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HPLC Determination of the Sugar Compositions of the Glycans on the Cationic Peanut Peroxidase

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Cationic peanut peroxidase has three N-linked glycans. Two glycoforms, CP− and CP+, are known to occur. In this study, the glycans of CP− and CP+ were sequentially separated and identified by trypsin digestion, Bio-Gel P-6 filtration, and reverse phase HPLC. Sugar composition analyses of the glycans were carried out by hydrolysis with TFA, labeling the released sugars with ABEE, and reverse phase HPLC of the ABEE-sugar derivatives. Five different sugars (GlcNAc, Gal, Man, Xyl, Fuc) were found in each of the six glycans investigated. Mannose residues accounted for 31 ± 1.8% of CP− and 40 ± 2.2% of CP+ glycans in term of molar content. The galactose content was 57% lower in CP+ glycans as compared to CP−. The Xyl and Fuc contents were also lower in CP+ than in CP− glycans. A hypothesis made has been confirmed one step further, that is the Gal is one of the terminal sugars of the glycans and the removal of the Gal as a terminal sugar residue in CP− leads to the exposure of Man residue, which is then able to bind to Con-A.

Keywords: *Cationic peanut peroxidases; glycans; HPLC; sugar compositions*

INTRODUCTION

Glycoproteins are widely distributed in nature. Carbohydrate content and configuration is often an important feature in the study of the functional and biological properties of glycoproteins (Kanshansky, 1987; Goochee et al., 1991). It is generally accepted that glycans modify the physicochemical properties of glycoproteins, maintain the protein configuration, protect the protein from degradation, serve as passports for protein secretion, and are important recognition markers (Dwek, 1996; Sharon and Lis, 1993). Whether and why a carbohydrate moiety is necessary for the biological activities of glycoproteins has attracted the most interest. Peanut (*Arachis hypogaea*) peroxidase, a glycoprotein, has been studied intensively (van Huystee, 1990). The cationic isozyme of peanut peroxidase may be separated into two fractions by Con-A affinity chromatography: CP−, the flow-through fraction of 40 KDa, and CP+, the binding fraction of 37 KDa. CP+ comprises only 10% of the total cationic isozyme concentration in the suspension culture medium of peanut cells (O'Donnell et al., 1992). A comparison of the two fractions in terms of Soret band absorption, calcium content (O'Donnell et al., 1992), formic acid cleavage pattern, and molecular weight of the peptide chain (Wan and van Huystee, 1993) did not reveal any difference. The information above suggests that CP− and CP+ are two glycoforms of the cationic isozyme that differ only by glycan masses (Wan and van Huystee, 1993). Moreover, since previous studies have proven that the two glycoforms have the same level of specific enzyme activity (O'Donnell et al., 1992), it will be of interest to determine the difference in sugar composition and content between the two glycoforms. In this study, we investigated the sugar composition of the individual N-linked glycans from both glycoforms by enzyme cleavage and HPLC techniques.

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MATERIALS AND METHODS

Materials. TPCK-treated trypsin, Con-A, methyl α-D-glucopyranoside, and ABEE were purchased from Sigma Chemical Company. Sodium cyanoborohydride, TFA, and standard sugars of the highest purity were obtained from Aldrich Chemical Company. Bio-Gel P-6 (50−150 mesh) was purchased from Bio-Rad Laboratories. Sephadex G-100 (40−120 μm) and CM Sephadex 50 were from Pharmacia LKB Biotechnology AB. Dialysis tubing with MWCO 3000 Da was from VWR Scientific. Acetonitrile and methanol were HPLC grade.

Peroxidase Purification. CP− and CP+ were purified from the spent media of peanut (*Arachis hypogaea*) cell suspension cultures (O'Donnell et al., 1992; Chibbar et al., 1984). Briefly, 14-day-old cell cultures were filtered through Whatman no. 1 filter paper. The filtrate was brought to 70% acetone and left at 4 °C overnight. The precipitate was collected by centrifugation at 9000*g* for 10 min and redissolved in 0.02 M sodium acetate buffer (pH 5.0). After spinning at 13000*g* for 15 min, the supernatant was brought to 80% ammonium sulfate and centrifuged again at 13000*g* for 10 min. The pellet was resuspended in double distilled H_2O and dialyzed against the same overnight. The dialysate was centrifuged to remove any insoluble materials then loaded onto a CM Sephadex 50 column. Cationic peroxidase was eluted in a gradient of 0.02−0.1 M sodium acetate buffer (pH 5.0) and applied to a Con-A affinity column. CP− flowed through with TCM-saline buffer whereas CP+ was eluted in TCM-saline buffer containing 0.1 M methyl α-D-glucopyranoside. Fractions pertaining to CP− and CP+ were applied to a size exclusion column (G-100, 2.5 × 80 cm), where they were purified further. The final purity of each sample was determined by RZ value and SDS−PAGE.

