



IMMUNOGENICITY OF THE N-GLYCANS OF PEANUT PEROXIDASE

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Key Word Index—*Arachis hypogaea*; Leguminosae; immunogenicity; glycans; peroxidase; ELISA.

Abstract—The three tryptic glycopeptides of cationic peanut peroxidase (C. PRX) and the sole one of anionic peanut peroxidase (A. PRX) were individually coupled to bovine serum albumin to raise antisera. The three categories of antibodies directed towards three *N*-glycans of C. PRX (anti-GLa, anti-GLb and anti-GLc) were isolated from antisera with glycan-conjugated ECH Sepharose 4B affinity columns and the distribution of epitopes on the *N*-glycans was investigated. The reactivity of anti-GLa, anti-GLb and anti-GLc is inhibited 25–40% by 1 M fucose, compared with a slight inhibition by *N*-acetylglucosamine and xylose. Mannose and galactose showed no inhibition to anti-GLa and only a slight inhibition to anti-GLb and anti-GLc. All of anti-GLa, anti-GLb and anti-GLc recognize A. PRX and horseradish peroxidase but do not recognize fetuin. Also, their reactivity is inhibited by bromelain by more than 70%. The three categories of antibodies present high homogeneity and appear to be directed mainly towards the core structure [Xyl] (Man)₃ [Fuc] (GlcNAc)₂. An effective and simple method to screen antibodies with carbohydrate specificities is described herein.

INTRODUCTION

Peanut cells in suspension culture secrete two peroxidase isozymes, a cationic (EC 1.11.1.7, 40 kD) and an anionic (EC 1.11.1.11, 42 kD) one [1]. Genes encoding peanut peroxidases have been cloned [2] and the amino acid sequence of cationic isozyme (C. PRX) was verified to be the same as predicted by the cDNA clone prxPNC1 [3, 4]. Of the five potential *N*-glycosylation sites in C. PRX, only three, Asn₆₀, Asn₁₄₄ and Asn₁₈₅, are used [4]. Both Asn₂₀₉ and Asn₂₇₅ have a proline residue at the C-terminal of the consensus sequence and the asparagines are, therefore, not glycosylated as shown. Probably the pyrrolidine side-chain of proline prevents the formation of a favourable conformation of the peptide domain for glycosylation [5]. The anionic isozyme (A. PRX) has a sole glycan of 8000 D [6]. Tunicamycin-supplemented culture medium does not inhibit the biosynthesis of the peptide moiety but dramatically decreases the secretion of peanut peroxidases [7, 8]. Glycans apparently protect against trypsin proteolysis of peanut peroxidases [8]. An immuno cross-reaction among peanut peroxidase isozymes and other plant glycoproteins of different sources and functions was observed [6, 9]. Such cross-reaction was also found with antibodies directed to other glycoproteins [10] and is suggested to be due to the carbohydrate moiety. As antibodies are widely used as molecular probes to identify antigens, cross-reaction may cause problems. To investigate the immunogenicity of the carbohydrate moiety of glycoproteins, we raised antisera against tryptic glycopeptides of peanut peroxidases, isolated antibodies directed towards the *N*-glycans and determined the distribution of epitopes on the glycans.

RESULTS

Raising of Abs against TPCK-tryptic glycopeptides

TPCK treatment eliminates the impurity of chymotrypsin activity in trypsin [11] so that TPCK-trypsin cleaves peptide bonds precisely at the carboxyl end of lysine and arginine as confirmed by amino acid sequence analysis [4]. All three TPCK-tryptic glycopeptides of C. PRX, GP_a, GP_b and GP_c have one free amino group at *N*-terminal and one at C-terminal lysine [2, 4]. These can be linked to a carrier protein by a bifunctional coupling reagent, glutaraldehyde. BSA is not a glycoprotein (66 kD) and has 59 lysine residues (30–35 are available for coupling) [12]. 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, (ca 60 kD) has three *N*-linkage and three *O*-linkage glycans [22]. There was a clear separation of its TPCK-tryptic glycopeptides from other peptides on a Bio-gel P10 column (Fig. 1). Approximately 0.5 μM glycopeptides (from 5 mg fetuin) were used for coupling to 3 mg BSA with different amounts of glutaraldehyde and then separated on a gel (Fig. 2). Lane 5 shows the least uncoupled BSA and lane 9 is strongly stained with PAS reagent, which demonstrates that 10 μM glutaraldehyde is sufficient for the coupling of 0.5 μM glycopeptides to 3 mg BSA.

