JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Qualitative and Quantitative Analysis of Carbohydrate Modification on Glycoproteins from Seeds of *Ginkgo biloba*

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Supporting Information

ABSTRACT: Recent progress in the relationship between carbohydrate cross-reactive determinants (CCDs) and allergic response highlights the importance of carbohydrate moieties in the innate immune system. Previous research pointed out that the protein allergen in *Ginkgo biloba* seeds is glycosylated, and the oligosaccharides conjugated to these proteins might also contribute to the allergy. The aim of this study was to analyze carbohydrate moieties, especially N-linked glycans, of glycoproteins from *Ginkgo* seeds originating from different places for detailed structures, to enable further research on the role played by N-glycans in *Ginkgo*-caused allergy. Results of monosaccharide composition and immunoblotting assays indicated the existence of N-glycans. Detailed structural elucidation of the N-glycans was further carried out by means of hydrophilic interaction ultraperformance liquid chromatography (HILIC-UPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In total, 14 out of 16 structures detected by UPLC were confirmed by MALDI-TOF-MS and tandem mass spectrometry, among which complex-type N-glycans bearing Lewis A determinants and high-mannose-type N-glycans were identified from *Ginkgo* seeds for the first time. Precise quantification of N-glycans was performed by use of an external standard, and both the absolute amount of each N-glycan and the percentage of different types of N-glycan showed significant diversity among the samples without any pattern of geographic variation.

KEYWORDS: *Ginkgo seeds, N-glycome, structure analysis, quantification*

1. INTRODUCTION

Food allergy is one of the most common health problems, affecting people of all ages and races and from all regions. Often patients are not only allergic to one particular food but also to a food group to a certain extent. For example, clinical statistics showed that children with hazelnut allergy might also be hypersensitive to other tree nuts and peanuts.[1] The concept of carbohydrate cross-reactive determinants (CCDs) was first proposed by Clemens and co-workers[2] decades ago and was once regarded as a promising reasonable explanation for the food allergy phenomenon. However, the contribution of these CCDs to allergic cross-reactions is still under dispute. Results of clinical statistical analysis pointed out that anti-CCD immunoglobulin E (IgE) might not be able to provoke severe immunological responses and clinical symptoms, and CCDs were regarded as being irrelevant to clinical symptoms.[3−6] Recently, Altmann and co-workers[7] found that removal of β-1,2-xylose substitution on N-glycans by silencing the xylosyltransferase gene in transgenic tomatoes reduced the responsiveness in tomato-allergic patients. It was also found that Gal-α1,3-Gal oligosaccharide in red meat can cause delayed anaphylactic reaction in adults.[8] Another study about N-glycans of kiwi allergens also revealed that sugar moiety could be recognized by dendritic cells (DCs) and then activated DCs.[9] Although in vivo observation of the function of CCDs still remains contradictory, more and more evidence manifested that carbohydrate epitopes on allergens play an important role in recognition of innate immune cells, activation of antigen-presenting cells, and production of IgE.[10]

Studies on structure−function relationships of N-glycans from plants revealed that the binding specificity and affinity of CCDs to IgG or IgE are structure-dependent. The moiety of α-1,3-fucose attached to innermost GlcNAc and the moiety of β-1,2-xylose linked to core mannose on asparagine-linked glycans are regarded as two major independent epitopes in IgE-mediated allergy of plant extraction or foodstuffs.[11] α-1,3-fucosylated and β-1,2-xylosylated core structure of N-glycan ($MMXF^3$) have been proved to be the dominant cross-reactive carbohydrate motif on allergens of plant food through biochemical and immune biochemical experiments.[12] Truncation on trimannose can cause decreased binding affinity of CCDs. For instance, MUX showed lower binding capacity to IgG compared to MMX. Artificial transferrin modified with MUX, instead of MMX, also exhibited weaker binding ability to rabbit anti-horseradish peroxidase (HRP) serum in Western blotting.[13] Besides truncation, an additional substituent on core trimannose might also shield core fucose or xylose epitopes. For example, either GnGnF or GnGnX, which both contained the terminal GlcNAc modification to core trimannose, exhibited poor affinity with rabbit anti-HRP serum.[13]

Analysis of N-linked oligosaccharides revealed the widespread existence of core α-1,3-fucose and/or β-1,2-xylose modification on glycoproteins from plant foodstuffs,[14,15] and $MMXF^3$ is particularly abundant in tree nuts. According to

Received: April 12, 2017
Revised: August 11, 2017
Accepted: August 11, 2017
Published: August 11, 2017

ACS Publications © XXXX American Chemical Society

DOI: 10.1021/acs.jafc.7b01690
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Wilson et al.,[14] the relative amount of $MMXF^3$ was 58.2% in pistachio and 32.8% in walnut. Although the percentage of $MMXF^3$ in peanut was only 2.5%, MMX and M4X together made up 38.8% of the total N-glycans.[14]

Seeds of *Ginkgo biloba* belong to tree nuts and are a popular food in East Asia. They also causes allergy for many consumers. Little is known about allergens from *Ginkgo* seeds and the contribution of CCDs. One report about the N-linked glycan structures in *Ginkgo* seeds showed the exceptional presence of xylose-containing N-glycans in mature *Ginkgo* seed. However, the absence of high-mannose-type N-glycans in this report failed to explain the existence of an *endo-β-N*-acetylglucosaminidase, which is active only on high-mannose-type N-glycans, in *Ginkgo* seeds, reported by the same group.[16] The gap between the two findings indicated that current data for N-glycome structures of *G. biloba* seeds are incomplete, and a more profound and comprehensive analysis of N-glycans from seeds of *G. biloba* is required.

For a long time, the analysis of N-glycans in plant has been restricted by lack of a desirable enzyme to liberate oligosaccharides from glycoprotein. Enzymatic release based on glycopeptide N-glycosidase (PNGase) A is a commonly used method in analysis of plant N-glycan, but it can only react on glycopeptides and requires a proteolytic pretreatment with protease. It also requires several steps of purification by ion-exchange or molecular sieve chromatography before further analysis, which might result in critical loss of N-glycans.[17] In our previous work, we discovered a novel N-glycanase, PNGase H^+, from *Terriglobus roseus* and developed a corresponding strategy for efficient structure analysis of N-linked glycans from plant and insect glycoproteins.[15,18] PNGase H^+ can directly hydrolyze glycoprotein under mild conditions, and one step of purification by solid-phase extraction is enough to remove salt and proteins. In this article, by employment of the novel PNGase H^+-based strategy, we analyze the carbohydrate components, especially N-glycans, from *Ginkgo* seeds and make a qualitative and quantitative comparison of N-glycans between *Ginkgo* seeds from different producing areas.

2. MATERIALS AND METHODS

2.1. Materials. Recombinant PNGase H^+ was expressed and purified as reported previously.[18] Monosaccharide standards including Glc, Gal, Man, Fuc, Xyl, Ara, Rha, GlcA, GalA, GlcN, and Api were purchased from J&K (Beijing, China). Anti-horseradish peroxidase (HRP) antibody, produced in rabbit, and anti-rabbit IgG−alkaline phosphatase antibody, produced in goat, were purchased from Sigma−Aldrich Co. 1-Phenyl-3-methyl-5-pyrazolone (PMP) was obtained from Aladdin (Shanghai, China). Anthranilic acid (2-AA) and 2-aminobenzamide (2-AB) were from J&K. All chemicals and reagents were of the highest grade purity available.

2.2. Sample Collection and Protein Extract Preparation. Fresh *Ginkgo* seeds were purchased at local markets from 10 geographically diverse regions of China (Taixing, Jiangsu, abbreviated as JSTX; Pizhou, Jiangsu, abbreviated as JSPZ; Kuming, Yunan, abbreviated as YNKM; Linyi, Shandong, abbreviated as SDLY; Suzhou, Hebei, abbreviated as HBSZ; Guilin, Guangxi, abbreviated as GXGL; Liupanshui, Guizhou, abbreviated as GZLPS; Huixian, Gansu, abbreviated as GSHX; Qingchuan, Sichuan, abbreviated as SCQC; and Nanxiong, Guangdong, abbreviated as GDNX). Twenty-five grams of shelled seed was blended with 20 mL of 50 mM Tris-HCl, pH 7.0 and centrifuged (20000*g* for 20 min, 4 °C). Supernatant (5 mL) was mixed with 2.5 mL of methanol and 5 mL of chloroform, and the mixture was vortexed for 10 min. The mixture was allowed to stand at 4 °C overnight to precipitate protein. Total protein was then collected by centrifugation at 20000*g* for 30 min. The pellet was carefully washed with deionized water five times, to remove free oligosaccharides and polysaccharides, and freeze-dried prior to further analysis.