Trypsin Digestion and Gel Filtration Chromatography. Either 30 mg of CP− or 30 mg of CP+ was treated with acidic acetone to remove the heme moiety (Chibbar et al., 1984). The pelleted apoprotein was resuspended in 3 mL of

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0.1 M NH_4HCO_3, pH 8.0 buffer, and digested with TPCK-treated trypsin (substrate:enzyme = 30:1, w/w) at 37 °C for 1 h (Wan and van Huystee, 1993). The glycopeptides and peptides produced by trypsin digestion were loaded onto a Bio-Gel P-6 column (1.5 × 90 cm, molecular mass range 1000−6000 Da, 50−150 mesh), which was equilibrated with 0.1 M ammonium acetate buffer (pH 7.0). Elution was carried out with the same buffer at room temperature, at a flow rate of 8 mL/h (Wan and van Huystee, 1993). Fractions of 2 mL were collected and then tested for glycopeptides using a modified phenol−sulfuric acid method. In this method, 50 μL of sample was mixed with 50 μL of 5% phenol in a clear 1.5-mL microtube, and then 400 μL of concentrated sulfuric acid was added. The color of the reaction solution was observed 15 min later (Dubois et al., 1956). The glycopeptide-containing fractions were pooled, dialyzed against double distilled H_2O, and lyophilized.

Separation of Glycopeptides by Reverse Phase HPLC. The system Gold apparatus (programmable solvent module 126, Beckman) and a Rheodyne injector with a 500-μL sample loop were used. Samples were injected onto a C-18 column (Ultrasphere 4.6 × 150 mm, Beckman) at room temperature. Elution was performed at a flow rate of 1.2 mL/min in an acetonitrile gradient in 0.1% TFA (0−5 min, 0.1% TFA; 5−10 min, 0−20% acetonitrile; 10−35 min, 20−25% acetonitrile; 35−50 min, 25−45% acetonitrile; 50−60 min, 45−100% acetonitrile). Fractions of 1.2 mL were collected. Glycan-containing fractions were identified using a modified phenol−sulfuric acid method. The fractions were pooled accordingly, dialyzed against double distilled H_2O, and lyophilized.

Acid Hydrolysis of Glycopeptides. Glycopeptides (1.0 mg ± 0.1 mg) were dissolved in 100 μL of double distilled H_2O in a Pierce Recti-vial (1.5 × 4 cm), and an equal volume of 4 M TFA was added to give a 2 M TFA solution. The vial was capped and placed in a heating block at 100 °C for 6 h (Kwon and Kim, 1993). The hydrolysate was cooled to room temperature and the remaining TFA was removed by keeping the vial in a vacuum desiccator till the hydrolysate was dried thoroughly. The dried hydrolysate was then resuspended in 10 μL of double distilled H_2O.

Labeling of Sugars with ABEE. The procedures for preparing the ABEE reagent and derivatizing sugars were those of Wang et al. (1984). Briefly, 165 mg of ABEE and 35 mg of sodium cyanoborohydride were mixed in 41 μL of glacial acetic acid and 0.35 mL of methanol. Before use, the reagent was warmed to dissolve any crystals formed during storage. To 10 μL of hydrolysate in a Reacti-vial, 40 μL of ABEE reagent was added, and the mixture was incubated at 80 °C for 50 min, and 0.2 mL of double distilled H_2O and 1 mL of chloroform were then added to the reaction mixture. The sugar derivatives partitioned into the upper aqueous phase after shaking the vial and letting the two phases separate. A 20-μL sample of the aqueous solution was subjected to HPLC for sugar analysis.

