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Large-Scale Preparation of Asn-Glycopeptide Carrying Structurally Homologous Antigenic N-Glycan

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In our previous report (M. Okano, *et al.*, *Clin. Exp. Allergy*, 34, 770–778 (2004)), we found that free plant complex type N-glycans suppressed the production of IL4 from Th2 cells of Japanese cedar pollinosis patients, suggesting that plant complex type N-glycan can be used as a leading compound for developing immuno-pharmaceuticals. Although immunoreactive plant complex type N-glycans occur ubiquitously on glycoproteins expressed in plants, an appropriate procedure has not been established to prepare non-labeled immunoreactive glycans or glycopeptides bearing structurally homologous immunoreactive glycans in large amounts. In this study, therefore, we developed a new preparative procedure for the large-scale preparation of Asn-glycopeptide bearing plant complex type N-glycan using a combination of gel-filtration and the hydrophilic partitioning method. By this new method, about 103 mg of Asn-glycopeptide bearing the antigenic N-glycans was obtained from 1.9 kg of shelled *Ginkgo biloba* seeds.

Key words: plant N-glycan; immunoreactive glycan; glycopeptide preparation; neoglycoconjugate; *Ginkgo biloba*

It is believed that highly antigenic N-glycans bearing β 1-2 xylosyl and/or α 1-3 fucosyl residues are ubiquitously expressed in higher plant cells.^{1–4)} As for the involvement of plant antigenic N-glycans in Japanese cedar pollinosis, we have reported that the allergy-associated IgE class antibodies of about 25% of allergy sufferers recognized β 1-2 xylose and/or α 1-3 fucose-containing complex-type glycans, though the clinical relevance of antigenic glycan-specific antibodies has been questioned.^{5–7)} On the other hand, we found that free plant complex type N-glycans suppressed the

production of IL4 from Th2 cells of Japanese cedar pollinosis patients, suggesting that plant N-glycan can be used as a leading compound in developing immuno-pharmaceuticals.⁷⁾ It is well known that immunoreactive plant complex type N-glycans occur ubiquitously on glycoproteins expressed in plant cells, but an appropriate method to prepare large amounts of non-labeled antigenic glycans or glycopeptides bearing structurally homologous immunoreactive glycans has not been established. When we prepared glycopeptides from protease digests of various plant glycoproteins, glycopeptides carrying high-mannose type N-glycans were obtained by Con A affinity chromatography, but those carrying plant complex type N-glycans only could hardly purified in large quantity due to a lack of the corresponding lectins or contamination by other oligosaccharides. Furthermore, even if glycopeptides having plant complex type N-glycans can be purified for analytical purposes, highly structural heterogeneities are often observed in N-glycan moieties, and this hampers investigation of the possible biological impact of specific N-glycans. For evaluation of the physiological activity of plant complex type N-glycans involved in the immune response and the development of glyco-pharmaceuticals, an effective method for the large-scale preparation of structurally homologous N-glycopeptides and non-labeled glycan remains to be established.

In our previous studies,^{8,9)} we found that a plant complex type N-glycan, Man₃Xyl₁Fuc₁GlcNAc₂, occurred predominantly (more than 90%) on storage glycoproteins of *Ginkgo biloba* seeds, suggesting that *Ginkgo* seeds are a good source for preparation of highly homologous N-glycopeptide and non-labeled glycan. In the present study, therefore, we used *Ginkgo biloba* seeds to prepare N-glycopeptides carrying antigenic N-glycans. Here we report on an effective method

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Abbreviations: PITC, phenyl isothiocyanate; RP-HPLC, reversed-phase HPLC; SF-HPLC, size-fractionation HPLC; PA-, pyridylamino; Endo-H, endo- β -N-acetylglucosaminidase from *Streptomyces plicatus*; Diplococcal β -GlcNAc₄ase, β -N-acetylglucosaminidase from *Diplococcus pneumoniae*; Hex, hexose; HexNAc, N-acetylhexosamine; Pen, pentose; Man, D-mannose; Xyl, D-xylose; Fuc, L-fucose; GlcNAc, N-acetyl-D-glucosamine; M3FX, Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA; GN1M3FX, GlcNAc β 1-2Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA or Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA; GN2M3FX, GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA

for large-scale (100 mg) preparation of glycopeptide with a structurally highly homologous plant antigenic *N*-glycan, $\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-Asn}$, from an actinase-digest of storage glycoproteins of *Ginkgo biloba* seeds.

