Ginkgo biloba α-fucosidase with activity towards plant complex type *N*-glycans containing the Lewis a epitope: Purification and characterization

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We have identified, and purified to homogeneity, a high molecular weight *Ginkgo biloba* α-fucosidase (α-fucosidase Gb, 120 kDa estimated by SDS-PAGE) with activity against α-fucosylated oligosaccharides. When a Lewis a epitope-containing *N*-glycan was used as a substrate, α-fucosidase Gb showed optimum activity at approximately pH 5.5, suggesting that it functions in acidic environments such as the vacuole. It remains uncertain, however, whether this *Ginkgo* α-fucosidase belongs to the GH29 family, since its *N*-terminal sequence could not be determined, probably due to a chemical modification. α-Fucosidase Gb showed substantial activity towards the α1,3-fucosyl linkage in Lacto-*N*-fucopentaose III and an α1,4-fucosyl linkage in the Lewis a epitope found in plant complex type *N*-glycans, indicating an involvement in the degradation process of α-fucosylated oligosaccharides or *N*-glycoproteins.

Key words : α-fucosidase, plant *N*-glycan, *N*-glycan degradation, *Ginkgo biloba*

Introduction

The occurrence of β1,2-xylosyl (Xyl) and α1,3-fucosyl (Fuc) residues are structural features of plant *N*-glycans. Interestingly, the Lewis a epitope (Galβ1-3(Fucα1-4) GlcNAc) often occurs on secreted plant type *N*-glycoproteins[1-3]. Recently Rips *et al*[4]. reported that plant complex type *N*-glycans linked to the *Arabidopsis* endo-β1,4-glucanase (KORRIGAN, KOR1) play a critical *in vivo* role involved in cell wall and root elongation. Furthermore, they found that deletion of the core α1,3-fucosyl residue in *N*-glycans and the appearance of high mannose type *N*-glycans linked to *Arabidopsis thaliana* glycoproteins significantly inhibited normal root elongation. Based on these findings, these authors speculated that a functional interplay exists between plant complex type *N*-glycans bearing the α1,3 fucosyl residue and the gene KOR1, thereby regulating cellulose biosynthesis in *Arabidopsis* seedlings. In order to understand the physiological significance of the core α1,3-fucosyl residue on plant complex type *N*-glycans, as well as the degradation mechanism for plant *N*-glycoproteins, the identification of a plant α-fucosidase that is active against the core α1,3-fucosyl linkage is an important goal in glycobiology. However, up until now, a plant α-fucosidase capable of releasing the α1,3-fucosyl residue from plant complex type *N*-glycans such as $GlcNAc_2Man_3Xyl_1Fuc_1GlcNAc_2$ has not been found. Although a plant α-fucosidase, which hydrolyzes the α1,4-fucosyl linkage in Lewis a-type and the 1,3-fucosyl linkage in lacto-*N*-fucopentaose III (LNFPIII), has been purified from almond meal and characterized (designated as almond α-fucosidase I)[5-8], this almond α-fucosidase could not release the core α1,3-fucose from plant complex type *N*-glycans[9]. Previously, we purified and characterized a rice α-fucosidase with activity toward Lewis a epitope-containing *N*-glycans, but this rice enzyme was also unable to hydrolyze the core α1,3-fucosyl residue in Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc[10], suggesting that plant GH29 α-fucosidases may be inactive toward the core α1,3-fucose in plant complex type *N*-glycans.

In a previous study[11], we found that *Ginkgo* seeds predominantly store *N*-glycoproteins bearing plant complex type *N*-glycans, suggesting that the seed should contain several kinds of glycosidases involved in the degradation of plant complex type *N*-glycans. Based on the finding, we have purified and characterized glycosidases involved in the degradation of *N*-glycoproteins from the seeds of *Ginkgo biloba*[11-14], indicating that *Ginkgo* seeds are a good source for the preparation of glycosidases that could be useful in analyzing the structures of bioactive glycans. In the course of purifications of α-mannosidase[12] and β-galactosidase[14], we uncovered α-fucosidase activity against the Lewis a epitope, confirming this assumption. The aim of this study was to attempt to purify and characterize an α-fucosidase from

