Changes in N-Linked Oligosaccharides during Seed Development of Ginkgo biloba

Yoshinobu KIMURA1 and Sayuri MATSUO

Department of Bioresources Chemistry, Faculty of Agriculture, Okayama University, Okayama 700-8530, Japan

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Structural changes in N-linked oligosaccharides of glycoproteins during seed development of Ginkgo biloba have been explored to discover possible endogenous substrate(s) for the Ginkgo endo-β-N-acetylglucosaminidase (endo-GB; Kimura, Y., et al. (1998) Biosci. Biotechnol. Biochem., 62, 253-261), which should be involved in the production of high-mannose type free N-glycans.

The structural analysis of the pyridylaminated oligosaccharides with a 2D sugar chain map, by ESI-MS/MS spectroscopy, showed that all N-glycans expressed on glycoproteins through the developmental stage of the Ginkgo seeds have the xylose-containing type (GlcNAc1→Man3Xyl1→Fuc1→GlcNAc2) but no high-mannose type structure. Man3Xyl1Fuc1GlcNAc2, a typical plant complex type structure especially found in vacuolar glycoproteins, was a dominant structure through the seed development, while the amount of expression of GlcNAc2Man3Xyl1Fuc1GlcNAc2 and GlcNAc2Man3Xyl1Fuc2GlcNAc2 decreased as the seeds developed. The dominantly occurrence of xylose-containing type structures and the absence of the high-mannose type structures on Ginkgo glycoproteins were also shown by lectin-blotting and immunoblotting of SDS-soluble glycoproteins extracted from the developing seeds at various developmental stages.

Concerning the endogenous substrates for plant endo-β-N-acetylglucosaminidase, these results suggested that the endogenous substrates might be the dolichol-oligosaccharide intermediates or some glycopeptides with the high-mannose type N-glycan(s) derived from misfolded glycoproteins in the quality control system for newly synthesized glycoproteins.

Key words: plant N-glycan; storage glycoprotein; endo-β-N-acetylglucosaminidase; seed development; Ginkgo biloba

In our previous paper,1) we purified and characterized an endo-β-N-acetylglucosaminidase (endo-GB) from mature Ginkgo biloba seeds. Endo-GB could hydrolyze the chitobiose linkage of various high-mannose types of N-glycans to release free N-glycans bearing one GlcNAc residue at the reducing end. Endo-GB as well as other some plant endoglycosidases2,3) were highly active towards the high-mannose type N-glycans having the Manα1→2Manα1→3Manβ1-structure unit, suggesting that the plant endoglycosidases have a specific binding site for the α1,2-mannosyl residue. This observation led us to assume that endo-GB would play a critical role to release the high-mannose type N-glycans from polypeptide chains in catabolism of N-linked glycoproteins during germination of the seeds.

As a first step to elucidate a deglycosylation mechanism working in germinating seeds and the physiological significance of plant endo-β-GlcNAc-ase, we tried to identify glycoprotein(s) that could be possible endogenous substrate(s) for the endoglycosidase in the Ginkgo seed. We encountered a puzzling finding, however, that storage glycoproteins in the mature Ginkgo seeds bear only xylose-containing type N-glycans,1,4) which cannot be endogenous substrates. In other words, there were no endogenous glycoprotein substrate(s) in the Ginkgo seeds, although the endo-β-N-acetylglucosaminidase occurred in the same seeds.