Reverse Phase HPLC of ABEE-Sugar Derivatives and Construction of Calibration Curves of Standard Sugars for Quantitative Analysis. Except for a 20-μL sample loop, the same apparatus was used as for separation of glycopeptides. A sample of 20 μL of ABEE-sugar derivatives was injected onto the C-18 column at room temperature. Chromatography was performed in an isocratic mode with 88% solvent A (50 mM sodium acetate buffer, pH 4.5) and 12% solvent B (50 mM sodium acetate buffer, pH 4.5/acetonitrile/methanol = 40/40/20, v/v/v) at a flow rate of 2.4 mL/min. ABEE-sugar derivatives were detected at 254 nm (Kwon and Kim, 1993).

To set up standard curves, a range of standard sugars from 50 to 200 nmol, in 10 μL of aqueous solution containing 200 nmol of lactose as internal standard, was derivatized with ABEE reagent, and 10% of the ABEE-sugar derivatives was analyzed as described above.

RESULTS

Peroxidase Purification. The CP− and CP+ samples purified through the steps described in methods reached the RZ value of 3.2. These samples were also confirmed to be homogeneous by SDS−PAGE.

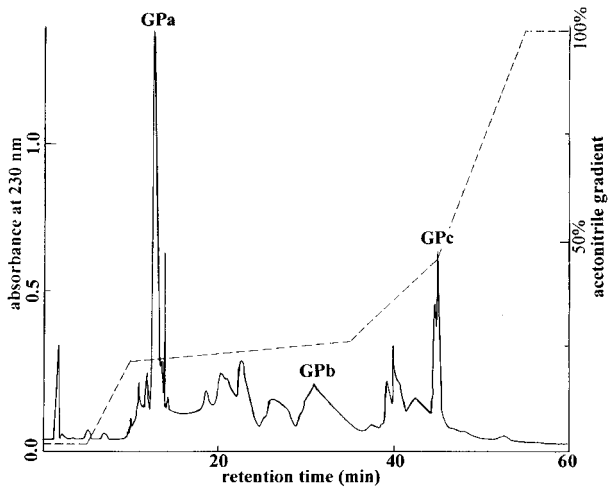

Figure 1. Separation of the three glycopeptides of CP− by reverse phase HPLC. The glycopeptides generated by trypsin digestion and primarily purified through Bio-Gel filtration were separated by HPLC with a C-18 bonded silica column as described under Materials and Methods. The acetonitrile gradient is indicated as dash lines. The effluents were monitored at wavelength 230 nm. The glycopeptide peaks are designated GPa, GPb, GPc, respectively.

HPLC Separation of Glycopeptides. There are four potential sites for N-glycosylation with the consensus sequence of Asn-X-Thr/Ser according to the deduced amino acid sequence of peanut cationic peroxidase cDNA clone (prx PNC-1) (Buffard et al., 1990). Prior data have shown that three of them (Asn-60, Asn-144, and Asn-185) are glycosylated (Wan and van Huystee, 1993). According to this amino acid sequence, trypsin treatment released the three glycopeptides and many peptides. The molecular mass of each of the glycopeptides was calculated based on the putative structures of the glycans (van Huystee et al., 1992) and their associated amino acid sequences. They were 4143, 5283, and 4814 Da. The molecular masses of peptides were much lower, ranging from 146 to 3656 Da. Prior to HPLC of the glycopeptides, the TPCK-trypsin treated sample was purified by a Bio-Gel P-6 filtration chromatography, through which most of the peptides were removed from the glycopeptides. In doing so, we eliminated a number of peaks that would have been brought about by the peptide fragments of trypsin treated sample on the profile of HPLC of the glycopeptides; therefore, the designation of glycopeptide peaks was simplified by a large extent. Fractions 12−21 from Bio-Gel P-6 column were phenol−sulfuric acid reaction positive (the reaction solution turned to brownish). They were then treated as described and subjected to HPLC. Figure 1 shows the elution pattern of the glycopeptides by HPLC. The combination of detection at 230 nm for peptides and identification of glycans by the modified phenol−sulfuric acid technique enabled us to locate the glycopeptide peaks in the HPLC elution profile. The first glycopeptides were eluted at retention time 12.5 min, and were designated as GPa. Corresponding to this peak, fractions 12 and 13 were phenol−sulfuric acid reaction positive and were collected. The second glycopeptide was eluted at 31−36 min, and was designated as GPb. Fractions 32−36 were the corresponding fractions (by phenol−sulfuric acid method) and pooled. The reason that GPb maintained such a long range of retention time is possibly due to the slow

