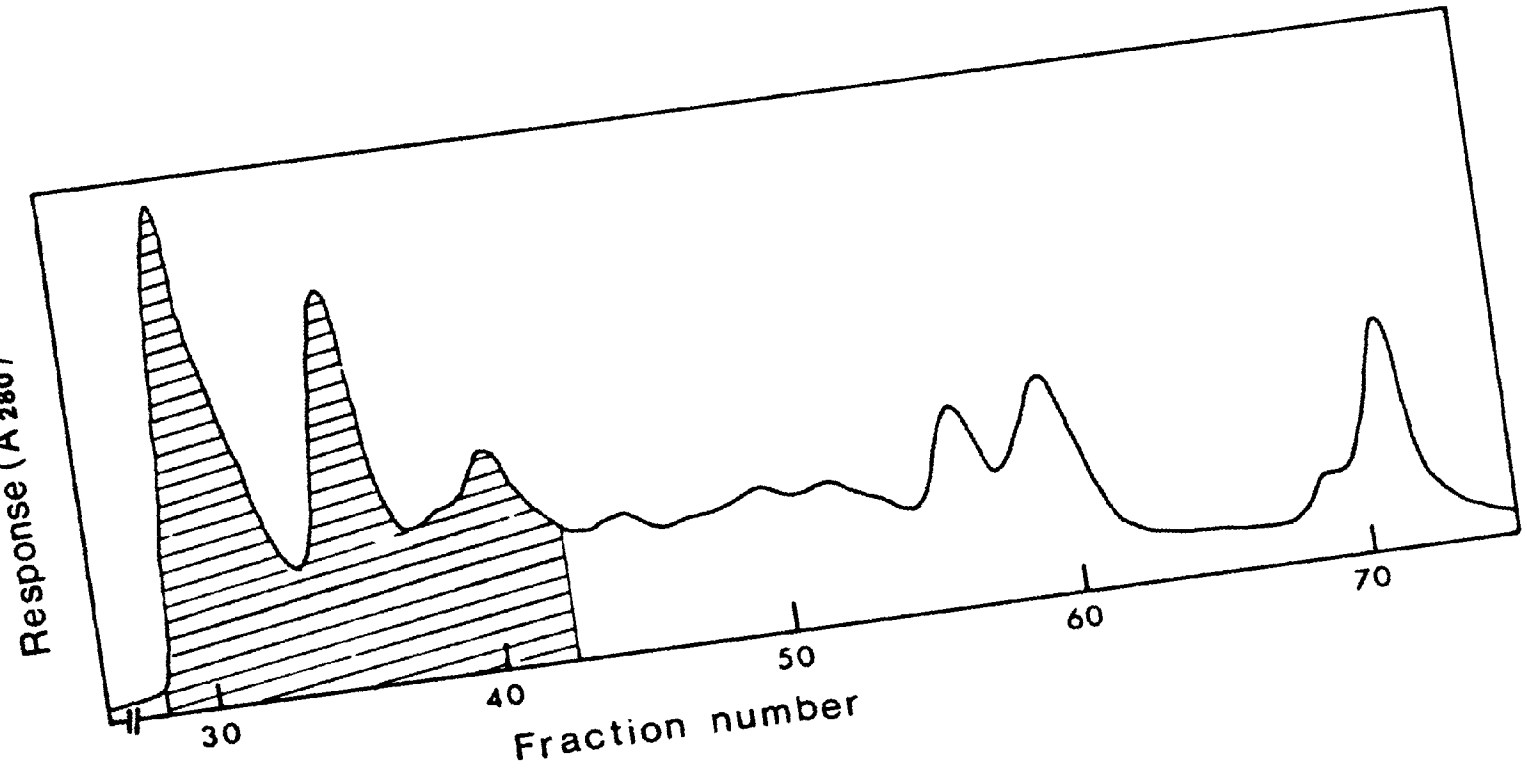

Response (A_{280})
30
40
50
60
70
Fraction number

Fig. 4.2. 12% SDS-PAGE of TPCK-tryptic glycopeptides of fetuin coupled to BSA using varying amounts of glutaraldehyde. Lane 1, 5 μg BSA; lane 2, 5 μg fetuin; lane 3-6, 10 μg coupled BSA; lane 7-10 40 μg coupled BSA. Lanes 1-6 were stained with 0.1% Coomassie blue R. Lane 7-10 were stained with PAS reagent. The arrows show standard protein molecular weight marks. TPCK-tryptic glycopeptides of 5 mg fetuin (around 0.5 μM of glycopeptide) were coupled to 3 mg BSA. For lanes 3 and 7, 2 μM (0.1 mL 0.2% glutaraldehyde); lane 4 and 8, 4 μM (0.2 mL of 0.2% glutaraldehyde); lanes 5 and 9, 10 μM (0.1 mL 1% glutaraldehyde); lane 6 and 10, 20 μM (0.2 mL 1% glutaraldehyde) were used, respectively.

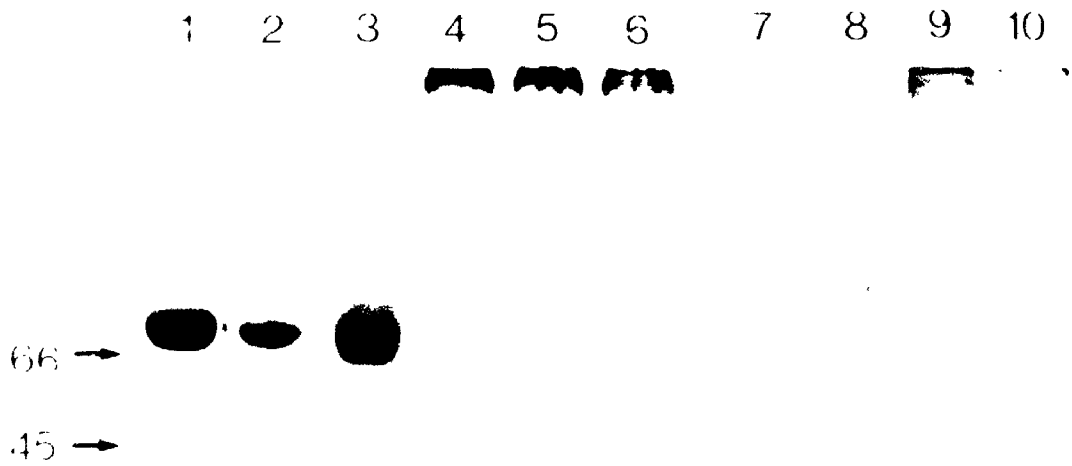

1 2 3 4 5 6 7 8 9 10
66
45

Fig. 4.3. Separation of glycopeptide of A.PRX on Bio-gel P10 column. Ten milligrams of heme depleted A.PRX were digested with TPCK-trypsin in a ratio of 1/30 (TPCK-trypsin/CP-, w/w) at 37^{0}C for 40 min.. The mixture was applied to a Bio-gel P10 column (1.5 cm x 90 cm) and eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% sodium azide at a flow rate 8 mL/h. The eluent was monitored at 280 nm using a UV-2 monitor (Pharmacia) and fractions of 2 mL were collected. Glycopeptide fractions were identified and are cross-hatched.

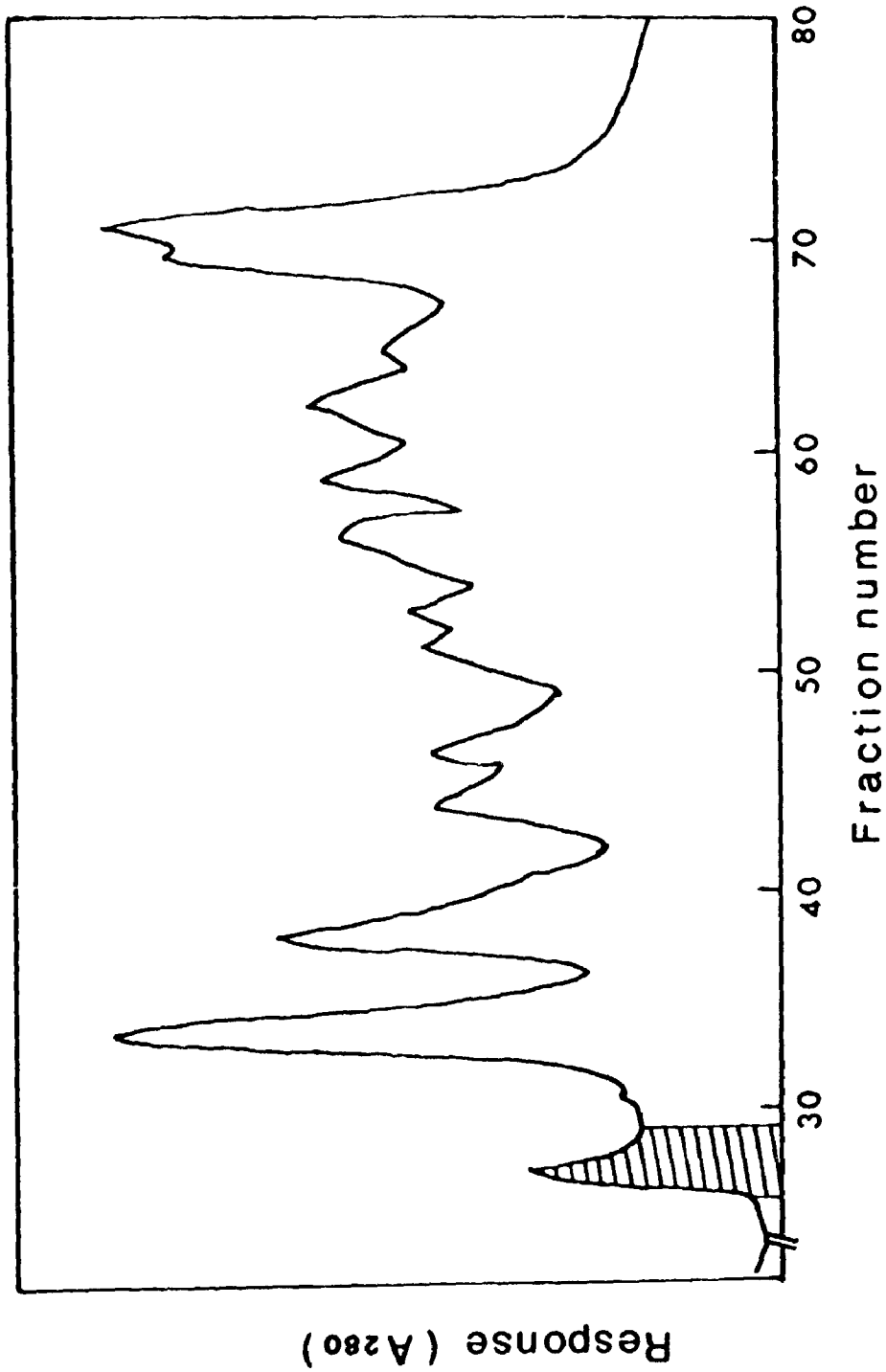

Response (A_{280})
30
40
50
60
70
80
Fraction number

Fig. 4.4. Purification of glycopeptides of A.PRX with reverse-phase HPLC. Glycopeptide fractions of 1 mg TPCK-tryptic A.PRX separated on Bio-gel P10 column (Fig. 4.3) were dissolved in 100 μL of 0.1% TFA, applied to a Waters u-Bondapak™ C_{18} column (3.9 x 300 mm) and eluted with an acetonitrile linear gradient at 1.2 mL/min. The concentration of acetonitrile was raised from zero to 30% in 30 min.. The elution was monitored by UV absorption at 230 nm. Only one glycopeptide peak was identified and named as GP.

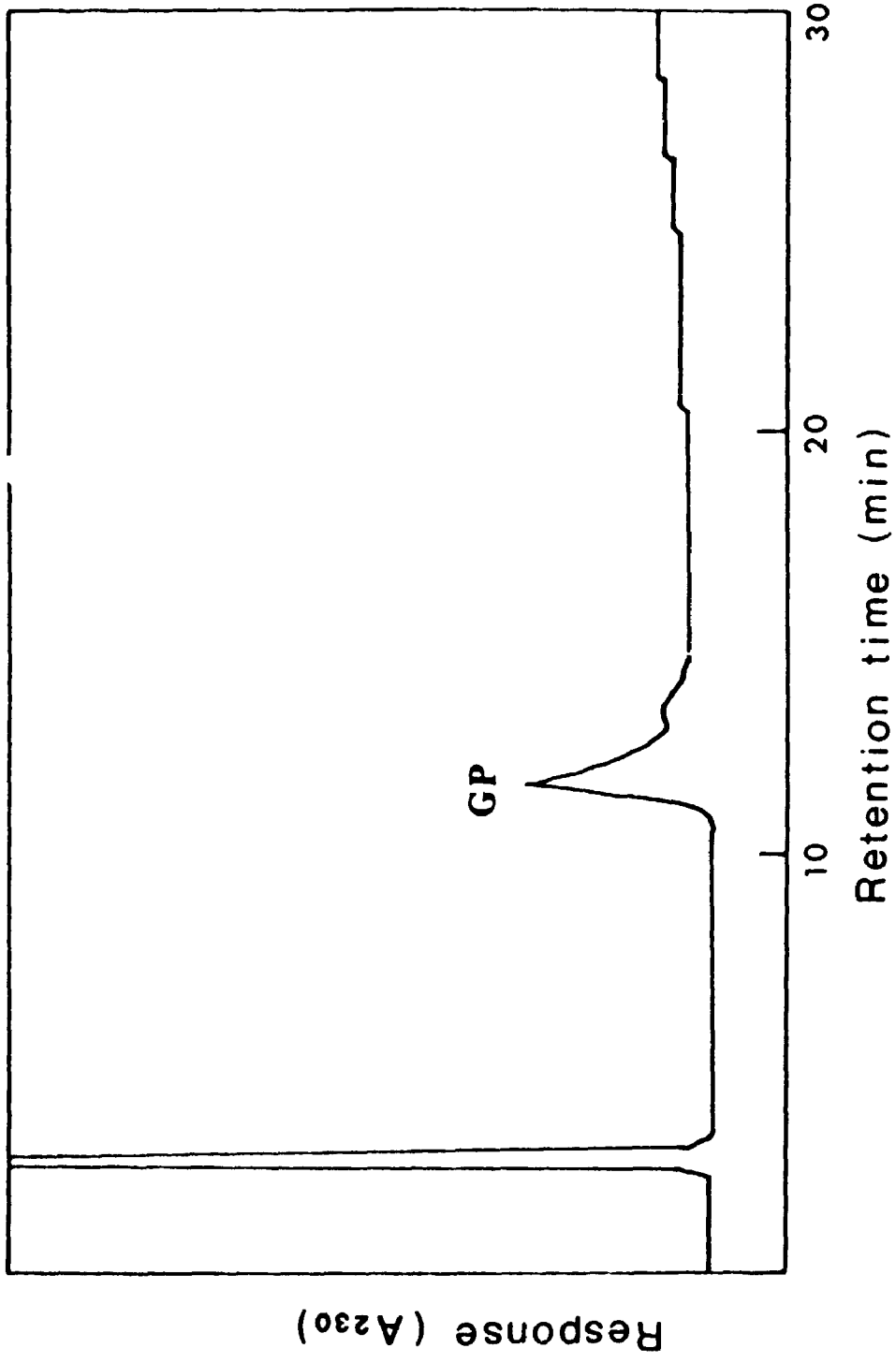

GP
10
20
30
Retention time (min)
Response (A230)

Table 4.1 Titre determination of antisera against TPCK-tryptic glycopeptides of C.PRX and A.PRX conjugated to BSA[a]

Coated Ag (ug /well)		Dilution times of antisera				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
GPa	2.5	1.162	1.052	0.540	0.245	0.022
	1.25	1.105	1.026	0.490	0.168	0.031
	0.63	1.029	0.962	0.425	0.128	0.020
	0.32	1.003	0.626	0.219	0.049	0.028
	0.16	0.845	0.572	0.135	0.051	0.026
GPb	2.5	*	*	*	1.842	0.689
	1.25	*	*	*	*	0.608
	0.63	*	*	*	1.956	0.596
	0.32	*	*	*	1.781	0.536
	0.16	*	*	*	1.741	0.524
GPc	2.5	*	*	*	1.422	0.420
	1.25	*	*	*	1.291	0.282
	0.63	*	*	*	1.264	0.255
	0.32	*	*	*	1.170	0.208
	0.16	*	*	*	0.933	0.134
GP	10	1.672	1.572	0.451	0.062	0.002
	5	1.899	1.693	0.582	0.084	0.014
	2.5	*	*	0.575	0.056	0.010
	1. 25	*	1.703	0.446	0.048	0.003
	0.63	1.364	1.169	0.282	0.026	0.005
	0.32	1.182	0.851	0.200	0.015	0.008

[a]The titre was measured using ELISA after 4 immunization injections. Each value represents the mean of three assays. The * means that the A_{405} was greater than 2.

Fig. 4.5. Typical response curves for the reaction between the optimized amounts of coated antigens and dilutions of purified antibodies against individual glycans of C.PRX. The amounts of coated antigens were: GPa, 1 μg/well; GPb and GPc, 0.5 μg/well. The stock concentration of purified antibodies was: anti-GLa, 0.64 μg/μL; anti-GLb, 0.02 μg/μL and anti-GLc, 0.08 μg/μL. The antibodies were diluted from the initial concentration as two-fold series. Anti-GLa (O---O), anti-GLb (●---●) and anti-GLc (▽---▽). Each point represents the mean of three assays.

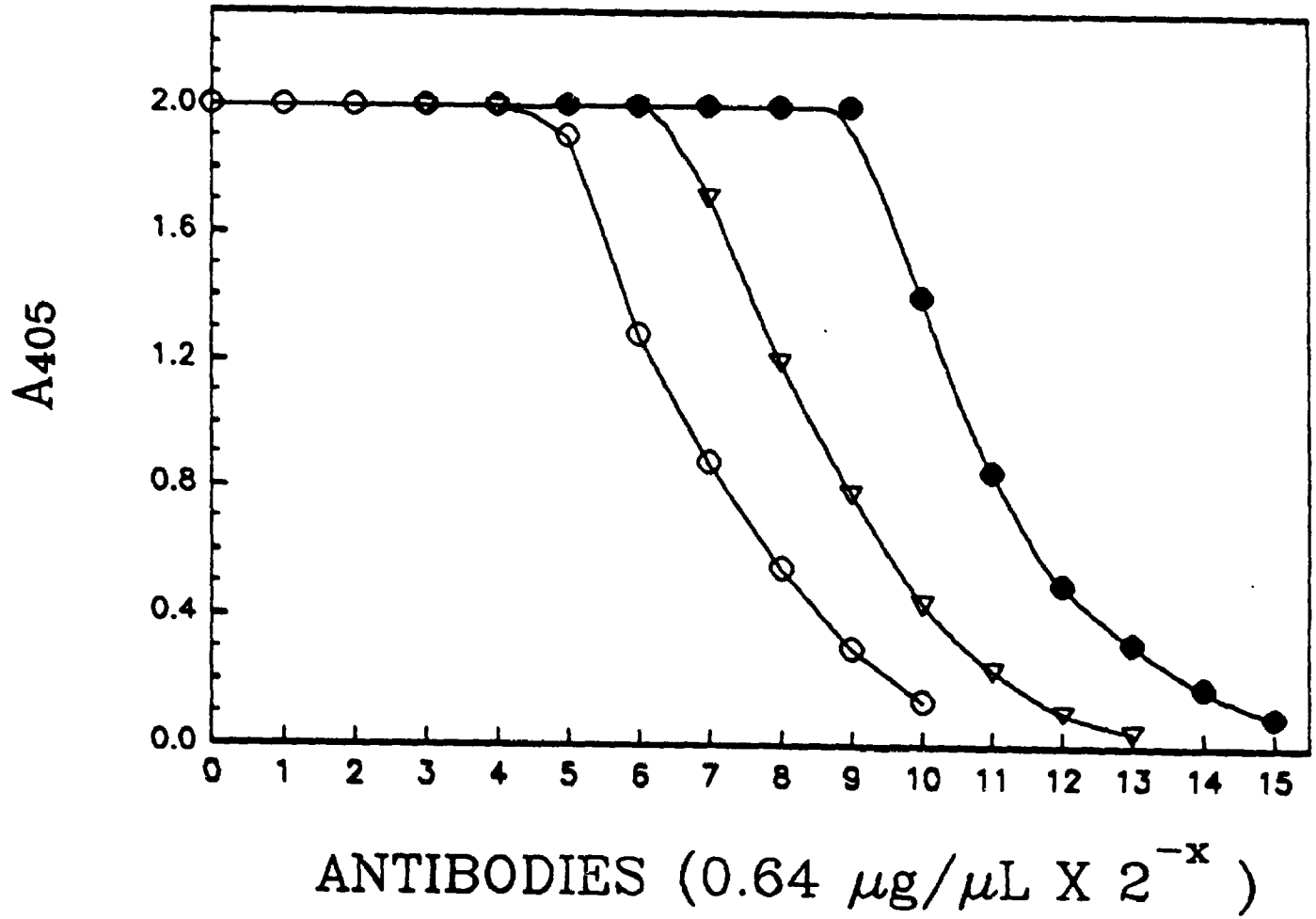

A405
2.0
1.6
1.2
0.8
0.4
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ANTIBODIES (0.64 μg/μL X 2^-x)

Fig. 4.6. Inhibition of the binding of antibodies (A, anti-GLa; B, anti-GLb and C, anti-GLc) to immobilised antigens (A, GPa; B, GPb and C, GPc) by preincubation with various monosaccharides. The concentration of antibodies employed was: anti-GLa, 5 ng/μL; anti-GLb, 0.3 ng/μL and anti-GLc, 2.5 ng/μL. The monosaccharides were diluted from the initial concentration of 1 M as five-fold series. Mannose ▽---▽, N-acetyl glucosamine ●---●, galactose ○---○, xylose □---□, fucose ▼---▼. Each point represents the mean of three assays.