1 To whom correspondence should be addressed. Tel: +81-86-251-8296; Fax: +81-86-251-8388; E-mail: yoshimar@cc.okayama-u.ac.jp
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Abbreviations: endo-β-GlcNAc-ase, endo-β-N-acetylglucosaminidase; endo-GB, endo-β-GlcNAc-ase from Ginkgo biloba seed; PA, pyridylamino; RP-HPLC, reverse-phase HPLC; SF-HPLC, size-fractionation HPLC; ESI-MS, electrospray ionization mass spectrometry; MS/MS, tandem mass; Con A-HRP, concanavalin A coupled with horseradish peroxidase; M3X, Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4GlcNAc-PA; M3FX, Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc-PA; M3FX, Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc-PA; M3FX, Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc-PA; M3FX, Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc-PA; G2M3FX, GlcNAcβ1→2Manα1→6(Manα1→3)(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc-PA.
Furthermore, we have recently found some high-mannose type free N-glycans in the developing Ginkgo biloba seeds and identified their structures (Man$_x$GlcNAc$_y$). This suggested that the endogenous substrates for endo-GB should occur at the early stage of seed development and the endo-$\beta$-GlcNAc-ase already worked to release the high-mannose type N-glycans from certain endogenous substrate(s) at a certain developmental stage. Taking account of the substrate specificity of endo-GB, the structures of N-glycans linked to the storage glycoproteins, and the occurrence of free N-glycans in the Ginkgo seeds, it seems that the Ginkgo biloba seed can be a good plant to identify the endogenous substrate(s) for the plant endo-$\beta$-N-acetylglucosaminidase. If the endogenous substrates are some glycoproteins bearing the high-mannose type N-glycans, such glycoprotein(s) should occur in an early stage of seed development. Therefore, we analyzed the structures of N-glycans linked to the storage glycoproteins synthesized in developing Ginkgo seed.

In this communication, we describe how no glycoprotein synthesized at the early stage of seed development carries the high-mannose type N-glycans. This fact seems to suggest that the endogenous substrate(s) for the plant endoglycosidase could be small molecular glycoconjugate(s), such as dolichololigosaccharide intermediates occurring in N-glycan biosynthesis or glycopeptide(s) derived from misfolded glycoproteins in the quality control system for newly synthesized glycoproteins, rather than glycoproteins bearing the high-mannose type N-glycans.

Materials and Methods

Materials. Developing seeds of Ginkgo biloba were collected on the campus of Okayama University from June to October 1997 or June to August 1998. A Cosmosil 5C18-AR column (0.60×25 cm) was purchased from Naicali Tesque, Inc., and an Asahipak NH$_2$P-50 column (0.46×25 cm) from Showa Denko Co. Concanaavalin A coupled to horseradish peroxidase (Con A-HRP) was purchased from Seikagaku Kogyo Co. An antiserum against $\beta_1$-2 xylose-containing N-glycans was a generous gift from Dr. Arnd Sturm (Friedrich Miescher-Institute, Basel, Switzerland). A goat anti-rabbit IgG coupled to horseradish peroxidase, 4-chloro-1-naphtol, and gelatin were purchased from Bio-Rad. Ricin, M3FX, GN2M3FX, GN1M3FX, and M3X were prepared described our previous reports.

Preparation of storage glycoproteins from developing Ginkgo seeds. Developing Ginkgo seeds (about 100~350 g) collected on June 7, August 1, and October 5, 1997 were homegenized and defatted in acetone (10 times the volume of seeds). The resulting defatted powder (about 35~120 g) was suspended in 500 ml of 25 mM Tris-glycine buffer (pH 8.5), containing 0.2 M NaCl. The slurry was stirred for 2 h at 4°C. The homogenate was squeezed through four layers of Miracloth (Calbiochem Co.) and centrifuged. After centrifugation (8,000×g for 20 min.), the supernatant was 100% saturated with ammonium sulfate. The resulting precipitate was dissolved in deionized water and dialyzed exhaustively against deionized water. Finally the dialyze, including the resulting precipitate, was lyophilized and the lyophilizate was used as the storage glycoproteins in the following experiments.

Pyridylation of the sugar chains. N-Glycans were released by hydrazinolysis (100°C, 12 hr, in 20 ml of anhydrous hydrazine) from the storage glycoproteins (about 100~500 mg). After N-acetylation of the hydrazinolysate with saturated ammonium bicarbonate (20 ml) and acetic anhydride (0.8 ml), the acetylated hydrazinolysate was desalted by Dowex 50×2 resin. Pyridylation of the sugar chains was done by the method of Kondo et al. Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus with a Jasco 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-AR column (0.6×25 cm) or an Asahipak NH$_2$P-50 column (0.46×25 cm). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 15% for 40 min at a flow rate 1.2 ml/min. In the case of size-fractionation HPLC using the Asahipak NH$_2$P-50 column, the PA-oligosaccharide was eluted by increasing the water content in the water-acetonitrile mixture from 30% to 50% linearly for 25 min at a flow rate of 0.7 ml/min.