Materials and Methods

Materials. *Ginkgo biloba* seeds were collected in Inazawa, Aichi, Japan. An Asahipak NH2P-50 4E column (0.46×25 cm) was purchased from Showa Denko (Tokyo), a Cosmosil 5C18-AR column (0.6×25 cm) and Cosmosil 5C22-AR column (4.6×250 mm) from Nacalai Tesque (Kyoto, Japan), and Sephadex G-25 Superfine from GE Healthcare Japan (Tokyo). M3FX, GN1M3FX, and GN2M3FX were prepared as described in our previous papers.⁵⁾ Actinase E was from Wakenyaku (Kyoto, Japan), α -mannosidase (jack bean) from Seikagaku Kogyo (Tokyo), and *Diplococcus pneumonia* β -N-acetylglucosaminidase (β -GlcNAc'ase) from Boehringer (Mannheim, Germany).

Preparation of glycopeptides from *Ginkgo biloba* seed proteins. Shelled *Ginkgo* seeds (1.8 kg) were homogenized and defatted in acetone (5 L). The defatted powder of the seeds (about 900 g) was homogenized in 10 L of 50 mM Tris-HCl buffer pH 8.6 containing 0.2 M NaCl (TBS, Tris buffered saline), and storage proteins were extracted at 4 °C over 3 d. The homogenate was filtered with gauze, and the filtrate was centrifuged at 8,000 *g* for 30 min. The resulting supernatant (TBS-extract) was saturated with ammonium sulfate. Insoluble materials obtained by filtration with gauze and centrifugation were suspended in 5 L of 5% acetic acid and basic proteins were extracted at 4 °C overnight. The homogenate was filtered with gauze and the filtrate was centrifuged at 8,000 *g* for 30 min. The resulting supernatant (acetic acid-extract) was saturated with ammonium sulfate. After centrifugation (8,000 *g*, 30 min), the ammonium sulfate precipitate obtained from the TBS extract and acetic acid extract was dissolved in a small volume of water, dialyzed against running tap water for several hours, and against deionized water (10 L) overnight. The resulting dialysate was lyophilized, and the resulting lyophilizate was used as crude *Ginkgo* glycoprotein (62 g).

The crude *Ginkgo* proteins (32.8 g) were reduced and carboxymethylated by the method of Crestfield *et al.*,¹⁰⁾ and exhaustively dialyzed against deionized water. The resulting alkylated *Ginkgo* proteins were digested with actinase E (500 mg in total) in 1.2 L of 0.1 M NH_4HCO_3 at 37 °C for 48 h. The actinase digest was concentrated to about 250 mL with a rotary evaporator and centrifuged at 8,000 *g* for 30 min to remove insoluble materials. The supernatant (about 40 mL) was subjected to gel filtration on a Sephadex G-25 Superfine column (4×85 cm) equilibrated with 0.1 N NH_4OH . The glycopeptide-fraction (elution volume from 430 mL to 790 mL), detected by the Phenol- H_2SO_4 method,¹¹⁾ was pooled and concentrated to about 40 mL by rotary evaporator. The glycopeptide-fraction was further purified by a second gel filtration on a Sephadex G-50 column (2×180 cm). As shown in Fig. 1A, two saccharide-containing fractions (F-I and F-II) were obtained. After concentration of the two glycopeptide-fractions by rotary evaporator, each fraction (50 mL) was digested again with actinase E (100 mg each) in 0.1 M NH_4HCO_3 at 37 °C for 48 h. Each actinase digest (Fig. 1F-I and F-II) was subjected to a third gel filtration on a Sephadex G-25 Superfine column (5.2×142 cm) equilibrated with 0.1 N NH_4OH . The actinase digest of F-I eluted in void volume of the Sephadex G-25, indicating that this saccharide-containing fraction was hardly digested by actinase E and might contain polysaccharides but not glycopeptides. On the other hand, the actinase digest of F-II separated into two oligosaccharide-containing fractions, as shown in Fig. 1B. Each fraction (F-II-1 and F-II-2) was pooled and lyophilized, and was used in further purification with Shodex Asahipak NH2P-50 resin.