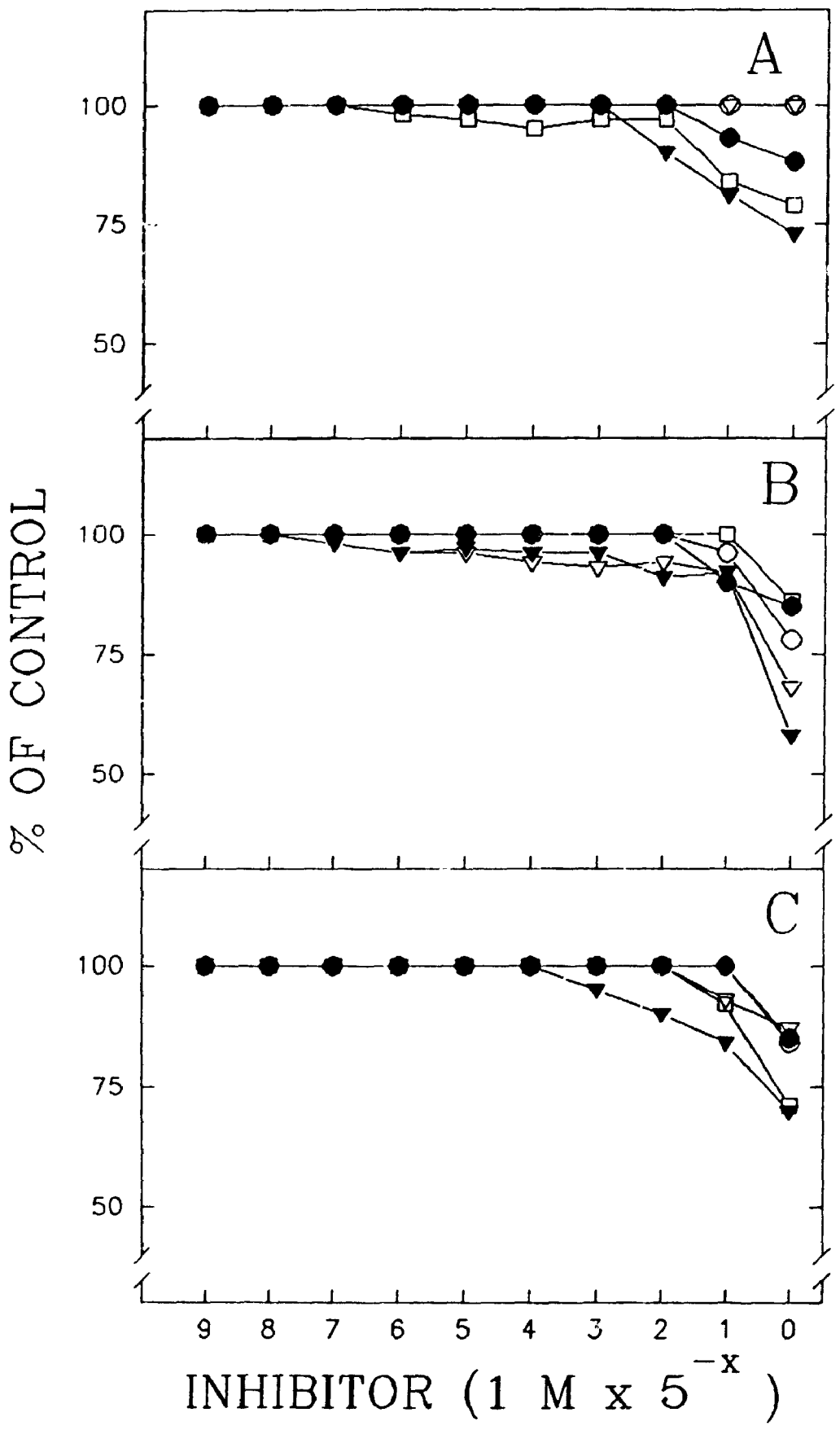

A
B
C
100
75
50
% OF CONTROL
9
8
7
6
5
4
3
2
1
0
INHIBITOR (1 M x 5 $^{-x}$)

Fig. 4.7. Inhibition of the binding of antibodies (A, anti-GLa; B, anti-GLb and C, anti-GLc) to immobilised antigens (A, GPa; B, GPb and C, GPc) by preincubation with glycopeptides from C.PRX. The concentration of antibodies employed was: anti-GLa, 5 ng/μL; anti-GLb, 0.3 ng/μL and anti-GLc, 2.5 ng/μL. The glycopeptides were diluted from the initial concentration of 10 μg/μL as two-fold series. GPa o---o, GPb ●---●, GPc ▽---▽. Each point represents the mean of three assays.

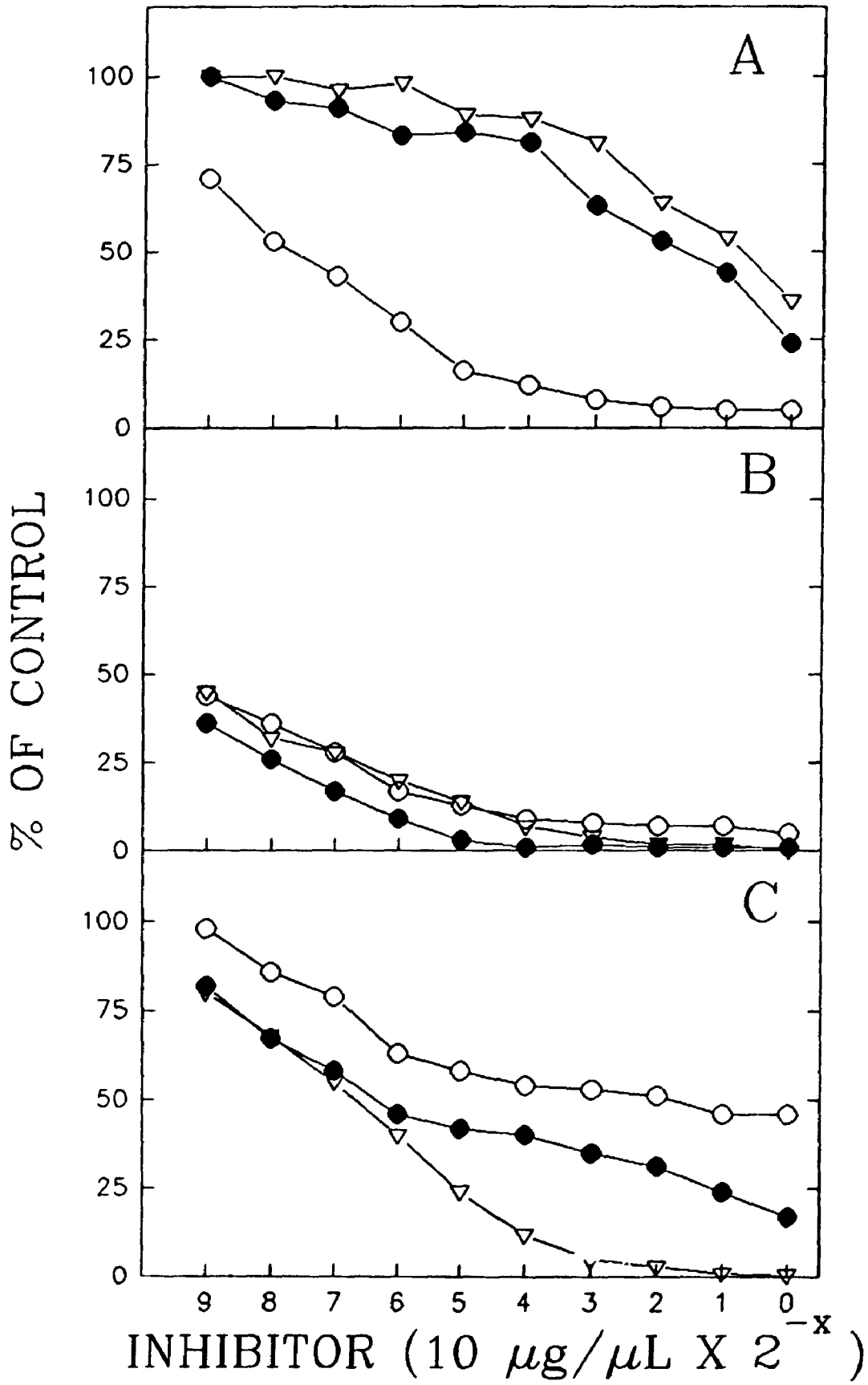
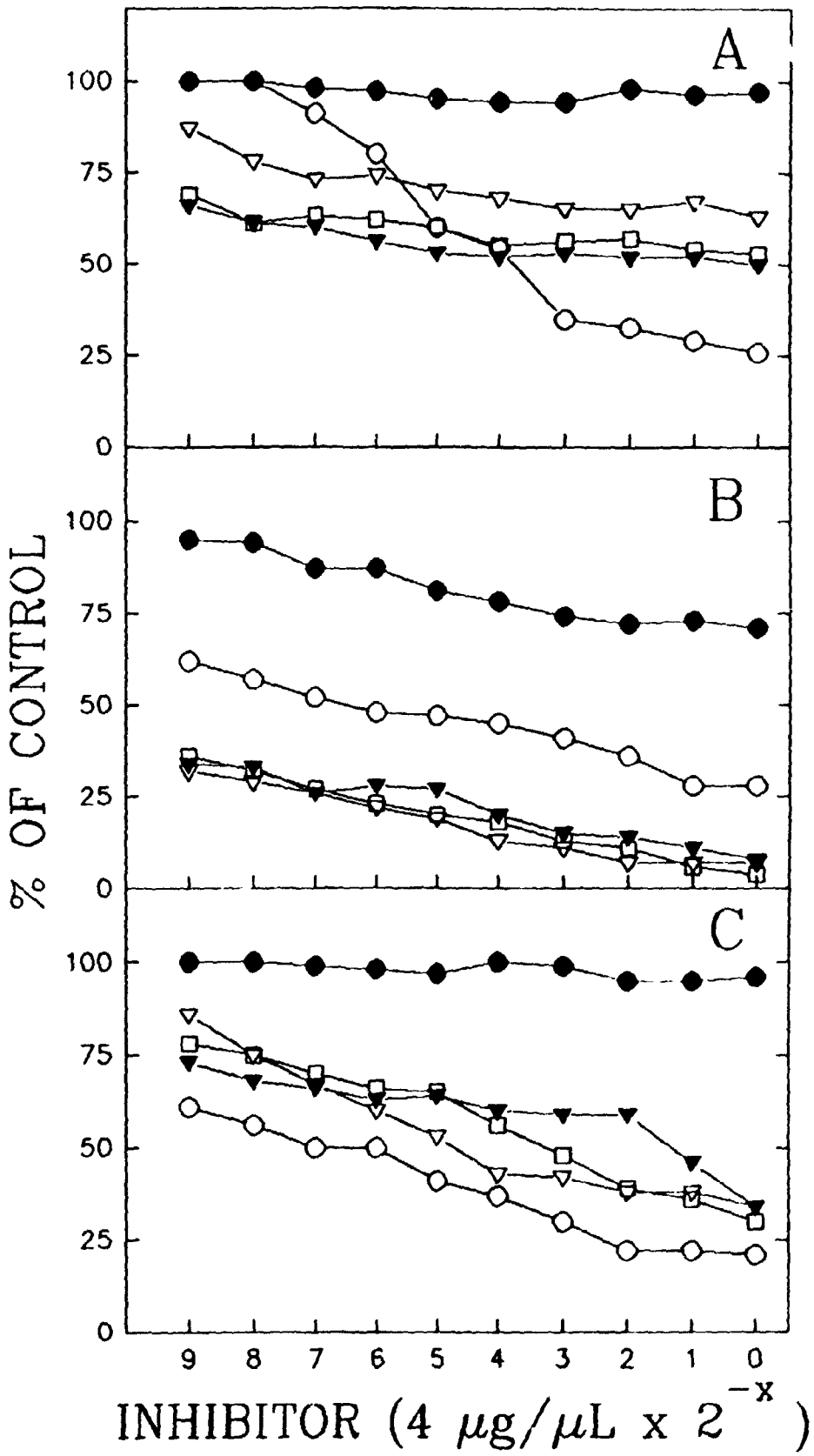

A
B
C
100
75
50
25
0
% OF CONTROL
9
8
7
6
5
4
3
2
1
0
INHIBITOR (10 μg/μL X 2^-X)

Table 4.2 Comparison of immunogenicity of glycans of C.PRX[a]

Antibodies	Affinity columns	Flow through proteins
Anti-GLa (3.7 mg)	GLb-ECH Sepharose 4B and GLc-ECH Sepharose 4B	49 μg (1.3%)
Anti-GLb (3.9 mg)	GLa-ECH Sepharose 4B and GLc-ECH Sepharose 4B	2 μg (0.05%)
Anti-GLc (3.9 mg)	GLa-ECH Sepharose 4B and GLb-ECH Sepharose 4B	31 μg (0.8%)

[a]Each of anti-GLa, anti-GLb and anti-GLc was passed through the two rival glycan-Asn-ECH Sepharose 4B affinity columns contiguously linked and the amount of flow-through proteins was measured and converted to the percentage of applied antibodies. The column running condition is the same as for the purification of antibodies described in "Materials and methods".

Fig. 4.8. Inhibition of the binding of antibodies (A, anti-GLa; B, anti-GLb and C, anti-GLc) to immobilised antigens (A, GPa; B, GPb and C, GPc) by preincubation with various glycoproteins. The concentration of antibodies employed was: anti-GLa, 5 ng/μL; anti-GLb, 0.3 ng/μL and anti-GLc, 2.5 ng/μL. The glycoproteins were diluted from the initial concentration of 4 μg/μL as two-fold series. Bromelain ○---○, fetuin ●--●, A.PRX ▽---▽, HRP ▼---▼, C.PRX □---□. Each point represents the mean of three assays.


A
B
C
100
75
50
25
0
% OF CONTROL
9
8
7
6
5
4
3
2
1
0
INHIBITOR (4 μg/μL x 2^-x)

CHAPTER 5

Carbohydrate Moiety of Peanut Peroxidase Necessary for Enzyme Activity

5.1. Introduction

The roles of the carbohydrate moiety of peanut peroxidases have been considered in several aspects. Their glycans are critical for the secretion of peroxidases. Tunicamycin-supplemented culture medium does not inhibit the biosynthesis of the peptide moiety but dramatically decreases the secretion of peanut peroxidases into the culture (Ravi et al., 1986; Hu and van Huystee, 1989a). Glycans apparently protect against trypsin proteolysis of peanut peroxidases (Hu and van Huystee, 1989a). This chapter focuses on whether glycans are necessary for enzyme activity. The effect of the removal of glycans on the enzyme structure and activity, and the effect of carbohydrate-directed antibody binding on enzyme activity were investigated. The results show that the carbohydrate moiety of peanut peroxidase probably is not directly involved in the catalytic centre but is essential to maintain the structure for the expression of enzyme activity.

5.2. Materials and methods

5.2.1. Cell culture and peroxidase purification

As described in section 1.2.

5.2.2. Raising of antisera directed against TPCK-tryptic glycopeptides of C.PRX and A.PRX

The isolation of the TPCK-tryptic glycopeptides derived from C.PRX (GPa, GPb and GPc) and A.PRX (GP), the coupling of the glycopeptides to BSA and the immunization are described in section 4.2.1, 4.2.2 and 4.2.3. The antisera directed against GPa, GPb, GPc and GP were named anti-GPa, anti-GPb, anti-GPc and anti-GP respectively.

5.2.3. Raising of antisera directed against intact C.PRX and A.PRX

Antisera against intact C.PRX and A.PRX were raised as reported (Chibbar and van Huystee, 1984b; Zeng and van Huystee, 1992). One milligram of either purified C.PRX or A.PRX was emulsified in complete (for initial injection) or incomplete (for subsequent boosting) Freund's adjuvant and was injected subcutaneously into New Zealand rabbits. The rabbits were boosted twice every two weeks and the blood was collected. The antisera directed against C.PRX and A.PRX were named anti-C.PRX and anti-A.PRX, respectively.

5.2.4. Antibody binding test

Three hundred μL antiserum of different dilutions (in 0.05 M sodium

phosphate buffer, pH 7.0) was mixed with 0.6 μg of either C.PRX or A.PRX and incubated at 37°C for 1 h.. Then 1.3 ml of the above buffer, 0.5 ml 1% (v/v) guaiacol and 1 ml 0.3% H_2O_2 were added to the incubant (van Huystee and Lobarzewski, 1982). The change of absorbance at 470 nm of the mixture was recorded on Shimadzu UV-160 spectrophotometer using the time scan mode. Within the straight line region of the time-course curve the reaction kinetics is zero order. The change of A_{470} within 30 seconds (ΔA_{470}/30 sec) was used to calculate C.PRX or A.PRX activity.

5.2.5. Treatment of C.PRX with PNGase F

Ten μg of C.PRX was incubated with PNGase F (Boehringer Mannheim, specific activity of PNGase F: 25,000 units/mg protein) in 100 μL 0.1 M sodium phosphate buffer, pH 8.0, with different ratio (units of PNGase F/mg C.PRX) from 0.12 to 6 at 37°C for increasing time by a modified method (Tarentino et al., 1985). After incubation 1 μg C.PRX was taken to measure enzyme activity as described in section 5.2.4. For calcium content, absorption and CD spectra determination, C.PRX was treated with PNGase F (0.6 units of PNGase F/mg C.PRX) at 37°C overnight and dialysed against distilled water at 4°C overnight. To check whether there is an impurity of protease activity in PNGase F, 15 units of recombinant product of ß-galactosidase in E.coli (around 50 μg protein, Boehringer Mannheim) was incubated with 0.03 unit of PNGase F (0.6 unit/mg protein) in 500 μL of 0.1 M sodium phosphate buffer, pH 8.0, at 37°C overnight.

To measure ß-galactosidase activity, 3 μL of mixture was taken and 200 μl of 0.1 M sodium phosphate buffer, pH 8.0, 200 μl 10 mM nitrophenol-galactopyranoside (Sigma) aqueous solution were added, mixed and incubated at 37°C for 30 min. To stop the reaction, 1.25 ml of 1 M sodium carbonate solution was added and the absorbance at 400 nm was measured (Montreuil et al., 1986b).

5.2.6. SDS-polyacryramide gel electrophoresis

C.PRX treated with PNGase F (0.6 units of PNGase F/mg C.PRX) at 37°C overnight was separated on SDS-polyacryramide gel. After electrophoresis the gel was stained with PAS reagent as described in section 3.2.4.

5.2.7. Reconstitution of PNGase F treated C.PRX

C.PRX treated with PNGase F (0.6 units of PNGase F/mg C.PRX) at 37°C overnight was reconstituted in 0.1 M Tris-HCl, pH 7.4, 0.1 mM heme, 10 mM $CaCl_2$ at 4°C overnight (Tams and Welinder, 1991) and then dialysed against distilled water at 4°C overnight to remove any extra heme, Ca^{2+} and salts. The activity of reconstituted C.PRX was checked before and after dialysis.

5.2.8. Absorption spectra

Absorption spectra of C.PRX, PNGase F treated C.PRX (F-C.PRX) and reconstituted C.PRX (R-C.PRX) were determined on a Shimadzu UV-160

spectrophotometer using the spectra mode.

5.2.9. Circular dichroism spectroscopy

The CD spectra of C.PRX and PNGase F treated C.PRX (F-C.PRX) were recorded on a Jasco J-500C spectropolarimeter controlled by the program CDSCAN operating on an IBM 90001 computer (Gasyna et al., 1989). The data were manipulated with the program Spectra Manager (Browett and Stillman, 1987). The work of CD spectra was done by Dr. M. Stillman of Department of Chemistry, UWO.

5.2.10. Calcium determination

The calcium content of C.PRX and PNGase F treated C.PRX was measured on an atomic absorption spectrophotometer (AA-5) using $CaCl_2$ as standard.

5.2.10. Protein determination

The technique of Bradford (Bradford, 1976) was employed for protein determination and bovine serum albumin (Sigma) as standard.

5.3. Results

The maximum inhibition of enzyme activity by the binding of anti-C.PRX and anti-A.PRX antibodies was 60% and 30%, respectively (Table 5.1).

However, the binding of antibodies raised against glycopeptides did not affect enzyme activity at any dilutions (Table 5.1). As shown in Table 5.2, the treatment with PNGase F decreased C.PRX activity and this effect was time and dose dependent. When C.PRX was treated with PNGase F at the ratio of 0.6 units of PNGase F/mg C.PRX for 24 h., the enzyme activity was completely eliminated. The incubation of a recombinant product ß-galactosidase in E. coli with PNGase F does not decrease galactosidase activity (A_{400} with treatment of PNGase F is 1.192, A_{400} without treatment is 1.182). This assay excludes the possibility that a protease impurity in PNGase F may degrade C.PRX during incubation. This conclusion is further supported by SDS-PAGE of PNGase F treated C.PRX (Fig. 5.1). PNGase F treated C.PRX only lost around 2-3 kilodaltons of mass. As PNGase F treated C.PRX was still stained with PAS reagent, it is concluded that the deglycosylation was not complete.

Fig. 5.2 displayed that both C.PRX and PNGase F treated C.PRX have a peak at 405 nm, which is typical of most heme proteins (Keilin and Hartree, 1951). But the RZ value of PNGase F treated C.PRX was only 43% of that of C.PRX (Table 5.3). Reconstituted C.PRX did not show any enzyme activity either before or after dialysing, but the RZ value was partially recovered to 65% of that of native C.PRX (Table 5.3). The loss of heme of C.PRX with the treatment of PNGase F was further confirmed by CD spectra. Fig. 5.3. showed that C.PRX has negative CD bands at 340, 375 mμ and a positive band at 408 mμ in the visible region. These CD bands occur at wavelengths where heme

absorption bands are evident (Strickland et al., 1968). But the occurrence of these bands was decreased in the spectra of PNGase F treated C.PRX, which indicates the loss of heme. In the ultraviolet region, the spectra of C.PRX and PNGase F treated C.PRX showed some differences in both the intensity and shape. This is probably contributed to by the alteration in the orientation of aromatic amino acid residues within the protein, which suggests a conformational change of PNGase F treated C.PRX (Marañon et al., 1993). The result of calcium content measuring of C.PRX and PNGase F treated C.PRX showed that the calcium content of PNGase F treated C.PRX is 50% of that of intact C.PRX. As C.PRX has two calcium ions each molecule (Hu et al., 1987), probably one calcium ion is lost in PNGase F treated C.PRX.

5.4. Discussion

As discussed in section 1.6, glycans of glycoproteins perform important biological functions, such as in the secretion and sorting of glycoproteins (Faye and Chrispeels, 1989; Machamer et al., 1985; Ravi et al., 1986), protection against proteolysis (Olden et al., 1987; Wilson et al., 1981; Hohmann et al., 1987), immunogenicity (see chapter 4) and recognition (Feizi, 1985; Hakomori, 1984). Probably the most controversial point is whether glycans are necessary for the biological activity of glycoproteins.