ESI-MS spectrometry. The mass spectrometer used was a Perkin Elmer ScieX API-III, triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source. The mass spectrometer was operated in the positive mode; the ion spray voltage was 4200 V. Samples were typically dissolved in 50% acetonitrile/water (containing 0.05% formic acid) and at a concentration of approximately 10 μmol/μl and introduced into the electrospray needle by mechanical infusion through a micro syringe at a flow rate of 5 μl/min. The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas. The collision energy was 60~100 eV. The scanning was done with a step size of 0.5 Da. and the CAD daughter ion spectrum was recorded from m/z 200.

SDS-PAGE, lectin-blotting, and immunoblotting of SDS-soluble glycoproteins in developing Ginkgo
seeds—Endosperms of developing Ginkgo seeds (55–100 g) were homogenized and defatted in acetone (10 times the volume of seeds). The resulting defatted powder (3.3–27 g) was suspended in boiled 25 mM Tris-glycine buffer (pH 8.8) containing 0.1% SDS (10–50 ml). The homogenate was squeezed through four layers of Miracloth (Calbiochem Co.) and centrifuged. After centrifugation of the filtrate, the resulting supernatant (10–20 ml) was used in the following analyses. SDS-PAGE was done by the method of Laemmli and Favre on a 15% acrylamide gel in 0.1 M Tris-glycine buffer system under reducing conditions with 5% 2-mercaptoethanol. Proteins on the gel were stained with Coomassie brilliant blue. Phosphorylase B (97.4 kDa), bovine serum albumin (BSA, 66.2 kDa), L-glutamate dehydrogenase (55.0 kDa), aldolase (40.0 kDa), ovalbumin (OVA, 42.7 kDa), carboxyl anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) were used as marker proteins (Promega) for the mass calibration. The proteins on the gel were transferred to a PVDF membrane for 30 min at 120 mA on Horize-blot (AE-6675 P/N, Atto Co.), using 0.1 M Tris-glycine containing 5% methanol (pH 8.8) as transfer buffer. Membranes were then probed with an antiserum that reacts with β1→2 xylose-containing N-glycans by the method of Laurière et al. or concanavalin A coupled with horseradish peroxidase by the method of Faye and Chrispeels. For detection of glycoproteins conjugated with antibodies specific for β1→2 xylose-containing N-glycans, we used goat anti-rabbit Ig-G coupled to horseradish peroxidase (Bio-Rad).

Results and Discussion

Structural analysis of N-glycans linked to storage glycoproteins in developing Ginkgo biloba seeds

In our previous papers, we had already analyzed the structures of two N-glycans linked to storage glycoproteins in mature Ginkgo seeds; one major structure (95%) was Manα1–6(Mana1–3)(Xyβ1–2)
Manβ1–4GlcNAcβ1–4(Fucα1–3)GlcNAc (M3FX) and the other minor structure (5%) was Manα1–6(Mana1–3)(Xyβ1–2)Manβ1–4GlcNAcβ1–4GlcNAc (M3X). These typical plant complex type structures have been found in many plant glycoproteins, especially storage glycoproteins accumulated in vacuoles. As shown in Fig. 1, four relevant PA-sugar chains; Peak-A corresponded to M3FX, Peak-B to GN2M3FX, Peak-C to GN3M3X, and Peak-D to M3X, respectively. Furthermore, ESI-MS analysis showed the molecular masses of these four PA-sugar chains; m/z 1267.5 ([MαnXγiFucβiGlcNAcβiPA+H]+) for Peak-A, m/z 1675.0 ([GlcNAcβiManβiXγiFucβiGlcNAcβiPA+H]+) for Peak-B, m/z 1471.0 ([GlcNAcβiManβiXγiFucβiGlcNAcβiPA+H]+) for Peak-C, and m/z 1121.5 ([GlcNAcβiFucβiGlcNAcβiPA+H]+) for Peak-D, respectively.

The structures of these PA-sugar chains were further analyzed by ESI-MS/MS. All relevant fragment ions derived from the parent ions of these four PA-sugar chains were well assigned as follows.