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Ginkgo biloba seeds capable of hydrolyzing the core α1,3-fucosyl linkage. The *Ginkgo* α-fucosidase (α-fucosidase Gb) purified in this study had a molecular weight of approximately 120 kDa, a pH optimum of 5.5, and strong activity toward the α1,4-fucosyl residue in the Lewis a epitope and the α1,3-fucosyl residue in lacto *N*-fucopentaose III (LNFP III). However, α-fucosidase Gb showed no activity toward the fucosyl linkage in Manβ1-4GlcNAc β1-4 (Fucα1-3) GlcNAc-PA, and Fucα1-3GlcNAc-PA under the same condition, in which the α1,4-fucosyl residue in the Lewis a epitope was completely hydrolyzed. These results suggest that α-fucosidase Gb is a different species from the well-characterized plant GH 29 α-fucosidases, which have a molecular weight of approximately 55 kDa; it is likely to be involved in the degradation of fucosylated oligosaccharides in the seeds of *Ginkgo biloba*, a living fossil plant[15].

Materials and Methods

Materials - *Ginkgo biloba* seeds were collected in Inazawa, Aichi Prefecture, Japan. A Cosmosil 5C18-AR column (6.0×250 mm) was purchased from Nakalai Tesque (Kyoto, Japan). A Shodex Asahipak NH2P-50-4E column (4.6×250 mm) and a Shodex IEC QA-825 column (8.0×75 mm) were from Showa Denko (Tokyo, Japan). DEAE cellulose and Superdex 200 (16×140 cm) were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Butyl-Toyopearl was purchased from Tosoh (Tokyo, Japan). PA-labeled lacto-*N*-fucopentaose III (LNFP III) was purchased from Takara Bio. Authentic PA-sugar chains (Gal2Fuc2GN2M3FX, M3FX, and MFX) were prepared from glycoproteins produced by cultured rice cells[16] and momordin-a[17]. MF and GNF were also prepared from MFX using tomato β-xylosidase[18], β-mannosidase (*Helix pomatia*, Sigma), and β-*N*-acetylhexosaminidase (Jack bean, Sigma). Their structures were confirmed by ESI-MS and MS/MS analyses.

Assay system for α-fucosidase activity during enzyme purification -α-Fucosidase activity was routinely measured using a pyridylaminated glycan, Gal2Fuc2GN2M3FX (5.6 pmol, 7 μL). The enzyme solution (10 μL) was added to 10 μL of 0.15 M acetate buffer (pH 5.0) containing the PA-sugar chain. After a 30 min incubation at 37℃, the enzymatic reaction was stopped by heating in boiling water for 5 min, and following centrifugation, an aliquot (10 μL) of the reaction mixture was analyzed by size-fractionation (SF-) HPLC using a Shodex Asahipak NH2P-50 column (4.6×250 mm) previously equilibrated with 80% (v/v) acetonitrile/water. The PA-sugar chains were eluted by increasing the water content of the water-acetonitrile mixture from 26 to 50% linearly at a flow rate of 0.7 mL/min. PA-sugar chains were detected with a Jasco FP-920 Intelligent Fluorescence detector (Jasco, Japan) (excitation at 310 nm, emission at 380 nm). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of fucosyl residue from Gal2Fuc2GN2M3FX per minute at 37℃ in Na-acetate buffe (pH 5.0).

Purification of α-fucosidase Gb - Unless stated otherwise, protein purification steps were carried out at 4℃. Protein concentrations were determined by measuring the absorbance at 280 nm with bovine serum albumin (BSA) as the standard.

Step 1. Preparation of crude α-fucosidase Gb - Mature *Ginkgo biloba* seeds (2.8 kg) were homogenized in acetone and the resulting defatted powder (1.3 kg) was suspended in 5 L of 50 mM Tris-HCl buffer (pH 8.0) and left at 4℃ overnight. The mixture was then squeezed through two layers of gauze and centrifuged at 10,000×*g* for 20 min. The supernatant was saturated with 100% ammonium sulfate saturation. The precipitate was collected after centrifugation and dialyzed against deionized water, and the resulting supernatant was used as a crude α-fucosidase solution.

Step 2. DEAE-cellulose column chromatography - The crude enzyme dialyzed against 50 mM Tris-HCl buffer (pH 8.0) was loaded onto a DEAE cellulose column (4×36 cm) previously equilibrated with the same buffer. The run-through fraction containing α-fucosidase activity was pooled as shown in Fig. 1-I and brought to 100% ammonium sulfate saturation and the precipitate was collected by centrifugation.