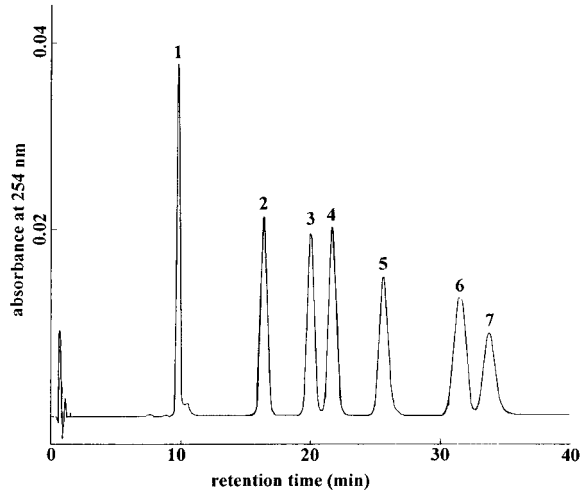

Figure 2. Chromatographic separation of standard monosaccharides by reverse phase HPLC. Twenty microliters of mixture containing 20 nmol of each ABEE-sugar derivatives was injected to a C-18 column. Elution was performed according to the procedure given under the Materials and Methods. The identities of the peaks are as follows: 1, GlcN; 2, Lac (internal standard); 3, Gal; 4, Man; 5, Xyl; 6, ABEE reagent; 7, Fuc.

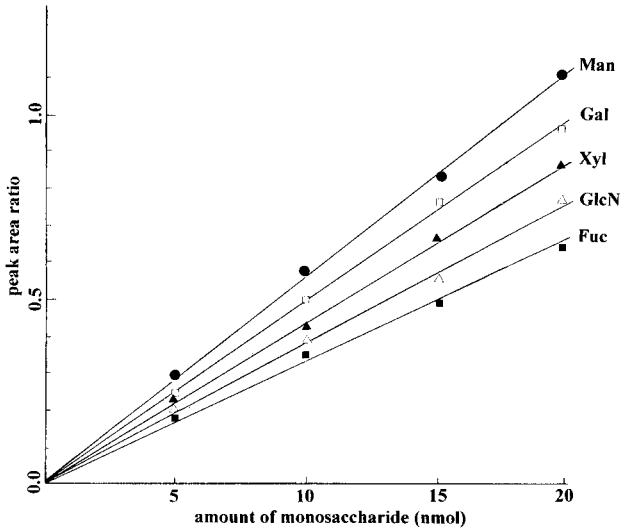

Figure 3. Calibration curves for quantitative analysis of monosaccharides. Varying amounts of standard sugars (50, 100, 150, 200 nmol) in 10 μL of aqueous solution containing 200 nmol of lactose were derivatized with ABEE reagent, and 10% of the derivatives was subjected to HPLC. The peak area ratios represent the ratios of integrated peak areas given by various amounts of sugars to those given by 20 nmol of lactose.

increase of acetonitrile gradient (Figure 1). The last glycopeptide displayed a retention time of 45.6 min, and was designated as GPc. In response to this peak, fractions 45 and 46 were found containing sugars and collected. Separation of the three glycopeptides of CP+ was carried out by the same approaches, and the results were the same as that of CP− (data not shown).