Hydrophilic partitioning of glycopeptides with Shodex Asahipak NH2P-50 resin. Glycopeptides (F-II-1, 350 mg; F-II-2, 520 mg) partially purified by gel-filtration was dissolved in 50% acetonitrile/water (2 mL) and mixed with 25 mL of settled Shodex Asahipak NH2P-50 4E resin equilibrated with 25 mL of 80% acetonitrile/water

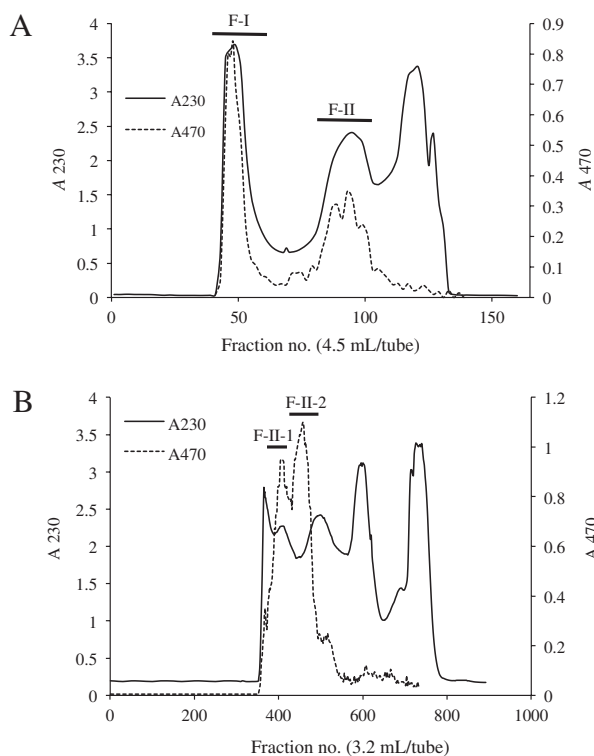


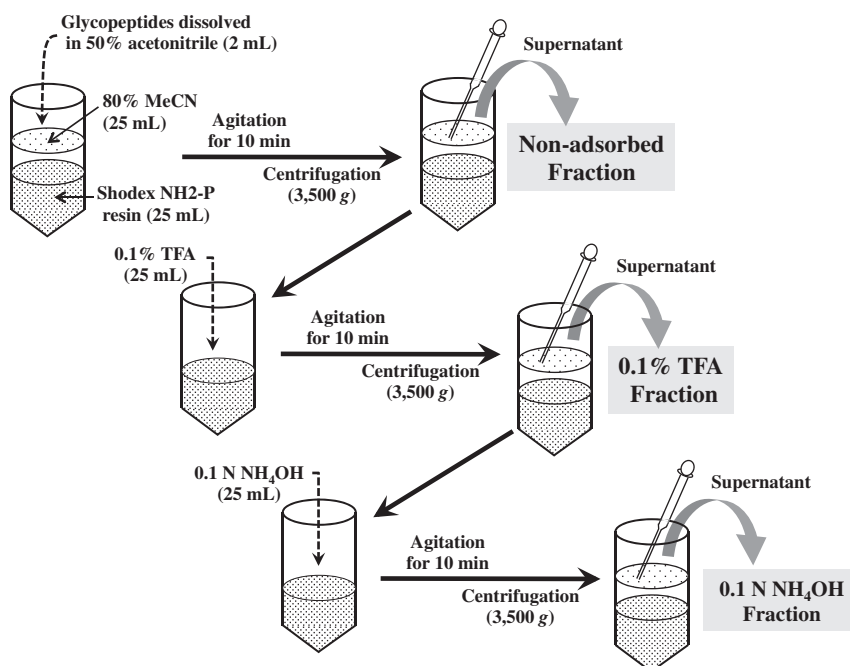
Fig. 1. Gel-Filtration of Pronase Digest of Alkylated *Ginkgo biloba* Seed Glycoproteins.