Step 3. Butyl-Toyopearl column chromatography - The precipitate obtained in Step 2 was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer. Solid ammonium sulfate was then added to obtain a final concentration of 1.5 M. The resulting enzyme solution was loaded onto a Butyl-Toyopearl column (3.5×35 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1.5 M ammonium sulfate. After washing the column with the same buffer, the adsorbed enzyme was eluted with a linear reverse gradient of ammonium sulfate (1.5-0 M) in the same buffer. Fractions containing the α-fucosidase activity (horizontal bar in Fig. 1-II) were collected and 100% saturated with ammonium sulfate and the precipitate collected by centrifugation.

Step 4. QA-825 HPLC - *Ginkgo* α-fucosidase was

further purified with a Jasco 880-PU HPLC apparatus equipped with a Shodex IEC QA-825 column (8.0×75 mm). The α-fucosidase fraction obtained in Step 3 was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) and applied to an anion exchange column previously equilibrated with the same buffer. The absorbed proteins were eluted using a linear gradient of NaCl from 0 to 0.15 M in the same buffer at a flow rate of 1.0 mL/min. The α-fucosidase fraction (run-through fraction) as indicated by a horizontal bar in Fig. 1-III was concentrated with a membrane filter (Vivaspin 15R, 30,000 MWCO, Sartorius Stedim Biotech).

Step 5. Gel filtration through a Superdex 200 column - The final purification of α-fucosidase was achieved by gel filtration HPLC using a Superdex S-200 column as shown in Fig. 1-IV. The concentrated enzyme obtained in step 4 was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, and the dialysate was loaded onto the column previously equilibrated with the same buffer. The column was developed at a flow rate of 0.5 mL/min.

SDS-PAGE and blotting of purified Ginkgo α-fucosidase - SDS-PAGE was carried out according to the method described by Laemmli *et al*[19]. on 12% acrylamide gel in 0.1 M Tris-glycine buffer using a slab gel apparatus under reducing condition with 1% 2-mercaptoethanol or under non-reducing condition. Precision Plus Protein™ Standards (Bio-Rad) (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa) were used as the marker proteins for molecular mass determination. Proteins on the gel were stained with a Silver staining kit (Silver Stain Kit II, Wako Co., Japan).

Effect of pH and temperature on Ginkgo α-fucosidase activity - The optimum pH for the enzyme's activity against Gal2Fuc2GN2M3FX was determined by incubating the enzyme (0.013 μU) in varying buffers ranging from pH 4.0 to 8.0 (Acetate buffer for pH 4.0-5.5, MES buffer for pH 6.0-6.5, HEPES buffer for pH 7.0-8.0) at 37℃ for 1 hr. The optimum temperature for the enzyme's activity against Gal2Fuc2GN2M3FX in Na-acetate buffer (pH 5.0) was determined at six different temperatures (20, 30, 40, 50, 60, and 70℃).

Substrate specificity of Ginkgo α-fucosidase - The substrate specificity of the purified *Ginkgo α-fucosidase* was analyzed using authentic PA-sugar chains: Gal2-Fuc2GN2M3FX, MFX, GNF, lacto-*N*-fucopentaose I, and lacto-*N*-fucopentaose III. PA-sugar chains (approximately 20 pmol) were incubated with purified *Ginkgo* α-fucosidase (0.014 μU) in 0.1 M Na-acetate buffer (pH 5.5) at 37℃ for 30 min or 2 h. When Gal2Fuc2GN2M3FX was used as a substrate, the reaction mixture was analyzed by SF-HPLC on a Shodex Asahipak NH2P-50-4E column (6.0×250 mm). When LNFP III, MFX, GNF were used as substrates, the reaction mixtures were analyzed by RP-HPLC using a Cosmosil 5C18-AR column (6.0×250 mm). The PA-sugar chains were eluted by increasing the acetonitrile content from 0 to 7% linearly at a flow rate of 1.2 mL/min. and detected with a Jasco FP-920 Intelligent Fluorescence detector (excitation at 310 nm, emission at 380 nm).