2.3. Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoretic Analysis. The protein precipitate was freeze-dried and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Each sample (400 μg) was incubated with 5× Laemmli buffer at 95 °C for 10 min before being loaded onto a 12% polyacrylamide gel. Bovine serum albumin (BSA, 10 μg) without glycosylation was used as the negative control and glycoprotein HRP (10 μg) as the positive control. After electrophoretic separation, the glycoprotein was first detected by periodic acid−Schiff method (PAS) and the gel was then stained again with Coomassie blue.[19]

2.4. Western Blot. SDS−PAGE was carried out by the same method described above except that 10% of the amount (40 μg) was applied to each sample. Samples were first separated by SDS−12% PAGE and then transferred to a nitrocellulose blotting membrane (Pall Corp.) and blocked with 1% (w/v) BSA for 2 h at room temperature. The membrane was then incubated with a primary antibody (rabbit anti-HRP antibody, 1:100 000 v/v) overnight at 4 °C. The membrane was thoroughly washed with TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20) and then probed with a secondary antibody (alkaline phosphatase-labeled goat anti-rabbit IgG, 1:200 000 v/v) for 2 h at room temperature. After extensive washing with TBST buffer, the blots were detected with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium solution (BCIP/NBT).

2.5. Monosaccharide Analysis. One milligram of dry *Ginkgo* seed protein extract was hydrolyzed with 1 mL of trifluoroacetic acid (TFA, 4 M) at 110 °C for 8 h. The hydrolyzed sample was divided into two equal parts and dried by vacuum concentration. Methanol (0.5 mL) was added to remove residual TFA via speed vacuum three times. The two portions of dried sample were derivatized with two kinds of labeling reagents, 2-AA and PMP,[20] respectively.

The 2-AA reaction mixture contained dried sample, 10 μL of NaAc (48.24 mg/mL), and 20 μL of 2-AA solution [3 mg of 2-AA and 30 mg of sodium cyanoborohydride in 1 mL of 2% (w/v) boric acid in methanol]. The mixture was incubated at 80 °C for 60 min. Samples were diluted to 1 mL with deionized water. The analysis was carried out on a Shimadzu Nexera UPLC system equipped with a RF-20Axs fluorescence detector and a reverse-phase column (Phenomenex Hyperclone, 5 μm ODS, 120 Å, 250 × 4.60 mm). UPLC conditions were as follows: solvent A was 1% tetrahydrofuran (THF), 0.5% phosphoric acid, and 0.2% *n*-butylamine in water; solvent B was 100% acetonitrile; flow rate was 1 mL/min; and excitation/emission were at 360/425 nm. After injection of 10 μL of sample, 2.5% solvent B was held for 7 min, and a linear gradient of 2.5−18% solvent B was applied from 7 to 25 min. Then solvent B was increased to 95% in 2 min and held for 5 min. Solvent B was then decreased to 2.5% in 1 min and maintained for 7 min for equilibration.

The PMP reaction mixture, containing dried sample, 20 μL of deionized water, 30 μL of NaOH (0.5 M), and 50 μL of PMP in methanol (0.5 M), was incubated at 70 °C for 100 min. After cooling to room temperature, the mixture was neutralized with 40 μL of HCl (0.5 M) and diluted with 860 μL of deionized water. The excess PMP was extracted five times with 0.5 mL portions of chloroform. The aqueous layer (10 μL) was analyzed on the same UPLC system equipped with a SPD-UV20AD absorbance detector and a reverse-phase column (Phenomenex Kinetex 1.7 μm C18, 100 Å, 150 × 2.10 mm). Solvent A was ammonium acetate in water (50 mM, pH 5.5), and solvent B was 100% acetonitrile. The flow rate was 0.4 mL/min, and the detection wavelength was 245 nm. After injection of 10 μL of sample, a linear gradient of 17.9−19% solvent B was applied from 0 to 12 min, followed by 19−26.7% B from 12 to 28 min, then increased to 95% B in 1 min and held for 2 min. Solvent B was then decreased to 17.9% in 1 min and maintained for 8 min for equilibration.

2.6. N-Glycan Release and Fluorescent Labeling. The total protein extract was suspended in water and denatured by heating at 95 °C for 10 min. According to the method described previously,[18] 0.2 milliunit of recombinant PNGase H^+ was used to release N-glycans

DOI: 10.1021/acs.jafc.7b01690
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

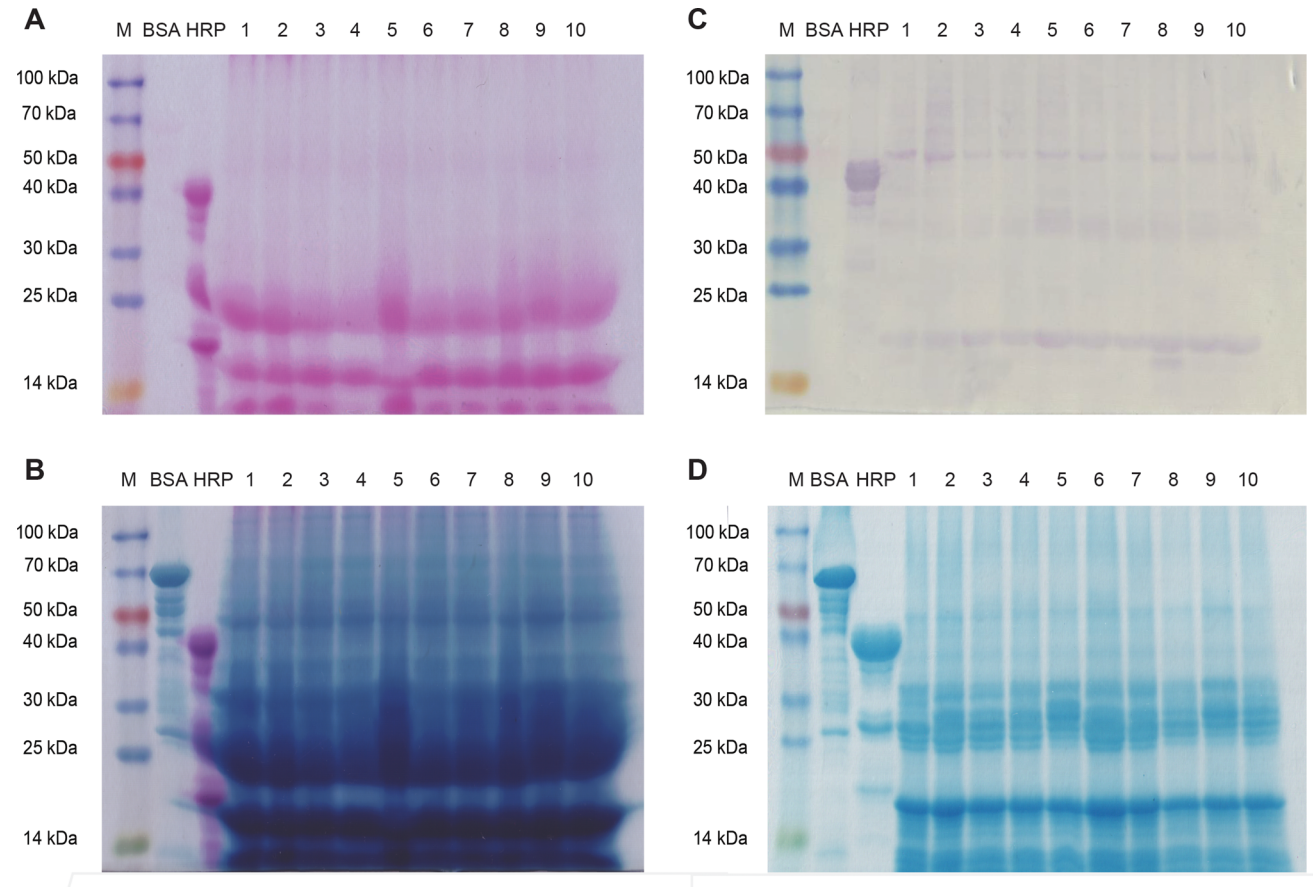

Figure 1. SDS−PAGE and Western blot analysis of crude protein extraction from *Ginkgo* seeds. (A) SDS−PAGE analysis of crude protein extract of 10 samples stained with PSA. (B) Gel from panel A restained with Coomassie blue. (C) Western blot analysis of crude protein extracts of 10 samples reacted with rabbit anti-HRP antibody. (D) SDS−PAGE corresponding to Western blot in panel C. Lane 1, JSTX; lane 2, YNKM; lane 3, JSPZ; lane 4, HBSZ; lane 5, SCQC; lane 6, GXGL; lane 7, GSHX; lane 8, GZLPS; lane 9, GDNX; lane 10, SDLY.