A, Gel-filtration of the first actinase digest of alkylated glycoproteins by Sephadex G-50 (2.0×180 cm) equilibrated with 0.1 N NH_4OH . The glycopeptide fraction (5 mL out of 40 mL), obtained by Sephadex G-25 column (4×85 cm), was loaded onto a Sephadex G-50 column. Solid line indicates the elution of peptides (A_{230}) and a dotted line indicates the elution of glycopeptides (A_{470}). The two glycopeptide-fractions (F-I and F-II) were pooled, as indicated by horizontal bars, and digested with actinase again. B, Gel-filtration of the actinase digest of F-II obtained in Fig. 1A through Sephadex G-25 Superfine Column (5.2×142 cm) equilibrated with 0.1 N NH_4OH . The solid line indicates the elution of peptides (A_{230}) and the dotted line indicates elution of the glycopeptides (A_{470}). Two glycopeptide-fractions (F-II-1 and F-II-2) were pooled, as indicated by horizontal bars, and analyzed separately.

in a centrifuge tube. After incubation for 10 min and centrifugation (3,500 *g*, 5 min), the supernatant was discarded and the resin was washed twice with 80% acetonitrile/water (12.5 mL). After centrifugation, the resulting supernatant was discarded, and the resin was washed with 0.1% TFA/water (25 mL) to elute the glycopeptide absorbed. The whole procedure is summarized in Scheme 1. The glycopeptide-fraction was concentrated to dryness by rotary evaporator, and the amino acid composition and *N*-glycan structure were analyzed.

Amino acid composition of *Ginkgo biloba* glycopeptide. Amino acid analysis of the *Ginkgo* glycopeptide was done as described by Henrickson and Meredith¹²⁾ after acid hydrolysis with 5.7 N HCl containing 0.02% 2-mercaptoethanol at 110 °C for 12 h. The phenyl isothiocyanate (PITC)-labeled amino acids were separated by RP-HPLC on a Hitachi L-6200 HPLC apparatus equipped with a Hitachi Diode Array Detector L-7455 using a Cosmosil 5C22-AR column (4.6×250 mm). Elution of PITC-derivatives were carried out as described by Bidlingmeyer *et al.*¹³⁾ The solvent system consisted of two solvents: solvent A was 0.14 M sodium acetate buffer pH 6.25 containing 0.05% triethylamine, and solvent B was 60% acetonitrile in water. PITC-labeled amino acids were eluted and separated by increasing the concentration of solvent B from 10% to 51% over 22 min at a flow rate of 1.0 mL/min.

Preparation of pyridylaminated *N*-glycans from *Ginkgo biloba* glycopeptide. *N*-Glycans were released by hydrazinolysis (100 °C,



Scheme 1. The Procedure for Purification of Glycopeptides Fractionation Using Shodex NH2-P Resin.

10 h) from the glycopeptides. After *N*-acetylation of the hydrazinolysate with saturated sodium bicarbonate and acetic anhydride, the acetylated hydrazinolysate was desalted with Dowex 50 $\times$ 2 resin.^{8,9} The oligosaccharides were pyridylaminated, and the resulting pyridylaminated oligosaccharides were partially purified by the method of Natsuka and Hase.¹⁴

Purification of pyridylaminated *N*-glycans. The PA-derivatives obtained by gel-filtration were evaporated to dryness. The resulting residue was dissolved in desalted water (100 μ L) and separated by HPLC on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer (excitation 310 nm, emission 380 nm), using a Cosmosil 5C18-AR column (4.6 $\times$ 250 mm). The PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 7% over 60 min at a flow rate 1.2 mL/min.^{8,9} *N*-Glycan-containing fractions (elution time 20–40 min) were pooled and evaporated to dryness. The partially purified PA-sugar chains were further separated by SF-HPLC on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer (excitation 310 nm, emission 380 nm) using a Shodex Asahipak NH2P-50 column (0.46 $\times$ 25 cm). The PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 26 to 50% linearly over 40 min at a flow rate of 0.7 mL/min.^{8,9}

Electrospray ionization (ESI) mass spectrometry. ESI-MS and MS/MS analyses of PA-oligosaccharides were done using an Agilent 6500 series HPLC-Chip/QTOF-MS system equipped with a micro-well-plate auto sampler (maintained at 10.0 $^{\circ}$ C), capillary sample loading pump, nanopump, HPLC-Chip interface, and an Agilent 6520 Q-TOF LC/MS.

Glycosidase digestion. Digestion of PA-sugar chains with diplococcal β -*N*-acetylglucosaminidase was done using about 200 pmol of PA-sugar chains under the conditions described in our previous report.¹⁵ The reactions were stopped by boiling the mixtures for 3 min. After centrifugation at 20,000 *g* for 10 min, the resulting glycosidase digests were analyzed by SF-HPLC using an Asahipak NH2P-50 column. In the case of the analysis of glycosidase digests, the PA sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 26 to 50% linearly over 40 min at a flow rate of 0.7 mL/min.^{8,9} Peaks were pooled and concentrated to dryness by rotary evaporator.