Although the antibodies directed against intact C.PRX or A.PRX inhibited enzyme activity, the antibodies directed against glycopeptides did not block

enzyme activity at all. Similar results were observed in the antibody binding test using antibodies against native HRP IX and its glycopeptide (Conroy et al, 1982; Clark et al., 1983). These suggested that the glycans are not directly involved in the active centre. The experiment of PNGase F treatment of C.PRX showed that PNGase F eliminated C.PRX activity and that the loss of the activity is not due to the protease impurity, but resulted in partial deglycosylation of C.PRX by PNGase F (as shown in Fig. 5.3). That glycans are necessary for the expression of biological activity of glycoproteins was also reported in some other cases. The deglycosylation of yeast acid phosphatase by tunicamycin (Mizunaga and Noguchi, 1982), the removal of N-glycans of thyrotropin by PNGase (Thotakura et al., 1992) and the elimination of carbohydrate of maize catalase inhibitor by glycosidase (Tsaftaris et al., 1980) either abolished or greatly affected the biological activity of the glycoproteins. The biological activities of human tissue plasminogen activator (Howard et al., 1991) and antibody towards α(1-6)dextran (Wright et al., 1991) have even been shown to be dependent on the structural features of the oligosaccharides at distinct positions within the peptide. Why are the biological activities of glycoproteins related to carbohydrate moiety? Probably the change of the glycan structure or the removal of glycans directly or indirectly induces a conformational alteration in the protein structure. It is noted that after reconstitution of PNGase F treated C.PRX with heme and calcium, the enzyme activity was not regained and the RZ value was only partially recovered. In contrast, the removal of heme by

acidic acetone and the loss of C.PRX enzyme activity can be restored with reconstitution (Chibbar et al., 1984). The removal of glycans from C.PRX may generate some subtle change of the spatial structure of the heme envelope and loosen the linkage between heme and peptide chain. This structural change is not as reversible as the removal of heme by acidic acetone (Chibbar et al., 1984). The structural change of the peptide chain with PNGase F treatment was demonstrated by the CD experiment. Antibodies against nonglycosylated envelope proteins of Semliki Forest virus do not react with their native glycosylated counterparts. This suggests that the carbohydrate chains confer conformational features on the glycoprotein not present in the nonglycosylated species (Schwarz et al., 1979).

When C.PRX is stored at dilute concentration the RZ value is readily decreased unless it is stored in 10 mM Ca^{2+} (van Huystee et al., 1990). Further CD spectra study revealed that Ca^{2+} plays an important role in maintaining protein structure (Marañon et al., 1993) of C.PRX. A similar case occurs for α-amylase. When it is stored in low calcium concentration the molecule changed conformation (Bush et al., 1989). The loss of one calcium ion in PNGase F treated C.PRX may strengthen the change of the conformation of the peptide moiety and the association of heme with peptide chain. As heme is the important prosthetic group in peroxidase catalysed redox reactions, the loss of heme or any shift of the orientation of heme in the heme environment obviously destroys enzyme activity. How glycans affect the spatial structure of proteins is

not yet clear. It can not be resolved definitively until the tertiary structure of glycosylated versus nonglycosylated (or modified) forms of glycoproteins are compared (Olden et al., 1985).

Table 5.1 Effect of antibody binding on anionic and cationic peroxidase activity. Three hundred μL of a variety of antisera with different dilutions was incubated with 0.6 μg C.PRX (in the case of anti-GPa, anti-GPb, anti-GPc and anti-C.PRX) or 0.6 μg A.PRX (in the case of anti-GP and anti-A.PRX) at 37^{0}C for 1 h.. Then enzyme activity was determined and the change of A_{470} within 30 seconds (ΔA_{470}/30 sec) was employed to calculate peroxidase activity. The effect of antibody binding on peroxidase activity was expressed as % of control = (ΔA_{470} with antiserum/ΔA_{470} with preimmune) x 100 %. The initial concentrations of IgGs of the antisera are anti-C.PRX, 20 mg/mL; anti-A.PRX, 18 mg/mL; anti-GPa, 16 mg/mL; anti-GPb, 20 mg/mL; anti-GPc, 18 mg/mL and anti-GP, 19 mg/mL respectively.

serum	dilution	ΔA_{470}/30 sec	% of control
anti-GP_a	1:1	0.129	109
	1:10	0.166	101
	1:100	0.195	113
anti-GP_b	1:1	0.120	102
	1:10	0.166	101
	1:100	0.180	104
anti-GP_c	1:1	0.128	108
	1:10	0.172	106
	1:100	0.174	101
anti-GP	1:1	0.120	102
	1:10	0.159	97
	1:100	0.173	100
preimmune	1:1	0.118	100
	1:10	0.164	100
	1:100	0.173	100
anti-C.PRX	1:1	0.053	38
	1:10	0.063	38
	1:100	0.155	84
anti-A.PRX	1:1	0.097	72
	1:10	0.123	75
	1:100	0.170	101
preimmune	1:1	0.135	100
	1:10	0.164	100
	1:100	0.168	100

Table 5.2. Inhibition of C.PRX activity by the treatment of PNGase F. C.PRX was incubated with PNGase F at 37°c for increasing time as described in section 5.2.5. One microgram of C.PRX was used to determine enzyme activity at different time and the change of absorbance at 470 nm within 30 seconds (ΔA_{470}/30 sec) was employed to calculate C.PRX activity.

Dose (units/mg C.PRX)	Incubation time	C.PRX activity (ΔA_{470}/30 sec.)		
		Control	Treatment	% of control
0.12	20 min	0.257	0.268	104
	1.5 h.	0.261	0.262	100
	3 h.	0.255	0.242	95
	6 h.	0.258	0.206	80
	25 h.	0.243	0.022	9
0.6	20 min	0.257	0.256	100
	1.5 h.	0.261	0.159	61
	3 h.	0.255	0.130	51
	6 h.	0.258	0.081	31
	25 h.	0.240	0.009	4
3	20 min	0.263	0.208	79
	1.5 h.	0.260	0.120	46
	3 h.	0.255	0.053	31
	6 h.	0.258	0.001	0.6
	25 h.	0.240	0.000	0
6	20 min	0.257	0.082	48
	1.5 h.	0.261	0.007	4
	3 h.	0.255	0.002	1
	6 h.	0.259	0.000	0

Fig. 5.1. 12% SDS-PAGE of PNGase F treated C.PRX. C.PRX was treated with PNGase F (0.6 unit/mg C.PRX) at 37^{0}C overnight and then applied on SDS-gel. Left, 10 μg native C.PRX; Right, 20 μg PNGase F treated C.PRX. The arrows show standard protein molecular weight marks (kd). After electrophoresis the gel was stained with PAS reagent.

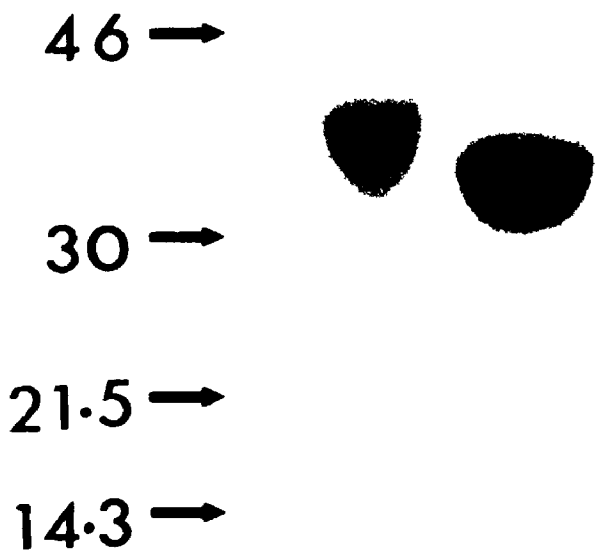

46
30
21·5
14·3

Fig. 5.2. Comparison of absorption spectra of C.PRX, PNGase F treated C.PRX and reconstituted C.PRX. The concentration of C.PRX, PNGase F treated C.PRX (F-C.PRX) and reconstituted C.PRX (R-C.PRX) used for absorption spectra was 0.4 mg/mL H_2O (10 μM). The absorption spectra were determined on Shimadzu UV-160 spectrophotometer.

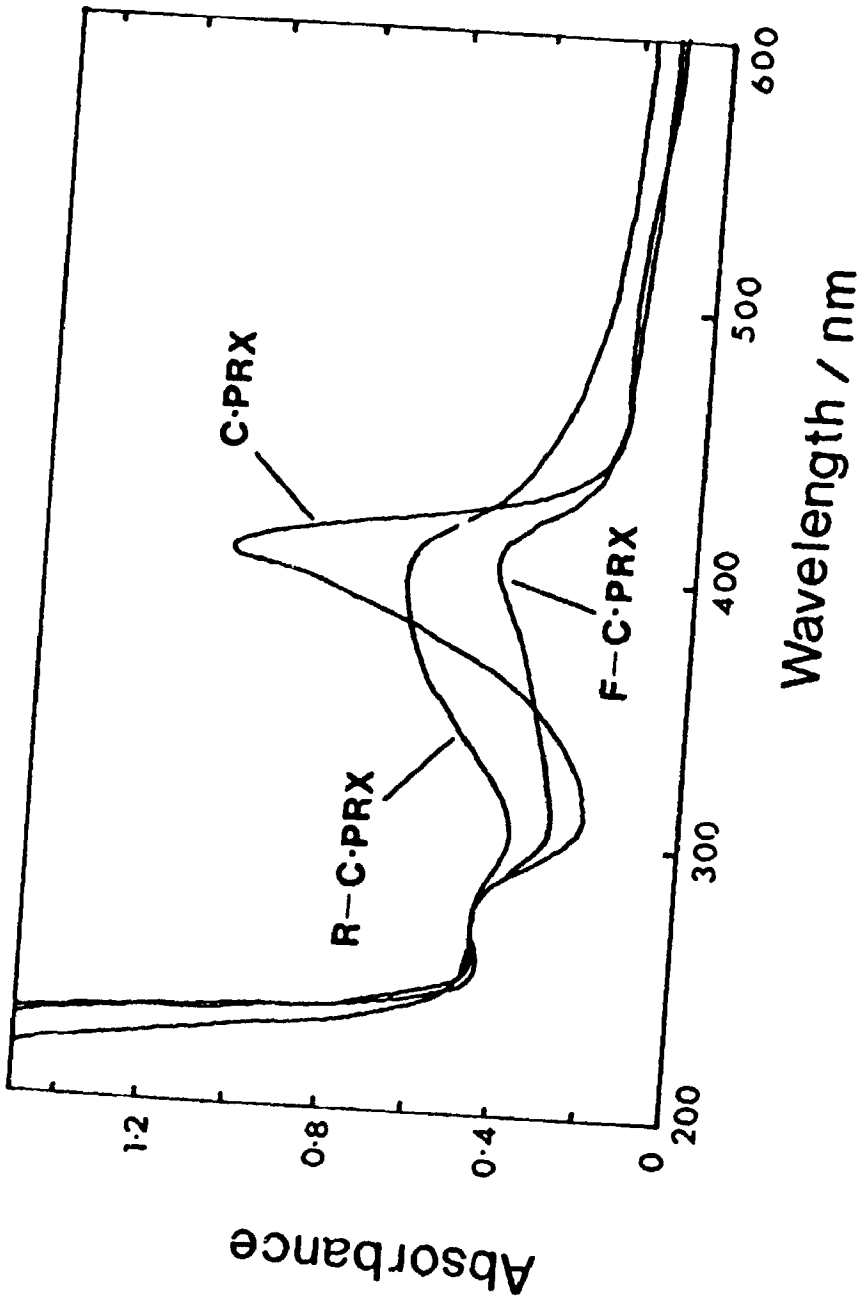
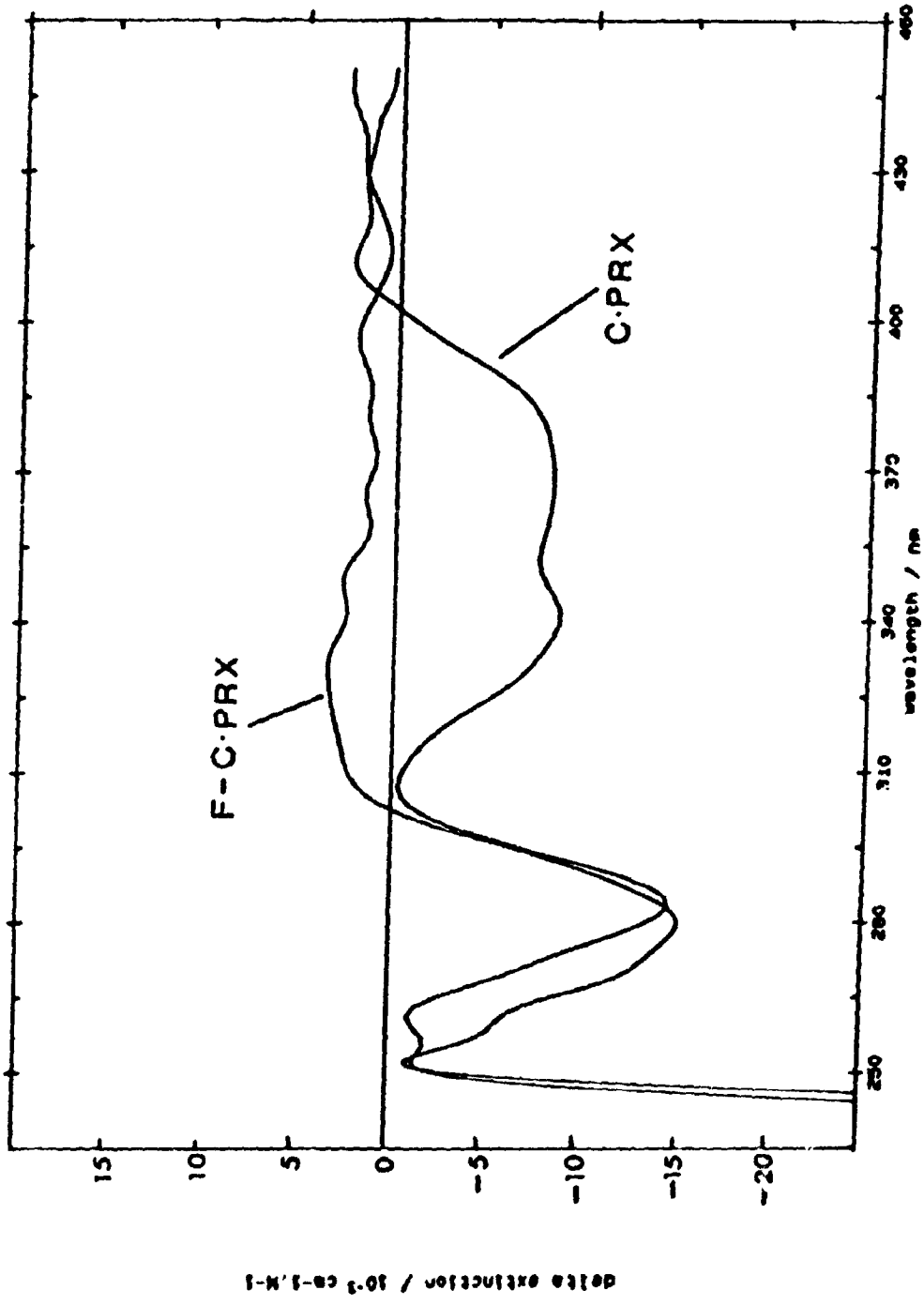

C·PRX
R—C·PRX
F—C·PRX
Absorbance
1·2
0·8
0·4
0
200
300
400
500
600
Wavelength / nm

Table 5.3. RZ value (A_{405}/A_{280}) of C.PRX, PNGase F treated C.PRX (F-C.PRX) and reconstituted C.PRX (R-C.PRX).

FRACTIONS	RZ	% of RZ of C.PRX
C.PRX	2.3	100
F-C.PRX	1.0	43
R-C.PRX	1.5	65

Fig 5.3. Comparison of CD spectra of C.PRX and PNGase F treated C.PRX. The concentration of C.PRX and PNGase F treated C.PRX (F-C.PRX) used for CD spectra determination was 0.4 mg/mL H_2O (10 μM). The CD spectra were determined on a Jasco J-500C spectropolarimeter.


C·PRX
F-C·PRX
15
10
5
0
-5
-10
-15
-20
250
280
310
340
370
400
430
460
wavelength / nm
delta extinction / 10^3 cm-1.M-1

CHAPTER 6

Heterogeneous Glycosylation of Cationic Peanut Peroxidase

6.1. Introduction

A minor form of cationic peroxidase was isolated during the purification of the major form of cationic peroxidase (known as C.PRX) (van Huystee et al., 1990b). In contrast to C.PRX which does not bind to a lectin, Con A, column, this minor form of cationic peroxidase does bind to Con A and is eluted from Con A with TCM-saline buffer (see Table 1.1) containing 0.1 M methyl-mannopyranoside. On the basis of Con A affinity, the major form of C.PRX is named CP- and the minor one, CP+. These two forms vary in mass. CP+ migrates more rapidly than CP- on SDS-gel. But the difference in the mobility of CP- and CP+ on SDS-gel is eliminated by the treatment of both fractions individually with trifluoromethane sulfonic acid (TFMS). Also, the measurement of the Soret band absorption, calcium concentration and specific enzyme activity did not reveal any differences between these two forms (O'Donnell et al. 1992), as compared to that shown in the true peanut isozymes (Hu et al., 1990). These results suggested that CP- and CP+ may be two differentially glycosylated forms of one isozyme. In this chapter I confirm further that CP-

and CP+ share the same peptide chain and both have 3 glycans. The difference between CP- and CP+ is due to the heterogeneous glycosylation at specific glycosylation sites, frequently called microheterogeneity of the glycans. The microheterogeneity between CP- and CP+ is caused by a co-secreted ß-galactosidase in the culture medium.

6.2. Materials and methods

6.2.1. Isolation of the two forms of cationic peroxidase

The major peak of cationic peroxidase passes through the Con A column as flow-through and is named CP-. After extensive washing of the column with TCM-saline buffer, the small peak that binds to Con A is eluted with TCM-saline containing 0.1 M methyl-mannopyranoside and is named CP+ (O'Donnell et al. 1992).

6.2.2. Peptide mapping of 70% formic acid cleaved CP- and CP+

The cleavage of CP- and CP+ with 70% formic acid and the separation on 15% SDS-gel is described in section 3.2.3 and 3.2.4.

6.2.3. Purification of TPCK-tryptic glycopeptides of CP- and CP+

The digestion of CP- and CP+ with TPCK-trypsin and the separation of glycopeptide fractions on Biogel P6 column are described in section 2.2.2 and

section 2.2.3. Further identification of the glycopeptides on HPLC was carried out with a Waters Model 510 system using a u-Bondapak™ C_{18} column (3.9 x 300 mm) (Waters). The mobile phase was 0.1% TFA in water and the mobile phase modifier was acetonitrile containing 0.1% TFA. The eluent was monitored at 230 nm.

6.2.4. Con A affinity chromatography of glycopeptides of CP- and CP+ and identification on HPLC

Glycopeptide fractions of 4.5 mg of CP- or CP+ separated on Bio-gel P6 column were dissolved individually in 0.5 ml of TCM-saline buffer and applied to a Con A column (1 cm x 8 cm) equilibrated with TCM-saline buffer. The eluate was monitored at 280 nm using a UV-2 monitor (Pharmacia) at a flow rate of 12 ml/h. and fractions of 3 ml were collected. The column was washed with TCM-saline until the absorbance returned to the baseline and then eluted with TCM-saline containing 0.01 M, followed by 0.3 M of methyl-mannopyranoside. Each eluate fraction was pooled separately, dialysed (MWCO, 1000) against distilled water overnight, lyophilized and then identified on HPLC as in section 3.2.6.

6.2.5. Mass determination of glycans of CP- and CP+

TPCK-tryptic glycopeptides, GPa, GPb and GPc of 120 mg CP- and 80 mg CP+ purified on HPLC (section 6.2.4) were extensively digested individually with pronase as described in section 4.2.4. The produced glycan-Asn

fragments, named GLa, GLb and GLc according to their derivation from GPa, GPb and GPc, were separated from amino acids on Bio-Gel P4 column (1.5 x 90 cm, 200-400 mesh, molecular weight range 800-4000 daltons, Bio-Rad). The column was equilibrated and eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% NaN_3 at room temperature at a flow rate 8 ml/h and the eluent was monitored at 280 nm using a UV-2 monitor (Pharmacia) and fractions of 2 ml were collected. Glycan fractions were identified by the phenol-sulphuric acid method (section 2.2.6). The P4 column was calibrated with C.PRX (40,000 daltons), large oligosaccharide from fetuin (2992 daltons) and its desialylated derivative (2119 daltons), stachyose (Sigma, 667 daltons). The large oligosaccharide was prepared by extensive digestion of fetuin with pronase at 37°C for 24 hours (Nilsson et all, 1979). The desialylated derivative of this large oligosaccharide was prepared by removing the peripheral sialic acid sugar residues by hydrolysis in 0.05 M H_2SO_4 at 80°C for 1 hour (Rupar and Cook, 1982). A simple linear correlationship exists between the distribution coefficient (Kav) of solute on gel chromatography and the logarithm of solute molecular weight (Ln M.W.) within the molecular weight range of the gel. Kav is calculated as: $Kav = (Ve - V_0) / (Vt - V_0)$. Here Vt = total bed volume, obtained by water calibration of the column prior to packing the bed.; V_0 = void volume, determined by measuring the elution volume of a solute which is excluded from the pores of the gel matrix; Ve = elution volume of solute (Gel chromatography, Bio-Rad laboratories, 1975). The equation of linear regression for the Biogel P4

column is: Kav = -0.31 Ln M.W. + 2.68. The correlation coefficient (r) is -0.99.

6.2.6. Glycosidase activity assay

A modified method (Montreuil et al., 1986b) was employed to measure the activities of ß-galactosidase, α-mannosidase and N-acetyl-glucosaminidase in culture medium. Two hundred μL of culture medium was incubated with 200 μL of McIlvaine buffer of different pH (or pH 3.5, the pH optimum for ß-galactosidase) and 200 μL of 10 mM aqueous substrate (p-nitrophenyl-α-D-mannopyranoside, p-nitrophenyl-ß-D-galactopyranoside or p-nitrophenyl-N-acetyl-ß-D-glucosaminide, Sigma) solution at 37°C for 30 min.. Then 1.25 mL of 1 M sodium carbonate aqueous solution was added to the incubant. The absorbance of nitrophenol liberated by glycosidases was measured at 400 nm.

One unit (U) ß-galactosidase is empirically defined as the amount of enzyme which releases 1 μmol of p-nitrophenol from p-nitrophenyl-ß-D-galactopyranoside in 30 min. at 37°C and pH 3.5. A range of p-nitrophenol (Sigma) concentration was used to calculate the amount of ß-galactosidase.