TPCK-trypsin digested A. PRX showed a sole glycopeptide on a Bio-gel P10 column and HPLC (data not shown), which is consistent with the data obtained by purification on ion-exchange columns [6].

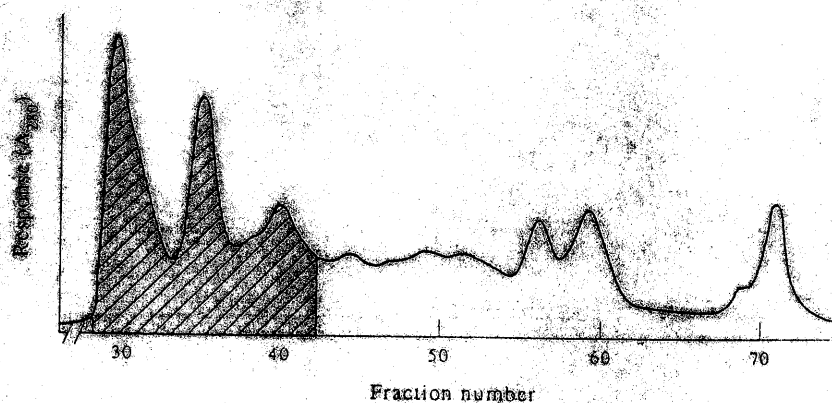


Fig. 1. Separation of glycopeptides of fetuin on Bio-gel P10 column. Fetuin (30 mg) was digested with TPCK-trypsin in a ratio of 1:30 (TPCK-trypsin/fetuin, w/w) at 37° for 15 hr. The mixture was applied to a Bio-gel P10 column (1.5 × 90 cm) and eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% sodium azide at a flow rate 7.2 ml hr⁻¹. The eluent was monitored at 280 nm and fractions of 1.8 ml were collected. Glycopeptide fractions were identified and indicated as cross-hatched.