Separation of ABEE-Sugar Derivatives by HPLC and Construction of Standard Curves for Sugar Content Analysis. Although more than 200 monosaccharides are found in nature, only a few of them are known to frequently occur in glycoproteins. They are GlcNAc, GalNAc, Gal, Glc, Man, Fuc, Rha, Ara, Xyl, and Sia. Among them, Sia and Glc do not exist in plant glycoproteins (Sharon and Lis, 1981; Vliegenthart and Montreuil, 1995). The C-18-bonded silica column resolved the rest of the eight sugars well when only employed for qualitative analysis. This means that although the overlaps between peaks were observed, the identification of retention time for each sugar was still achieved. To begin with, we determined through qualitative analysis that out of the eight kinds of sugars, only five were present in each of the glycans investigated. They were GlcNAc, Gal, Man, Xyl, and Fuc. This result was consistent with what we have obtained using trimethylsilyl methylglycosides and gas chromatography analysis at the Complex Carbohydrate Research Center in Athens, GA (van Huystee et al., 1992). Then we focused on the quantitative resolution of the five sugars. Reducing the hydrophobicity of mobile phase by decreasing the proportion of solvent B gave rise to a better separation between the sugars. When 88% solvent A and 12% solvent B was used as a mobile phase isocratically, the five sugars were separated completely, but their retention times were retarded and signal peaks were broadened accordingly. This could result in a lengthy running time and inaccurate readings of peak area values (Kwon and Kim, 1993). To overcome the problems, we tested different flow rates and found that, at 2.4 mL/min, the total running time was shortened to 40 min from 120 min at a flow rate of 1.0 mL/min and that the peaks were narrowed considerably (Figure 2). More importantly, the linear relationship between peak area ratio (integrated peak area of each sugar divided by that of 20 nmol of lactose) and amount of sugar (nmol) was still being maintained (Figure 3). A higher flow rate could break the linearity. Since the peptides contained in the hydrolysate interfere with the sugar detection (Weitzhandler et al., 1996), the ABEE reagent was used to label the sugars specifically. The ABEE-sugar derivatives hence produced could be monitored at 254 nm. But this raised a concern about the degree of completion of derivatization reaction. Introducing an internal standard correction factor by addition of a constant amount of internal sugar to varying amounts of sugars to be derivatized eliminated the effect of incomplete reaction. We found lactose, being eluted between GlcN and Gal, was a good internal standard sugar in our system. Column temperature can be adjusted to optimize resolution (Kwon and Kim, 1993; Snyder and Kirkland, 1979). However, in our case, the HPLC analyses were undertaken at room temperature while we were still able to get desired results. ABEE-sugar derivatives were proven to be stable for at least 24 h at 25 °C (Kwon and Kim, 1993).

Sugar Composition Determination of Glycans. Hydrolysis of glycans with 2 M TFA caused deacetylation of amino sugars. No signal peaks corresponding to those of amino sugars were observed in the TFA treated samples, which showed that all amino sugars in the glycans have been deacetylated completely. GlcNAc was the only amino sugar detected in the glycans, its molar content was determined by that of GlcN, which was the deacetylated form. Since it was only the relative sugar contents of the glycans (i.e., the molar percentages of sugars of the glycans) that were measured, with or without the addition of the internal standard sugar would not make a difference to the values; therefore, the internal standard sugar was abandoned while glycan samples were analyzed. Figure 4 shows the profile of reverse phase HPLC of ABEE-sugar derivatives of glycans. The ABEE reagent peak can be diminished or even eliminated by extensive extraction with chloroform. The molar percentages of sugars are presented in Table 1. Each of the three glycans of CP+ has a lower content of galactose in

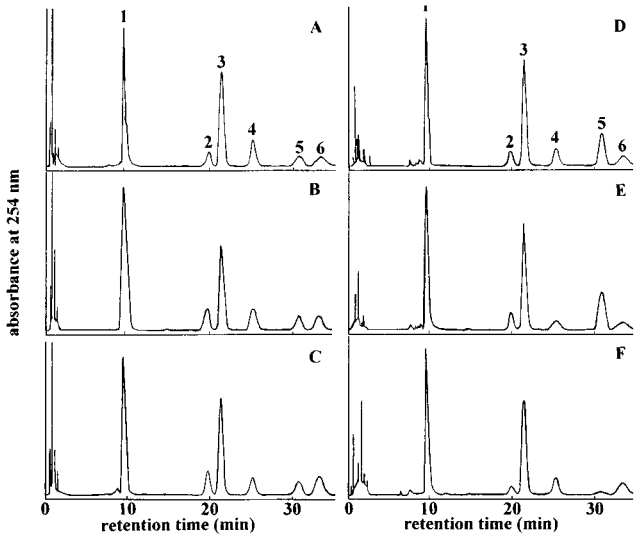

Figure 4. HPLC separation of the monosaccharides liberated from glycans by TFA hydrolysis. About 1 mg of glycopeptides was hydrolyzed with 2 M TFA, and the released sugars were derivatized with ABEE reagent as described under Materials and Methods. 10% of the ABEE-sugar derivatives was separated by HPLC: A, GPa of CP−; B, GPb of CP−; GPc of CP−; D, GPa of CP+; E, GPb of CP+; F, GPc of CP+. The identities of the peaks are as follows: 1, GlcN; 2, Gal; 3, Man; 4, Xyl; 5, ABEE reagent; 6, Fuc.