A ß-galactosidase staining method (Erickson and Steers, 1970) was improved to detect the enzymatically active zones or spots on IEF gel or NC membrane (Schleicher and Schuell, BA 85, 0.45 μm). The gel or membrane was incubated in 0.025% 6-bromo-2-naphthyl-ß-D-galactopyranoside (BNG, Sigma) in 10% methanol in a buffer (McIlvaine buffer, pH 3.5 for culture medium ß-galactosidase; 0.1 M sodium phosphate buffer, pH 8.0 for commercial ß-

galactosidase, the recombinant product of ß-galactosidase in E.coli, Boehringer Mannheim) containing 0.01 M NaCl, 0.01 M $MgCl_2$ for 5 min. at room temperature, followed by 2 min in diazo-blue B (1 mg/mL double distilled water, Sigma). To enhance the development of the purple band or spots, the gel or membrane was treated additionally with 0.01 M sodium carbonate for 30 sec.. The reaction was terminated in 7.5% acetic acid.

6.2.7. Measuring of peroxidase activity in culture medium

Thirty μL of culture medium was mixed with 1.5 mL 0.05 M sodium phosphate buffer, pH 7.0, 0.5 mL 1% (V/V) guaiacol solution and 1 mL 0.3% H_2O_2. The absorbance at 470 nm was immediately measured.

6.2.8. Isoelectrofocusing on flat gel

Isoelectrofocusing gel was prepared with a gel mould according to the company's instruction (LKB, Application Note 250). An LKB Multiphor plastic sheet (24 x 10 cm) was placed on a 3 mm glass plate, hydrophillic side up and hydrophobic side down. The rubber gasket (0.7 mm thick) was coated with a thin film of silicone grease (Dow Corning Corporation) and placed around the edge of the plastic sheet. Another 3 mm glass plate, whose lower side was coated with a thin film of Repel-silane (LKB) to give water repellent properties to the glass, is placed on the top of the gasket. The sandwich is clamped together, leaving the open end of the gasket unclamped. To prepare one piece

of gel, 5 mL 29.1% acrylamide solution, 5 mL 0.9% bis solution, 3.5 mL 87% glycerol solution, 1.9 mL Pharmalyte (pH 3-10, Sigma) and 14.6 mL double distilled water were mixed, de-gased for 10 min., then 120 μL 10% freshly prepared ammonium persulphate were added and mixed by swirling the flask. The gelling solution was filled into the mould through the open end. The gasket was sealed and the mould clamped and left in a vertical position for 1 hour to polymerize. Then the clamps were removed from the mould and the mould was laid horizontally at 4^0C for 30 min. to facilitate dismantling. The gel was wrapped in 2 layers of cling wrap and stored at 4^0C until it was needed. The gel could be stored for 2 weeks. Isoelectrofocusing was processed with a LKB 2117 Multiphor II electrophoresis unit and LKB 2197 power supply. The plate of Multiphor was precooled for 30 min and then a thin film of paraffin oil was placed on the plate. The gel was placed on the plate. Trapping air bubbles was avoided. Cathode electrode strip soaked in 1 M NaOH and anode electrode soaked in 1 M H_3PO_4 were put on the gel. The gel was prefocused for 1 hour. The power was raised from 1.1 W to 1.8 W at the first 10 min. and then kept constant 1.8 W. Samples were dissolved in 1% glycine and applied to the gel with a sample frame. The gel was running for 30 min. at a constant power of 1.8 W, then the frame was removed and the excess liquid on the gel was mopped up. Then the power was raised and maintained at 5 W for 2 hours. At the end of the run, 10 X 5 mm pieces of gel were excised at 1 cm intervals down one side and each was soaked in 1 mL double distilled water at

4°C overnight. The pH value of each tube was measured. The gel was refocused for 10 min. To show proteins the gel was fixed in 3.46% (w/v) sulphosalicylic acid, 11.5% trichloroacetic acid (w/v) aqueous solution for 30 min. The remainder of pharmalyte was washed out with destaining solution for 15 min, stained in Coomassie Brilliant Blue R250 solution (0.460 g/400 mL destaining solution) at 60°C for 10 min and destained with destaining solution (25% ethanol, 8% acetic acid). The ß-galactosidase band on the gel was detected as described in section 6.2.6.

6.2.9. Purification of culture medium ß-galactosidase

The first steps in the procedure for the purification of ß-galactosidase are the same as those for peroxidases. Briefly, the 14 day culture was filtered through Whatman No.1 filter paper and the spent medium was brought to 70% acetone. After centrifugation at 9000 X g for 15 min. the pellet was suspended in 0.02 M sodium acetate buffer, pH 5.0. The insoluble material was removed by centrifugation at 13000 x g for 10 min. and the supernatant was brought to 80% ammonium sulphate. After another centrifugation, the pellet was resuspended in 0.02 M sodium acetate buffer, pH 5.0 and dialysed against the same buffer. The crude medium proteins were applied to a cationic exchange column of carboxymethyl Sephadex-50 (2.5 x 15 cm, Pharmacia) at a flow rate of 1.5 mL/min and the eluent was monitored at both 280 nm and 405 nm. Fractions of 7.5 mL were collected and ß-galactosidase activity of each fraction

was analysed. The flow-through fractions of A.PRX were washed off with 0.02 M sodium acetate buffer, pH 5.0; the weakly bound fractions of C.PRX were eluted with a linear gradient of 0.02 M - 0.1 M sodium acetate buffer, pH 5.0 and the strongly bound fractions, named as CPz, were eluted with a linear gradient of 0.1 - 0.5 M sodium acetate buffer, pH 5.0. The ß-galactosidase activity-containing fractions of CPz were pooled and precipitated with 70% acetone, redissolved in 0.5 mL 7% isopropanol and applied to a Sephadex G75 column (2.5 x 80 cm, Pharmacia). The flow rate was 0.3 mL/min and the eluent was monitored at both 280 nm and 405 nm. Fractions of 4.5 mL were collected and ß-galactosidase activity of each fraction was analysed using dot blotting assay. The ß-galactosidase activity-containing fractions were pooled, lyophilized and subjected to preparative isoelectrofocusing.

6.2.10. Preparative isoelectrofocusing

Preparative isoelectrofocusing was performed using a Rotofor cell (Bio-Rad). The cylindrical focusing chamber of the Rotofor cell has a capacity of approximately 50 mL and is divided into 20 discrete compartments by a membrane core. The ß-galactosidase preparation from G75 column was dissolved in 50 mL Milli-Q water containing 20% glycerol and 1.5% pharmalyte (pH 3-10, Sigma) and loaded into the focusing chamber. The focusing was run at a constant power 12 W for 5 h. at 4^0C with a power supply 3000Xi (Bio-Rad). The contents of the focusing chamber were collected into 20 fractions and each

fraction was analyzed for pH, ß-galactosidase activity, absorbances at 280 nm and 405 nm. The ß-galactosidase containing fractions were pooled, brought to final volume of 50 mL with 20% glycerol and focused once more using the same procedure as for the first focusing. The purity of resulted ß-galactosidase was checked with 12% SDS-PAGE using the same procedure described in section 4.2.6. To remove the pharmalyte from the purified protein solution, the sample was brought to 1 M NaCl and left for 20 min. before dialysation against distilled water overnight.

6.2.11. Treatment of CP- and TPCK-tryptic glycopeptides of CP- with purified medium ß-galactosidase

Four milligrams of CP- (RZ>3) or TPCK-tryptic glycopeptides derived from 4 mg of CP- from Bio-gel P6 column were treated with 50 U (see section 6.2.6 for the definition of U) of purified medium ß-galactosidase in 0.4 mL of McIlvaine buffer, pH 3.5 at 37^0C for 2 days. After dialysing against TCM-saline buffer (MWCO, 1000), the treated glycopeptides and CP- were applied to the Con A column (1 cm x 8 cm) individually and the glycopeptides from Con A were subjected to the reverse-phase HPLC analysis as described in section 6.2.4.

6.3. Results

The peptide maps in Fig. 6.1 illustrate that both CP- and CP+ treated

with 70% formic acid shared the same cleavage pattern. Furthermore, when TPCK-trypsin digested CP- and CP+ were passed through a Bio-gel P6 column to separate glycopeptide fractions and then were identified on a reverse-phase HPLC, both CP- and CP+ presented 3 phenol-sulphuric acid reactive glycopeptide peaks with similar retention time (Fig. 6.2). These results prove that the two forms of cationic peanut peroxidase seemingly have the same peptide chain corresponding to the base sequence of cDNA clone prxPNC1 and that the same number of glycans is attached to identical glycosylation sites.

To find the source of the difference of carbohydrate moiety between CP- and CP+, the Con A binding property of the glycopeptides was tested. The results (Fig. 6.3) showed that the glycopeptides of CP- do not bind to Con A affinity column and are present in the flow-through with TCM-saline solution. In contrast, the glycopeptides of CP+, except GPa do mostly bind and elute with TCM-saline, 0.01 M methyl-mannopyranoside. In the cases of both CP- and CP+, no peptides are found in the fraction eluted with TCM-saline, 0.3 M methyl-mannopyranoside. By comparing the corresponding peak areas, most of GPa in CP+ still does not bind to Con A, and only a small part does bind (Fig. 6.3).

The heterogeneous glycosylation of cationic peroxidase at specific glycosylation sites was further demonstrated by the mass determination of the glycans. The individual glycopeptides, GPa, GPb and GPc, were extensively digested with pronase and then passed through a calibrated Biogel P4 column.

Fig. 6.4 shows that GLa-CP- has only one peak and GLa-CP+, two. GLa-CP- is the largest glycan with 14 sugar residues (Table 6.1). Compared to the result of Con A affinity chromatography of TPCK-tryptic glycopeptides (Fig. 6.3), peak I of GLa-CP+ (Fig. 6.4) is probably the major component that does not bind on Con A column while the minor one, peak II does. GLb and GLc indicate greater diversity. GLb-CP- has three peaks while GLb-CP+, two (Fig. 6.5). Both GLc-CP- and GLc-CP+ showed three peaks (Fig. 6.6). The molecular weights and putative lengths of the heterogeneous forms of glycans of both CP- and CP+ are calculated and summarized in Table 6.1.

To study the source of heterogeneous glycosylation, some glycosidase activities in the culture medium were investigated. Fig. 6.7 showed that in 12 day culture medium α-mannosidase and N-acetyl-ß-glucosaminidase activities were not detectable at a pH range of 2.6-7.5, but a ß-galactosidase activity certainly occurs and the pH optimum is around 3.5. The culture time curve revealed that the ß-galactosidase is secreted mainly on the eighth day and reaches a peak in 11 day culture medium. The secretion of ß-galactosidase and peroxidase is synchronous (Fig. 6.8). No α-mannosidase and N-acetyl-ß-glucosaminidase activities were found in the entire culture cycle of two weeks (Fig. 6.8). The ß-galactosidase has a higher activity at 37°C than at 25°C (Fig. 6.9). The enzyme is very stable. After storage at 37°C for 24 h., 80% of ß-galactosidase activity remains. The recovery rate of ß-galactosidase activity after precipitation with 70% acetone is around 70%.

ß-Galactosidase can be stained using 6-bromo-2-naphthyl-ß-D-galactopyranoside (BNG) as substrate (Erickson and Steers, 1970). Fig. 6.10 demonstrates that the sensitivity of this staining method is pH dependent and the pH optimum for the culture medium ß-galactosidase is around 3.5, the same as the pH optimum for substrate of p-nitrophenyl-ß-D-galactopyranoside (Fig. 6.7). An additional treatment with 0.01 M sodium carbonate enhances the development of the purple spots and increases the sensitivity of this staining method more than ten times (Fig. 6.11). This improved staining technique was used to stain enzymatically active bands or spots of ß-galactosidase on IEF gel or NC membrane.

When the crude medium proteins were passed through the CM column, they were fractionated into three parts (Fig. 6.12): flow-through fractions (A.PRX), weakly bound fractions (C.PRX) and strongly bound fractions (CPz). All the three parts have peroxidase activity, although the characteristics of the peroxidase in CPz fractions are unknown. But the ß-galactosidase activity was only found associated with CPz fractions. To purify the ß-galactosidase, the CPz fractions were pooled, precipitated with 70% acetone and passed through a size exclusion column of Sephadex G75 (Fig. 6.13). Then the ß-galactosidase containing fractions were subjected to isoelectrofocusing. Fig. 6.14 shows the isolation of ß-galactosidase on IEF flat gel. There are two major ß-galactosidase bands with pI of 7.3 and 7.6. The unidentified peroxidase presented a minor curved brown band with a pI of 9 and most of it exceeded

the cathodic end of the gel (the pI is higher than 9.5). The preparative isoelectrofocusing was performed with a Rotofor cell, which has 20 discrete compartments separated by a membrane core. The ß-galactosidase containing fractions 14-18 of the first fractionation (Fig. 6.15) were pooled and subjected to the second fractionation without any addition of pharmalyte. The narrower pH range produced better separation and yielded purified ß-galactosidase (Fig. 6.16). The purity of ß-galactosidase in fractions 11-17 from the second preparative IEF was checked by SDS-PAGE (Fig. 6.17). Fractions 11-13 have only one protein band with mass around 60 kd and fractions 14-17 have an additional protein band with mass of 66 kd. As fractions 11-13 have lower pH than fractions 14-17, the 60 kd band should be corresponding to the ß-galactosidase band with pI of 7.3 on IEF flat gel and the 66 kd band has pI of 7.6 (Fig. 6.14). As pharmalyte can be stained by Coomassie blue as seen on the front of the gel in Fig. 6.17, the pharmalyte was removed from pooled fractions 14-18 from the first IEF fractionation and fractions 11-17 from the second IEF fractionation by dialysation. Fig. 6.18 verified that the second preparative IEF yielded two ß-galactosidase isozymes with masses of 60 kd and 66 kd at near purity.

The amount of ß-galactosidase was empirically calculated by the reaction to release p-nitrophenol from p-nitrophenyl-ß-D-galactopyranoside and commercial p-nitrophenol was used as standard (Fig. 6.19). One unit (U) ß-galactosidase is defined as the amount of enzyme which releases 1 μmol of p-

nitrophenol from p-nitrophenyl-ß-D-galactopyranoside in 30 min. at 37°C and pH 3.5. As 200 µL of a 12 day cell culture yielded an absorbance of 0.6 at 400 nm (Fig. 6.7 and Fig. 6.8), around 60 nMol of p-nitrophenol was released in this reaction (Fig. 6.19). The level of ß-galactosidase activity in a 12 day cell culture is around 0.3 U / mL cell culture. To examine whether the medium ß-galactosidase is responsible for the heterogeneous glycosylation of cationic peanut peroxidase, purified CP- (RZ>3) and the glycopeptides of CP- were treated with purified medium ß-galactosidase separately and then their Con A binding property was checked. Fig. 6.20 exhibited that after incubation with ß-galactosidase, 80% of CP- was converted to CP+ based on a comparison of the occurrence of heme protein (absorbance at 405 nm) in flow-through and bound fractions. After incubation with purified medium ß-galactosidase, the trypsin-released glycopeptides of CP- were no longer present in the flow-through fractions as seen in Fig. 6.3., but instead were bound to Con A and eluted with TCM-saline, 0.01 M methyl-mannopyranoside (Fig. 6.20). These data confirmed that the medium ß-galactosidase is indeed able to convert CP- to CP+. The microheterogeneity of glycans between CP- and CP+ is probably caused by the co-secreted ß-galactosidase.

6.4. Discussion

N-glycosylation is a major post-translational modification of proteins. The processing of glycosylation and the determination of the structure of mature

glycans are reviewed in section 1.1. In contrast to the polypeptide chain of a glycoprotein, which presents precise homology in amino acid sequence and length, the glycans linked at specific amino acids of the peptide chain often present a structural heterogeneity. The heterogeneity is produced by the variation in the number and positions of the external sugar residues or the number of antennae on a similar core structure (Montreuil et al. 1986a, Beely 1985b). The heterogeneity of glycans causes a polymorphism of glycoproteins and poses difficulties in the identification of glycoproteins and the primary structure determination of glycans. The heterogeneous glycosylation of glycoproteins has attracted much attention and has been reported to be present in flax peroxidases (Gaudreault and Tyson 1986, 1988), secreted carrot glycoproteins (Sturm 1991), murine transferrin receptor (van Driel and Goding 1985), bovine pancreatic DNase (Kijimoto-Ochiai et al. 1989), bean phaseolin (Sturm et al. 1987), protozoan invertase (Dauvrin and Thines-Sempoux 1989), and human serum glycoproteins (Timmann et al., 1991). But there are very few data on the differences of individual glycans in heterogeneously glycosylated proteins and the explanation how these differences are produced.

CP+, the minor component of cationic peanut peroxidase that binds to Con A column, was observed in the late 1980's in our lab and was first investigated by Sesto (Sesto, 1989). The late attention to the occurrence of CP+ was probably due to the fact that CP+ accounts for only 10% of the cationic peroxidase (O'Donnell et al., 1992). Only when a large amount of

protein (more than 20 mg) was passed through the Con A column, did Sesto note and collect the CP+ fraction. The polymorphism of cationic peanut peroxidase was once suggested to be due to the difference in the number of glycans. CP- was considered to have four glycans but CP+ only three (van Huystee et al., 1992).

As presented in chapter 3, my experiments show that out of five potential N-glycosylation sites in CP-, only three are used. Fig. 6.2 further verifies that both CP- and CP+ have the same number of glycans at the same glycosylation sites.

The distinguishing feature of CP+ from CP- is mainly based on the lectin binding property. Lectins can bind to specific carbohydrate determinants and are widely used in the study of glycoproteins. Con A has the specificity for any glycoconjugate with terminal mannose or glucose residues. Although the interactions between glycoconjugates and lectins have a great deal in common with the interactions of carbohydrate-specific antibodies with glycoconjugates (Beely 1985c, Montreuil et al. 1986a), sometimes non-specific reactions, such as hydrophobic and ionic absorption of glycoproteins on the immobilized lectins have been observed (Montreuil et al. 1986a, Sonnewald et al., 1989) and restrict the use of immobilized lectins. But, the non-specific interactions as mentioned above are rare in the cases of glycopeptides and glycans so that these compounds interact specifically with immobilized lectins based on an 'actual' affinity chromatography (Montreuil et al. 1986a). In order to study the nature of

reactions of Con A with CP- or CP+, the Con A binding property of TPCK-tryptic glycopeptides of CP- and CP+ were tested. These results authenticate the Con A binding properties of CP- and CP+ on the basis of 'actual' affinity and confirm that the difference between CP- and CP+ is caused by the heterogeneous structures of glycans attached to all three glycosylation sites of Asn_{60}, Asn_{144} and Asn_{184}. Gel filtration chromatography of GLa, GLb and GLc further revealed the heterogeneous glycosylation pattern of cationic peroxidase.

N-linked glycans of high mannose type bind strongly to Con A and are eluted with high concentration (0.3 M) of the haptenic sugar derivative. Complex type glycans either present no reaction or weak reaction with Con A and are eluted with low concentration (0.01 M) of the haptenic sugar derivative (Montreuil et al., 1986a). As all the glycans of CP- and CP+ either do not bind to Con A column or are eluted with 0.01 M methyl-mannopyranoside (Fig. 6.3), they are of the complex type. This is consistent with the sugar composition and linkage analyses of glycans done at the Complex Carbohydrate Research Centre, Athens, GA, USA. The glycans of cationic peroxidase are composed of mannose, N-acetyl glucosamine, galactose, xylose and fucose (van Huystee et al., 1992). It is obvious that the shift of the N-linked complex type glycans from non-binding type in CP- to weakly binding type in CP+ means that most external sugar residues at one or more antennae are changed to mannose or glucose from other sugars. Since the dominant terminal sugar residues of the glycans of CP- are galactose (van Huystee et al., 1992), the formation of CP+

type glycans may be due to the lack of terminal galactoses and thus the exposure of internal mannoses. The lack of terminal galactose in CP+ type glycans may be produced before secretion because of different processing of the oligosaccharide precursor by galactosyltransferase controlled by some biosynthetic factors as reviewed by Schachter (Schachter, 1986). Alternatively, it may be produced after secretion caused by a secondary modification by a co-secreted galactosidase in the culture.

The investigation of glycosidase activity in the culture medium revealed the occurrence of ß-galactosidase using either p-nitrophenyl-ß-D-galactopyranoside or 6-bromo-2-naphthyl-ß-D-galactopyranoside as substrate. There are mainly two ß-galactosidase isozymes, which are synchronously secreted into the medium together with peroxidases (Fig. 6.8). One has a mass of 60 kd and pI 7.3. The other has a mass of 66 kd and pI 7.6 (Fig. 6.14, Fig. 6.17 and Fig. 6.18). These medium ß-galactosidases do not bind to p-aminophenyl-ß-D-thiogalactopyranoside-Sepharose 4B affinity column (data not shown), which is widely used for the purification of ß-galactosidase (Steers et al., 1971). A combination of ion exchange chromatography, size exclusion chromatography and isoelectrofocusing achieved a good separation of the medium ß-galactosidases from other medium proteins as demonstrated by SDS-PAGE (Fig. 6.18).

BNG as substrate to stain ß-galactosidase is useful because its development of purple colour is significant. My experiments showed that this

staining method is pH dependent and the pH optimum for the medium ß-galactosidase is 3.5. A range of 0.001-0.1 M sodium carbonate improves the sensitivity of the staining. However, dissolving diazo-blue B directly in 0.01 M sodium carbonate causes a brown precipitant and does not enhance colour development. Probably a basic environment is favourable for the colour development of the reaction product of diazo-blue B and 6-bromo-2-naphthyl released by ß-galactosidase from BNG. This improved staining method could be used for dot blotting assay to screen ß-galactosidase after chromatography or preparative IEF as shown in Fig. 6.13, Fig. 6.15 and Fig. 6.16. Also, it could be used to stain ß-galactosidase on gel as shown in Fig. 6.14.

The *in vitro* experiments of the conversion of glycans from CP- type to CP+ type (Fig. 6.20 and Fig. 6.21) indicated that the co-secreted ß-galactosidase causes most likely the heterogeneous glycosylation of cationic peanut peroxidase. In the *in vitro* experiments, 80% of CP- was converted to CP+ (Fig. 6.20) and all the glycopeptides of CP- were converted to Con A binding type (Fig. 6.21). In contrast, in the cell culture CP+ accounts only 10% of the cationic peroxidase (O'Donnell et al., 1992). The reason for the difference is that the *in vitro* incubation condition is more favourable for the formation of CP+. The concentration of ß-galactosidase in the incubation (50 U / 0.4 mL buffer) is approximately 400 times higher than in the 12 day cell culture medium. The pH of cell culture medium is around 4.5, instead of the pH optimum 3.5 for ß-galactosidase. And the incubation temperature is 37^{0}C, not

25°C as for cell culture. Usually the hydrolysis of galactoses by ß-galactosidase from glycopeptides is almost 10-fold faster than from glycoproteins (Montreuil et al., 1986b) so that all the glycopeptides from CP- were converted to Con A binding type as shown in Fig. 6.21.