from extraction in 10 mM acetic acid at 37 °C overnight. The supernatant was collected by centrifugation at 12 000 rpm for 20 min and purified with Supelclean ENVI Carb solid-phase extraction (SPE) tubes. The SPE tube was activated with 3 mL of 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA, v/v) and equilibrated with the same volume of deionized water. The sample was loaded to the SPE tube and then washed with 3 mL of deionized water. Released N-glycans were eluted with 20% and 40% acetonitrile containing 0.1% TFA (v/v), collected, dried, and fluorescently labeled with 2-AB. An aliquot (10 μL) of 2-AB derivatization solution [35 mM 2-AB and 0.1 M sodium cyanoborohydride in dimethyl sulfoxide/acetic acid (7:3 v/v)] was added, and the mixture was incubated at 65 °C for 4 h.

2.7. N-Glycan Profiling and Quantification by Ultraperformance Liquid Chromatography. Before hydrophilic interaction ultraperformance liquid chromatographic (HILIC-UPLC) analysis, the 2-AB labeled product was diluted 5-fold with deionized water. Diluted sample was mixed with 40 μL of acetonitrile and 40 μL of the mixture was used for UPLC analysis. The sample (10 μL) was separated by an Acquity ethylene-bridged hybrid (BEH) glycan column (Waters, 1.7 μm, 2.1 × 150 mm) with the same UPLC system described earlier. The flow rate was 0.5 mL/min, and effluent was monitored by a fluorescence detector (Ex/Em: 330/420 nm). Solvent A was aqueous 50 mM ammonium formate buffer, pH 4.5, and solvent B was acetonitrile. A linear gradient of 5−12% solvent A was applied from 0 to 6 min and then increased to 44% over 39 min, followed by further increase to 100% over 3 min and held for 1 min. Solvent A was then decreased to 5% in 7 min and maintained for 7 min to equilibrate the column. In order to quantify N-glycans, a commercial maltopentaose was used as an external standard with amounts from 0.1 to 12 nmol, labeled and analyzed under the same UPLC conditions. Quantification of each N-linked oligosaccharide was performed on the basis of a linear equation of the amount of 2-AB-maltopentaose and its corresponding peak area.

2.8. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometric Analysis. After separation by normal-phase UPLC, fractions were collected, dried by vacuum concentration, and then analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). A Bruker Autoflex Speed instrument (equipped with a 1000 Hz Smartbeam-II laser) with 6-aza-2-thiothymine as matrix was used. Mass spectra were analyzed by use of Bruker Flexanalysis software version 3.3.80, followed by manual analysis with the assist of GlycoWorkbench (version 1.1).

2.9. Statistical Analysis. Results of N-glycan quantification are reported as mean ± standard deviation (SD) of triplicate determinations. Differences among 10 samples were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test via PASW Statistics 18 (Chicago, IL). *P*-values less than 0.05 were defined as being significant.

3. RESULTS AND DISCUSSION

3.1. Analysis of Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis and Western Blot. Crude protein extracts from *Ginkgo* seeds collected from 10 different locations in China were separated by SDS−12% PAGE and stained by PAS, which is specific to carbohydrate moieties (Figure 1A). Only proteins with glycosylation could be detected by PAS and showed bright red color on SDS−PAGE. The PAS-stained gel was then restained with Coomassie blue to display the overall distribution of all proteins (Figure

DOI: 10.1021/acs.jafc.7b01690
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

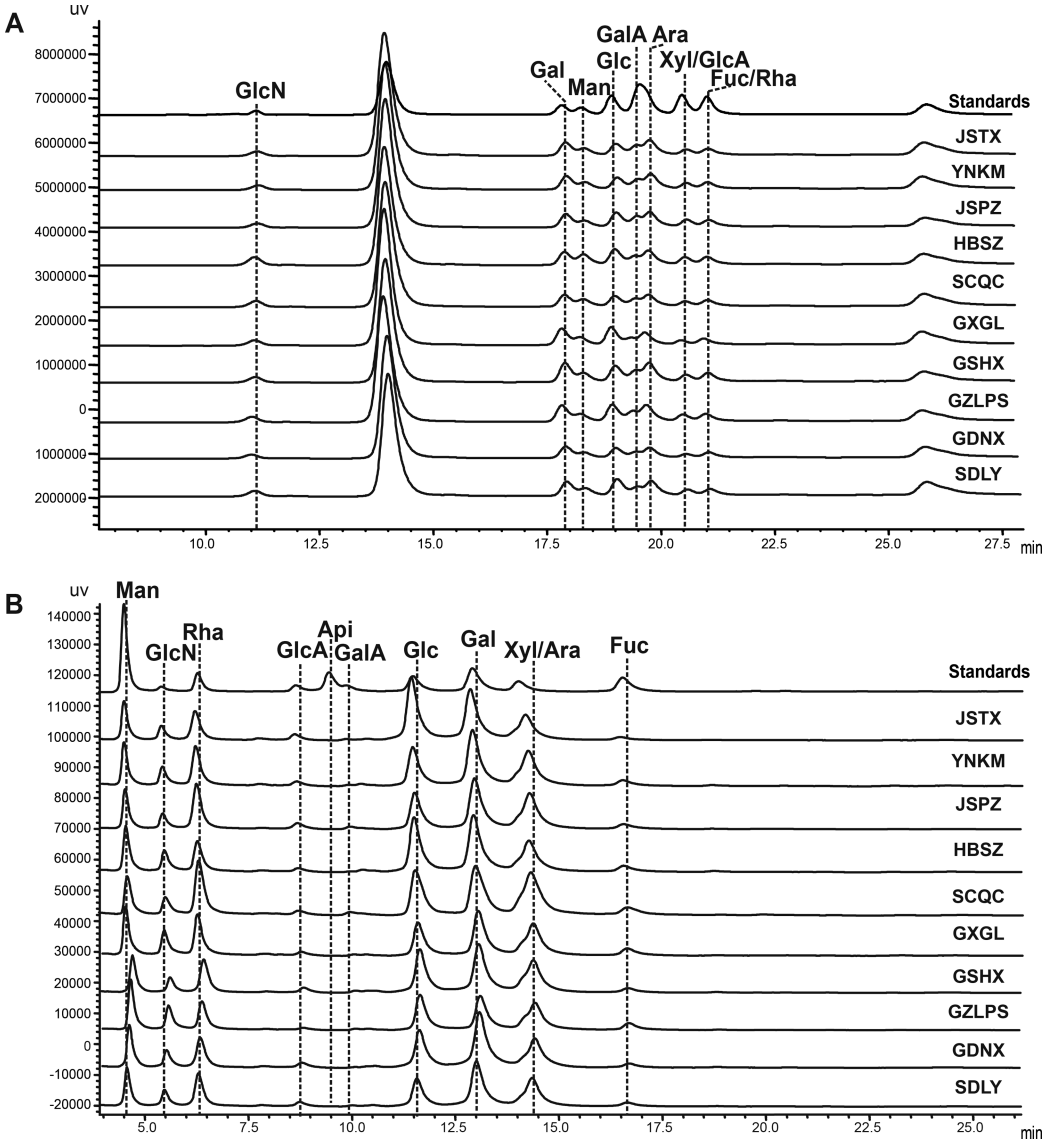

Figure 2. Monosaccharide composition assay of crude protein extraction from *Ginkgo* seeds. (A) Chromatograms of monosaccharides labeled with 2-AA, detected by fluorescence. (B) Chromatograms of monosaccharides labeled with PMP, detected by UV absorption.

1B). Most of the *Ginkgo* proteins were distributed at positions around molecular masses 50, 25, 20, and 12 kDa (Figure 1B), whereas only three major fractions were detected by PAS with molecular masses around 25, 20, and 12 kDa (Figure 1A), indicating the partial glycosylation of proteins in *Ginkgo* seeds. Furthermore, all 10 samples showed a similar distribution pattern of proteins in both gels, suggesting the consistency of protein type regardless of geographic variation.