Results and Discussion

Purification of Ginkgo *α-fucosidase.*

The *Ginkgo* α-fucosidase (α-fucosidase Gb) was purified to homogeneity approximately 304-fold (the initial specific activity in the crude extract was 0.12 μU/mg, and the final activity was 36 μU/mg). When the *Ginkgo* α-fucosidase was fractionated using DEAE cellulose, the α-fucosidase activity against Gal2Fuc2GN2M3FX was detected in the non-absorbed fraction (Fig. 1-I). Hence, we used the non-absorbed fraction in further purification of α-fucosidase. The active fraction was further purified using a Butyl-Toyopearl column (Fig. 1-II), followed by QAE-HPLC (Fig. 1-III). In this second anion-exchange chromatography step, two distinct α-fucosidase active fractions were separated as shown in Fig. 1-III, one that was bound to the anion-exchange column and was salt-elutable and one that did not bind to the anion-exchange column (run-through). The run-through fraction (indicated by a horizontal bar,) having the predominant activity, was used in the subsequent purification step, since the molecular weight of this α-fucosidase activity was significantly larger than known plant GH29 α-fucosidases[8-10] as described below. The α-fucosidase in the second fraction (i.e. the salt-elutable fraction), being active against α1,4-fucosyl residue, seemed to be a similar enzyme to the almond α-fucosidase I[6-9] and rice α-fucosidase[10] that belong to the common plant GH29 α-fucosidase family, although further purification and characterization of this α-fucosidase activity was not carried out in this study.

Molecular mass and N-terminal amino acid sequence

Gel filtration using the Superdex S-200 column revealed that the molecular mass of native α-fucosidase Gb was approximately 120 kDa, being slightly smaller than that of the marker protein, aldolase (158 kDa). When the purity of α-fucosidase Gb was checked by SDS-PAGE on a 12% gel under both reducing and non-