The discovery of the co-secreted ß-galactosidases in the culture medium and the confirmation of their role in converting CP- to CP+ may explain the heterogeneous glycosylation of C.PRX. As shown above, the glycans of C.PRX exhibited a high heterogeneity in both Con-A binding and length. The Con-A flow-through type glycans are mostly seen in CP-. They have mainly terminal galactose residues on the antennae as revealed by sugar composition and linkage analyses (van Huystee et al., 1992). The shorter CP- type glycans (GLb-CP- II and III, GLc-CP- II and III) and all the CP+ type glycans (which do bind to Con-A and are mainly seen in CP+, Table 6.1) lack one or more terminal galactose residues on the antennae caused by the co-secreted ß-galactosidase, and so inner mannose residues are exposed. As a result the Con-A affinity of glycans is gradually increased with the decrease of galactose on the glycans at specific glycosylation sites. The Con-A affinity of the entire enzyme molecules is determined by all three glycans. Indeed, there are a number of forms of C.PRX with varying glycans and thus varying Con A affinity. That may be a reason that when the Con-A column was washed with TCM-saline buffer the flow-through fraction of C.PRX presented a long tailing on the elution profile (O'Donnell et al., 1992). CP- and CP+ are only two groups of

isoforms of C.PRX separated on the basis of Con-A binding. During the preparation of this thesis, a 2-D NMR structure analysis of GLa-CP-, the largest and the least heterogeneous glycan (Table 6.1) is emerging in Dr. G. Shaw's lab. The preliminary results show that GLa-CP- has 14 sugar residues and a core structure [Xyl](Man)$_3$[Fuc](GlcNAc)$_2$. There are 3 terminal galactoses, one terminal fucose, two inner N-acetyl-glucosamines and one inner mannose on the antennae and some linkages among peripheral sugar residues are still under investigation (personal communication). These data are consistent with the mass determination of GLa-CP- and implicate that the GLa-CP+ I and GLa-CP+ II are derived from GLa-CP- with the loss of terminal galactose residues.

The heterogeneity of the carbohydrate moiety of peroxidase caused wider bands of glycoproteins and glycopeptides on SDS-gel as shown in Fig. 3.1, Fig. 3.3 and Fig. 6.1. Instead of three, four peaks of TPCK-tryptic glycopeptides of CP- were observed on gel filtration chromatography (Fig. 3.5), although CP- has only three glycosylated asparagines. Obviously this is due to the diversification of the glycans.

The study on the heterogeneous glycosylation of cationic peanut peroxidase presented here demonstrates an example of interaction between two co-secreted enzymes in a cell culture. It depicts individual glycans in heterogeneously glycosylated proteins and interprets how the microheterogeneity of glycans could be created after secretion of glycoproteins. From this study, we hypothesize that if an ß-galactosidase inhibitor, which

should not affect the synthesis and secretion of peroxidase, is added to the culture medium, the homogeneity of peroxidase molecules will be increased. That would help the crystallization of peroxidase and X-ray diffraction of the crystal of peroxidase.

Fig. 6.1. Comparison of a 70% formic acid cleavage pattern of CP- and CP+ on 15% SDS-polyacrylamide gel. Lane 1, 10 μg of standard protein molecular weight markers: bovine albumin, 66 kd; egg albumin, 45 kd; glyceraldehyde-3-phosphate dehydrogenase, 36 kd; carbonic anhydrase, 29 kd; trypsin inhibitor, 20.1 kd and α-lactalbumin, 14.4 kd. Lane 3, CP-, 10 μg. Lane 4, formic acid cleaved CP-, 40 μg. Lane 6, CP+, 10 μg. Lane 7, formic acid cleaved CP+, 40 μg. After electrophoresis the gel was stained with 0.1% Coomassie blue R, 40% methanol, 10% acetic acid and destained with 20% methanol, 7.5% acetic acid.

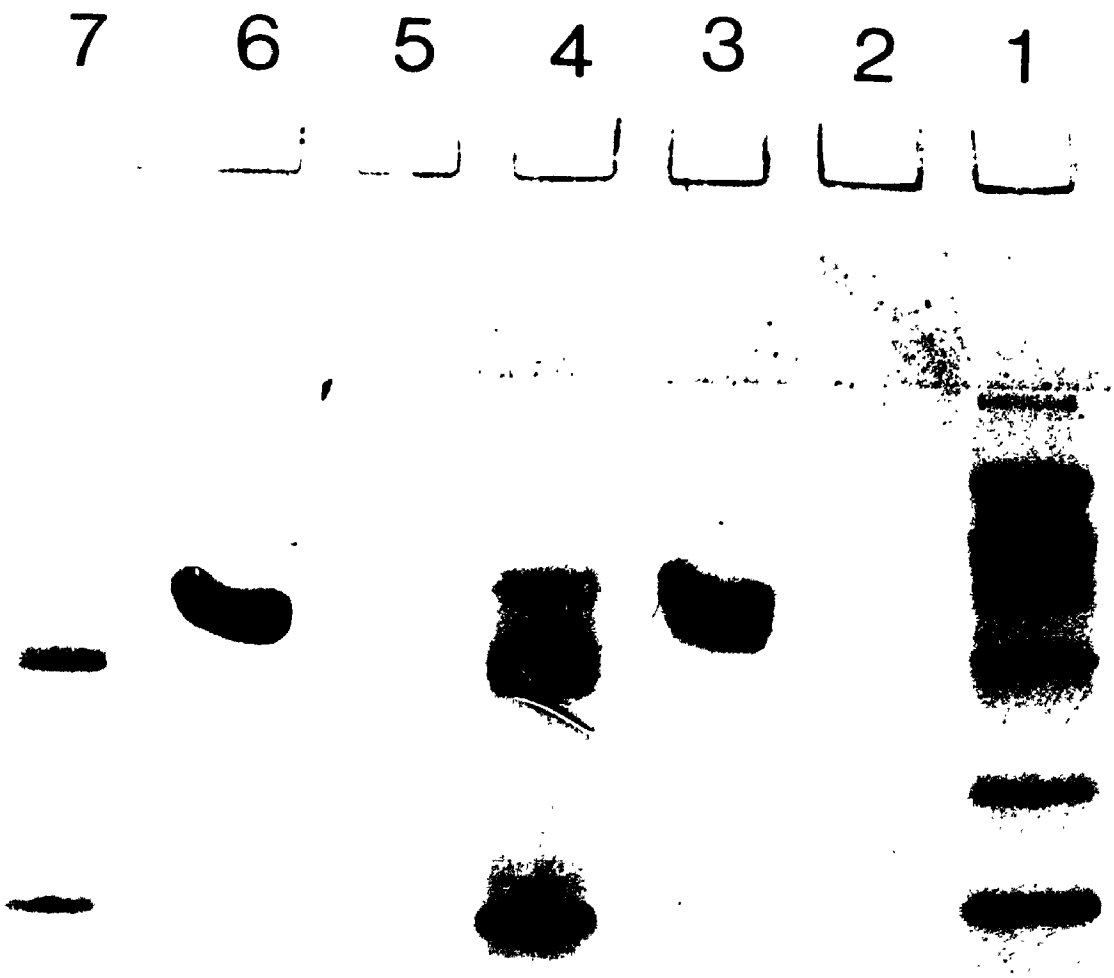

7
6
5
4
3
2
1

Fig. 6.2. Purification of glycopeptides of CP- and CP+ with reverse-phase HPLC. Glycopeptide fractions of 8 mg of TPCK-tryptic CP- or CP+ separated on Biogel P6 column were individually pooled, lyophilized, and dissolved in 1 ml of 0.1% TFA in water. Two hundred and fifty microliters of the solution was applied to a u-Bondapak™ C_{18} column (3.9 x 300 mm, Waters) and eluted with an acetonitrile gradient (containing 0.1% TFA) at 1.2 ml/min. The gradient was programmed with a digital professional 350 computer. The concentration of acetonitrile was raised from zero to 32% in 50 min using gradient curve 4 on the computer and then raised to 80% in 20 min, curve 3. The elution was monitored by UV absorption at 230 nm using LC spectrophotometer Lambda-max Model 481 detector. The glycopeptide peaks were identified with phenol-sulphuric acid technique, and were designated as GPa, GPb, GPc, respectively.

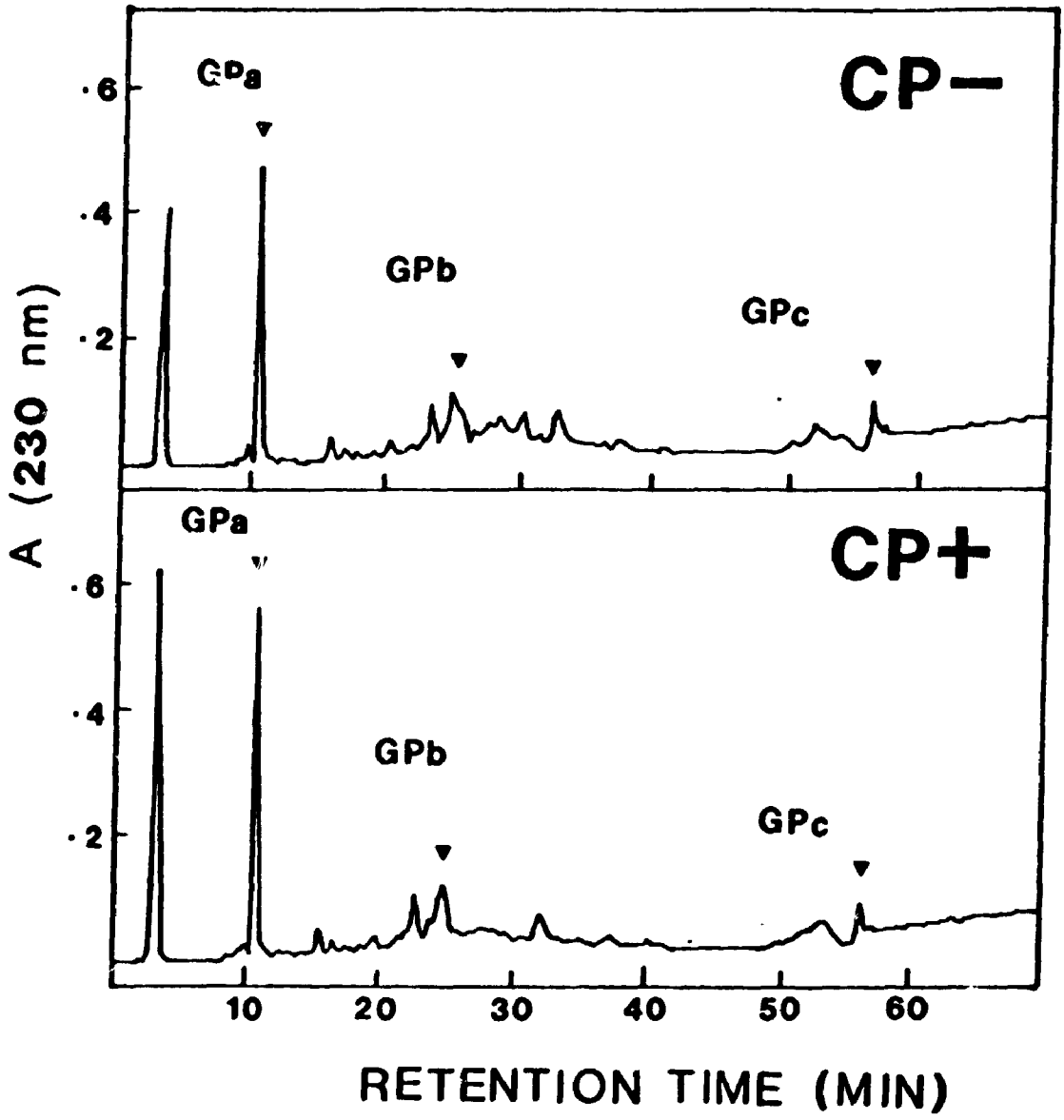

CP−
GPa
GPb
GPc
CP+
GPa
GPb
GPc
A (230 nm)
.6
.4
.2
.6
.4
.2
10
20
30
40
50
60
RETENTION TIME (MIN)

Fig. 6.3. Con A affinity chromatography and reverse-phase HPLC of glycopeptides of CP- and CP+. Panel A, Con A affinity chromatography of TPCK-tryptic glycopeptides of CP- or CP+. The arrows indicate the starting points of different washing buffers: B. TCM-saline; C. TCM-saline, 0.01 M methyl-mannopyranoside; D. TCM-saline, 0.3 M methyl-mannopyranoside. Panels B-D, reverse-phase HPLC of each fraction eluted with different washing buffers from Con A column as indicated in panel A. Each fraction from Con A was dialysed, lyophilized, and dissolved in 0.5 ml of 0.1% TFA. One hundred microliters of the solution was subjected to HPLC analysis. The gradient of acetonitrile is shown as a dashed line. GPa, GPb and GPc were allocated on the same basis as for Fig 6.2.

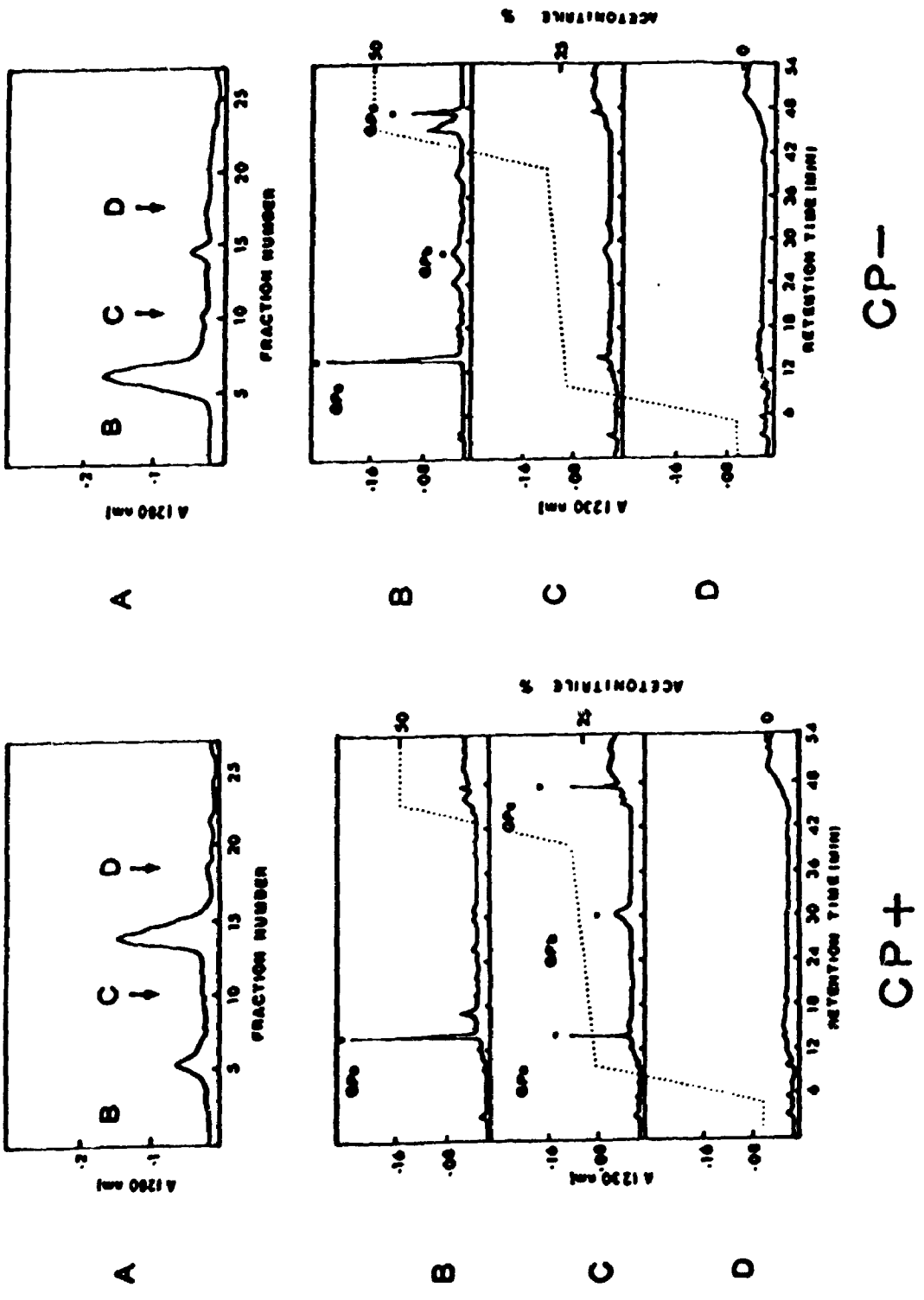

CP−
CP+
A
B
C
D
FRACTION NUMBER
ACETONITRILE %
RETENTION TIME (MIN)
A 1280 nm
A 1230 nm

Fig. 6.4. Gel filtration chromatography of GLa of CP- (GLa-CP-) and CP+ (GLa-CP+) on Biogel P4 column. GPa of CP- or CP+ was extensively digested with pronase for 72 h and the resulting GLa was separated from amino acids on P4 column. Two hundred microliter of eluent of each fraction was used to detect sugars using the phenol-sulphuric acid (A_{490}) method (section 2.2.6). The detected heterogeneous forms of GLa were labelled with Roman numerals.

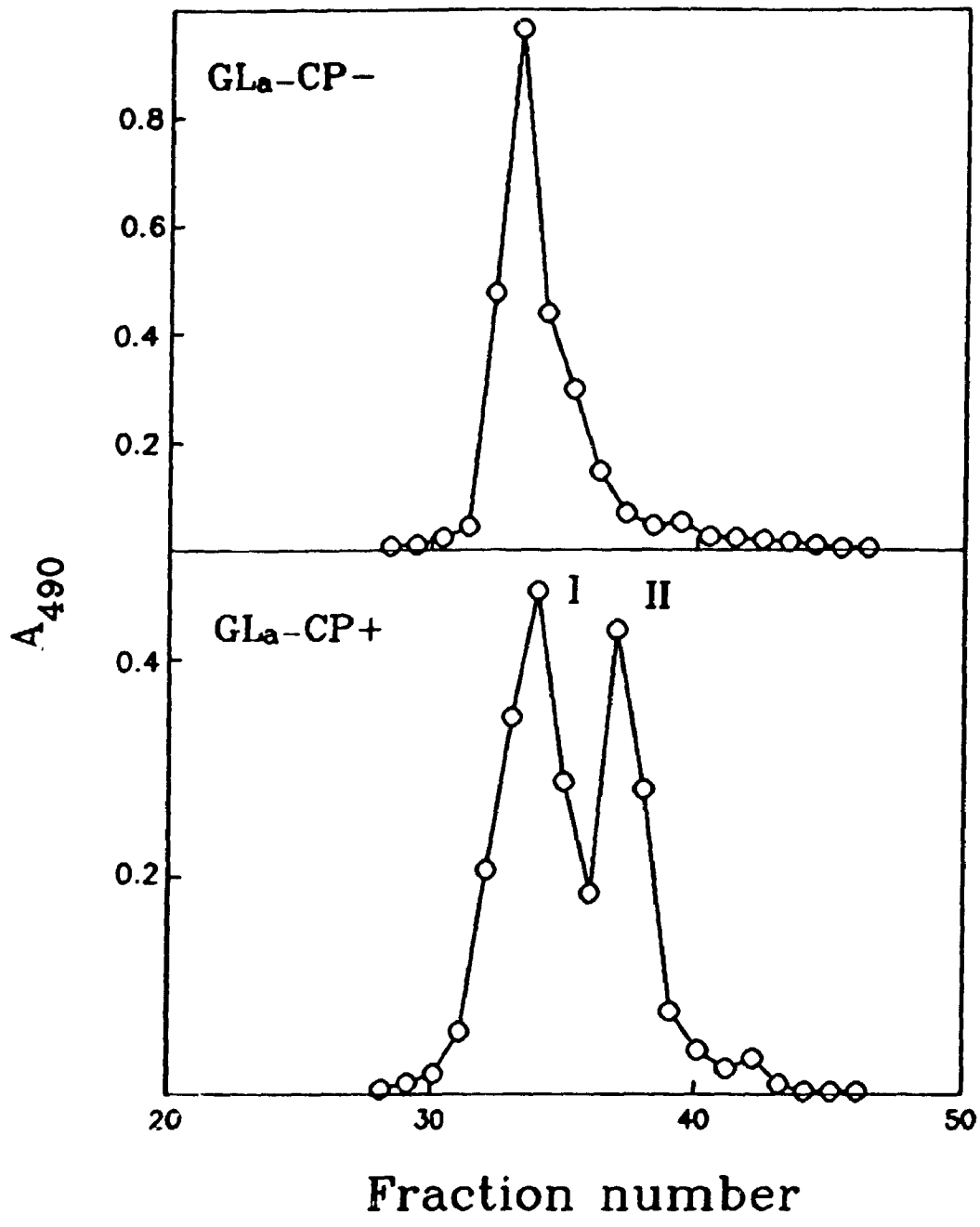

GLa-CP−
GLa-CP+
I
II
0.8
0.6
0.4
0.2
0.4
0.2
A490
20
30
40
50
Fraction number

Fig. 6.5. Gel filtration chromatography of GLb of CP- (GLb-CP-) and CP+ (GLb-CP+) on Biogel P4 column. GPb of CP- or CP+ was extensively digested with pronase for 72 h and the resulting GLb was separated from amino acids on P4 column. Two hundred microliter of eluent of each fraction was used to detect sugars using the phenol-sulphuric acid (A_{490}) method (section 2.2.6). The detected heterogeneous forms of GLb were labelled with Roman numerals.