The relevant signals observed by MS/MS analysis of Peak-A could be reasonably assigned as fragment ions derived from the M3FX (Fig. 2 Panel A); m/z 1121.5 ([MαnXγiGlcNAcβiPA+H]+, m/z 989.5 ([MαnGlcNAcβiPA+H]+, m/z 959.5 ([Manα1XγiGlcNAcβiPA+H]+), m/z 827.5 ([Manα1GlcNAcβiPA+H]+), m/z 690.0 ([Manα1GlcNAcβiPA+H]+, m/z 665.0 ([Manα1GlcNAcβiPA+H]+, m/z 503.0 ([GlcNAcβiPA+H]+), m/z 446.0 ([FucβiGlcNAcβiPA+H]+), m/z 300.0 (GlcNAc-PA).
The relevant signals from Peak-B could be reasonably assigned as fragment ions derived from the GN1M3FX (Fig. 2, Panel B); m/z 1528.5 ([GlcNAc\_Man\_Xyl\_GlcNAc\_PA + H]\(^{+}\)), m/z 1471.5 ([GlcNAc\_Man\_Xyl\_Fuc\_GlcNAc\_PA + H]\(^{+}\)), m/z 1397.0 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 1326.0 ([GlcNAc\_Man\_Xyl\_GlcNAc\_PA + H]\(^{+}\)), m/z 1309.5 ([GlcNAc\_Man\_Xyl\_GlcNAc\_PA + H]\(^{+}\)), m/z 1194.0 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 1163.5 ([GlcNAc\_Man\_Xyl\_GlcNAc\_PA + H]\(^{+}\)), m/z 1132.5 ([GlcNAc\_Man\_Fuc\_GlcNAc\_PA + H]\(^{+}\)), m/z 989.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 959.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 929.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 899.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 869.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 839.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 732.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 692.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 662.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 632.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)). The relevant signals from Peak-C could be reasonably assigned as fragment ions derived from the GN1M3FX (Fig. 2, Panel C); m/z 1325.0 ([GlcNAc\_Man\_Xyl\_GlcNAc\_PA + H]\(^{+}\)), m/z 1265.7 ([Man\_Fuc\_Xyl\_GlcNAc\_PA + H]\(^{+}\)), m/z 1192.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 1121.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 989.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 959.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 929.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 899.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)), m/z 869.5 ([GlcNAc\_Man\_GlcNAc\_PA + H]\(^{+}\)). Concerning the structure of Peak-C, GlcNAc1Man3Xyl1Fuc1GlcNAc2-PA structure occurs in two isomers; one is Man\_1-6(GlcNAc\_1 - 2Man\_1 - 3)(Xyl\_1 - 2)Man\_1 - 4GlcNAc\_1 - 4(Fuc\_1 - 3)GlcNAc\_PA (G1M3FX), the other one is GlcNAc\_1 - 2Man\_1 - 6(Man\_1 - 3)
Fig. 3. Proposed Structures of N-Glycans of Storage Glycoproteins in Developing Ginkgo biloba Seeds.

(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA ($^{33}$M3FX). It has already been found that on the ODS column the former structure is eluted before GN2M3FX and the latter structure is eluted after GN2M3FX. Considering the elution position of Peak-C which was eluted after GN2M3FX (Peak-B) on the ODS column, the structure of Peak-C should be GN3M3FX.

The relevant signals observed by MS/MS analysis of Peak-D could be reasonably assigned as fragment ions derived from the M3X (Fig. 2 Panel D); m/z 989.5 ([Man$_3$GlcNAc$_2$-PA + H$^+$]), m/z 959.5 ([Man$_3$-Xyl$_1$GlcNAc$_2$-PA + H$^+$]), m/z 827.5 ([Man$_3$GlcNAc$_2$-PA + H$^+$]), m/z 665.5 ([Man$_3$GlcNAc$_2$-PA + H$^+$]), m/z 503.0 ([GlcNAc-GlcNAc-PA + H$^+$]), m/z 300.1 ([GlcNAc-PA + H$^+$]).

Taking account of these data described above, the structures of four N-glycans linked to the storage glycoproteins expressed in developing Ginkgo biloba seeds should be as shown in Fig. 3.

