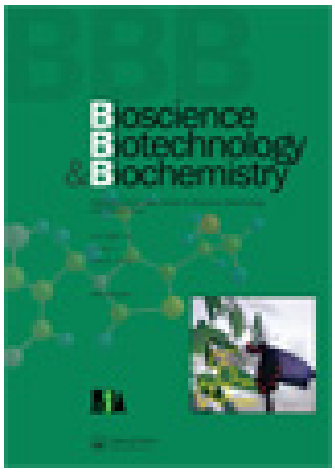



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# β-Galactosidase from Ginkgo biloba seeds active against β-galactose-containing N-glycans: purification and characterization

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# β-Galactosidase from *Ginkgo biloba* seeds active against β-galactose-containing *N*-glycans: purification and characterization

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**In this study, we purified an acidic β-galactosidase to homogeneity from *Ginkgo biloba* seeds (β-Gal'ase Gb-1) with approximately 270-fold purification. A molecular mass of the purified β-Gal'ase Gb-1 was estimated about 35 kDa by gel filtration and 32 kDa by SDS-PAGE under non-reducing condition, respectively. On the other hand, β-Gal'ase Gb-1 produced a single band with a molecular mass of 16 kDa by SDS-PAGE under reducing condition. The *N*-terminal amino acid sequences of 32 kDa and 16 kDa molecules were the same and identified as H-K-A-N-X-V-T-V-A-F-V-M-T-Q-H-, suggesting that β-Gal'ase Gb-1 may function as a homodimeric structure *in vivo*. When complex-type *N*-glycans containing β-galactosyl residues were used as substrates, β-Gal'ase Gb-1 showed substantial activity for β1-4 galactosyl residue and modest activity for β1-3 galactosyl residue with an optimum pH near 5.0. Based on these results, the involvement of β-Gal'ase Gb-1 in the degradation of plant complex-type *N*-glycans is discussed.**

**Key words:** β-galactosidase; plant *N*-glycan; *N*-glycan degradation; glycosidase; *Ginkgo biloba*

One of the structural features of plant *N*-glycosylation is the occurrence of a Lewis a epitope (Galβ1-3(Fucα1-4)GlcNAcβ1-).[1,2] In sharp contrast to the occurrence of β1-4 galactosyl residues in animal complex-type *N*-glycans, plant *N*-glycoproteins, especially secreted-type glycoproteins, often carry a β1-3 galactosyl residue in the Lewis a epitope; however, the physiological significance of the Lewis a epitope for plant development or differentiation remains to be elucidated. Although the β1,3-galactosyltransferase (β1-3GalT) gene responsible for the biosynthesis of the Lewis a epitope unit in plant *N*-glycans has been identified,[3] the plant β-galactosidase responsible for the degradation of plant complex-type *N*-glycans harboring the Lewis a epitope remains to be identified. As for the physiological function(s) of the Lewis a epitope in plant *N*-glycans, *Arabidopsis thaliana* with an inactivated β1-3GalT gene was reported to show normal morphology and growth under normal culture conditions, suggesting that the plant specific β1-3 galactosyl residue may not be essential for plant growth or development.[3] On the other hand, the physiological significance of β-galactosidase activity, which is associated with the turnover or rapid clearance of plant complex-type *N*-glycans, has not been reported to date.

Most of plant β-galactosidases (EC 3.2.1.23) belong to the glycoside hydrolase (GH) family 35, and are characterized by its ability to hydrolyze β-galactosyl residues from the non-reducing terminal β-galactosides.[4] In higher plants, β-galactosidases are mainly considered to be involved in the release of galactosyl residues from cell wall polysaccharides in an endo- or exo-cleavage manner.[5] The ability of β-Gal'ase to release galactosyl residues from cell wall components and other galactosylated glycoconjugates, such as galactolipids or glycoproteins, has led to the hypothesis that β-Gal'ase must be involved in plant growth (cell expansion), senescence, and fruit ripening[6–8] For example, in the case of tomato (*Lycopersicon esculentum*), β-galactosidase/exo β1-4 galactanase activity significantly increases during fruit ripening due to specific expression of the enzyme proteins at this stage, indicating that they play a role in the degradation of β1-4 galactan side chains of pectins as part of the ripening process.[5,9,10] Some other β-galactosidase activities toward β1-4 galactosyl[11–13] or β1-3/1-6 galactosyl linkages[14] of cell wall polysaccharides have been reported. On the other hand, the activity or expression level of the second class of β-galactosidase (active only on *p*NP-β-D-Gal) does not markedly change during

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*Abbreviations*: β-Gal'ase, β-galactosidase; β-Gal'ase Gb, β-galactosidase from *Ginkgo biloba*; PA, pyridylamino; SF-HPLC, size-fractionation HPLC; Man, D-mannose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; (Galβ1-4)2GN2M3, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; (Galβ1-3)2GalN2GN2M3, Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA; (Galβ1-3)2GN2M3FX, Galβ1-3GlcNAcβ1-2Manα1-6(Galβ1-3GlcNAcβ1-2Manα1-3)(Xylβ1-2) Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA.

ripening,[5,15,16] and thus their functions in plant growth and development remain elusive. From the view point of turnover mechanisms of the cell wall associated with plant differentiation or fruit ripening, many plant β-Gal'ases involved in the degradation of cell wall polysaccharide components from many types of plant materials (tomato,[5,9,10] carambola,[16] chick-pea,[17] Japanese pear,[18] pepper,[19] mango,[20] rice,[21] *A. thaliana*,[22] strawberry,[23] and orange[24]) have been characterized, and their genetic identifications have been reported.