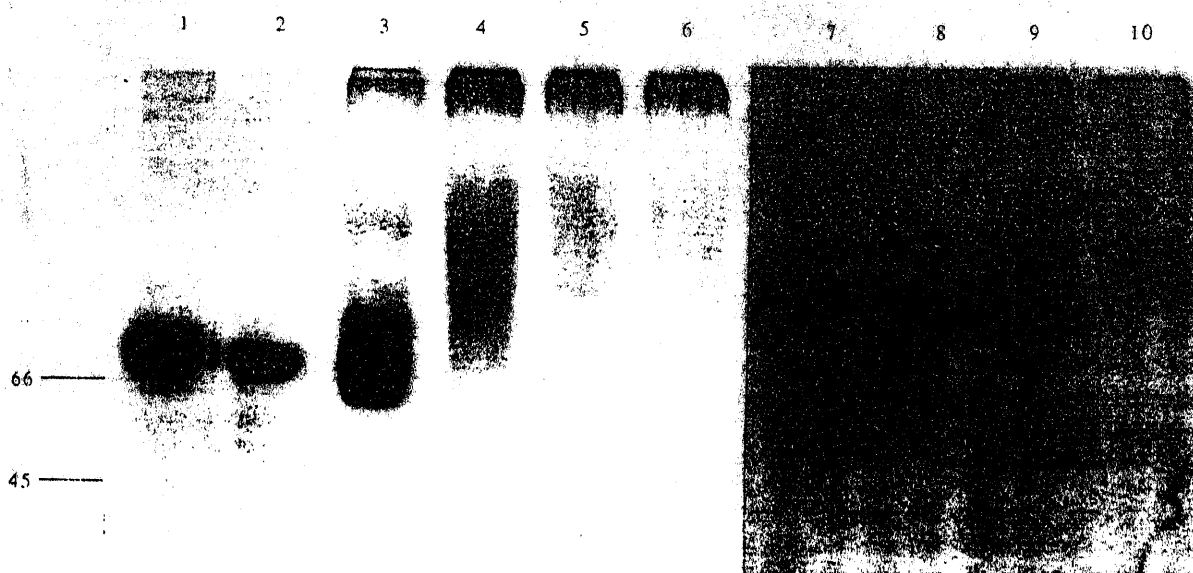


Fig. 2. 12% SDS-PAGE of TPCK-tryptic glycopeptides of fetuin coupled to BSA using varying amounts of glutaraldehyde. lane 1, 5 μ g BSA; lane 2, 5 μ g fetuin; lanes 3-6, 10 μ g coupled BSA; lanes 7-10, 40 μ g coupled BSA. Lanes 1-6 were stained with 0.1% Coomassie blue R. Lanes 7-10 were stained with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

Antisera were collected after four immunizations. Titre determination demonstrated that antisera to GP_a and GP showed titres ca 100 times lower, compared with antiserum of GP_b and GP_c. Since GP_a has the largest glycan and the shortest tryptic peptide chain (13 amino acid residues) among the three glycopeptides of C. PRX, and GP has a very large glycan of 8000 D [6], the lower titres were probably caused by the poor binding of glycopeptides to the ELISA plate. GP coated at higher amounts presented an even lower reading. This may be caused by overlapping of glycopeptides on the plate so

that absorbed antigen molecules became detached during the test and thus reduced the sensitivity. The optimal amount of antigen coated on ELISA plate was chosen as: GP_a, 1 μ g of equivalent C. PRX/well; GP_b and GP_c, 0.5 μ g of equivalent C. PRX/well and GP, 2 μ g of equivalent A. PRX well.

Competitive ELISA

In order to investigate the distribution of epitopes on individual glycans, the antibodies specific to a carbohy-

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drate domain were purified by passing IgGs precipitated at 50% ammonium sulphate through a corresponding conjugated ECH Sepharose 4B affinity column. The bound antibodies are specific to carbohydrate and are named anti-GLa, anti-GLb and anti-GLc, respectively. Figure 3 shows the sigmoidal response curves of isolated antibodies specific to the carbohydrate moiety. The dilutions that gave a linear response (when A_{405} is 1) were used as working concentrations for competitive ELISA (for anti-GLa, $5 \text{ ng } \mu\text{l}^{-1}$; anti-GLb, $0.3 \text{ ng } \mu\text{l}^{-1}$ and anti-GLc, $2.5 \text{ ng } \mu\text{l}^{-1}$). Sugar composition and linkage analyses demonstrated that the glycans of C. PRX are composed of *N*-acetylglucosamine, mannose, galactose, xylose and fucose and that all the glycans share a common core structure $[\text{Xyl}] (\text{Man})_3 [\text{Fuc}] (\text{GlcNAc})_2\text{-Asn}$ [14]. Competitive ELISA showed that the reactivity of antibodies anti-GLa, anti-GLb and anti-GLc is inhibited by *ca* 25–40% by 1 M fucose (Fig. 4). As fucose occurs only in the core structure of glycans [14], this suggests that all the glycans may have an epitope on the fucose-directed core structure. *N*-Acetylglucosamine and xylose slightly inhibit the reactivity of all 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 inhibit almost completely the reactivity of anti-GLb, while anti-GLa and anti-GLc are inhibited to a lesser degree by the other two rival glycopeptides (Fig. 5). 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 1). This indicated that none of the three classes of antibodies has any specific antibody that is not recog-