The most important finding in this structural analysis is that no high-mannose type N-glycan bearing α-1,2-mannosyl residue(s) was detected, at least in analyzable concentration, from matured or processed glycoproteins synthesized in the developing Ginkgo biloba seeds. This finding clearly suggested that endogenous substrate(s) bearing high-mannose type N-glycan for the Ginkgo endo-β-GlcNAc-ase do not occur as glycoprotein, although the real endogenous substrate(s) remains unknown.

It is also noteworthy that GlcNAc$_2$-Man$_3$Xyl$_1$Fuc$_2$GlcNAc$_2$ structures disappeared as the seeds matured. This observation seems to support the hypothesis$^{[4,15]}$ that, in plant cells, the N-glycans transferred to newly translated polypeptides and processed in the ER and the Golgi apparatus were further modified by an acidic N-acetylgalactosaminidase to make the Man$_3$Xyl$_1$Fuc$_2$GlcANc$_2$ in vacuoles or proteinbodies. Furthermore, the removal of terminal GlcNAc residue(s) at the non-reducing end seems to occur as the seeds develop or differentiate, since some glycoproteins expressed at early stages of seed development carry the GlcNAc residue(s) at the non-reducing end (Figs. 1 and 3).

Lectin-blotting and immunoblotting of SDS-soluble glycoproteins in developing Ginkgo seeds

To confirm the absence of glycoprotein bearing the high-mannose type N-glycan in the developing Ginkgo seeds, we analyzed the structural feature of N-glycans linked to SDS-soluble glycoproteins by aminoblotting with Con A-HRP and immunoblotting with an antiserum against β-1,2-xylosyl residue.
As shown in Fig. 4 (Panel-A Lane 1–6), no glycoprotein, to which Con A could bind through the high-mannose type glycans, was detected through every stage of seed-development, while the B-chain of ricin² was clearly detected by the Con A-affinoblotting (Panel-A, lane 7). On the contrary, the glycoproteins with xylose-containing N-glycans were detected by the immunoblotting with an antiserum against β-1,2-xylosyl residue from early stage of development to the final stage (Panel-B, lane 1–6). It is noteworthy that a few bands observed on the immunoblot membrane could not be observed on SDS-gel stained with Coomassie blue (Panel-C), suggesting the high sensitivity of immunoblotting analysis with β-1,2-xylose antibody.

These facts also showed that glycoproteins bearing high-mannose type N-glycans, which can be one of candidates of endogenous substrates for endo-GB, do not occur through the seed development or seed differentiation.

Concerning the processing of storage glycoproteins during seed development, it is noteworthy that some storage glycoproteins having xylose-containing N-glycan(s) (60.4 kDa and 51.4 kDa) changed to be smaller molecules (around 22 kDa) as the Ginkgo seeds developed. This change in the molecular weight of the storage glycoproteins having the xylose-containing N-glycans showed that processing of polypeptide chains or glycoprotein maturation, at least in the case of storage glycoproteins in Ginkgo seeds, occurs after the processing of N-glycans from high-mannose type to complex-type structures in the Golgi apparatus.

Panel-D shows the sample Ginkgo seeds used at each developmental stage. The seeds collected at middle of July were already covered with hard shells, even though the surface of seeds was still green.

In this communication, we demonstrated that almost no glycoproteins bearing the high-mannose type N-glycans occur through the development or differentiation of Ginkgo seeds, suggesting that the endogenous substrate for the Ginkgo endo-β-GlcNAc-ase might be other type(s) of small molecular glycoconjugates bearing the high-mannose type.
N-glycans rather than glycoproteins. Since we have already found that the high-mannose type free N-glycans (as the pyridylaminated derivatives) occur at the concentration of about 2.2 nmoles in one gram of the developing Ginkgo biloba seed, the endogenous substrate(s) should exist at nearly μm level at early stage of seed development. These circumstantial evidences led us to postulate that the possible endogenous substrates might be (1) the dolichol-oligosaccharide intermediates occurring in N-glycan biosynthesis or (2) glycopeptide(s) derived from misfolded glycoproteins in the quality control system for newly synthesized glycoproteins. Therefore, to evaluate these working hypotheses, structural analysis of the dolichol-oligosaccharide intermediates occurring in the plant cells and the characterization of glycopeptides bearing the high-mannose type N-glycans are in progress.

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