Crude protein extracts were also analyzed by Western blots with anti-HRP antibody for determination of CCDs. Figure 1C shows the Western blot and Figure 1D shows the corresponding SDS−PAGE. It is apparent that the negative control BSA shows no visible signal in anti-HRP blotting in Figure 1C and the positive control HRP exhibits a strong signal in both Figure 1C,D. Again, a similar blotting pattern in Western blot assay was shown among the 10 samples (Figure 1C). Blots with apparent molecular masses around 50, 30, and 20 kDa were observed in all samples, indicating the presence of N-glycans with CCDs in all three bands (Figure 1C).

By putting together the results of SDS−PAGE with PAS staining and the results of Western blot, it was apparent that part of the proteins from *Ginkgo* seeds were glycosylated and CCDs could be detected in all samples from different geographic regions. However, the proteins with immunoblotting signals from bands of 50 and 30 kDa were not able to be detected by the PAS method. This is possibly because the carbohydrate content of these bands was lower than the detection limit of the PAS method, although the amount of sample used in the PAS assay was much higher. It was also notable that the bands of 25 and 12 kDa, detected by PAS staining, had no binding to anti-HRP antibody in the Western blot assay. One possible reason is that, instead of N-linked glycans, the two fractions contain O-linked glycans. It was reported that anti-HRP serum from rabbit contains two types of antibodies: one antibody specific for N-glycans containing core α-1,3-fucose and second antibody specific for N-glycans containing β-1,2-xylose.[21] Only N-glycans with one of these two modifications can be detected by anti-HRP serum. Additionally, truncation or addition of substitutions on the

DOI: 10.1021/acs.jafc.7b01690
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

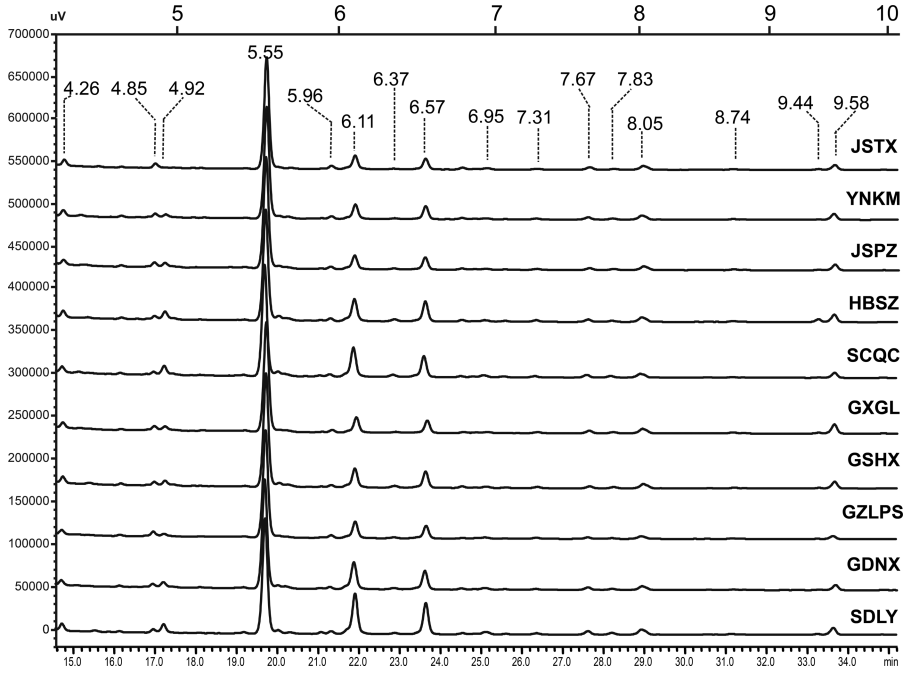

Figure 3. N-Glycan profiles of *Ginkgo* seed proteins from 10 different regions, marked with calculated GU value.

core trimannose can both decrease the binding affinity of antibody. Therefore, other possible reasons for the missing 25 and 12 kDa bands on Western blot are that either these fractions contain the N-glycans without α-1,3-fucose and β-1,2-xylose modification or they contain the N-glycans with truncation or additional substitutions on the core trimannose.[13]

3.2. Monosaccharide Composition. To determine the type of glycosylation on *Ginkgo* seed proteins, analysis of monosaccharide composition of crude protein extracts from 10 samples was performed via reverse-phase HPLC with two types of derivative reagents, PMP and 2-AA. According to Figure 2A, the released 2-AA-labeled monosaccharides of total protein extracts from different samples were composed of Gal, Man, Glc, GalA, Ara, Xyl/GlcA, Fuc/Rha, and GlcN. Two pairs of sugars (Xyl/GlcA and Fuc/Rha) failed to be distinguished within the pair by the 2-AA method. As shown in Figure 2B, the monosaccharide composition of protein extracts from 10 samples mainly comprised Man, Rha, GlcA, Glc, Gal, Xyl/Ara, Fuc, and GlcN. PMP-labeled xylose and arabinose had similar elution behavior and were not able to be separated from each other under the described conditions. Furthermore, HPLC profile of samples showed weak intensity of the sixth peak corresponding to PMP-GalA. This is possibly due to the low derivatization efficiency of GalA in the mixture or low sugar content of GalA in the hydrolysate of samples. The combination of results from the two different methods indicated that carbohydrate monosugar composition of crude extracts from *Ginkgo* seeds included Gal, Man, Glc, GalA, Ara, GlcA, Fuc, Rha, and GlcN. Due to the limit of the technologies used, the existence of xylose in *Ginkgo* seeds protein cannot be confirmed by either of the methods.

N- and O-glycosylation are the two major types of carbohydrate modification in plants. N-Glycan from plant glycoproteins contains a core $Man_3GlcNAc_2$ structure with further modification by fucose, xylose, galactose, and *N*-acetylglucosamine. Because GlcNAc is present only in N-glycans but not in O-glycans in plants and can be converted to GlcN by hydrolysis with TFA,[20] the detection of GlcN from the samples can therefore testify the existence of N-glycosylation on *Ginkgo* seed proteins.

The structure of O-glycan in plants is of higher complexity. For example, arabinogalactan proteins (AGPs) have highly heterogeneous O-glycans that consist mostly of galactose and arabinose and also minor amounts of rhamnose, fucose, glucuronic acid, and galacturonic acid.[22,23] Of all these, rhamnose, galacturonic acid, and glucuronic acid are uniquely found in plant O-glycans.[24] Hence the detection of GalA and Rha indicated that the carbohydrate complex of protein from *Ginkgo* seeds also contained O-linked glycan, especially arabinogalactan. This finding can also be used to explain the differential results between PAS stained SDS−PAGE and Western blot: the former reacts with all glycans, whereas the latter specifically detects only N-glycans. In brief, the results of monosugar determination elucidated that *Ginkgo* seed glycoproteins were modified by both N- and O-linked glycans.

3.3. N-Glycan Profiling of *Ginkgo* Seed Proteins by Ultraperformance Liquid Chromatography. N-Glycans were enzymatically released from *Ginkgo* seed proteins by recombinant PNGase H^+ and labeled with 2-aminobenzamide (2-AB). An analysis was performed via HILIC-UPLC to profile the structural nature of these glycans. A 2-AB-labeled dextran standard (2−20 glucose units) was used to generalize the elution time of each fraction into standard glucose unit (GU) value. As shown in Figure 3, glycoprofiles of all samples showed an analogous elution pattern in terms of the number of peaks and the corresponding retention time. In total, 16 different fractions were monitored and marked with GU values (Figure 3).