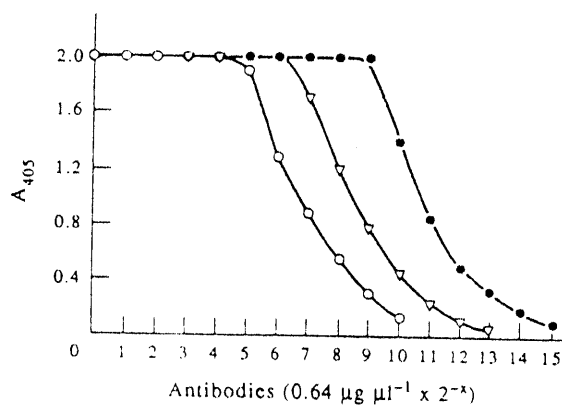


Fig. 3. Typical response curves for the reaction between optimized amounts of coated antigens and two-fold dilutions of purified antibodies against individual glycans of C. PRX. The amounts of coated antigens were: GPa, $1 \mu\text{g well}^{-1}$; GPb and GPc, $0.5 \mu\text{g well}^{-1}$. Anti-GLa ($\bigcirc$), anti-GLb ($\bullet$), and anti-GLc (∇). The x represents the number of dilutions indicated on the x axis. Each point represents the mean of three assays.

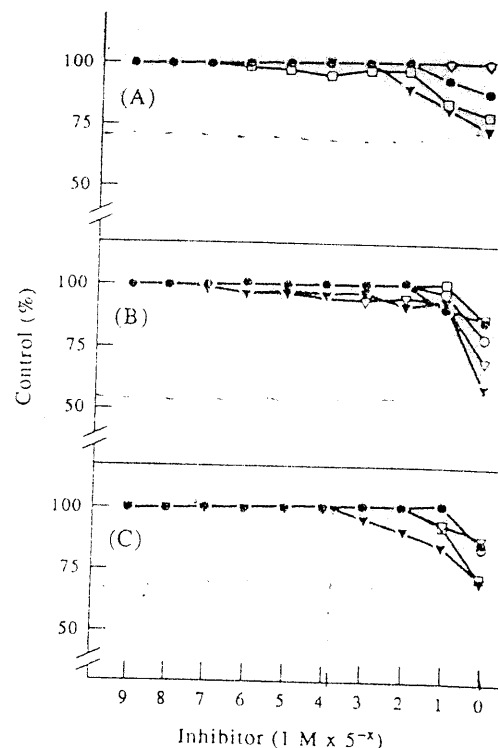


Fig. 4. Inhibition of the binding of antibodies (A, anti-GLa; B, anti-GLb and C, anti-GLc) to immobilized antigens (A, GPa; B, GPb and C, GPc) by preincubation with various monosaccharides. The amounts of coated antigens are as in Fig. 3. Mannose ∇ , *N*-acetylglucosamine $\square$, galactose $\bullet$, xylose $\bigcirc$, fucose $\blacktriangledown$. The x represents the number of dilutions indicated on the x axis. Each point represents the mean of three assays.

nized by epitopes on the other two rival glycans. All three categories of glycan-specific antibodies recognize epitopes on both A. PRX and HRP (Fig. 6). Also, their reactivity is inhibited more than 70% by bromelain at $4 \mu\text{g } \mu\text{l}^{-1}$. In contrast, at the same concentration, fetuin does not compete with anti-GLa and anti-GLc and only slightly with anti-GLb (Fig. 6). The detailed structure of glycans of bromelain, horseradish peroxidase (HRP) and fetuin are well established. Bromelain has a short *N*-linkage glycan $[\text{Xyl}] (\text{Man})_2 [\text{Fuc}] (\text{GlcNAc})_2\text{-Asn}$ [15, 16] 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 $[\text{Xyl}] (\text{Man})_3 [\text{Fuc}] (\text{GlcNAc})_2\text{-Asn}$ [10, 17]. Fetuin has three *O*-linkage and three *N*-linkage glycans, whose structure is much different from the structure of glycans of C. PRX. Neither fucose nor xylose are attached to the core structure of *N*-glycans, and the peripheral structure of all six glycans has a terminal *N*-acetylneuraminic acid [13]. 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 exhibited an even stronger inhibition than C. PRX (Fig. 6), probably because of the easier access of the shorter glycan of bromelain to the antibodies. The speci-