Results and Discussion

*Purification of antigenic *N*-glycan-containing glycopeptide from Ginkgo biloba seeds*

In this study, we used *Ginkgo biloba* seeds as a source of glycopeptides containing plant antigenic *N*-glycan, since we had found that the glycoproteins in *Ginkgo biloba* seeds exclusively carry the pauci mannose type *N*-glycan, Man₃Xyl₁Fuc₁GlcNAc₂, which is known as a typical plant antigenic *N*-glycan.^{8,9} First, alkylated (*S*-carboxymethylated) *Ginkgo* proteins were digested with actinase, and the resulting glycopeptides were partially purified by gel filtration using Sephadex G-25 and Sephadex G-50 columns, as shown in Fig. 1. Since the F-I fraction obtained by the second gel-filtration (Sephadex G-50 column) was not digested by actinase, suggesting that it must be soluble polysaccharides but not glycopeptides, we used the F-II fraction for purification of glycopeptides bearing M3FX. After actinase E digestion of the glycopeptide-fraction (F-II-2), the glycopeptides containing M3FX were further purified by a third gel filtration using a large size Sephadex G-25 column. As shown in Fig. 1B, two fractions were partially separated, and these two fractions (F-II-1, 350 mg; F-II-2, 520 mg) were used separately at the next purification step. Glycopeptides containing M3FX were finally purified by hydrophilic partitioning using Shodex Asahipak NH2P-50 resin. The glycopeptides that bound to the resin in 80% acetonitrile/water were eluted by 0.1% TFA/water. After elution of glycopeptides by 0.1% TFA, the Shodex Asahipak resin was washed away with 0.1 *N* NH₄OH. In the NH₄OH fraction, however, oligosaccharide was hardly detected, suggesting that almost all the glycopeptides were bound to the resin in 80% acetonitrile/water and were eluted by 0.1% TFA/water. The yields of the two glycopeptides obtained in the 0.1% TFA fraction were 32.5 mg for F-II-1 and 103.7 mg for F-II-2.

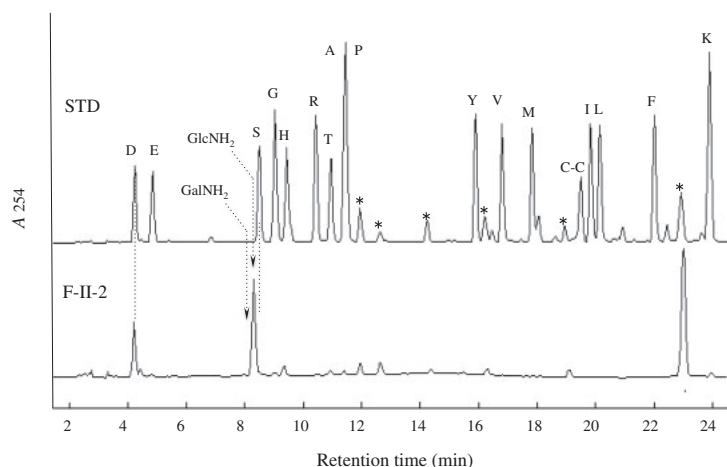


Fig. 2. Amino Acid Analysis of the M3FX-Glycopeptide F-II-2.

The amino acids and *N*-acetyl-hexosamine in the purified glycopeptides were analyzed as PITC-derivatives by reverse-phase HPLC after acid hydrolysis. The column used was Cosmosil 5C22-AR (4.6 × 250 mm). Several peaks, marked with asterisks, were not amino acids but unknown derivatives produced during PITC labeling.