The GU values of each UPLC fraction in this study were then compared to those in other reports for further inference of structure. According to data reported by Kimura and Matsuo,[25] N-linked glycans attached to storage glycoprotein from *Ginkgo* seeds only contain xylose-containing N-glycans, and the relative amount of $MMXF^3$ continuously increased during the whole

DOI: 10.1021/acs.jafc.7b01690
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

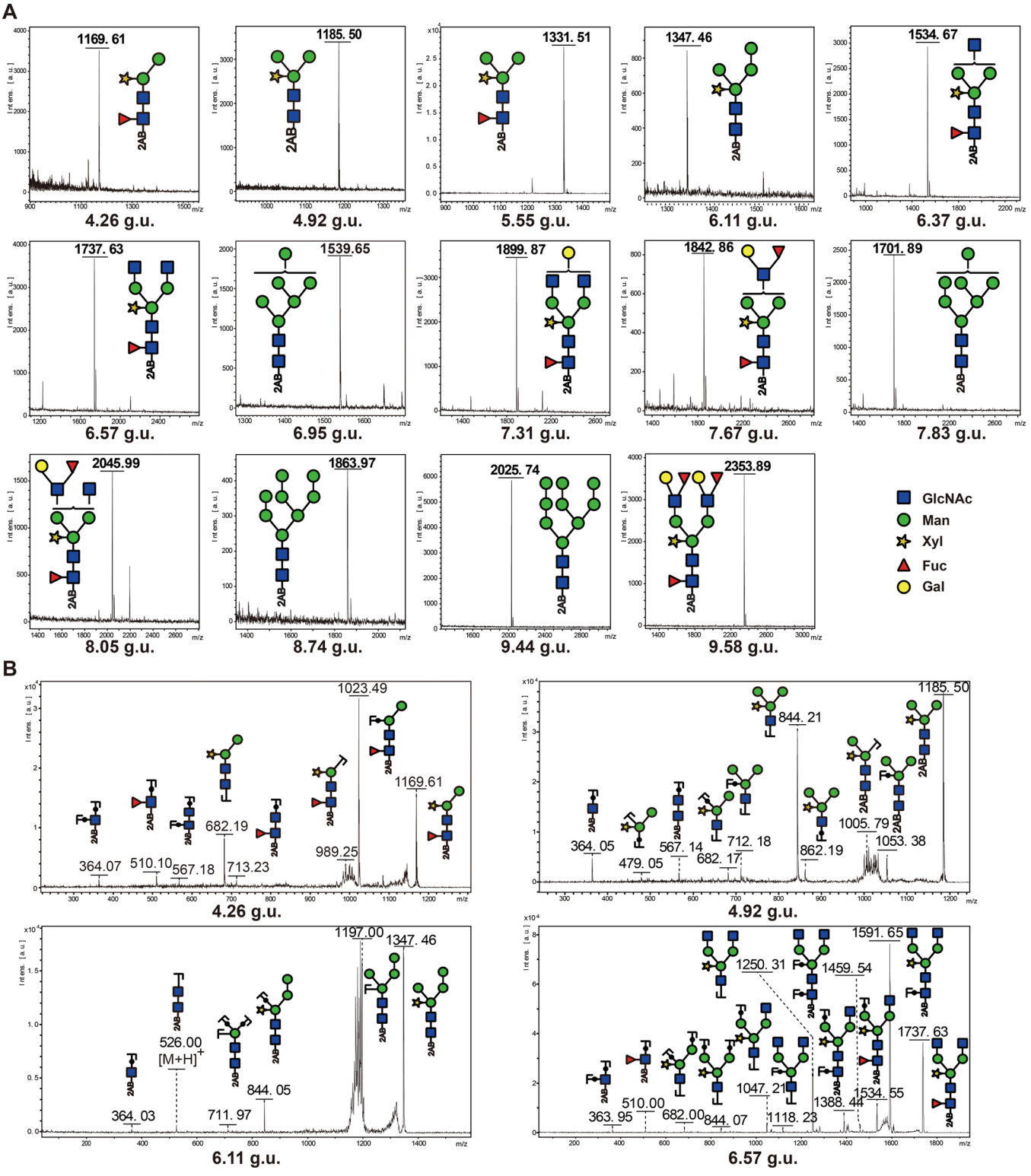

Figure 4. Structural analysis of 2-AB-labeled N-glycans from *Ginkgo* seed proteins by MALDI-TOF-MS and MS/MS. (A) MS analysis of fractions separated by HILIC-UPLC, marked with corresponding GU value. (B) MS/MS analysis of N-glycans with ion *m*/*z* values 1169.61, 1185.50, 1347.46, and 1737.63 $[M + Na]^+$. (F or F● indicates the position of fragmentation.)

period of seed development. $MMXF^3$ was also reported to be the most predominant structure in mature seed (more than 90%).[26] The abundance of peak 5.55 gu (GU value) was consistent with the abundance of $MMXF^3$ found by Kimura and Matsuo,[25] and its GU value also matched the reported data for $MMXF^3$ in our previous work.[15] Peak 5.55 gu was therefore proposed to be 2-AB-derivatized $MMXF^3$. From the comparison of GU values with reported data from Wilson et al.[14] and our previous work,[15] the structures of the remaining marked UPLC peaks were proposed as follows: 4.26 (M3), 4.85 ($MUXF^3$), 4.92 (MMX, M4), 5.96 (GnMX), 6.11 (M5, GnGnX, M4X), 6.37 ($GnMXF^3$), 6.57 ($Man4XF^3$, $GnGnXF^3$),

DOI: 10.1021/acs.jafc.7b01690
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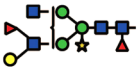
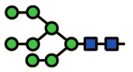
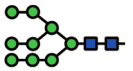
6.95 (M6), 7.31 (GGnXF^3), 7.67 [(GF)MXF^3], 7.83 (M7), 8.05 [(GF)GnXF^3], 8.74 (M8), 9.44 (M9), 9.58 [(GF)(GF)-XF^3]. The nomenclature system from Wilson et al.[14] was applied to name our structures.

3.4. N-Glycan Structural Characterization by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry and Tandem Mass Spectrometry. In order to confirm the proposed structures, each individual fraction from UPLC was collected separately and further analyzed by MALDI-TOF-MS and MS/MS. Mass spectra of 14 peaks that matched 2-AB-derivatized oligosaccharides from plants are shown in Figure 4A, and their MS/MS profiles are given in Figure 4B and Figure S1. No MS peak was detected from fractions of 4.85 and 5.96 gu. This might be because of the low abundance of these two fractions. On the basis of the interpretation from GU value, a proximate m/z value of 1053 responsible for 2-AB-M3 should be observed in the MS spectrum of peak 4.26 gu. However, only an ion m/z value of 1169.61 Da $[M + Na]^+$ was observed, which corresponds to 2-AB-MUXF (1169.43 $[M + Na]^+$). The MS/MS fragment ions (m/z 364.07 $[M + Na]^+$, 510.10 $[M + Na]^+$, 567.18 $[M + Na]^+$, 682.18 $[M + Na]^+$, 713.23 $[M + Na]^+$, 989.25 $[M + Na]^+$, and 1023.49 $[M + Na]^+$) derived from the parent m/z 1169.61 spectra were assigned as fragments derived from 2-AB-MUXF^3. The inconsistency of results predicted from GU value and mass spectrum indicated that MUXF^3 might be a structure which is beyond the applicable extent of GU methodology. There were two potential structures for peak 4.92 gu according to the GU value: 2-AB-MMX (theoretical m/z 1185.43 $[M + Na]^+$) and 2-AB-M4 (theoretical m/z 1215.44 $[M + Na]^+$). The MS result showed a m/z value of 1185.50 Da $[M + Na]^+$, which was consistent with the calculated mass value of 2-AB-MMX (1185.42 Da $[M + Na]^+$). A linkage of xylose to the trimannoside position of 2-AB-MMX was further determined by the MS/MS fragmentation results (m/z 364.05 $[M + Na]^+$, 479.05 $[M + Na]^+$, 567.14 $[M + Na]^+$, 682.17 $[M + Na]^+$, 713.18 $[M + Na]^+$, 844.21 $[M + Na]^+$, 862.19 $[M + Na]^+$, 1005.79 $[M + Na]^+$, and 1053.38 $[M + Na]^+$). The structural candidates for peak 6.11 gu were 2-AB-M5 (m/z 1377.49 $[M + Na]^+$), 2-AB-GnGnX (m/z 1591.58 $[M + Na]^+$), or 2-AB-M4X (m/z 1347.48 $[M + Na]^+$). From the mass spectrum, only a m/z value of 1347.46 Da $[M + Na]^+$ was observed, which is consistent with the theoretical mass charge ratio of 2-AB-M4X (1347.48 $[M + Na]^+$). This was further confirmed by the MS/MS fragment ions (m/z 364.03 $[M + Na]^+$, 526.00 $[M + H]^+$, 711.97 $[M + Na]^+$, 844.05 $[M + Na]^+$, and 1197.00 $[M + Na]^+$) derived from the parent m/z 1347.46 $[M + Na]^+$. Although two possible structures, 2-AB-Man4XF^3 (m/z 1493.54 $[M + Na]^+$) and 2-AB-GnGnXF^3 (m/z 1737.64 $[M + Na]^+$), were inferred from the GU value (peak 6.57 gu), only one mass value of 1737.63 Da $[M + Na]^+$ was detected by MALDI-TOF-MS. It was consistent with the calculated monoisotopic sodiated mass value of 2-AB-GnGnXF^3 (1737.65 $[M + Na]^+$). Assignment of this structure was confirmed by the MS/MS fragment ions (m/z 363.95 $[M + Na]^+$, 510.00 $[M + H]^+$, 682.00 $[M + Na]^+$, 844.07 $[M + Na]^+$, 1047.21 $[M + Na]^+$, 1118.23 $[M + Na]^+$, 1250.31 $[M + Na]^+$, 1388.44 $[M + Na]^+$, 1459.54 $[M + Na]^+$, 1534.55 $[M + Na]^+$, and 1591.65 $[M + Na]^+$) derived from the parent m/z 1737.63 $[M + Na]^+$ spectra. Similarly, the remaining structures interpreted from GU values were all confirmed by MALDI-TOF-MS and MS/MS (data shown in Figure 4A and Figure S1). All identified structures are summarized in Table 1. The Consortium for Functional Glycomics (CFG) notation system was used for depicting N-glycans.[27]