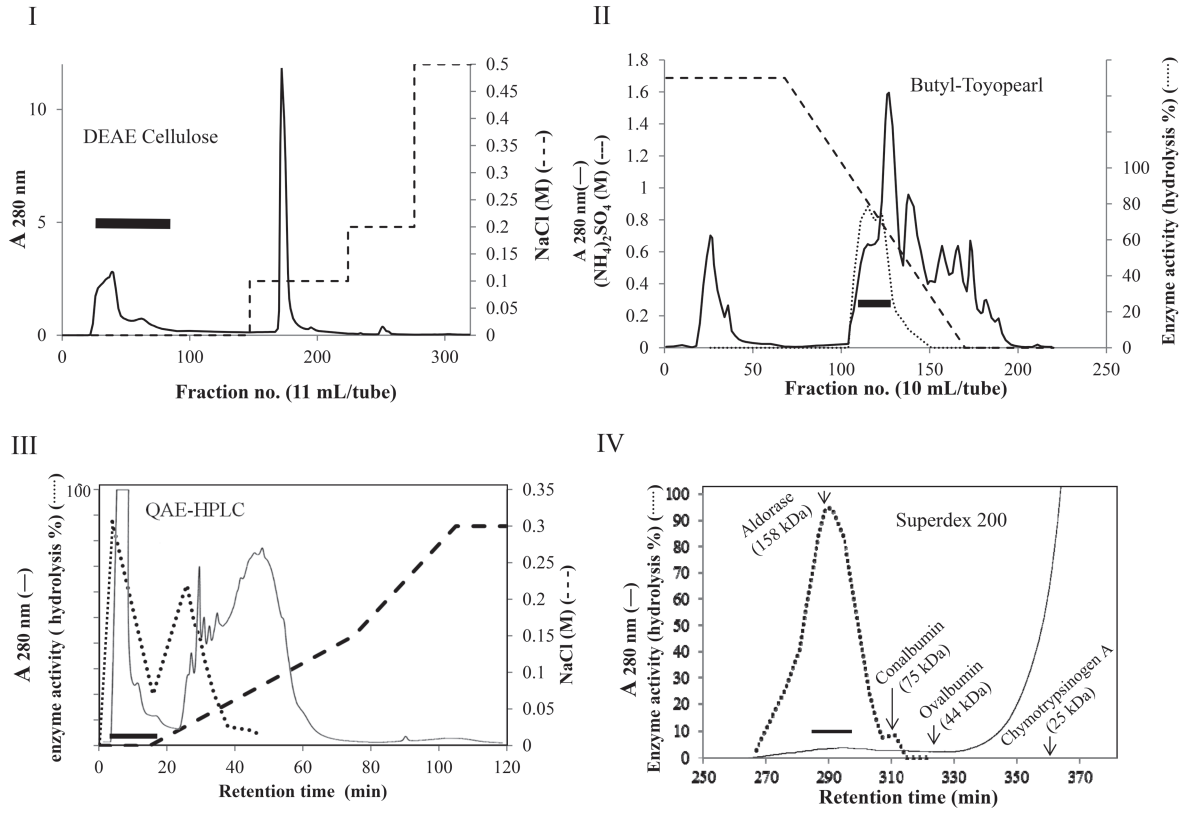

Fig. 1 Purification profile of α-fucosidase Gb
I, DEAE cellulose chromatography. α-Fucosidase activity was detected in the run-through fraction and the protein fractions indicated by a horizontal bar were pooled for the next step. II, Butyl-Toyopearl chromatography. Proteins were eluted using a reverse linear gradient of ammonium sulfate from 1.5 M to 0 M in the same buffer. The protein fractions indicated by the horizontal bar were pooled for further steps. III, QAE-HPLC. Proteins were separated on a Shodex IEC QA-825 column by linear gradient of NaCl from 0 M to 0.15 M in the 20 mM Tris-HCl buffer (pH 8.0). IV, Gel filtration profile on a Superdex S-200 (1.6×140 cm) column. The column was developed with 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl at a flow rate of 0.5 mL/min. Aldolase (158 kDa), Conalbumin (75 kDa), Ovalbumin (44 kDa), and Chymotrypsinogen A (25 kDa) were used as molecular size markers.

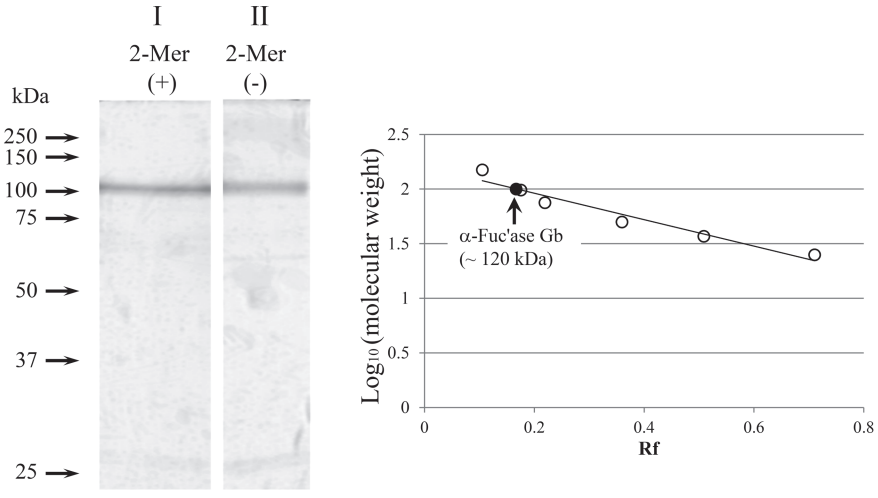

Fig. 2 SDS-PAGE of purified α-fucosidase Gb
Proteins were separated by SDS-PAGE using a 12% polyacrylamide gel and stained with a silver staining kit. I. α-Fucosidase Gb with 2-mercaptoethanol (2-Mer); II. α-Fucosidase Gb without 2-mercaptoethanol.

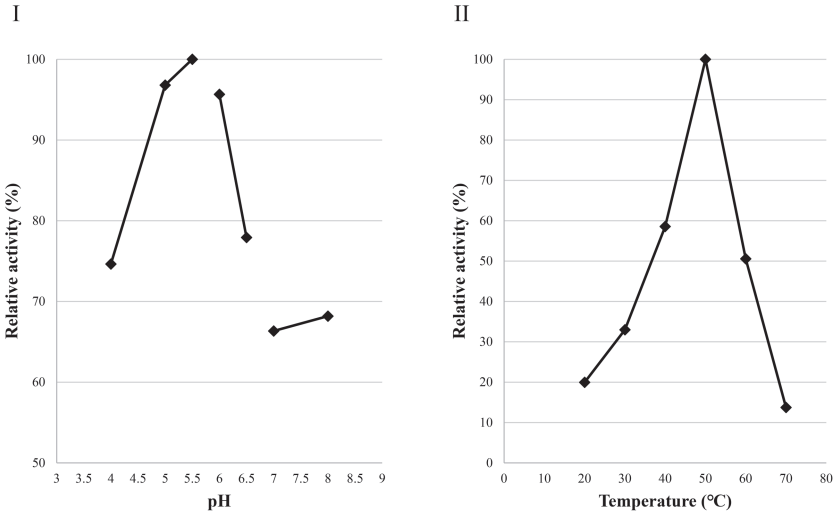

Fig. 3 Effects of pH and temperature on the activity of α-fucosidase Gb
Gal2Fuc2GN2M3FX was used as a substrate for analyzing the effects of pH and temperature on the activity of α-fucosidase Gb. I. Various buffers, Na-acetate buffer (pH 4.0-5.5), MES buffer (pH 6.0-6.5), and HEPES buffer (pH 7.0-8.0), were used in the 30 μL reaction mixture containing 5.6 pmol of substrate and 0.013 μU enzyme. The resulting products were analyzed by SF-HPLC. II. Effects of temperature on the activity of α-fucosidase Gb. A reaction mixture containing Gal2Fuc2GN2M3FX (5.6 pmol) and α-fucosidase Gb (0.013 μU) was incubated in Na-acetate buffer (pH 5.0) at various temperatures (20, 30, 40, 50, 60, and 70℃).