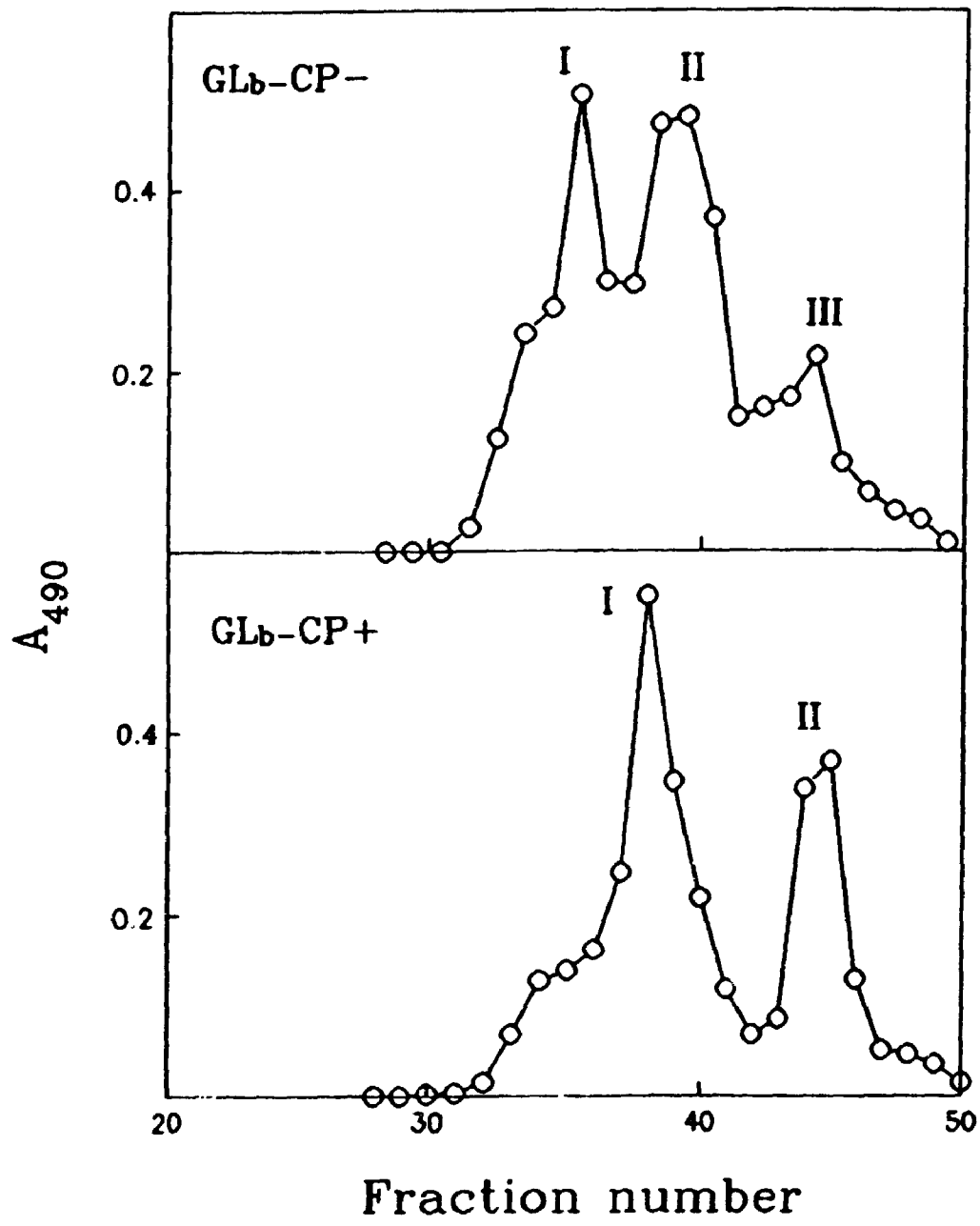

GLb-CP−
I
II
III
0.4
0.2
GLb-CP+
I
II
0.4
0.2
A_{490}
20
30
40
50
Fraction number

Fig. 6.6. Gel filtration chromatography of GLc of CP- (GLc-CP-) and CP+ (GLc-CP+) on Biogel P4 column. GPc of CP- or CP+ was extensively digested with pronase for 72 h and the resulting GLc was separated from amino acids on P4 column. Two hundred microliter of eluent of each fraction was used to detect sugars using the phenol-sulphuric acid (A_{490}) method (section 2.2.6). The detected heterogeneous forms of GLc were labelled with Roman numerals.

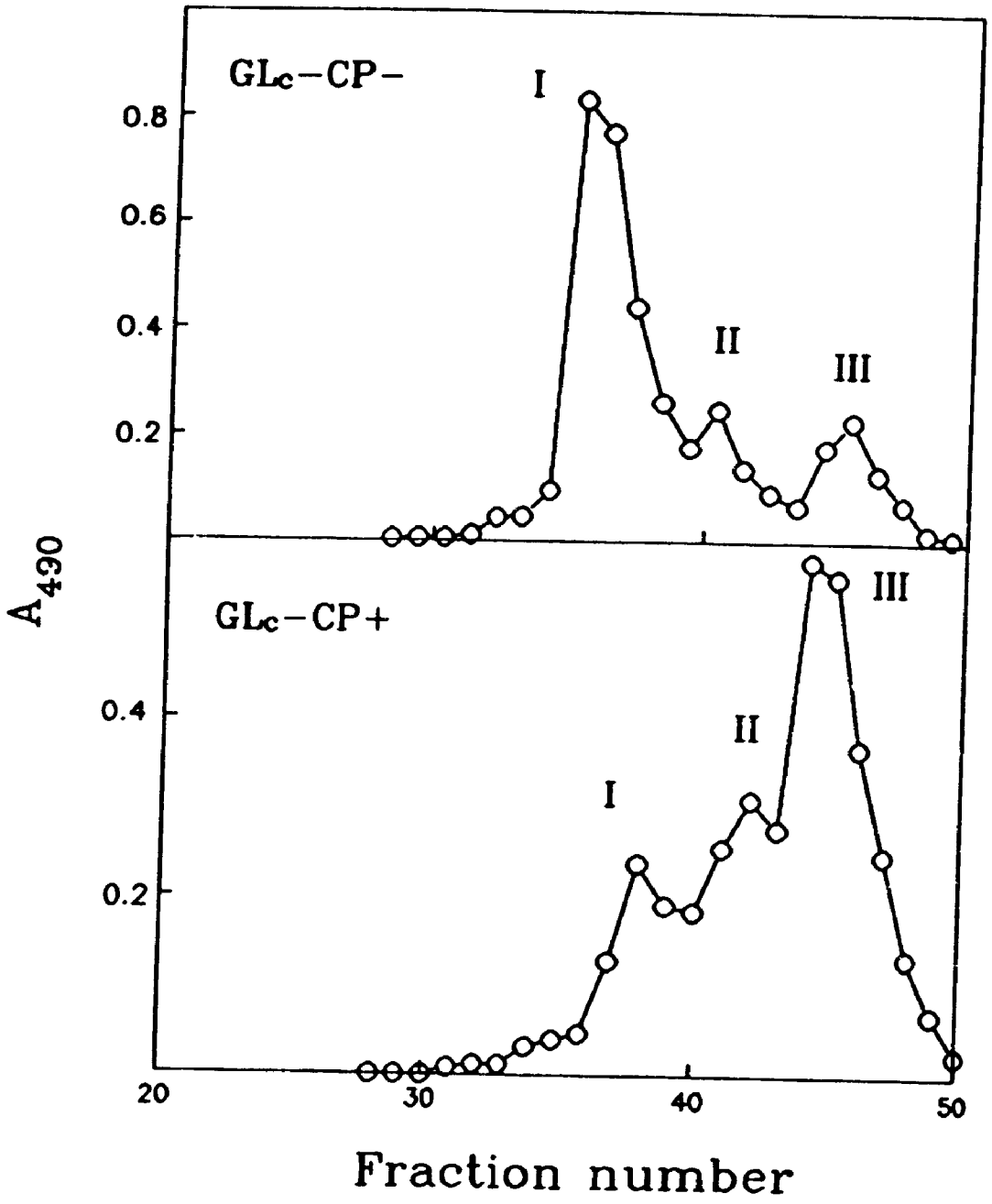

GLc−CP−
I
II
III
0.8
0.6
0.4
0.2
GLc−CP+
III
II
I
0.4
0.2
A490
20
30
40
50
Fraction number

Table 6.1. Molecular weights and putative lengths of the heterogeneous forms of glycans of CP- and CP+.

	CP-			CP+		
	mass[a]	M.W.[b]	#sugars[c]	mass[a]	M.W.[b]	#sugars[c]
GLa	2579	2456	14	I-2481*	2358	13
				II-2102	1979	11
GLb	I-2284*	2161	12	I-2011*	1888	11
	II-1831	1708	10	II-1367	1244	7
	III-1389	1266	7			
GLc	I-2203*	2080	12	I-1951	1828	10
	II-1671	1548	9	II-1564*	1441	8
	III-1268	1145	7	III-1400	1277	7

a The mass is calculated from the calibrated Biogel P4 column.

b Assuming the glycopeptides produced by pronase digestion contain only one asparagine residue (Mr=123 D), the molecular weights of glycans equal to (mass - Asn).

c Molecular weights of glycans are converted to the numbers of sugar residues using the average molecular weight of sugar residues in the core structure $GlcNAc_2(Fuc)Man_3(Xyl)$ (Mr=1235 D), 176 D.

* major sugar peak.

Fig. 6.7. Determination of glycosidase activities in 12 day culture medium at different pH. Two hundred μL of 12 day cell culture was incubated with 200 μL of McIlvaine buffer of different pH and 200 μL of 10 mM aqueous substrate (p-nitrophenyl-α-D-mannopyranoside, p-nitrophenyl-β-D-galactopyranoside and p-nitrophenyl-N-acetyl-β-D-glucosaminide) solution separately at 37°C for 30 min.. Then 1.25 mL of 1 M sodium carbonate aqueous solution was added. The absorbance of liberated nitrophenol by glycosidases was measured at 400 nm. Galactosidase activity ●--●, mannosidase activity ○--○, N-acetyl-glucosaminidase activity ▽--▽.

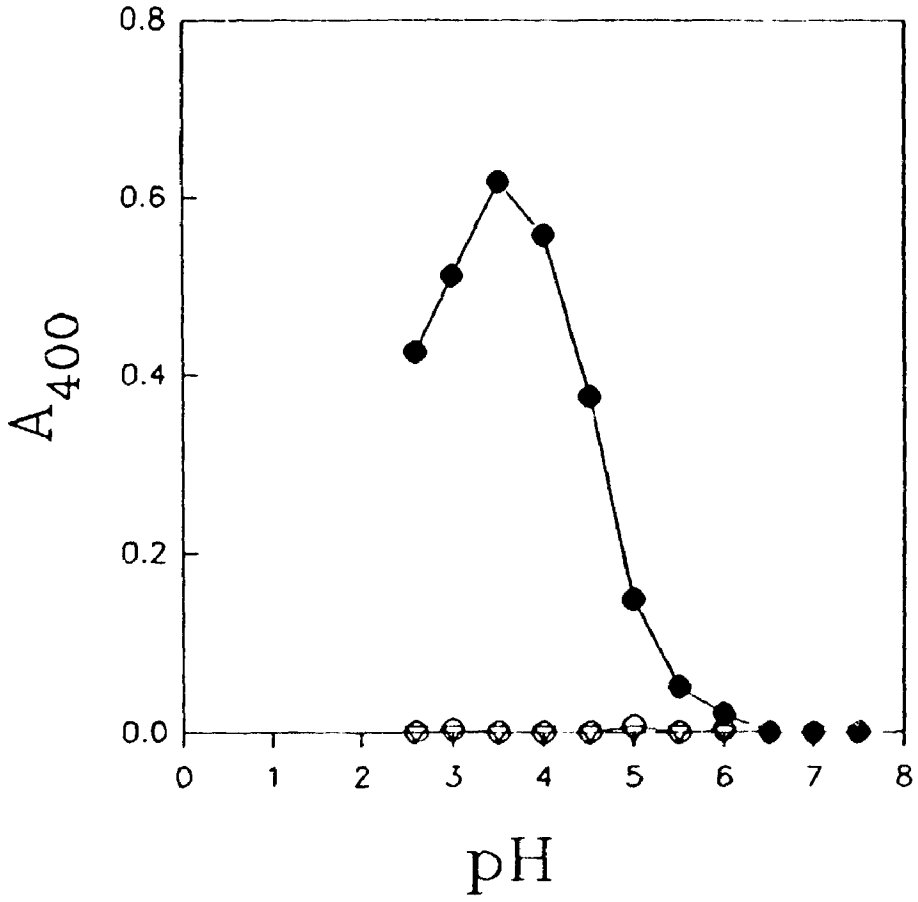

0.8
0.6
0.4
0.2
0.0
A_{400}
0
1
2
3
4
5
6
7
8
pH

Fig. 6.8. Glycosidase and peroxidase activities in culture medium within two weeks. Glycosidase and peroxidase activities in culture medium were checked daily. Two hundred μL of culture medium was used for the determination of glycosidase activities at pH 3.5 (see legend for Fig. 6.7). Thirty μL of culture medium was used for the determination of peroxidase. Galactosidase activity ◆--◆, mannosidase activity ▲--▲, N-acetyl-glucosaminidase activity □--□, C.PRX activity ○--○.

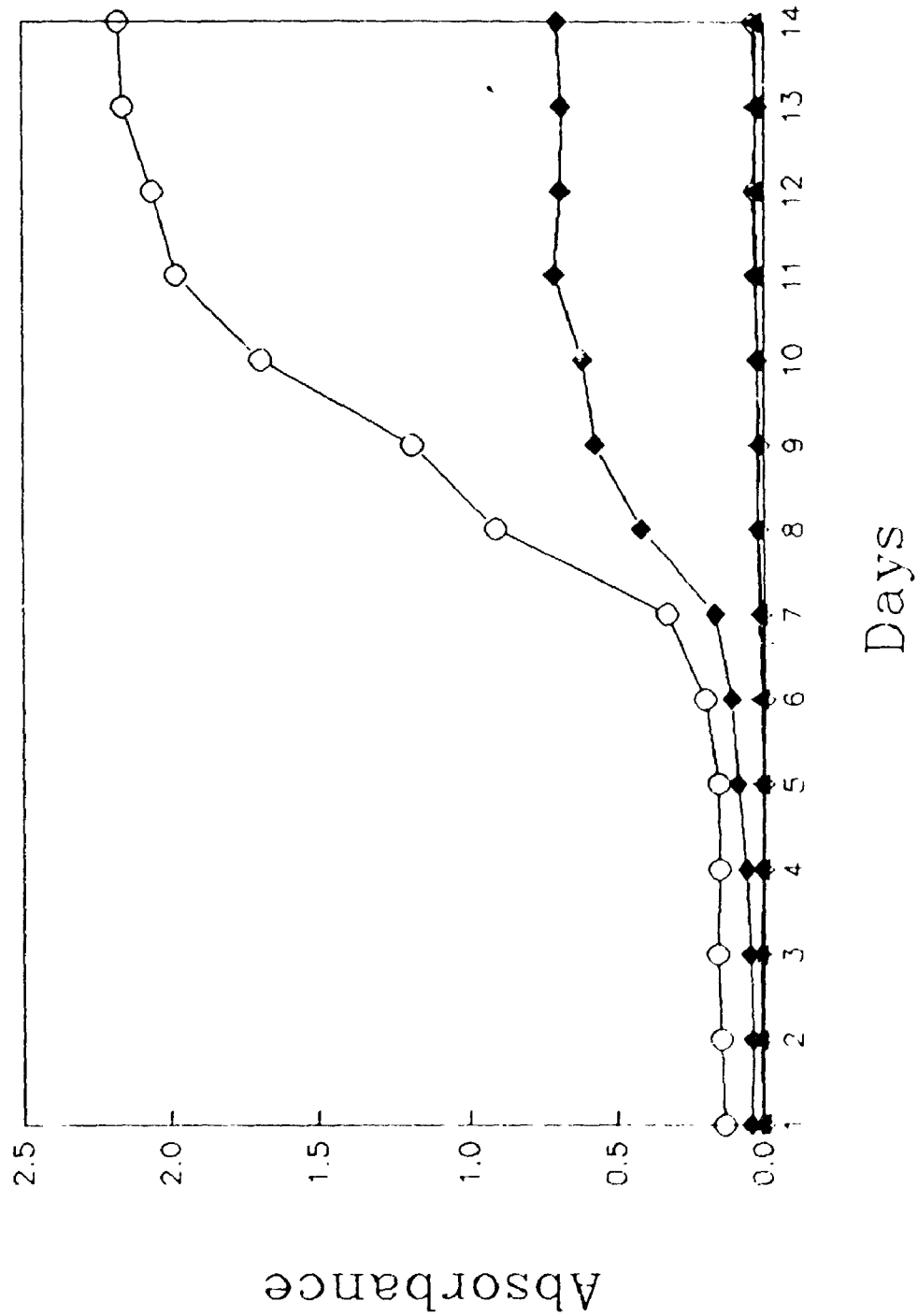

Absorbance
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days

Fig. 6.9. **The effect of temperature on ß-galactosidase activity.** Two hundred μL of 12 day cell culture was used to measure ß-galactosidase activity at pH optimum 3.5 with different incubation time at either 25°C (o--o) or 37°C (● --●).

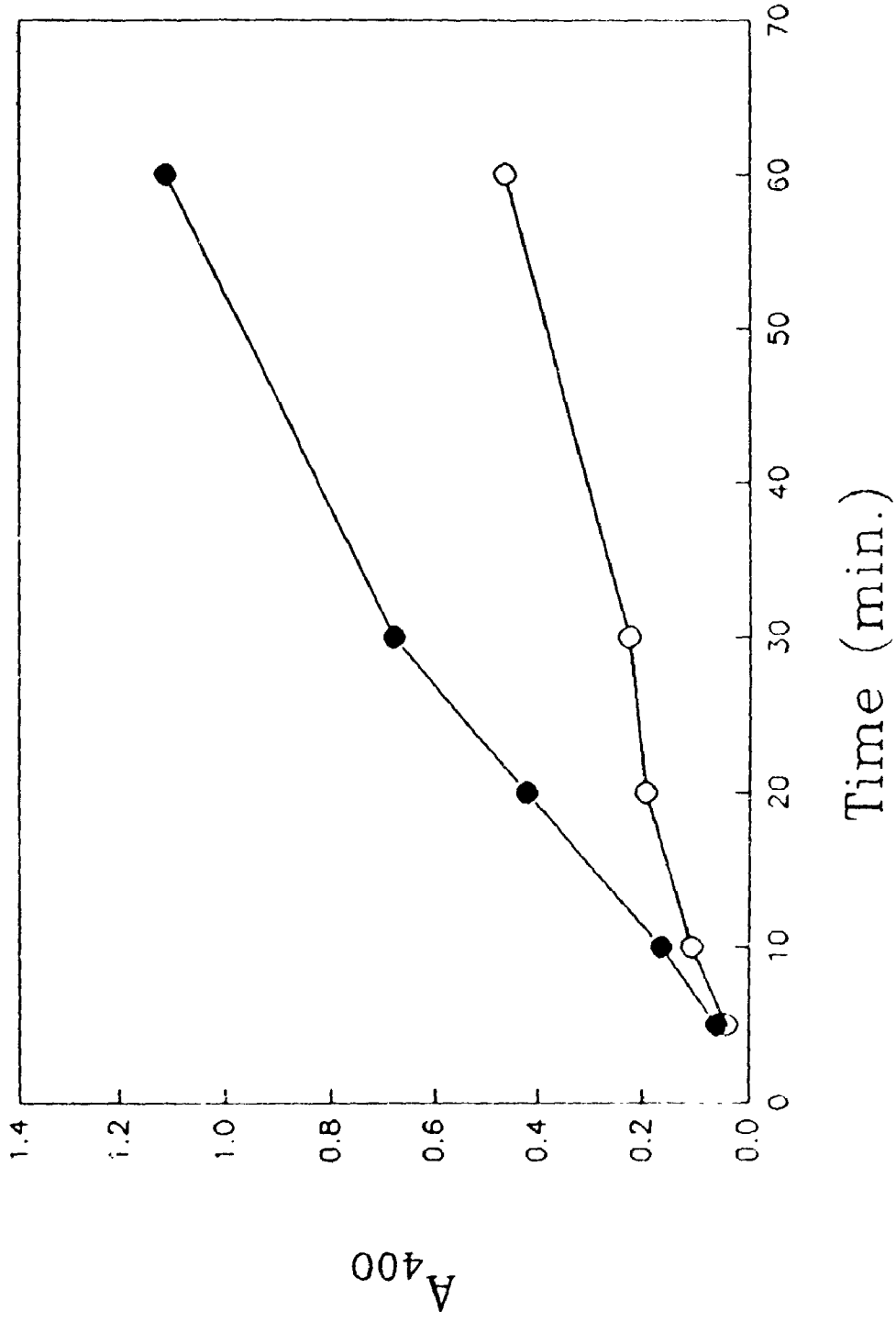

A_{400}
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Time (min.)

Fig. 6.10. The effect of pH on the sensitivity of β-galactosidase staining. Five microliters of aqueous solution containing 0.005 U of medium β-galactosidase were dot-blotted on NC membrane (0.45 μm). The membrane was incubated in 0.025% BNG in 10% methanol in McIlvaine buffer of different pH containing 0.01 M NaCl, 0.01 M $MgCl_2$ for 5 min., followed by 2 min in diazo-blue B (1 mg/mL double distilled water) and 30 sec. in 0.01 M sodium carbonate. The reaction was terminated in 7.5% acetic acid.

pH 2·6 3·0 3·5 4·0 4·5

pH 5·0 5·5 6·0 6·5 7·0 7·5

Fig. 6.11. The enhancement of β-galactosidase staining by the treatment of 0.01 M sodium carbonate. Five microliters of aqueous solution containing different amounts of either medium or commercial β-galactosidase were dot-blotted on NC membrane (0.45 μm). The initial amount of both β-galactosidases was 0.1 U and was diluted as two-fold series. The membrane was incubated in 0.025% BNG in 10% methanol in certain buffer (McIlvaine buffer, pH 3.5 for medium β-galactosidase; 0.1 M sodium phosphate buffer, pH 8.0 for commercial β-galactosidase) containing 0.01 M NaCl, 0.01 M $MgCl_2$ for 5 min., followed by 2 min in diazo-blue B (1 mg/mL double distilled water) and with or without an additional treatment of 0.01 M sodium carbonate for 30 sec.. The reaction was terminated in 7.5% acetic acid. A, medium β-galactosidase; B, commercial β-galactosidase; I, with an additional treatment of 0.01 M sodium carbonate; II, without the treatment of 0.01 M sodium carbonate.