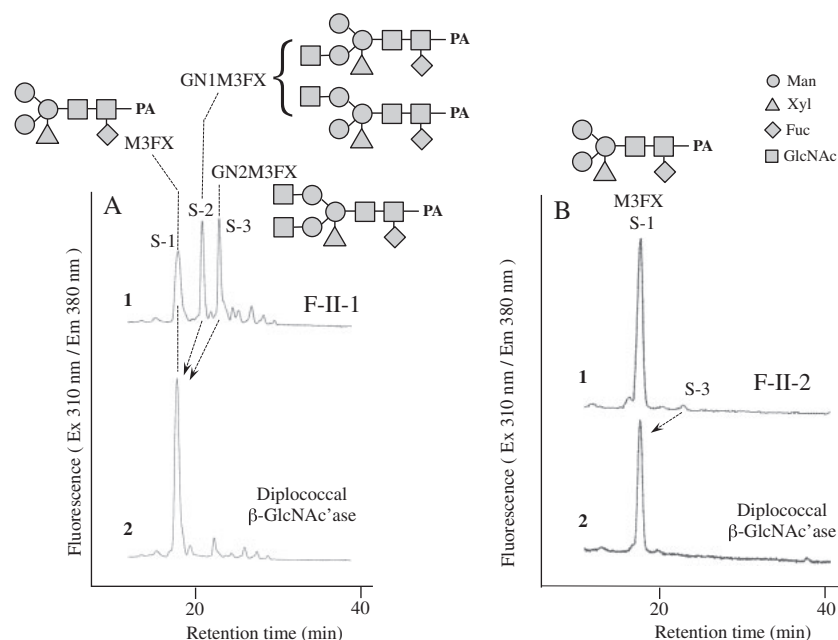


Fig. 3. SF-HPLC of PA-Sugar Chains Obtained from Two Purified Glycopeptides (F-II-1 and F-II-2).

A, SF-HPLC of PA-sugar chains obtained from F-II-1 (top) and their diplococcal β -GlcNAc'ase digests (bottom). Dotted lines indicate elution positions of authentic M3FX, GN1M3FX, and GN2M3FX. B, SF-HPLC of PA-sugar chains obtained from F-II-2 (top) and their diplococcal β -GlcNAc'ase digests (bottom).

Structural analysis of *Ginkgo biloba* glycopeptides purified with Shodex Asahipak NH2P-50 resin

The amino acid compositions of the two glycopeptides purified from F-II-1 and F-II-2 were analyzed by reversed-phase HPLC as PITC-labeled amino acids after acid-hydrolysis. From F-II-1, Asp, *S*-carboxymethylated Cys, Thr, and GlcNH₂ (eluted before Ser) were detected as the main amino acids and amino sugar at a ratio of 1.0:0.9:0.7:1.4, but some other amino acids such as Ser (0.5) and Gly (0.6) were also detected as minor components (data not shown). This result suggests that F-II-1 contains the *N*-glycosylated glycopeptide while the glycopeptide consisted of several amino acids in addition to the Asn residue. On the other hand, from F-II-2, only Asp and GlcNH₂ were detected at an area ratio of 1.0:1.5, suggesting that the purified glycopeptide, F-II-2, must have been *N*-glycosylated Asn (Fig. 2).

These results suggest that the batch method using a specific hydrophilic interaction between the hydroxyl groups (–OH) of the sugar moiety and the amino groups (–NH₂) of the Shodex Asahipak NH2P-50 resin is highly effective to purify Asn-glycopeptides, or short glycopeptides dissolved in 50–80% acetonitrile/water. Since it was confirmed that both glycopeptides obtained from F-II-1 and F-II-2 by the hydrophilic partitioning method were *N*-glycopeptides, the structures of the *N*-glycans linked to these glycopeptides were analyzed.

In structural analysis of the *N*-glycans linked to the two purified glycopeptides (F-II-1 and F-II-2) by size-fractionation (SF-) HPLC, three main PA-sugar chains, S1 (36%), S2 (32%), and S3 (32%), were obtained from glycopeptide F-II-1, while one major PA-sugar chain (about 95%) was obtained from glycopeptide F-II-2, as shown in Fig. 3A-1, and B-1 respectively. As shown in