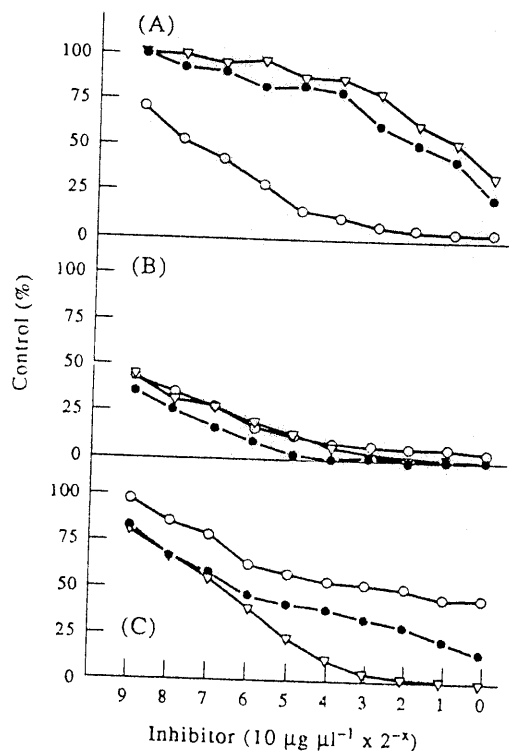


Fig. 5. Inhibition of the binding of antibodies (A, anti-GLa; B, anti-GLb and C, anti-GLc) to immobilized antigens (A, GPa; B, GPb and C, GPc) by preincubation with glycopeptides from C. PRX. The amounts of coated antigens are as in Fig. 3. GPa ○---○, GPb ●---●, GPc ▽---▽. The x represents the number of dilutions indicated on the x axis. Each point represents the mean of three assays.

cities of antibodies toward the common core structure were recognized by HRP but not by fetuin. Although the sugar composition of the glycan of A. PRX is known [14], the detailed structure is difficult to establish because of its large size. According to the competition test, the glycan of A. PRX probably has a similar core structure.

DISCUSSION

In the last decade, antibodies against varieties of glycoproteins have been raised and antigenic analyses showed

that oligosaccharides are the major antigens of glycoproteins [6, 10, 18–26]. In the present study, it is 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 inhibition on 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. 4B), in contrast with 95% inhibition by GPa at a concentration of $10 \mu\text{g } \mu\text{l}^{-1}$ (equal to $250 \mu\text{M}$, Fig. 5B) and 92% inhibition by HRP at $4 \mu\text{g } \mu\text{l}^{-1}$ (equal to $100 \mu\text{M}$, Fig. 6B). This is due to the small size of the monosaccharides and that monosaccharides may be a part of an epitope recognized by an antibody directed towards carbohydrate. Despite the disadvantage, monosaccharides are still useful competitors for epitope mapping of carbohydrate [22], especially because of the difficulty of obtaining specific oligosaccharides.

The three categories of polyclonal antibodies directed towards the three individual N-glycans of C. PRX exhibit high homogeneity as proved by competitive ELISA. Obviously, the similar immunogenicity of the three N-glycans of C. PRX is due to the similarity of sugar composition and core structure. It was reported that antiserum against C. PRX recognizes A. PRX [27] and that monoclonal antibodies directed to A. PRX recognize C. PRX [9]. 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 [22, 24, 28, 29].

The reactivity of the three categories of antibodies, anti-GLa, anti-GLb and anti-GLc is inhibited 25–40% 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 [15]. It was the first N-linkage glycan in higher plants whose primary structure was well established [15, 16]. As bromelain inhibits by more than 70% the reactivity of the three classes of antibodies directed to glycans of C. PRX, the core structure

Table 1. Comparison of immunogenicity of glycans of C. PRX*

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%)

*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 was the same as for the purification of antibodies described in the Experimental.

MONOSACCHARIDES
CORES
INHIBITION
OF
ANTIBODY
BINDING

Fig. 6. anti-GLb and anti-GLc to immobilized antigens (A, GPa; B, GPb and C, GPc) by preincubation with glycopeptides from C. PRX. The amounts of coated antigens are as in Fig. 3. GPa ○---○, GPb ●---●, GPc ▽---▽. The x represents the number of dilutions indicated on the x axis. Each point represents the mean of three assays.