Table 1. Monosaccharide Compositions of Glycans Separated from CP− and CP+ Fractions[a]

	GlcNAc	Gal	Man	Xyl	Fuc
GPa of CP−	39.6 ± 0.4	9.0 ± 0.1	29.2 ± 0.1	14.9 ± 0.2	7.3 ± 0.3
GPb of CP−	41.1 ± 0.4	8.1 ± 0.0	32.8 ± 0.4	10.7 ± 0.2	7.3 ± 0.2
GPc of CP−	36.2 ± 0.6	8.6 ± 0.1	31.0 ± 0.2	8.6 ± 0.1	15.6 ± 0.2
GPa of CP+	38.7 ± 0.5	4.4 ± 0.1	41.7 ± 0.4	8.6 ± 0.1	6.6 ± 0.2
GPb of CP+	49.5 ± 0.6	3.7 ± 0.0	38.0 ± 0.3	5.6 ± 0.2	3.2 ± .01
GPc of CP+	43.5 ± 0.4	2.9 ± 0.2	40.6 ± 0.5	7.5 ± 0.1	5.4 ± 0.2

[a] Monosaccharide contents are given as mean ($n = 3$) values ± SD and presented in mol %.

comparison with its counterpart of CP−. Con-A binds specifically to glucose and mannose (Montreuil et al., 1986). In the glycans of peanut peroxidase no glucose is present; therefore, the binding property of CP+ attributes to mannose. The β-galactosidase, co-secreted with the peroxidase into the culture media by peanut cells, could convert the non-binding CP− fraction into the Con-A binding CP+ fraction (Wan et al., 1994). According to this, it is reasonable to suggest that removal of galactose as a terminal sugar from the CP− glycans results in the exposure of a mannose site, which is then able to bind to Con-A. If this hypothesis is correct, the galactose content in CP+ should be lower than that in CP−. Indeed, we proved this to be the case. The ultimate confirmation of the hypothesis and the whole glycan structures will rely on ^{1}H-NMR spectroscopy. Xyl and Fuc also occur in lower molar percentages in the glycans of CP+. The reason for this needs to be studied.

DISCUSSION

Microheterogenity has been suggested to occur in the two glycoforms of cationic peroxidase (Wan et al., 1994). Preliminary studies have detected mannosidase, *N*-acetylglucosaminidase, and xylosidase activities in the cell culture medium (data not published). Whether the microheterogenity is due to the hydrolysis of mature glycoproteins by these enzymes or whether some sugars are prevented from being added to nascent glycoproteins remains a question to be explored.

One major difference of plant complex N-glycans from animal complex N-glycans is in that the former have a fucose residue α-1,3-linked to the proximal GlcNAc residue. PNGase F, which usually releases N-glycans from animal glycoproteins, does not act on plant glycoproteins because of the linkage of fucose residue. However, PNGase F treatment of CP− did cause a loss of molecular mass of 2−3 kDa, and the treated protein lost its entire enzymatic activity (van Huystee and Wan, 1994; Hu and van Huystee, 1989). These results lead us to study which sugars are cleaved by the PNGase F, since these cleaved sugars seem involved in the maintaining of enzyme activity. We are also pursuing the resolution of glycan configuration by ^{1}H-NMR spectroscopy, which has been proposed to be the best alternative to X-ray diffraction (MacArthur et al., 1994). The system we set up for unraveling the sugar composition of glycans is essential, and the data obtained in the study enable us to carry on further researches.

ABBREVIATIONS USED

ABEE, *ρ*-aminobenzoic ethyl ester; Ara, arabinose; CM, carboxyl methyl; Con-A, concanavalin-A; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GPa, glycopeptide a; GPb, glycopeptide b; GPc, glycopeptide c; HPLC, high performance liquid chromatography; Lac, lactose; Man, mannose; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance; PNGase F, peptide−*N*-acetyl-*β*-D-glucosaminyl asparagine amidase F; Rha, rhamnose; RZ, Reinheits Zahl value of absorbance at 405 nm/absorbance at 280 nm; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sia, sialic acid; TCM-saline buffer, 0.01M Tris-HCl (pH 7.5) containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$; TFA, trifluoroacetic acid; TPCK, *γ*-1-tosylamido-2-phenylethylchloromethyl ketone; Xyl, xylose.

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