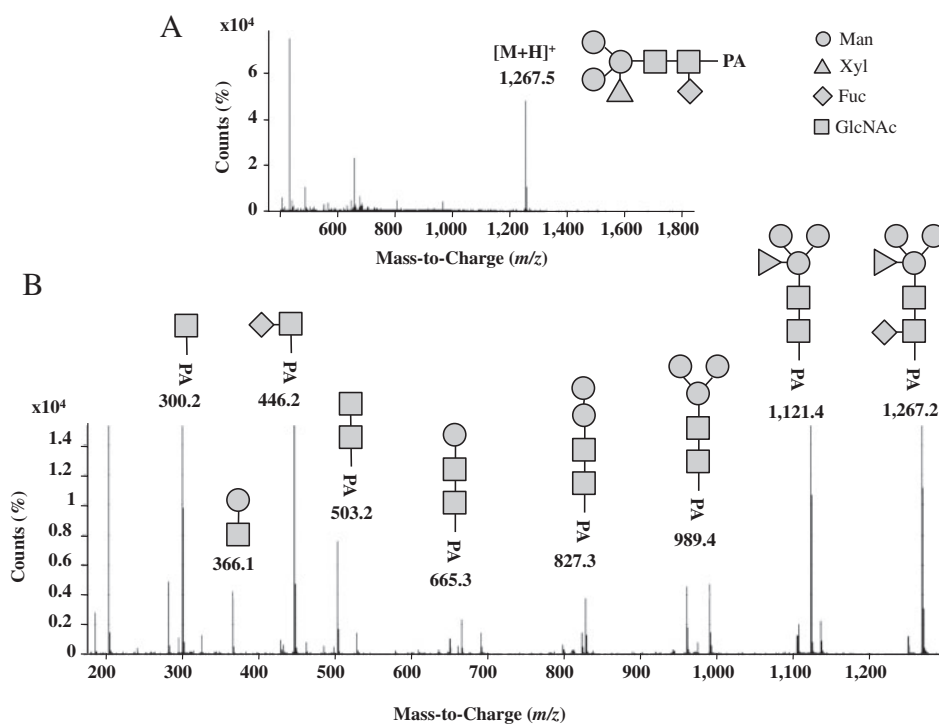


Fig. 4. ESI-MS and MS/MS Analysis of PA-Sugar Chain S-1 Obtained as in Fig. 3. A, MS-analysis of S-1. B, MS/MS analysis of the signal at m/z 1,267.5.

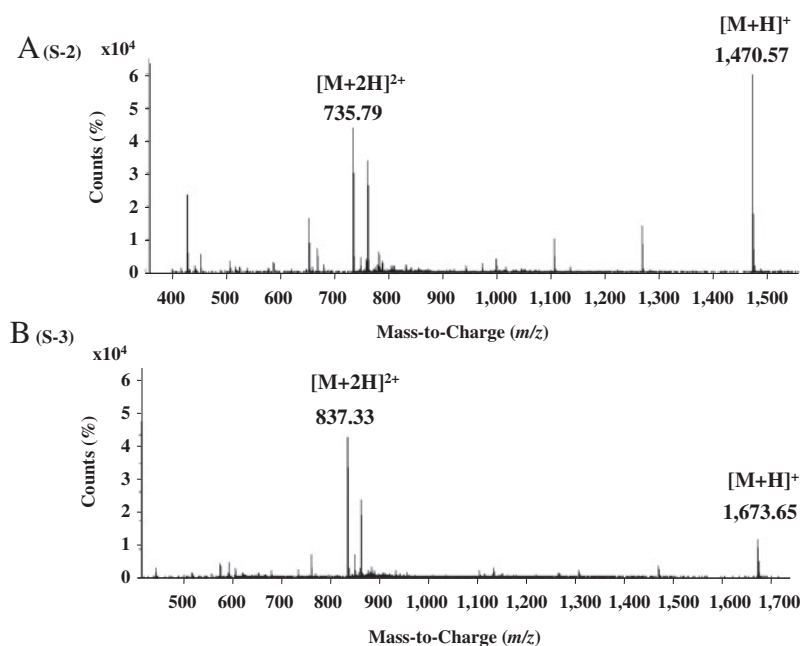


Fig. 5. ESI-MS Analysis of S-2 and S-3 Obtained as in Fig. 3A-1. A, MS spectrum of S-2. B, MS spectrum of S-3.

Fig. 3, the elution position of S-1 coincided with that of authentic $\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}$ (M3FX), that of S-2 with that of $\text{GlcNAc}_1\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}$ (GN1M3FX), and that of S-3 with that of $\text{GlcNAc}_2\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}$ (GN2M3FX). S-2 and S-3 were converted to M3FX by diplococcal β -GlcNAc'ase, as shown in Fig. 3A-2 and B-2. The predominant occurrence of $\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2$ (M3FX) in the purified glycopeptides agreed well with the past finding that storage glycoproteins in *Ginkgo biloba* seeds carry pauci mannose type *N*-glycans, M3FX, as a major (about 90%) component.