Table 1. Assigned Structures of N-Glycans from *Ginkgo* Seed Proteins[a]

GU	Assigned Structure	Name	Expected m/z	Detected m/z
4.26		MUXF^3	1169.43	1169.61
4.92		MMX*	1185.43	1185.50
5.55		MMXF^3*	1331.49	1331.51
6.11		M4X	1347.48	1347.46
6.37		GnMXF^3*	1534.57	1534.67
6.57		GnGnXF^3*	1737.64	1737.63
6.95		M6	1539.54	1539.65
7.31		GGnXF^3	1899.70	1899.87
7.67		(GF)MXF^3	1842.68	1842.86
7.83		M7	1701.60	1701.89
8.05		(GF)GnXF^3	2045.76	2045.99
8.74		M8	1863.65	1863.97
9.44		M9	2025.70	2025.74
9.58		(GF)(GF)XF^3	2353.87	2353.89

[a]Based on UPLC and MALDI-TOF-MS/MS. Asterisks indicate structures that were reported in previous work by Kimura and Matsuo.[25]

According to publications about plant glycoforms, three types of N-glycans, high-mannose, complex, and hybrid, have been distinguished. Apart from the hybrid glycoform, which is unique to papaya, the other two types are common and widespread in plants.[14,15] N-Linked carbohydrate modification from different species of plants presented distinct patterns. In this study, in total 14 structures of N-glycans were identified from glycoproteins of *Ginkgo* seeds. Four of them (M6, M7, M8, and M9) belong to the high-mannose type, which was distinguished for the first time in *Ginkgo*, and all others are complex-type N-glycans. Lewis A determinants, including (GF)MXF^3, (GF)GNXF^3 and (GF)(GF)XF^3, were also found in *Ginkgo* for the first time. No complex N-glycans with only fucosylation, like MMF, were detected.

It was reported by Kimura and Matsuo[25] that only four structures, MMXF^3, GnGnXF^3, GnMXF^3, and MMX^3, were detected from *Ginkgo* seeds. According to their report, N-glycans were released by hydrazinolysis and labeled with 2-PA, followed by the HPLC and ESI-MS/MS spectroscopic analysis

DOI: 10.1021/acs.jafc.7b01690
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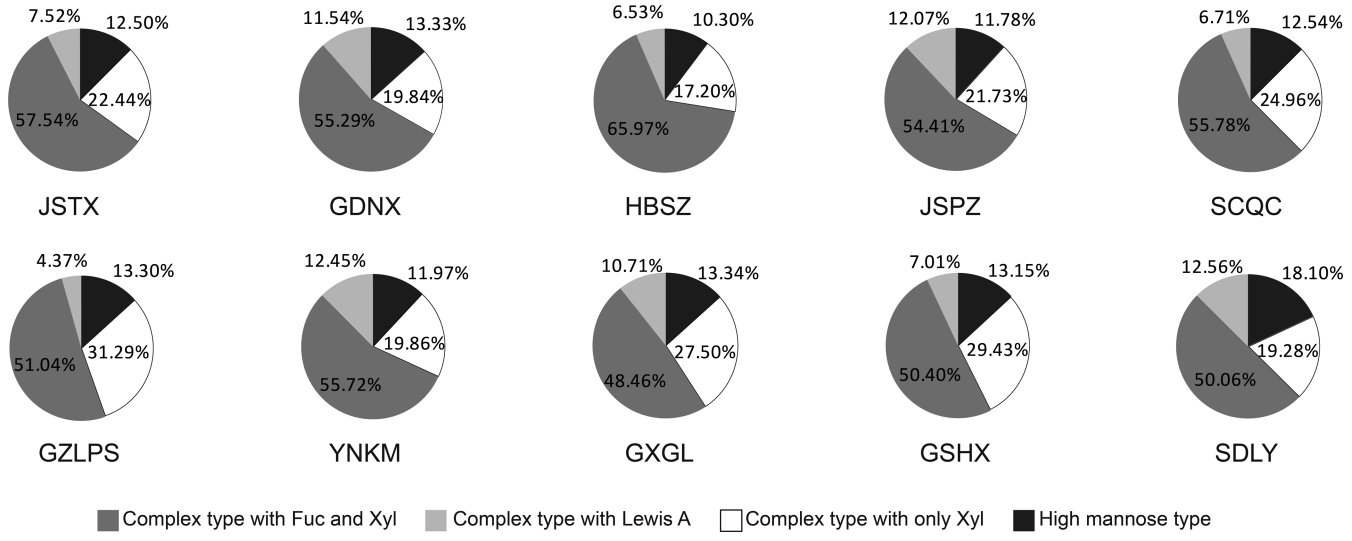

Figure 5. Relative quantity of N-glycans accounted by structural features from *Ginkgo* seeds.

for structure elucidation. They concluded that *Ginkgo* seeds had mainly xylose-containing oligosaccharides instead of high-mannosidic glycans, as no high-mannose-type N-glycans were detected by either immunoassays or lectin-blotting assays. Compared to enzymatic liberation, the reaction conditions of hydrazinolysis are harsh and can easily cause glycan degradation. Meanwhile, multiple purifications used in their approach prior to HPLC analysis could cause critical loss of N-glycans. In comparison, the novel PNGase H^+-based strategy discovered by our group allows minimal steps of purification and maximal recovery of low-abundance oligosaccharides under much milder conditions.[18] Many more novel structures, including high-mannose-type N-glycans, were thus found in *Ginkgo* seed proteins in our study, and this has made great contributions to the N-glycan database of *Ginkgo* seeds.

It is worth pointing out that Kimura and Matsuo[16] also discovered an *endo-β-N*-acetylglucosaminidase from *Ginkgo* seeds and found that this enzyme is only active on glycoproteins with high-mannose-type N-glycan. The existence of the potential endogenous protein substrate containing high-mannose-type N-glycans in *Ginkgo* seeds is thus expected, and the absence of high-mannose-type N-glycan in their report was inexplicable. Our finding of high-mannose-type N-glycans in *Ginkgo* seeds therefore explains the necessity of existence of the *endo-β-N*-acetylglucosaminidase and fills the gap between the two results from Kimura and Matsuo.

3.5. Quantification of N-Glycans. All samples were separately treated by enzymatic hydrolysis with recombinant PNGase H^+, 2-AB derivation, and HILIC-UPLC analysis four times to assure complete release of all N-glycans. The UPLC results of released N-glycans showed that the amount of N-glycan decreased with each round of enzymatic digestion; that is, no N-glycan was detected by UPLC after the fourth digestion (data are shown in Figure S2B). SDS−PAGE analysis was also performed to investigate the degree of deglycosylation. From the SDS−PAGE results, it was clear that bands at about 50, 30, and 20 kDa, which were deduced to bear N-glycans, all shifted after PNGase H^+ digestion, confirming the N-glycosylation of these proteins (data are shown in Figure S2A). By putting together the results of SDS−PAGE and UPLC analysis, the maximum deglycosylation of protein extract by PNGase H^+ was confirmed.