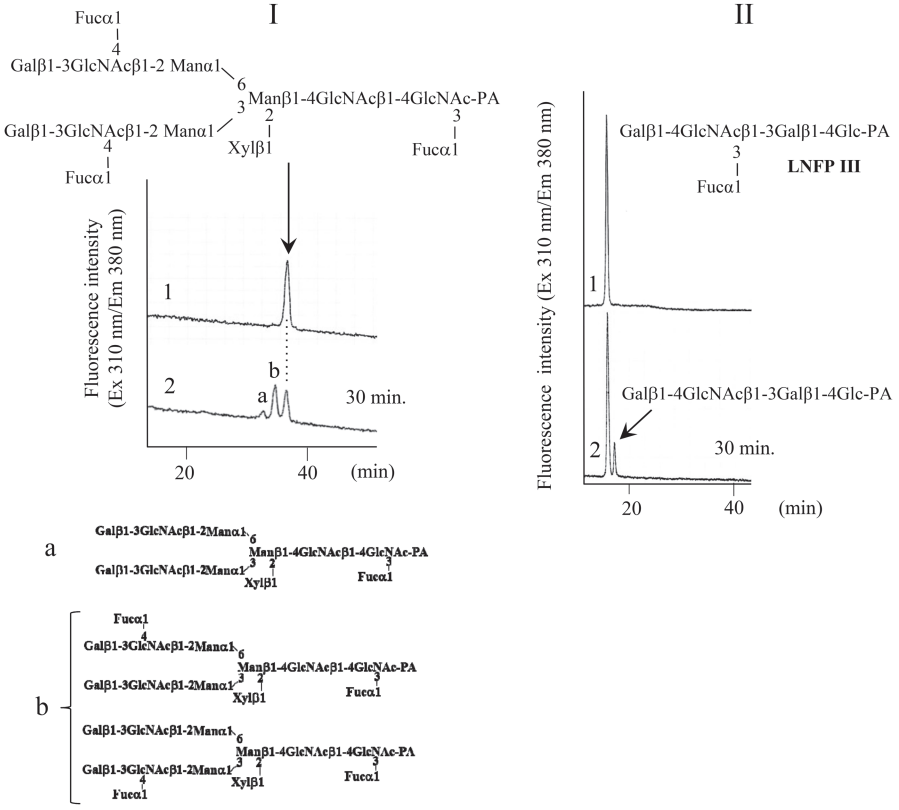

Fig. 4 SF-HPLC profile of Gal2Fuc2GN2M3FX-PA and oligosaccharides, LNFP III, incubated with α-fucosidase Gb
I. A reaction mixture containing Gal2Fuc2GN2M3FX (approximately 20 pmol) and α-fucosidase Gb (0.014 μU) was incubated in Na-acetate buffer (pH 5.0) at 37℃ for 30 min and analyzed by SF-HPLC. II. LNFP III (approximately 20 pmol) was incubated under the conditions described above and the reaction mixture was analyzed by SF-HPLC.