Furthermore, the structures of these *N*-glycans were confirmed by ESI-MS (Agilent 6500 Series HPLC-Chip/QTOF-MS System). Parent ions were detected at m/z 1,267.5, corresponding to $[\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}+H]^+$ from S-1 (Fig. 4A), m/z 1,470.6, $[\text{GlcNAc}_1\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}+H]^+$ and m/z 735.8 $[\text{GlcNAc}_1\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}+2H]^{2+}$ from S-2 (Fig. 5A), and m/z 1,673.7 $[\text{GlcNAc}_2\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}+H]^+$ and m/z 837.3 $[\text{GlcNAc}_2\text{Man}_3\text{Xyl}_1\text{Fuc}_1\text{GlcNAc}_2\text{-PA}+2H]^{2+}$ from S-3 (Fig. 5B). As shown in Fig. 4B, MS/MS analysis of S-1 provided the following fragments related to

Man₃Xyl₁Fuc₁GlcNAc₂-PA; *m/z* 300.2 (GlcNAc-PA), *m/z* 366.1 (GlcNAc-Man), *m/z* 446.2 (Fuc-GlcNAc-PA), *m/z* 503.2 (GlcNAc-GlcNAc-PA), *m/z* 665.3 (Man₁GlcNAc₂-PA), *m/z* 827.3 (Man₂GlcNAc₂-PA), *m/z* 989.4 (Man₃GlcNAc₂-PA), and *m/z* 1,121.4 (Man₃Xyl₁GlcNAc₂-PA). MS/MS analysis of S-2 provided the following fragments related to GlcNAc₁Man₃Xyl₁Fuc₁GlcNAc₂-PA; *m/z* 300.2 (GlcNAc-PA), *m/z* 366.1 (GlcNAc-Man), *m/z* 446.2 (Fuc-GlcNAc-PA), *m/z* 503.2 (GlcNAc-GlcNAc-PA), *m/z* 827.3 (Man₂GlcNAc₂-PA), *m/z* 959.4 (Man₂Xyl₁GlcNAc₂-PA), *m/z* 1,121.4 (Man₃Xyl₁GlcNAc₂-PA), *m/z* 1,267.5 (Man₃Xyl₁Fuc₁GlcNAc₂-PA), and *m/z* 1,324.5 (GlcNAc₁Man₃Xyl₁GlcNAc₂-PA) (data not shown). MS/MS analysis of S-3 provided the following fragments related to GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂-PA; *m/z* 300.2 (GlcNAc-PA), *m/z* 366.1 (GlcNAc-Man), *m/z* 446.2 (Fuc-GlcNAc-PA), *m/z* 528.2 (Man-Man-GlcNAc), *m/z* 1,470.6 (GlcNAc₁Man₃Xyl₁GlcNAc₂-PA), and *m/z* 1,527.6 (GlcNAc₂Man₃Xyl₁GlcNAc₂-PA) (data not shown). These results, obtained by MS-analysis and β -GlcNAc'ase digestion, clearly indicate that S-1 is Man₃Xyl₁Fuc₁GlcNAc₂-PA, S-2 is GlcNAc₁Man₃Xyl₁Fuc₁GlcNAc₂-PA, and S-3 is GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂-PA.

Conclusion

We established an effective method for large-scale preparation of Asn-glycopeptide (F-II-2) bearing a highly homologous plant antigenic *N*-glycan, Man₃Xyl₁Fuc₁GlcNAc₂, from the actinase digest of storage glycoproteins in *Ginkgo biloba* seeds. The yield of the *Ginkgo* glycopeptides from crude *Ginkgo* glycoproteins (32.8 g) was 103.7 mg as lyophilized sample (F-II-2). Although another *Ginkgo* *N*-glycopeptide (F-II-1, 32.5 mg as lyophilized sample) consisted of several amino acids and carried heterogenous plant complex *N*-glycans (M3FX, GN1M3FX, and GN2M3FX), the *N*-glycan moiety can be converted to M3FX by β -GlcNAc'ase digestion and the glycopeptide should be also useful in analyzing the immunological activity of plant antigenic *N*-glycan. The immunological activity of a neoglycopeptide, which consists of the *Ginkgo* glycopeptide (Asn-M3FX, F-II-2) and γ -polyglutamic acid, to stimulate Th2 or Th1 cells to produce cytokines

or to activate immature dendritic cell (iDC) will be described in elsewhere.

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