To quantify each N-glycan, a calibration curve was generated from the peak area of UPLC and the amount of 2-AB-maltopentaose by a linear equation [$y = (2 \times 10^6)x - 48\,195$, $R^2 = 0.996$]. The complex-type N-glycans were divided into three different subclasses manually, depending on the presence of fucose, xylose, and Lewis A determinants. Figure 5 shows the proportion of different types of N-glycan in detail. The relative quantity of complex-type N-glycans in seeds from 10 separate locations varied from 81.90% to 89.70%, and the sample from SDLY has a statistically lower value than others. The proportion of complex N-glycan with Fuc and Xyl ranged from 48.46% to 65.97%, the proportion of complex N-glycan with just xylose ranged from 17.20% to 31.29%, and the proportion of complex N-glycan with Lewis A determinants ranged from 4.37% to 12.56%. The percentage of high-mannose-type N-glycans varied from 10.30% to 18.10%. *Ginkgo* seeds from SDLY seemed to have the highest relative quantity of high-mannose-type N-glycans with a significant difference ($P > 0.05$).

The absolute amounts of different N-glycans were also calculated (Table 2). The complex type with Fuc and Xyl was the most abundant class, ranging from 1.39 ± 0.08 to 2.47 ± 0.77 μg/mg of dried protein extract. The sample from GSHX presented the lowest amount of fucosylated and xylosylated N-glycans, and the sample from SDLY had the highest amounts. No significant difference was observed between GDNX, JSPZ, SCQC, GZLPS, YNKM, and GXGL. Moreover, GZLPS has the highest amount of $MMXF^3$ (1.11 ± 0.06 μg/mg). Amounts of N-glycans bearing xylose but not fucose varied from 0.60 ± 0.20 to 1.20 ± 0.09 μg/mg; the highest amount was observed in GXGL sample. It is noteworthy that the absolute amount of high-mannose-type N-glycan (0.32 ± 0.01 to 0.87 ± 0.26 μg/mg) was apparently lower than that of complex-type N-glycan (2.28 ± 0.51 to 3.92 ± 1.17 μg/mg). The highest amount was observed in SDLY. The total sum amount for all N-glycans was also calculated (from 2.60 ± 0.52 to 4.79 ± 1.43 μg/mg), and the sample from SDLY was statistically higher than others ($P > 0.05$).

The structure and content of N-glycans are distinctive in different plants. Studies of plant N-glycomes reveal that different classes of plants show unique fingerprints of N-glycans, which are considered to be relevant to cross-

DOI: 10.1021/acs.jafc.7b01690
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Table 2. Quantification of N-Glycans from Extracted *Ginkgo* Seed Proteins[a]

	JSTX	GDNX	HBSZ	JSPZ	SCQC	GZLPS	YNKM	GXGL	GSHX	SDLY
Complex Type with Xyl but Not Fuc										
MMX	0.32 ± 0.04 a	0.44 ± 0.08 a	0.30 ± 0.00 a	0.32 ± 0.02 a	0.40 ± 0.05 a	0.85 ± 0.32 bc	0.27 ± 0.00 a	0.88 ± 0.07 c	0.56 ± 0.01 ab	0.39 ± 0.06 a
M4X	0.28 ± 0.19 ab	0.25 ± 0.03 a	0.31 ± 0.01 ab	0.35 ± 0.06 ab	0.29 ± 0.05 ab	0.29 ± 0.09 ab	0.34 ± 0.04 ab	0.32 ± 0.02 ab	0.25 ± 0.01 a	0.53 ± 0.18 b
sum	0.60 ± 0.23 a	0.69 ± 0.06 a	0.61 ± 0.00 a	0.67 ± 0.05 a	0.69 ± 0.01 a	1.13 ± 0.23 bc	0.61 ± 0.04 a	1.20 ± 0.09 c	0.81 ± 0.01 ab	0.91 ± 0.24 abc
Complex Type with Fuc and Xyl										
$MUXF^3$	0.15 ± 0.02 a	0.14 ± 0.01 a	0.59 ± 0.01 c	0.19 ± 0.00 a	0.17 ± 0.00 a	0.33 ± 0.06 b	0.18 ± 0.00 a	0.70 ± 0.07 c	0.17 ± 0.01 a	0.24 ± 0.07 ab
$MMXF^3$	0.99 ± 0.17 cd	0.97 ± 0.03 cd	0.60 ± 0.02 a	0.81 ± 0.04 abc	0.81 ± 0.03 abc	1.11 ± 0.06 d	0.86 ± 0.09 bc	0.81 ± 0.08 abc	0.65 ± 0.01 ab	0.81 ± 0.08 abc
$GnMXF^3$	0.17 ± 0.10 a	0.45 ± 0.06 abc	0.56 ± 0.03 c	0.34 ± 0.08 abc	0.34 ± 0.12 abc	0.21 ± 0.14 a	0.35 ± 0.05 abc	0.25 ± 0.00 ab	0.25 ± 0.02 ab	0.52 ± 0.18 bc
$GnGnXF^3$	0.16 ± 0.01 a	0.34 ± 0.05 ab	0.45 ± 0.01 a	0.29 ± 0.08 a	0.23 ± 0.10 a	0.18 ± 0.10 a	0.28 ± 0.04 a	0.30 ± 0.00 a	0.25 ± 0.03 a	0.55 ± 0.22 b
$GnGXF^3$	0.03 ± 0.00 a	0.04 ± 0.01 abc	0.13 ± 0.04 bc	0.05 ± 0.0 ab	0.03 ± 0.00 ab	0.03 ± 0.00 a	0.05 ± 0.01 ab	0.06 ± 0.00 abc	0.07 ± 0.03 ab	0.28 ± 0.22 c
sum	1.49 ± 0.27 ab	1.94 ± 0.13 abc	2.33 ± 0.07 bc	1.69 ± 0.20 abc	1.57 ± 0.25 abc	1.85 ± 0.25 abc	1.72 ± 0.18 abc	2.11 ± 0.15 abc	1.39 ± 0.08 a	2.41 ± 0.77 c
Complex Type with Lewis A Determinants										
$(GF)MXF^3$	0.05 ± 0.00 ab	0.09 ± 0.00 bc	0.06 ± 0.01 ab	0.08 ± 0.00 bc	0.04 ± 0.00 ab	0.03 ± 0.00 a	0.07 ± 0.00 abc	0.08 ± 0.00 abc	0.04 ± 0.01 ab	0.12 ± 0.05 c
$(GF)GNXF^3$	0.07 ± 0.00 a	0.16 ± 0.05 a	0.09 ± 0.00 ab	0.14 ± 0.00 a	0.08 ± 0.00 a	0.06 ± 0.00 a	0.14 ± 0.00 a	0.19 ± 0.00 a	0.08 ± 0.00 a	0.26 ± 0.12 b
$(GF)(GF)XF^3$	0.07 ± 0.01 a	0.16 ± 0.00 bc	0.08 ± 0.00 a	0.15 ± 0.00 b	0.07 ± 0.00 a	0.07 ± 0.00 a	0.17 ± 0.00 c	0.20 ± 0.00 d	0.07 ± 0.00 a	0.22 ± 0.01 d
sum	0.19 ± 0.01 a	0.41 ± 0.05 bc	0.23 ± 0.02 ab	0.37 ± 0.00 bc	0.19 ± 0.00 a	0.16 ± 0.00 a	0.38 ± 0.00 bc	0.47 ± 0.00 cd	0.19 ± 0.01 a	0.60 ± 0.18 d
High Mannose Type										
M6	0.15 ± 0.01 a	0.24 ± 0.04 ab	0.17 ± 0.01 a	0.20 ± 0.06 a	0.18 ± 0.06 a	0.19 ± 0.03 a	0.19 ± 0.03 a	0.23 ± 0.00 ab	0.18 ± 0.03 a	0.42 ± 0.17 b
M7	0.09 ± 0.01 ab	0.14 ± 0.04 a	0.10 ± 0.00 ab	0.08 ± 0.00 ab	0.09 ± 0.00 ab	0.12 ± 0.00 bc	0.09 ± 0.00 ab	0.15 ± 0.00 cd	0.09 ± 0.00 ab	0.20 ± 0.07 d
M8	0.05 ± 0.00 b	0.04 ± 0.00 e	0.05 ± 0.00 c	0.05 ± 0.00 c	0.05 ± 0.00 a	0.07 ± 0.00 f	0.05 ± 0.00 c	0.09 ± 0.00 h	0.05 ± 0.00 d	0.11 ± 0.03 i
M9	0.03 ± 0.00 ab	0.05 ± 0.00 abc	0.04 ± 0.00 ab	0.04 ± 0.00 abc	0.03 ± 0.00 ab	0.10 ± 0.00 a	0.04 ± 0.00 ab	0.11 ± 0.00 bc	0.04 ± 0.00 ab	0.15 ± 0.00 c
sum	0.32 ± 0.01 a	0.47 ± 0.08 a	0.36 ± 0.01 a	0.37 ± 0.06 a	0.35 ± 0.06 a	0.48 ± 0.03 a	0.37 ± 0.03 a	0.58 ± 0.01 a	0.36 ± 0.03 a	0.87 ± 0.26 b
total sum	2.60 ± 0.52 a	3.51 ± 0.20 abc	3.53 ± 0.10 abc	3.10 ± 0.31 ab	2.81 ± 0.31 a	3.63 ± 0.04 abc	3.08 ± 0.25 ab	4.36 ± 0.24 bc	2.76 ± 0.13 a	4.79 ± 1.43 c

[a]Amount of each N-glycan is expressed as mean ± SD (n = 3) in micrograms per milligram of dry protein extract. Different letters indicate significant differences ($p < 0.05$) in amount of N-glycan among different samples.