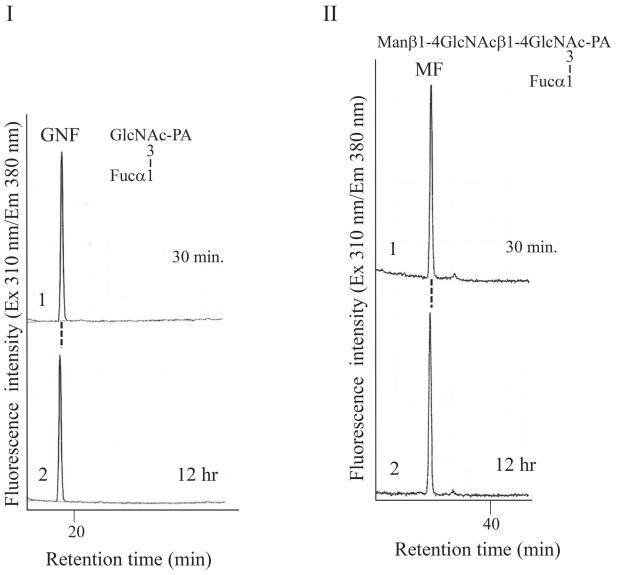

Fig. 5 RP-HPLC profile of MF and GNF incubated with α-fucosidase Gb for 2hrs
I. A reaction mixture containing MF (approximately 20 pmol) and α-fucosidase Gb (0.014 μU) was incubated in Na-acetate buffer (pH 5.0) at 37℃ for 2 hr and analyzed by RP-HPLC on a Cosmosil 5C18-AR column (6.0×250 mm). II. GNF (approximately 20 pmol) was incubated under the conditions described above and the reaction mixture was analyzed by RF-HPLC.

reducing conditions, as shown in Fig. 2, the purified α-fucosidase (α-fucosidase Gb) gave a single band with a corresponding molecular mass of 120 kDa. These results suggest that α-fucosidase Gb functions as a monomeric protein. Since it has been reported that the molecular masses of plant GH29 α-fucosidases from *Arabidopsis thaliana* (AtFUC1)[13], almond[13], and rice (α-Fuc'ase Os-1)[14] are approximately 55 kDa, the *Ginkgo* α-fucosidase purified in this study appears to be a different family from the plant GH29 α-fucosidases based on its molecular size. However, the *N*-terminal amino acid sequence could not be identified, probably due to a chemical modification, and at this point, it is unclear whether α-fucosidase Gb has any sequence homology with other plant α-fucosidases belonging to the GH29 family.

Effects of pH and temperature on α-fucosidase Gb *activity*

When α-fucosidase Gb was incubated with Gal2Fuc2-GN2M3FX at various pHs, the maximum activity was obtained at pH 5.5 (Fig. 3-I), suggesting that this *Ginkgo* α-fucosidase functions in a weak acidic environment such as the vacuole or cell wall, similar to other plant GH29 α-fucosidases[5-10]. Optimum activity was obtained

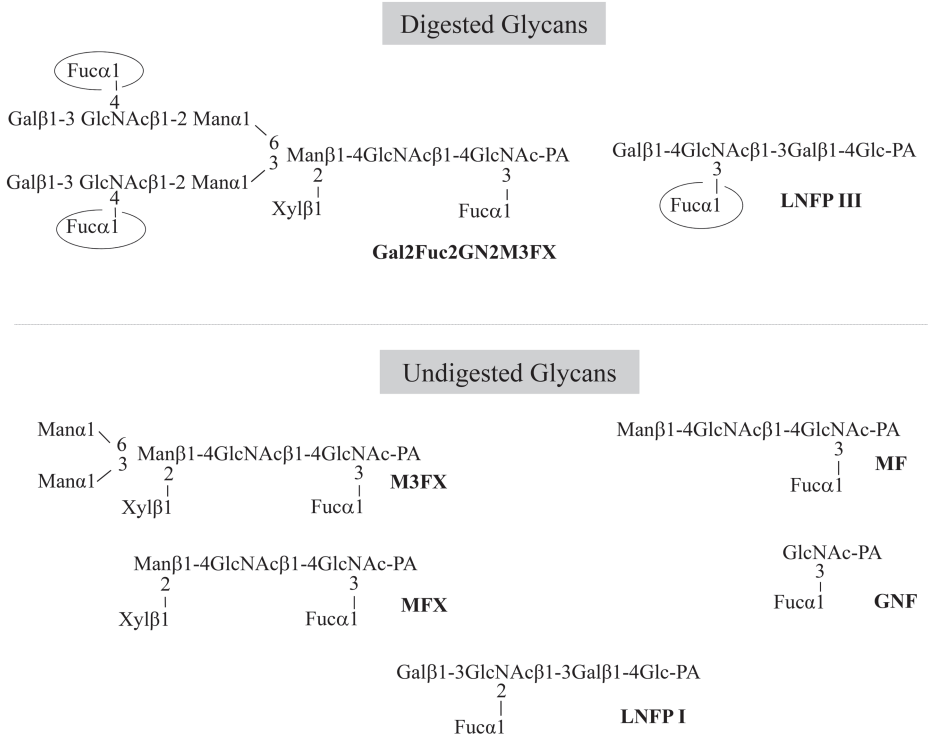

Fig. 6 Summary of reactivity of α-fucosidase Gb toward α-fucosylated glycans
L-Fucose residues susceptible to α-fucosidase Gb (0.014 μU) are circled. "*Undigested Glycans*" means that the enzymatic products were not found by HPLC-analysis after 2 hr incubation in Na-acetate buffer (pH 5.0) at 37℃.

at approximately 50℃ as shown in Fig. 3-II.