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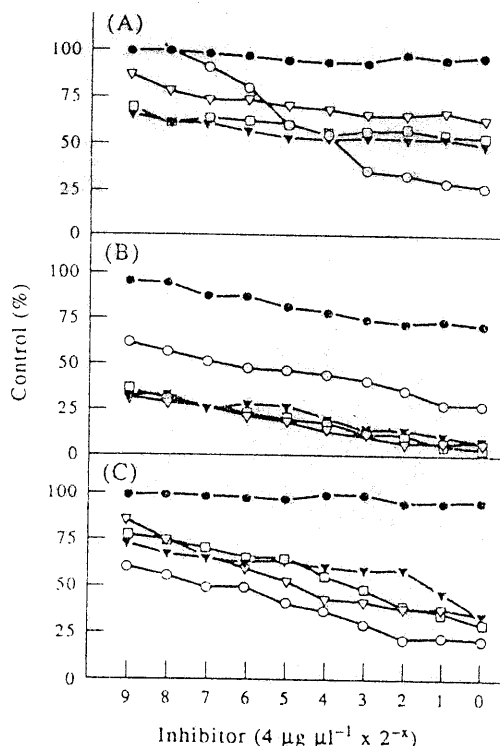


Fig. 6. Inhibition of the binding of antibodies (A, anti-GLa; B, anti-GLb and C, anti-GLc) to immobilized antigens (A, GPa; B, GPb and C, GPe) by preincubation with various glycoproteins. The amounts of coated antigens are as in Fig. 3. Bromelain ○---○, fetuin ●---●, A. PRX ▽---▽, HRP ▼---▼, C. PRX □---□. The x represents the number of dilutions indicated on the x axis. Each point represents the mean of three assays.

[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 [30], phaseolin from bean [31], *Sophora japonica* lectin [32], protease inhibitor from barbados pride seeds [33], *Clerodendron trichotomum* lectin [34], *Erythrina cristagalli* lectin [17], *S*-glycoproteins from *B. campestris* [35], 52/54-kDa medium protein and β -fructosidase from carrot [36], laccase from sycamore cells [37] and HRP [10, 17]. The homogeneity of the 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 [6, 9, 10].

Antibodies, including monoclonal antibodies and 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 [18, 28, 38, 39]. Critical for the proper application of antibodies in such studies is the awareness of potential cross-reactions based on the carbohydrate moiety, especially the core structure of oligosaccharide chains. In order to determine whether antibodies have carbohydrate specificity, or

to eliminate the immunogenicity of the carbohydrate moiety of glycoproteins, various methodologies have been developed. Deglycosylation of antigens of glycoproteins are widely used. For example, enzymatic deglycosylation by exo- or endo-glycosidases, chemical deglycosylation with trifluoromethane sulphonic 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 [40, 41]. Tunicamycin treatment may decrease the secretion of proteins [7, 8] and cause degradation of proteins before they are sorted [42]. In our work we have devised 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 probes to elucidate the structures of other carbohydrate chains.

EXPERIMENTAL

Cell culture and peroxidase purification. Peanut cells, derived from cotyledon slices (*Arachis hypogaea* L. var. Virginia 56R), were routinely subcultured for 14 days [43]. The C. PRX and A. PRX in the spent growth medium were purified as described in refs [44, 45].

TPCK-tryptic digestion. The heme of C. PRX or A. PRX was removed with acidic Me₂CO [46]. The apoproteins of C. PRX and A. PRX, and fetuin (Sigma) were incubated with 1-1-tosylamido-2-phenylethylchloromethyl ketone-treated trypsin (TPCK-trypsin, Worthington Biochemical Corporation) in 0.1 M ammonium bicarbonate, pH 8 at 37° for 40 min (C. PRX and A. PRX) or 15 hr (fetuin). The ratio of substrate: TPCK-trypsin was 30:1 (w/w). The trypsin was inactivated by boiling for 3 min.