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DOI: 10.1021/acs.jafc.7b01690

allergy.[14,28] The edible parts of legumes, like soya and pea, normally have high levels of oligomannosidic glycosylation.[29] Fruits like apple and kiwi have high proportions of complex-type N-glycans with Lewis A determinants.[30] Hybrid-type N-glycans have been found only in papaya.[14] Tree nuts contain high levels of complex-type Asn-linked oligosaccharides with Fuc and Xyl, especially $MMXF^3$. From the N-glycan profiles of 10 samples, it was clear that only complex and high-mannose and no hybrid glycosylation were observed in *Ginkgo* seeds. The complex type with Fuc and Xyl was the major type of N-glycan in different samples, with proportions ranging from 48.5% to 66.0%. This is in agreement with the findings in tree nuts, like almond (58.7%), hazelnut (48.4%), and pistachio (67.1%).[14] The high proportion of $MMXF^3$ found in *Ginkgo* also fits the profile of N-glycans from tree nuts. In brief, the N-glycome of *Ginkgo* seeds exhibited a similar pattern as tree nuts, not only in structure but also in content of glycans.

It had been difficult to measure the absolute amount of N-glycans due to limitations of the analytic technique. Neither chemical release nor enzymatic release by PNGase A can keep the intact structure of the protein, and it is therefore difficult to isolate glycans from the reaction mixture.[14,31,32] Multiple steps of purification are necessary before fluorescent labeling or HPLC analysis, which causes great loss of glycans, especially the ones in low abundance. With the help of PNGase H^+, the sample was able to be subjected to multiple hydrolysis until no freed glycans were detected, to enable the complete release of the sugar. Just one step of purification by carbon solid-phase extraction was needed in the whole procedure. The absolute amount of each N-glycan was thus able to be calculated. The differences in N-glycan content among 10 samples seemed to be random, and no systematic pattern of geographic variation was concluded from the data. Because $MMXF^3$ is considered to be the most important indicator for allergenicity, we especially investigated and compared the relative and absolute amounts of $MMXF^3$ among different samples. Although the highest proportion of $MMXF^3$ (38.1%) was observed in JSTX, the sample from GZLPS had the highest absolute content (1.11 ± 0.06 μg/mg). The differences between relative and absolute amounts were also observed in other glycans, indicating different levels of glycosylation for each sample. Quantification of N-glycans from different regions provided an analytical basis for further elicitation of the relationship between allergy and CCDs of *G. biloba*. Whether these variations in N-glycans could cause the differences in immune response still needs further biochemical or immunobiochemical studies in vivo and in vitro.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b01690.

Two figures showing MS/MS, SDS−PAGE, and UPLC analyses (PDF)

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Funding

This work was supported by the National Natural Science Foundation of China [Grants 31401648 (to T.W. and L.L.) and 31471703, A0201300537 and 31671854 (to J.V. and L.L.)] and the Natural Science Foundation of Jiangsu Province, China [Grants BK20140719 (to T.W. and L.L.) and Q0201500580 (to L.L.)], and the 100 Foreign Talents Plan (Grant JSB2014012 to J.V.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Glc, glucose; Man (M), mannose; Xyl (X), xylose; Rha, rhamnose; GalA, galacturonic acid; Api, apiose; Hex, hexose; Gal (G), galactose; Fuc (F), fucose; Ara, arabinose; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc (Gn), *N*-acetylglucosamine; HexNAc, *N*-acetylhexosamine

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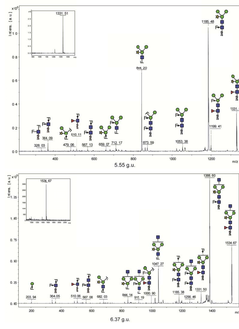
DOI: 10.1021/acs.jafc.7b01690
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

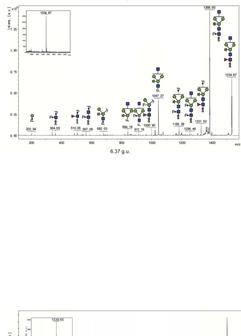
Supporting Information

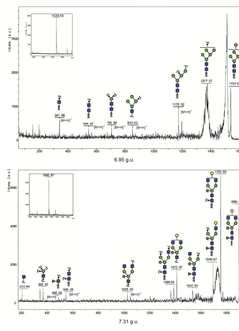
Qualitative and Quantitative Analysis of Carbohydrate Modification on Glycoproteins from seeds of *Ginkgo biloba*

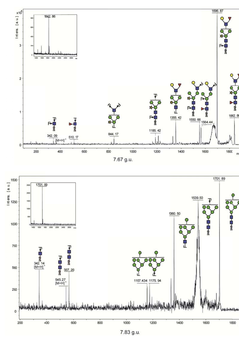
Ting Wang, Xiao-Chun Hu, Zhi-Peng Cai, Josef Voglmeir and Li Liu

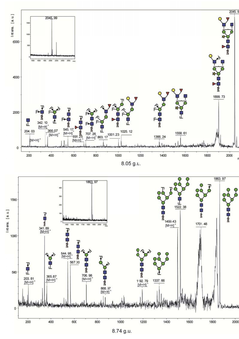
Supplementary Figure 1. MS/MS analysis of N-glycans with ion *m/z* value of 1331.51, 1534.67, 1539.65, 1899.87, 1842.86, 1701.89, 2045.99, 1663.97, 2025.74 and 2353.89 $[M+Na]^+$.

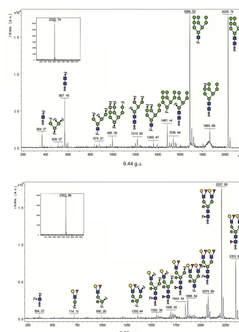
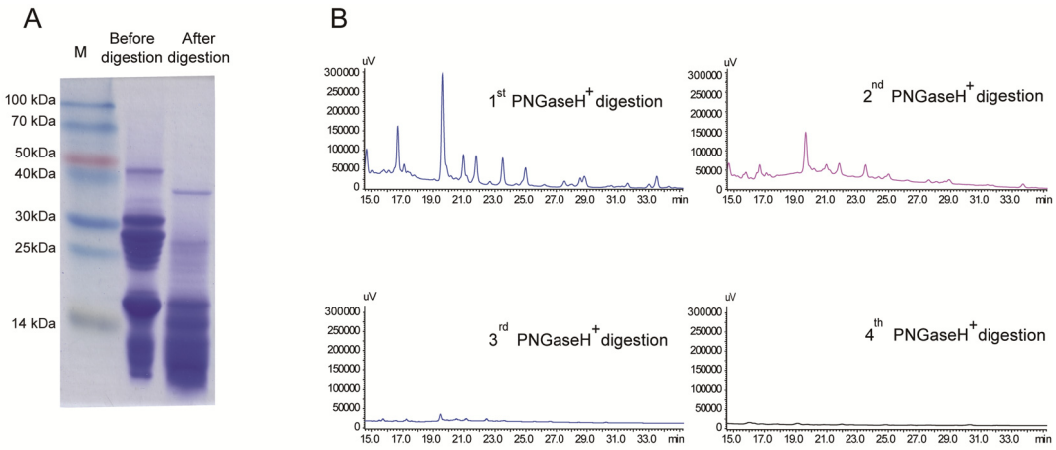












Supplementary Figure 2. A: SDS-PAGE analysis of *Ginkgo* protein and deglycosylated protein after 4th digestion with PNGase H^{+}. B: UPLC analysis of N-glycans released by PNGase H^{+}.