***Substrate specificity of* α-fucosidase Gb**

The substrate specificity of α-fucosidase Gb was analyzed with various plant complex type PA-sugar chains. As shown in Figs. 4, 5, and 6, α-fucosidase Gb possessed significant activity towards the α1,3/4-fucosyl linkages in the Lewis a epitope of Gal2F2GN2M3FX and LNFP III but not the α1-3 fucosyl linkages in M3FX, MFX or the α1,2-fucosyl linkage in LNFP I. Furthermore, MF and GNF were not hydrolyzed by this α-fucosidase Gb even after 2 hr-, 12 hr-incubation, while the α1,3-fucosyl residue of LNFP-Ⅲ was substantially digested. In this study, we could not prepare unlabeled oligosaccharides or glycopeptides bearing these small oligosaccharides (GlcNAcβ1-4(Fucβ1-3)GlcNAc (GN2F) or GNF), and it remains obscure whether this α-fucosidase Gb is active toward glycopeptides such as GN2F-Asn or GNF-Asn. Based on these results, we concluded that the substrate specificity of α-fucosidase Gb must be very similar to those of plant GH29 α-fucosidases[9, 10]. Ashida et al[20]. and Sakurama *et al*[21]. reported that a microbial α-fucosidase, *B. thetaiotaomicron* GH29-B enzyme (BT_2192), required a β-Gal residue adjacent to an α1,3-fucosyl residue in Lewis a epitopes or in LNFP-III. In the case of BT_2192, it has also been found that a Gal-binding pocket consisting of W230, E254, and D277 plays a critical role in the hydrolytic activity toward the Lewis a epitope. Considering the substrate specificities of α-fucosidase Gb together with plant GH29 α-fucosidases[9, 10] and *B. thetaiotaomicron* GH29-B enzyme[20, 21], the catalytic mechanism of these α-fucosidases seem to be similar, but it is unclear at this time whether plant GH29 α-fucosidases (AtFuc1 or α-Fuc'ase Os-1) and α-fucosidase Gb are involved in the hydrolysis of the core α1,3-fucosyl residue in plant *N*-glycans.

As a conclusion, in this study we found a high-molecular weight plant α-fucosidase with activity against α1,3/4-fucosyl residues in plant *N*-glycans and LNFP-Ⅲ. It is now necessary to identify the mRNA/amino acid sequence of this α-fucosidase Gb, along with its structure, in order to clarify its catalytic mechanism.

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Abbreviations: PA, pyridylamino; PCT, plant complex type; SF-HPLC, size-fractionation HPLC; RP-HPLC, reverse-phase HPLC; Le[a]; Lewis a, Fuc, L-fucose; Man, D-mannose; Gal, D-galactose; Xyl, D-xylose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; GNF, Fucα1-3GlcNAc-PA; MF, Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA: MFX, Xylβ1-2Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA: M3FX, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; Gal2Fuc2GN2M3FX, Galβ1-3(Fucα1-3)GlcNAcβ1-2Manα1-6[Galβ1-3(Fucα1-3)GlcNAcβ1-2Manα1-3](Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA; LNFP-I: Lacto-*N*-fucopentaose-I, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA; LNFP-III; Lacto-*N*-fucopentaose-III, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-PA.

ルイス a 抗原含有の植物複合型糖鎖に活性を示す銀杏種子由来の α-フコシダーゼ：精製と性質検討

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銀杏種子から高分子量（SDS-PAGE で120 kDa）を有し，α-フコース含有オリゴ糖に活性を示すα-フコシダーゼ（α-fucosidase Gb）を均一に精製した．ルイス a エピトープ含有 *N*-グリカンを基質とした場合，α-fucosidase Gb の至適 pH は 5.5 付近であることから，本酵素は液胞のような酸性環境で機能していることが示唆された．*N*-末端アミノ酸配列が化学修飾のため同定できなかったため，本酵素が GH29 ファミリーに属するかどうかは不明である．α-Fucosidase Gb は，Lacto-*N*-fucopentaose III の α1,3-フコース残基やルイス a エピトープ含有の植物複合型*N*-グリカンのα1,4-フコース残基を加水分解することから，α-フコース含有オリゴ糖や*N*型糖タンパク質の分解プロセスに関与することが示唆された．

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