Purification of TPCK-tryptic glycopeptides. The TPCK-trypsin digested proteins were applied to either Biogel P6 (C. PRX) or P10 (A. PRX and fetuin) columns (1.5 cm x 90 cm, 200–400 mesh, Bio-Rad). The column was equilibrated and eluted with 0.1 M ammonium acetate, pH 7, containing 0.02% sodium azide at room temp. at a flow rate of 8 ml hr⁻¹. Frs of 2 ml were collected. Glycopeptide frs were identified by either dot-blotting on NC membrane [47] or the pHOH-H₂SO₄ method [48]. TPCK-tryptic glycopeptide frs of A. PRX from Biogel P10 column were subsequently purified by reversed-phase HPLC using a μ -Bondapak™ C₁₈ column (3.9 x 300 mm, Waters). HPLC was carried out at a flow rate of 1.2 ml min⁻¹. The mobile phase was 0.1% aq. TFA and the mobile phase modifier was MeCN containing 0.1% TFA. The concn of MeCN was raised from zero to 30% in 30 min. The eluent was monitored at 230 nm. The sole glycopeptide was identified using the pHOH-H₂SO₄

technique [48] and named GP. The three TPCK-tryptic glycopeptides of C. PRX, GP_a, GP_b and GP_c were identified on HPLC as described previously [4]. GP_a contains glycosylation sites Asn₁₈₅; GP_b, Asn₆₀ and GP_c, Asn₁₄₄ [4].

Coupling of glycopeptides to bovine serum albumin (BSA). To raise antisera against individual tryptic glycopeptides of C. PRX and A. PRX, a modified coupling procedure [12] was employed. For one injection, each glycopeptide from 20 mg (0.5 μ M) C. PRX (ca 2 mg glycopeptide) or 8 mg (0.2 μ M) A. PRX (ca 2 mg glycopeptide) and 3 mg BSA (Sigma) were dissolved in 1 ml 0.01 M NaPi buffer, pH 7.2, containing 0.15 M NaCl, and vortexed for 5 min. Then 0.1 ml 1% (v/v) glutaraldehyde (Eastman Kodak Company) was added dropwise with constant agitation. The mixt. was incubated with shaking at 4° overnight. The coupling reaction was stopped by addition of 250 μ l of 1 M glycine. After incubation with continuous shaking for 3 hr at room temp., the glycopeptide-BSA conjugate was sepd from excess glutaraldehyde by dialysis against 0.01 M NaPi buffer, pH 7.2 using Spectro/Por membrane tubing (Spectrum Medical Industries Inc.) overnight and lyophilized. In order to optimize the coupling condition, the glycopeptides of TPCK-tryptic fetuin was used for a trial assay.

Immunization. Lyophilized glycopeptide-BSA conjugate was dissolved in 0.6 ml double-distilled H₂O 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 into New Zealand rabbits subcutaneously. The rabbits were boosted every 3 weeks until the titre was sufficiently high.

Affinity purification of antibodies specific to carbohydrate moiety of C. PRX. Each of the TPCK-tryptic glycopeptides, GP_a, GP_b and GP_c of 40 mg C. PRX isolated by HPLC was extensively digested with 0.5 mg pronase (Boehringer Mannheim) in 1 ml 50 mM Tris-HCl buffer, pH 8 containing 20 mM CaCl₂ and 0.02% sodium azide at 37° for 72 hr with additions of 0.2 mg pronase every 24 hr. To eliminate any possible impurity of endoglycosidase activity, pronase was dissolved in Tris-HCl buffer and preincubated at 37° for 30 min [49]. The glycan-Asn fragments produced, namely GL_a, GL_b and GL_c, were sepd from amino acids on Bio-Gel P4 column (1.5 $\times$ 90 cm, Bio-Rad) and coupled to ECH Sepharose 4B (Pharmacia) according to the manufacturer's instruction. Briefly, GL_a, GL_b, or GL_c or 40 mg C. PRX was dissolved in 1 ml double-distilled H₂O and mixed with 2 ml ECH Sepharose 4B gel suspension. *N*-Ethyl-*N'*-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC, Sigma) (1 ml, 0.4 M) was added dropwise to the mixt. with agitation. After 1 hr, the pH was adjusted to 5 using 0.2 M NaOH and left to agitate at 4° overnight. To block the remaining carboxyl groups, 200 μ l ethanolamine (Sigma) was added and incubated at room temp. for 5 hr. The gel was packed (5 $\times$ 40 mm) and washed alternatively $\times$ 3 with acetate buffer (0.1 M, pH 4) and Tris-HCl buffer (0.1 M, pH 8) each containing 0.5 M