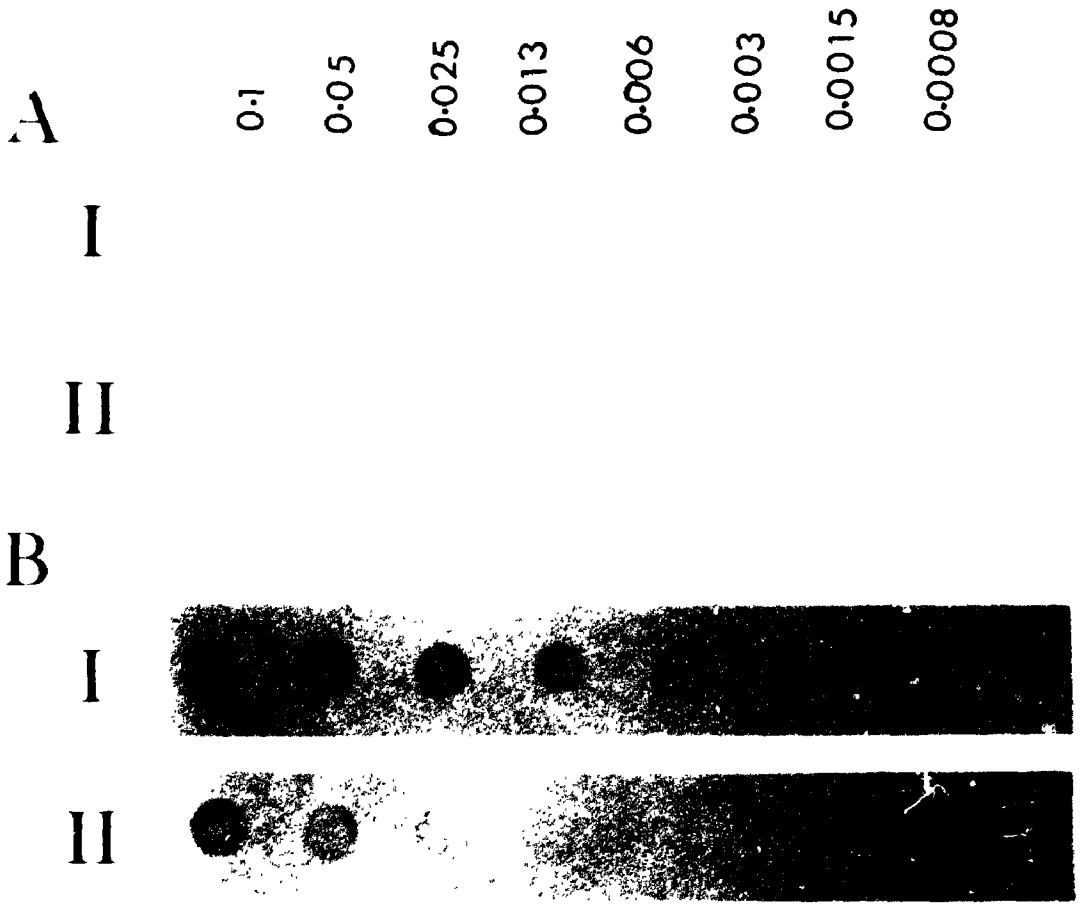

A
0·1
0·05
0·025
0·013
0·006
0·003
0·0015
0·0008
I
II
B
I
II

Fig. 6.12. Detection of β-galactosidase activity in the eluent from CM column. Crude medium proteins were applied to a CM column (2.5 x 15 cm). The gradient of the concentration of sodium acetate buffer, pH 5.0 is shown in dash line. The flow rate is 1.5 mL/min and the eluent was monitored at both 280 nm and 405 nm. Fractions of 7.5 mL were collected and 200 μL of each fraction were used to measure β-galactosidase activity. Only those denoted as the strongly bound fractions of CPz have β-galactosidase activity.

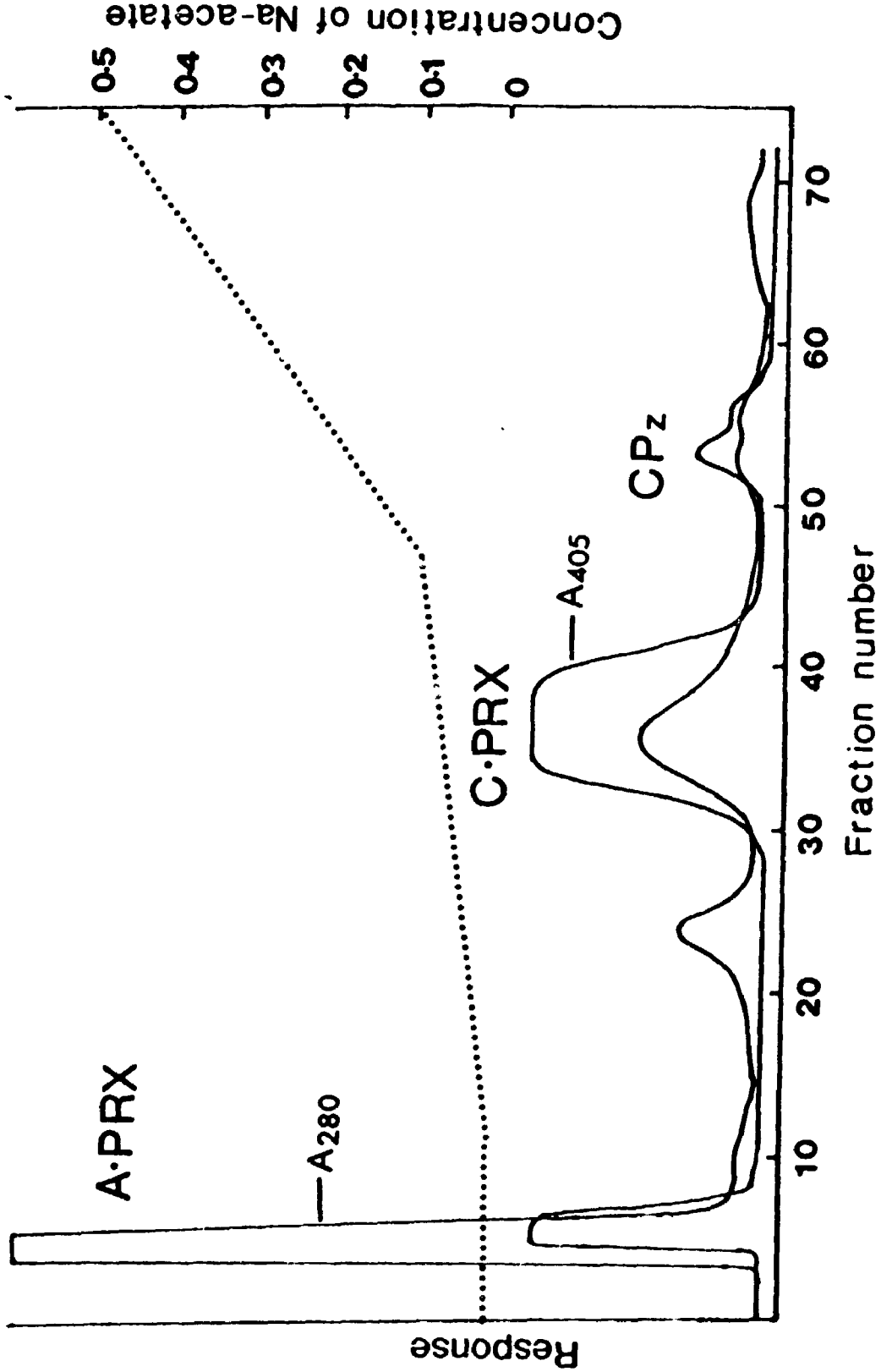
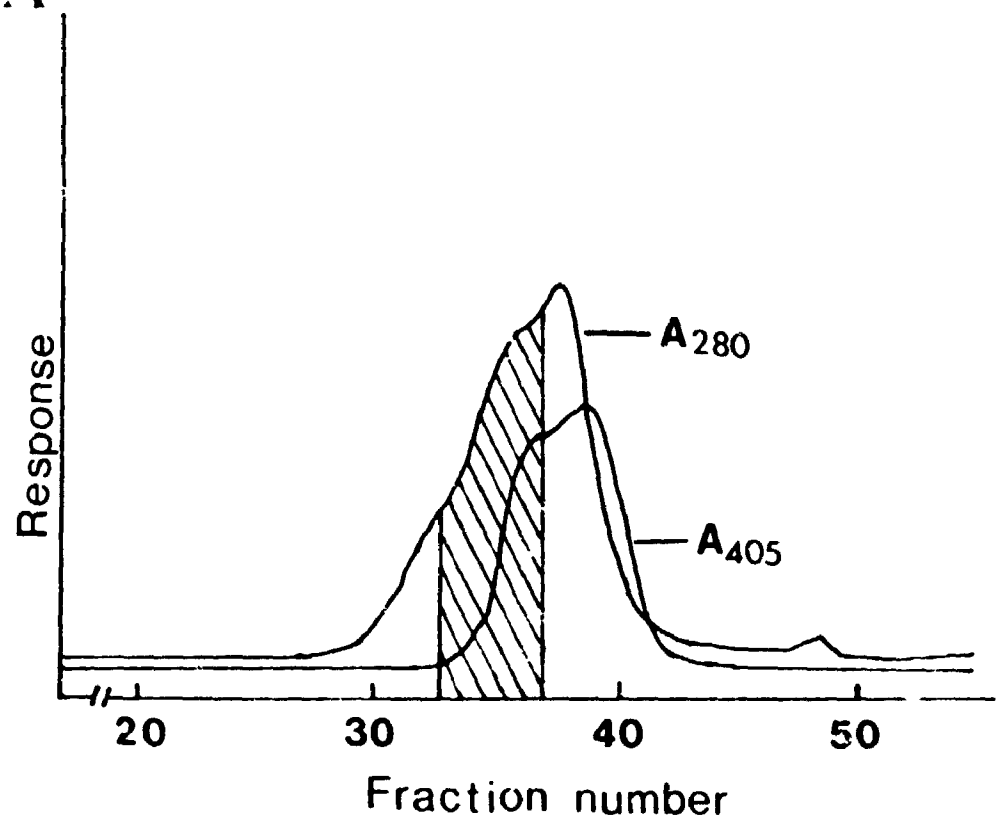

Concentration of Na-acetate
0·5
0·4
0·3
0·2
0·1
0
A·PRX
A280
C·PRX
A405
CPz
Response
10
20
30
40
50
60
70
Fraction number

Fig. 6.13. Preliminary purification of β-galactosidase on Sephadex G75 column. β-Galactosidase activity containing fractions (CPz) from CM column were precipitated with 70% acetone, redissolved in 0.5 mL 7% isopropanol and applied to a G75 column (2.5 x 80 cm). A, elution profile. The flow rate was 0.3 mL/min and the eluent was monitored at both 280 nm and 405 nm. Fractions of 4.5 mL were collected. B, dot blotting assay of β-galactosidase. Five μL of each fraction (fraction 26-45) was dot blotted on NC membrane for β-galactosidase staining as described in section 6.2.6. Fractions 33-36 showed purple dots and 37-43, brown dots on membrane. The β-galactosidase containing fractions is cross-hatched on elution profile.

A



B

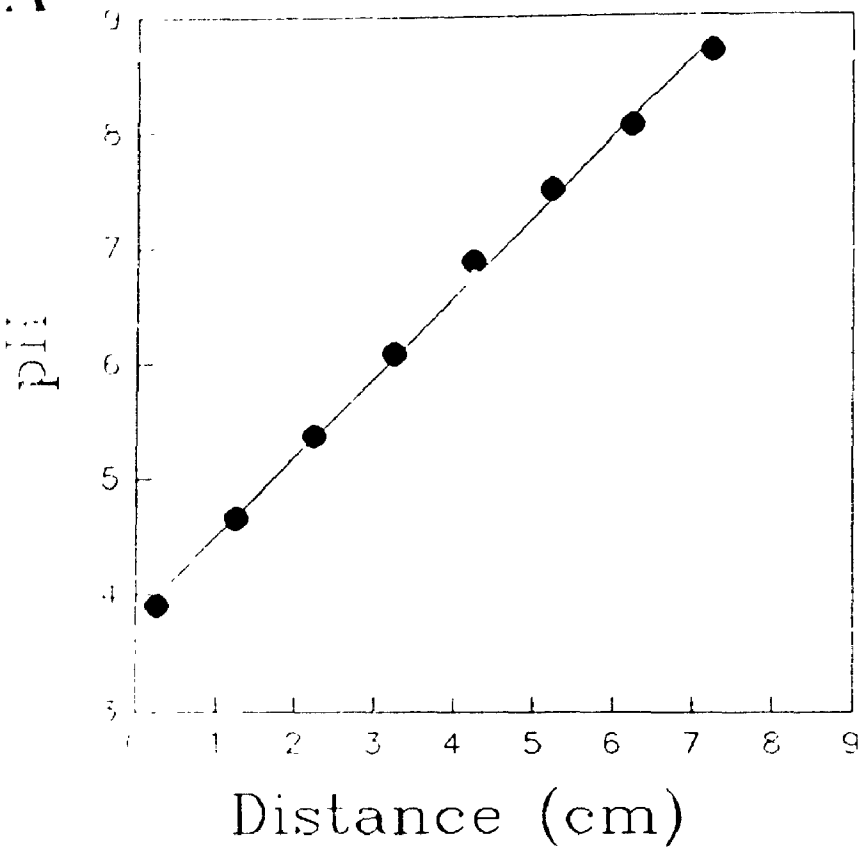
available for coupling) (Harlow and Lane, 1988). Therefore BSA is a good carrier for raising antibodies against glycopeptides as haptens. In order to establish the optimal coupling condition, TPCK-tryptic glycopeptides of fetuin were used to probe the amount of glutaraldehyde needed. Fetuin, with a mass of around 60 kd, has 3 N-linkage and 3 O-linkage glycans (Nilsson et al., 1979). Fig. 4.1 shows a clear separation of its TPCK-tryptic glycopeptides from other peptides on Bio-gel P10 column . Approximately 0.5 μM glycopeptides (from 5 mg fetuin) was used for coupling with 3 mg BSA using different amounts of glutaraldehyde and then separated on gel (Fig. 4.2). Lane 5 shows the least uncoupled BSA and lane 9 is strongly stained with PAS reagent, which demonstrates that 10 μM glutaraldehyde is a sufficient amount for the coupling of 0.5 μM glycopeptides to 3 mg BSA.

TPCK-trypsin digested A.PRX shows a sole glycopeptide on Bio-gel P10 column and HPLC (Fig. 4.3 and Fig. 4.4), which is consistent with the data obtained by purification on ion-exchange columns (Xu and van Huystee 1991).

Antisera were collected after 4 immunizations and ELISA readings of the titre determination are summarized in Table 4.1 Antisera to GPa and GP showed titres of around 100 times lower, compared with antiserum to GPb and GPc. Since GPa has the largest glycan and the shortest peptide chain (13 amino acid residues) among the three glycopeptides of C.PRX, and GP has a very large glycan of 8000 daltons (Xu and van Huystee 1991), the lower titres are probably caused by the poor binding of glycopeptides to the ELISA plate.

Fig. 6.14. Determination of pI of β-galactosidases by isoelectrofocusing on flat gel. A, pH gradient of the IEF gel. B, isoelectrofocusing of β-galactosidase activity-containing fractions from G75 column. Lane 1, 100 μg proteins stained with Coomassie blue; lane 2, 300 μg proteins stained for β-galactosidase activity as described in section 6.2.6. Two purple bands of β-galactosidase with pI 7.3 and 7.6 are indicated by arrows.

A



B

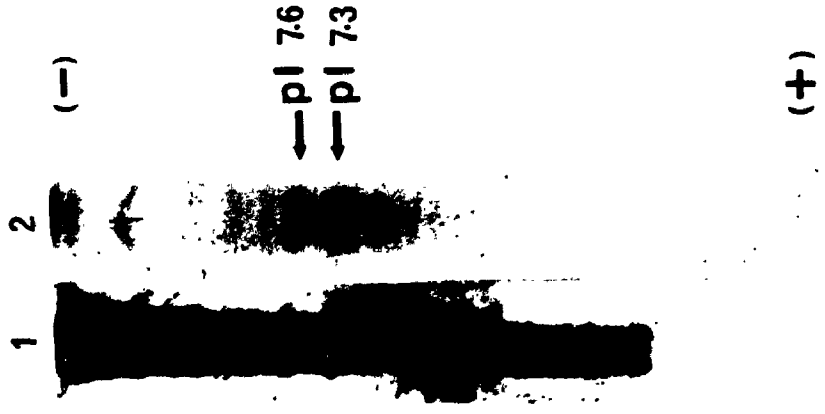
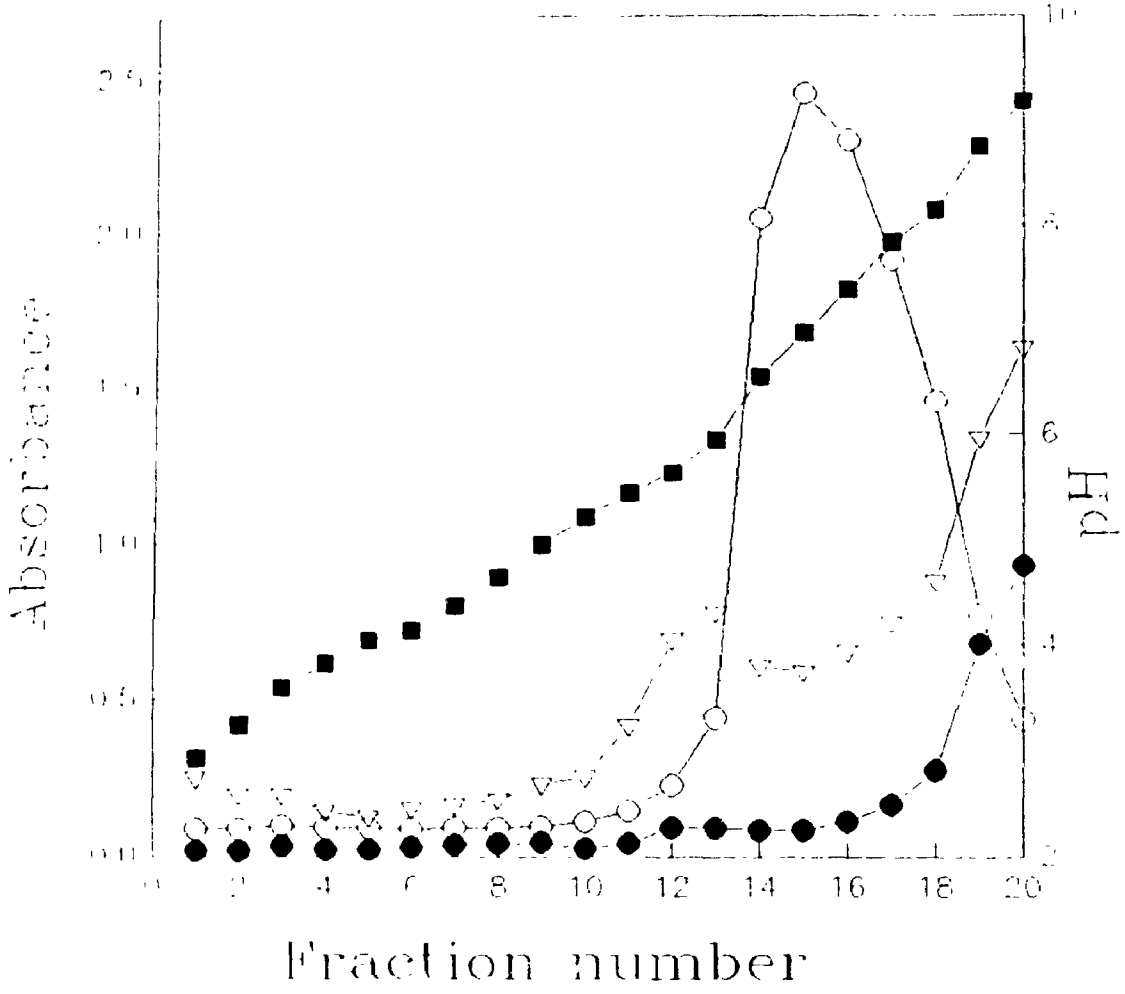


Fig. 6.15. Preparative isoelectrofocusing of Sephadex G75 purified β-galactosidases (first fractionation). A, A_{280}, A_{405} and pH of each fraction were determined. Ten μL of each fraction was used to measure β-galactosidase activity using p-nitrophenyl-β-D-galactopyranoside as substrate. A_{280} ▽--▽, A_{405} ●--●, A_{400} ○--○ and pH ■--■. B, five μL of each fraction was dot blotted on NC membrane and stained with β-galactosidase activity using BNG as substrate.

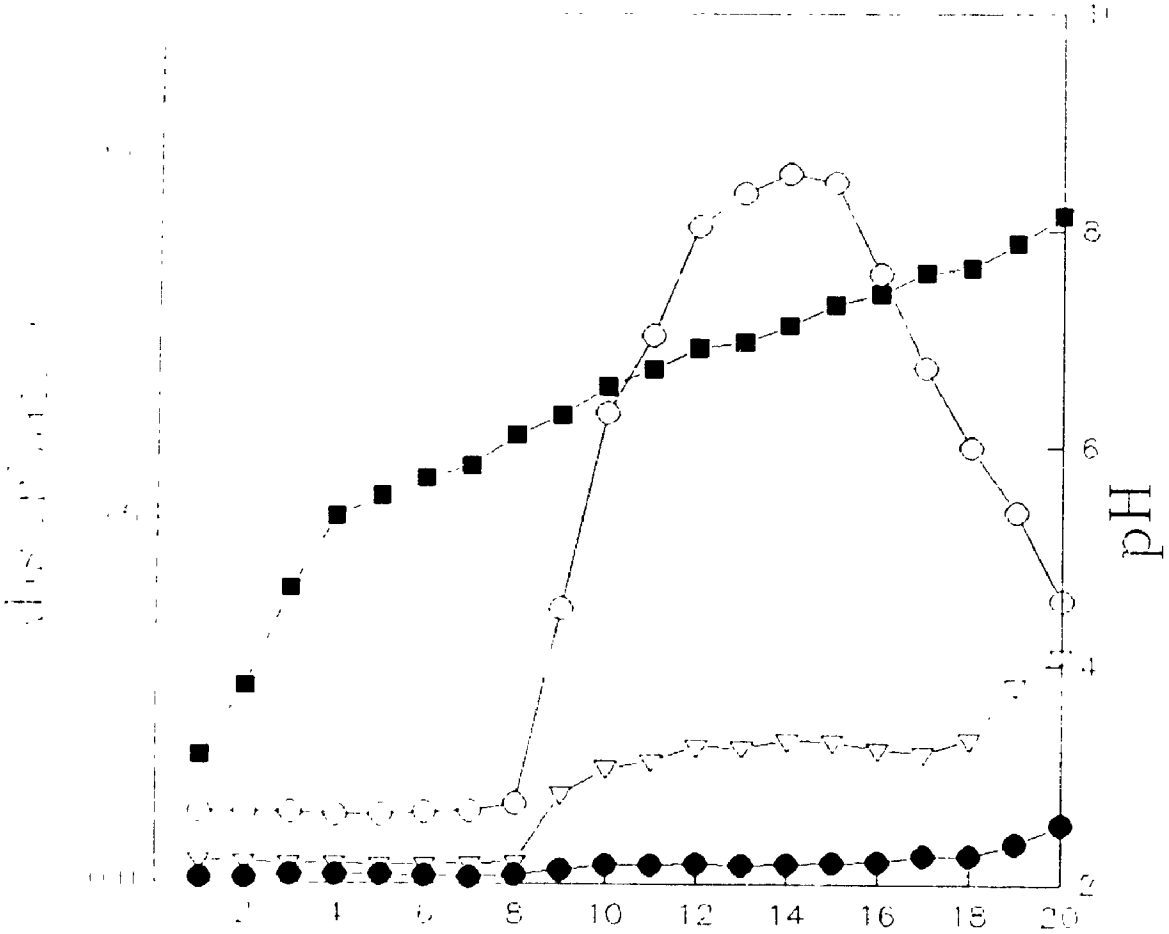
A



B

Fig. 6.16. Refractionation of fractions 14-18 from the first fractionation. Fractions 14-18 of the first fractionation (Fig. 6.15) were pooled, brought to final volume of 50 mL with 20% glycerol and refractionated. The absorbances, pH and β-galactosidase activity assays were processed as in Fig. 6.15. A_{280} ▽–▽, A_{405} ●--●, A_{400} o–o and pH ■--■.