NaCl to remove any uncoupled ligand. The columns were equilibrated with 10 mM Tris-HCl, pH 7.5. Under the above conditions, the coupling efficiency was ca 20% based on the measurement of the amount of uncoupled ligand recovered in the washings. Antiserum against GP_a, GP_b or GP_c was mixed with an equal vol. of satd (NH₄)₂ SO₄ and left at 4° overnight. After centrifugation, the pptd IgGs were dissolved in and dialysed against 10 mM Tris-HCl, pH 7.5 at 4° overnight. Then, the IgGs were passed twice through a corresponding conjugated ECH Sepharose 4B affinity column at a flow rate of 6 ml hr⁻¹. After washing the column with 10 mM Tris-HCl pH 7.5 until A₂₈₀ returned to the baseline, bound antibodies against individual glycans, designated anti-GL_a, anti-GL_b and anti-GL_c, respectively, were eluted with 3 M MgCl₂ and dialysed against H₂O to remove salt. The columns were washed immediately with 10 mM Tris-HCl, pH 7.5 to be regenerated.

Enzyme-linked immunosorbent assay (ELISA). Modified procedures for ELISA [9] were employed. Briefly, each well of a round-bottom polystyrene plate (Corning) was coated with 50 μ l of HPLC purified TPCK-tryptic glycopeptide, GP_a, GP_b, GP_c or GP, in 0.05 M Na₂CO₃ buffer pH 9.6 containing 0.02% sodium azide at 37° for 1 hr and then at 4° overnight. The amount of coated glycopeptides was given either as indicated in the case of titre determination of antisera or optimized as GP_a, 1 μ g of equivalent C. PRX/well, GP_b and GP_c, 0.5 μ g of equivalent C. PRX/well in competitive ELISA. The unbound glycopeptides were washed off with washing buffer (0.01 M Pi buffer pH 7.4 containing 0.15 M NaCl, 0.1% gelatin, 0.02% NaN₃ and 0.05% Tween-20). Then, 50 μ l of antibodies appropriately diluted with washing buffer was added to each well. After incubation at 37° for 1 hr, the plate was washed with washing buffer and incubated with 50 μ l of alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma, 1:5000 diluted in washing buffer) at 37° for 1 hr. Following washing, the plate was incubated with 50 μ l *p*-nitrophenylphosphate (Sigma, 0.5 mg ml⁻¹ in 9.7% diethanolamine pH 9.8) at 37° for 1 hr. The reaction was stopped by addition of 50 μ l of 3 M NaOH. The A 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° for 1 hr and then at 4° overnight. Mixtures (50 μ l) were transferred to each well coated with antigen and ELISA was processed as above. The concn of antibodies employed was anti-GL_a, 5 ng μ l⁻¹; anti-GL_b, 0.3 ng μ l⁻¹ and anti-GL_c, 2.5 ng μ l⁻¹ prepd in washing buffer. The initial concn of the competitors was glycopeptides (GP_a, GP_b, GP_c) 10 μ g μ l⁻¹, glycoproteins (bromelain from pineapple stem, Sigma; fetuin; A. PRX; C. PRX; HRP, Sigma) 4 μ g μ l⁻¹ and monosaccharides (mannose, galactose, fucose, xylose, *N*-acetylglucosamine, Sigma), 1 M. Each competitor tested was diluted from the initial concn as either 5-fold for monosaccharides or 2-fold for glycoprotein and glycopeptide series.

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4 mg / ml

SDS-electrophoresis. In order to probe the suitable amount of glutaraldehyde, 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 sepd on 12% SDS-gel [50]. After electrophoresis, the gel was stained with either periodic acid-Schiff's (PAS) reagent [47] to show carbohydrate or Coomassie Blue R to show protein.

Protein determination. The technique of ref. [51] was employed for protein determination with BSA as standard.

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