A



B

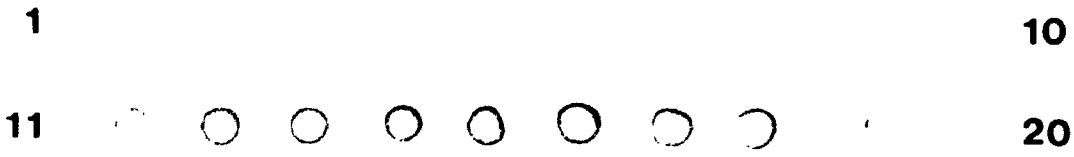


Fig. 6.17. 12% SDS-PAGE of β-galactosidase-containing fractions 11-17 from the second preparative isoelectrofocusing. Ten μL of each fraction 11-17 from second preparative isoelectrofocusing was directly (without dialyzing) applied to 12% SDS-PAGE and stained with Coomassie blue. Molecular weight marks were: phosphorylase B (rabbit muscle), 97.4 kd; bovine serum albumin, 66.2 kd; ovalbumin (hen egg white), 45 kd; carbonic anhydrase (bovine), 31 kd; soybean trypsin inhibitor, 21.5 kd. From left to right, fraction 11-17. The arrows indicated two β-galactosidase bands.

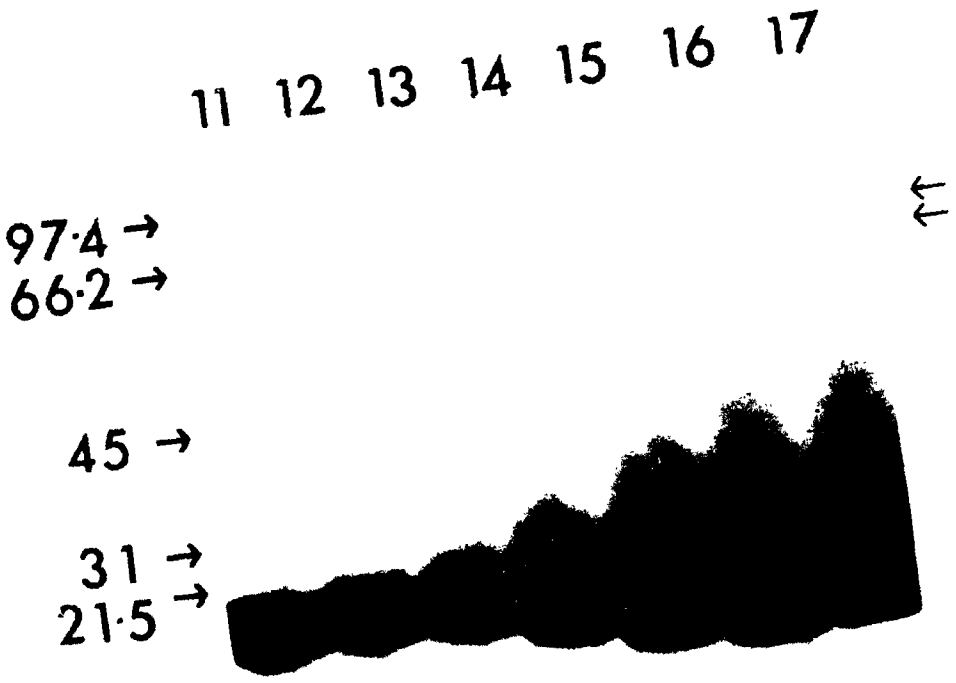

11 12 13 14 15 16 17
97·4 →
66·2 →
45 →
31 →
21·5 →

Fig. 6.18. 12% SDS-PAGE of β-galactosidase-containing preparations at various purification stages. Lane 1-3, 40 μL, 20 μL and 10 μL of pooled fractions 11-17 from refractionation (Fig. 6.16, dialysed); lane 4, 15 μL of pooled fractions 14-18 from first fractionation (Fig. 6.15, dialysed); lane 5, pooled β-galactosidase-containing fractions from G75 column (Fig. 6.13), 15 μg. The molecular weight marks were the same as in Fig. 6.17. After electrophoresis the gel was stained with Coomasie blue.

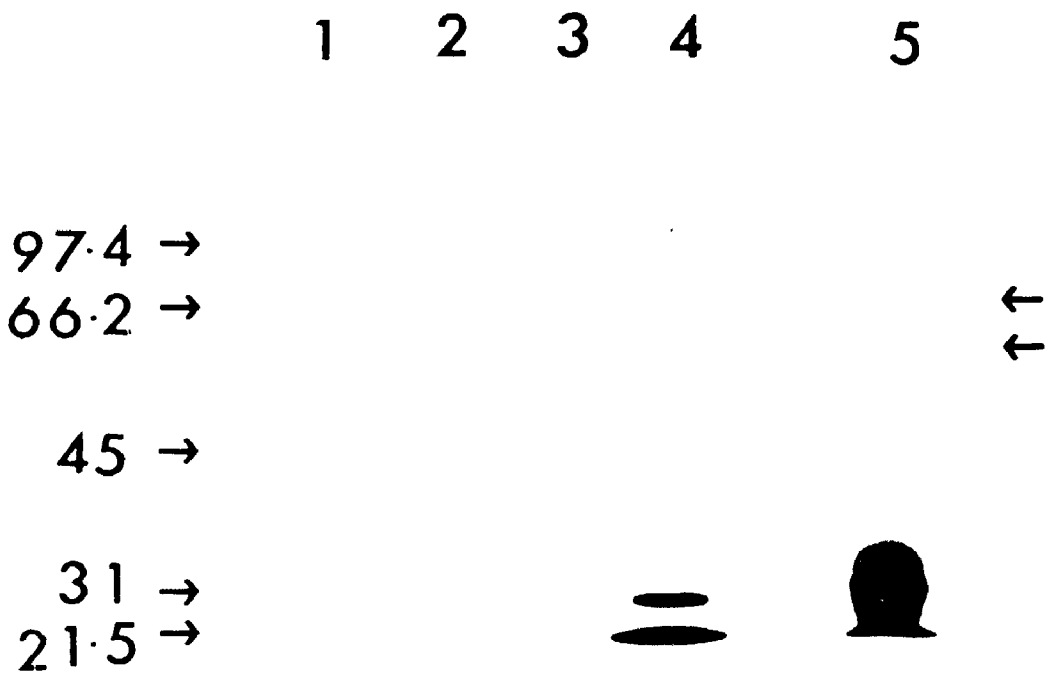

1
2
3
4
5
97·4 →
66·2 →
45 →
31 →
21·5 →
←
←

Fig. 6.19. Standard curve of p-nitrophenol for quantitative determination of β-galactosidase activity.

Four hundred μL of aqueous solution of different amounts of p-nitrophenol was mixed with 200 μL of McIlvaine buffer, pH 3.5 and 1.25 mL of 1 M sodium carbonate aqueous solution. The absorbance was measured at 400 nm.

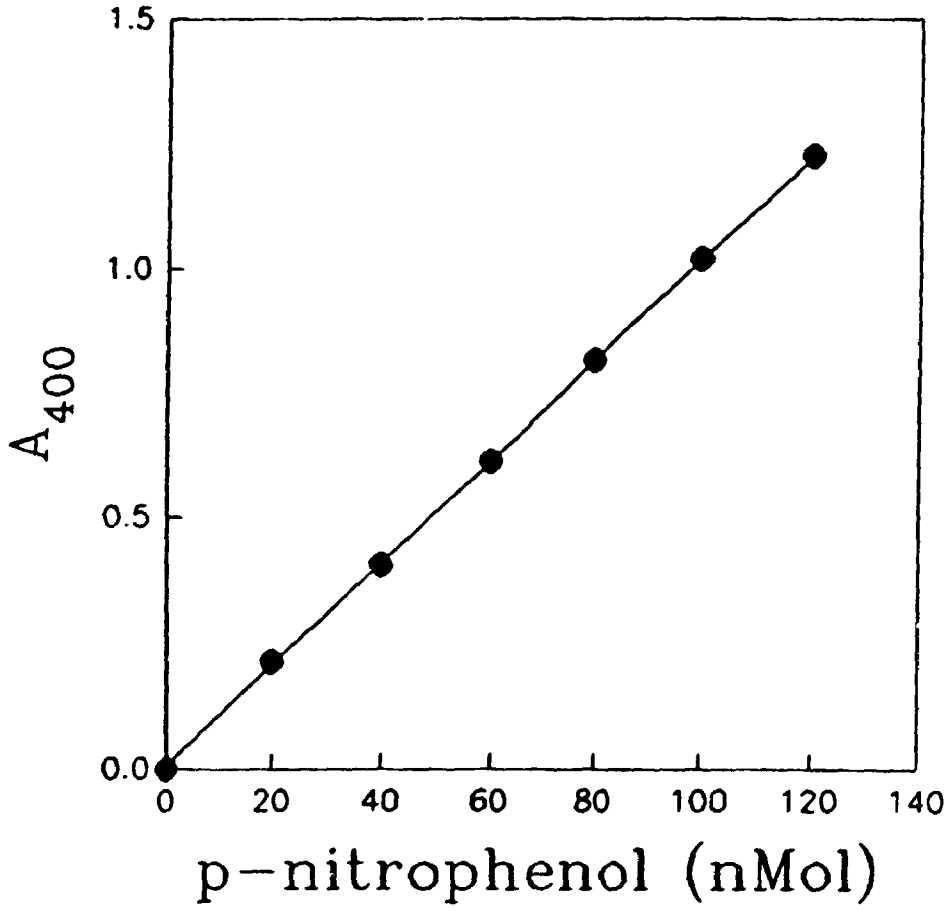

1.5
1.0
0.5
0.0
A_{400}
0
20
40
60
80
100
120
140
p-nitrophenol (nMol)

Fig. 6.20. Con A affinity chromatography of β-galactosidase treated CP-. Four milligrams of CP- were treated with 50 U of purified medium β-galactosidase in 0.4 mL of McIlvaine buffer, pH 3.5 at 37°C for 2 days, dialysed against TCM-saline buffer overnight and then applied to a Con A column (1 cm x 8 cm) at a flow rate of 1 mL/4 min.. The eluent was monitored at 405 nm using a UV-2 monitor (Pharmacia). The arrow indicates the addition of TCM-saline, 0.1 M methyl-mannopyranoside.

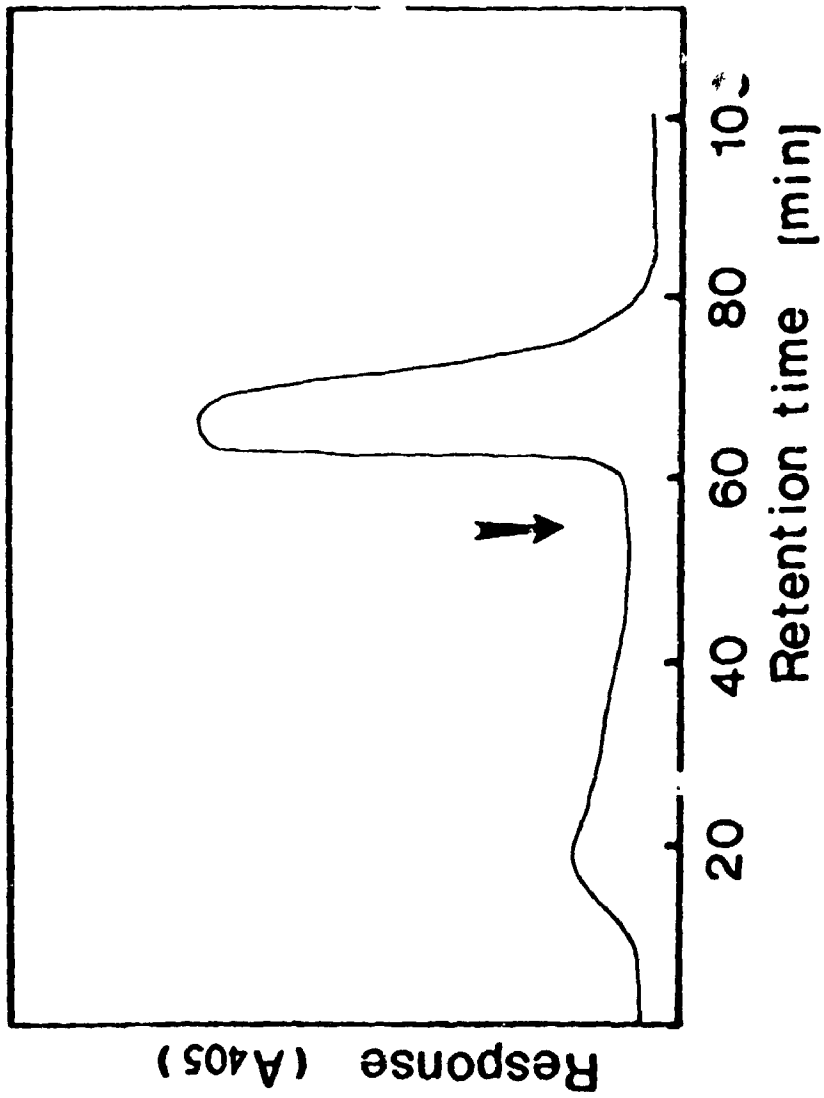
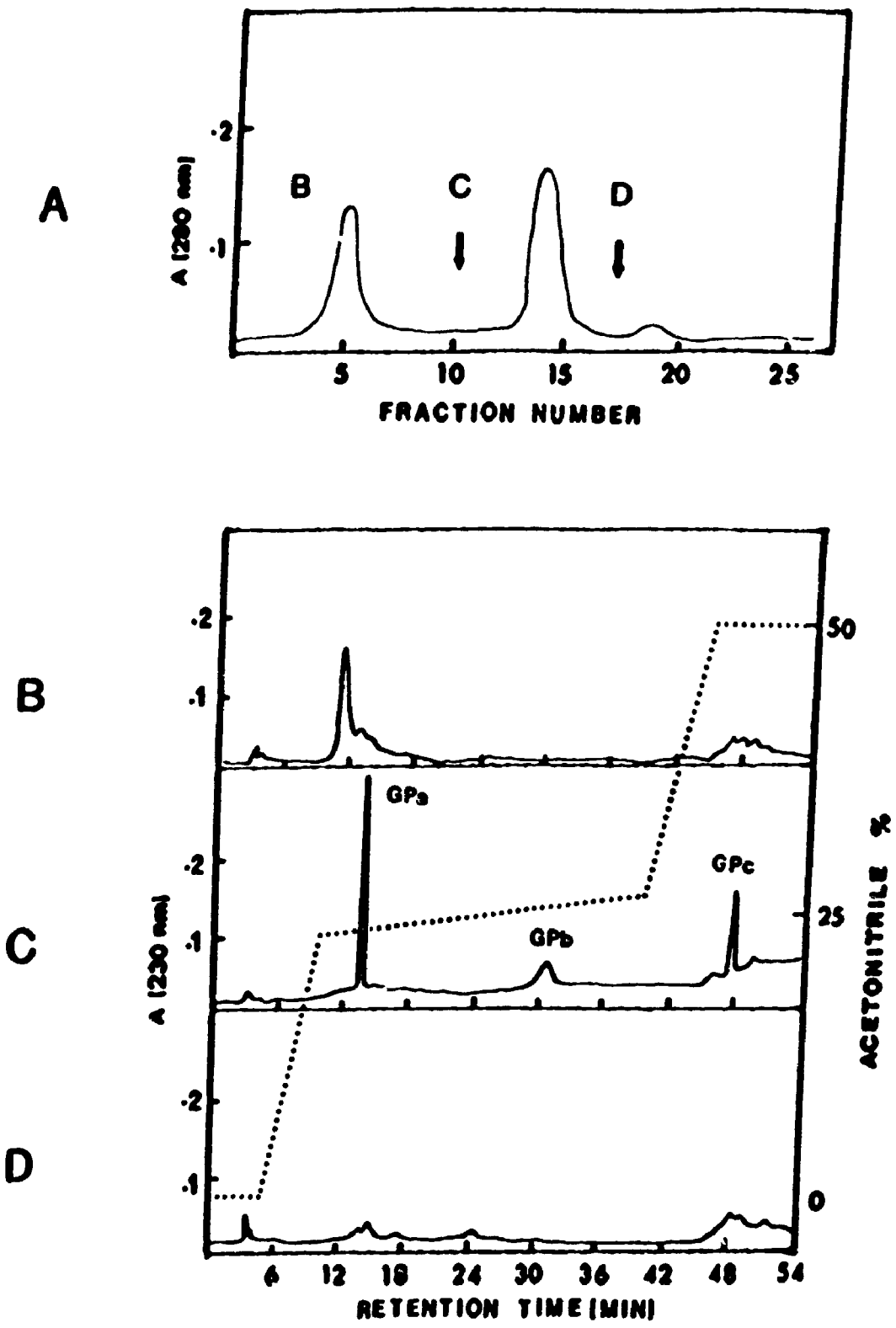

Response (A405)
20
40
60
80
Retention time [min]

Fig. 6.21. Con A affinity chromatography and reverse-phase HPLC of β-galactosidase treated glycopeptides of CP-. Panel A, Con A affinity chromatography of β-galactosidase treated glycopeptides of CP-. The arrows indicate the starting points of different washing buffers: B. TCM-saline; C. TCM-saline, 0.01 M methyl-mannopyranoside; D. TCM-saline, 0.3 M methyl-mannopyranoside. Panels B-D, reverse-phase HPLC of each fraction eluted with different washing buffers from Con A column as indicated in panel A. Each fraction from Con A was dialysed, lyophilized, and dissolved in 0.4 ml of 0.1% TFA. One hundred microliters of the solution was subjected to HPLC analysis. The gradient of acetonitrile is shown as dash line. GPa, GPb and GPc were allocated on the same basis as for Fig. 6.2.


A
B
C
D
A (280 nm)
.2
.1
B
C
D
5
10
15
20
25
FRACTION NUMBER
A (230 nm)
.2
.1
.2
.1
.2
.1
GPa
GPb
GPc
50
25
0
ACETONITRILE %
6
12
18
24
30
36
42
48
54
RETENTION TIME (MIN)

CHAPTER 7

Conclusions and Prospects

7.1. Conclusions

The studies presented in this thesis focus on the structure and function of the glycans of peanut peroxidases. An improved staining method with membrane-PAS reagent is proposed to detect glycoproteins and glycopeptides more quickly and more sensitively. The amino acid sequence of C.PRX was verified for the first time to be the same as the base sequence of cDNA clone prxPNC1. The short length of C.PRX glycans makes such a study easier than for the glycans of A.PRX. In contrast, the amino acid sequencing of the sole TPCK-tryptic glycopeptide of A.PRX does not match the base sequence of cDNA clone prxPNC2 (data not presented) and the size of the glycan is very large, so more attention was paid to C.PRX than to A.PRX.

Of the five potential glycosylation sites on C.PRX only three are used. The glycosylation of asparagine is not only dependent on the consensus sequence, but is also determined by the three dimensional structure of peptide nearby. The Chou-Fasman ß-turn is the preferred structure of peptides for N-glycosylation, probably because it favours the access of the oligosaccharide precursor and the oligosaccharyltransferase.

The glycans of C.PRX exhibited a high heterogeneity in both Con-A binding and length, and so caused two forms of CP- and CP+. It was verified that the heterogeneity of the glycans is generated from the hydrolysis of terminal galactoses by a co-secreted ß-galactosidase. The medium ß-galactosidase has mainly two isozymes with mass 60 kd, pI 7.3 and mass 66 kd, pI 7.6 respectively.

An improved ß-galactosidase staining method was developed, which could be used to detect enzymatically active zone of ß-galactosidase on gel or membrane.

Competitive ELISA revealed that the epitopes of the glycans of C.PRX are specially directed to the core structure. This result gives an indication that immuno-cross reaction among glycoproteins from different sources. The carbohydrate moiety of peanut peroxidases are necessary to maintain a spatial structure of the molecule for the enzyme activity. Removal of glycans by PNGase F caused the loss of enzyme activity.

7.2. Prospects

The fine structure analysis of the glycans of C.PRX is well in progress using 500 MZ NMR. The result will be a benefit for the further study on the glycosylation of C.PRX. There are several aspects emerging from the present studies:

1. Fine epitope mapping

From the present studies with polyclonal antibodies it was concluded that the main epitopes are on the core structure. Fine epitope mapping may be achieved using monoclonal antibodies, endo- and exo- glycosidases (Vautherot et al., 1992; Judelson et al., 1987; Masutani et al., 1990; McManus et al., 1988).

2. Quality control of C.PRX

As the co-secreted ß-galactosidase caused the heterogeneity of glycans of C.PRX, the inhibition of the activity of ß-galactosidase may increase the homogeneity of C.PRX and benefit the crystallization of C.PRX and X-ray diffraction of the crystal of C.PRX. The inhibitor should not affect the biosynthesis and secretion of C.PRX.

3. Is there any other isoform of peanut peroxidase?

As described in Chapter 6, after the separation of A.PRX and C.PRX on CM column, a tightly bound protein fraction, CPz, was eluted from the column. This fraction contains both the ß-galactosidase activity and peroxidase activity. This unidentified peroxidase isozyme has a pI higher than 9.5. Purification and characterization of this isozyme will be of interest to see whether the amino acid sequence of this isozyme matches the base sequence of the cDNA clone prxPNC2.

4. Glycosylation engineering of peanut peroxidases

Site-directed mutagenesis may be used, for example, to reduce or eliminate glycosylation sites by altering the consensus sequence, or to add glycosylation sites by changing the proline residues near the C-terminal of

Asn_{209} and Asn_{275}. The relationship of peptide structure and glycosylation, and the role of glycans could be studied using such mutants.

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