In addition to the well-characterized activity of plant β-Gal'ases on cell wall polysaccharide metabolism, it appears that β-Gal'ase must also be involved in the turnover of *N*- and *O*-glycoproteins (*N*-glycans and *O*-glycans linked to secreted- or vacuolar-type proteins) and other β-galactosylated glycoconjugates.[25] However, the genes encoding the β-galactosidase that is specifically involved in the degradation of plant *N*-glycans have not been identified to date. Although many putative genes of the β-galactosidase family have been reported, including those in tomato (at least 7 members), rice (15 members), and *Arabidopsis* (17 members),[9,12,26,27] it seems to be difficult to identify the one or two β-galactosidase genes that are responsible for the degradation of plant complex-type *N*-glycans based on *in silico* analyses.

In our previous report,[28] we purified and characterized a β-xylosidase from *Ginkgo biloba* seeds (β-Xyl'ase Gb-1), which hydrolyzed the β1-2 xylose linkage in truncated-type plant *N*-glycan ($Xyl_1Man_1Fuc_1GlcNAc_2$ or $Xyl_1Man_1GlcNAc_2$). In the course of purification of β-Xyl'ase Gb, we screened several exoglycosidase activities (α-fucosidase, α-mannosidase,[29] and β-galactosidase) and confirmed that *G. biloba* seeds exhibited some of β-Gal'ase's activities, suggesting that *Ginkgo* seed would be a good material to purify or identify the β-Gal'ase(s) responsible for the degradation of β-galactosylated *N*-glycans. Furthermore, Wilson et al., reported that Lewis a epitope-containing *N*-glycans occur on glycoproteins expressed in *G. biloba* seeds,[30] although we did not detect the Lewis a epitope-containing *N*-glycans from soluble glycoproteins in the seeds.[31,32] These results suggest that β-galactosylation and α-fucosylation of *N*-glycans occur in *Ginkgo* seeds and the complex-type *N*-glycans bearing the Lewis a epitope formed in Golgi apparatus may be trimmed by β-Gal'ase and α-fucosidase in the vacuole or protein body. Therefore, in this study, we used the seeds of *G. biloba* to purify and characterize the β-Gal'ase involved in the degradation of plant complex-type *N*-glycans. Purified β-Gal'ase Gb-1 showed significant activity against animal complex-type *N*-glycans bearing a β1-4 galactosyl residue and modest activity against plant complex-type *N*-glycans bearing a β1-3 galactosyl residue, indicating that β-Gal'ase Gb-1 could be involved in the turnover of β1-3 galactosylated *N*-glycoproteins in plants.

## Materials and methods

*Materials.* *G. biloba* seeds were collected in Inazawa, Aichi, Japan. An Asahipak NH2P-50-4E column (0.46 × 25 cm) and a Shodex IEC QA-825 column were purchased from Showa Denko (Tokyo, Japan). DEAE cellulose and Q-Sepharose were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Butyl-Toyopearl and TSK-Gel G3000SWXL (0.78 × 30 cm) were purchased from Tosoh (Tokyo, Japan). Hydroxylapatite CHT5-I (1.0 × 6.4 cm) was obtained from Bio-Rad (CA, USA). *p*NP-β-D-Gal was from Sigma Co. (St. Louis, USA). Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA ((Galβ1-4)2GN2M3) was prepared from egg yolk glycopeptide after sialidase digestion.[33] Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA ((Galβ1-3)2GalN2GN2M3) was prepared from royal jelly glycoprotein.[34] Galβ1-3GlcNAcβ1-2Manα1-6(Galβ1-3GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA ((Galβ1-3)2GN2M3FX) was prepared from rice *N*-glycans after α-fucosidase digestion.[35]

*Assay system for β-Gal'ase activity during enzyme purification.* β-Gal'ase activity was routinely measured using a synthetic substrate, *p*NP-β-D-Gal. The enzyme solution (10 μL) was added to 30 μL of 5 mM *p*NP-β-D-Gal (in 5 μL of 0.5 M glycine–HCl buffer, pH 3.5). After 2–4 h incubation at 37 °C, the reaction was stopped by adding 1.0 mL of 1.0 M glycine–NaOH buffer, pH 10.5. The released *p*-nitrophenol was measured by taking the absorbance at 420 nm. One unit of enzyme activity was defined as the amount required for releasing 1 μmol of *p*-nitrophenol per min under standard conditions.

*Assay method for pyridylaminated animal complex-type N-glycan, (Galβ1-4)2GN2M3.* Activity toward an animal complex-type PA-sugar chain, (Galβ1-4)2GN2M3, was measured in 12 μL of reaction mixture containing 1 μL substrate (5 pmol of PA-sugar chain), 1 μL of 0.5 M Na-citrate buffer (pH 5.0), and 10 μL enzyme solution. After incubation at 37 °C for 4–6 h, the enzymatic reaction was stopped by heating in the boiling water for 5 min, and following centrifugation, an aliquot (10 μL) of the reaction mixture was analyzed by size-fractionation (SF-) HPLC as described in our previous papers.[36]

*Purification of β-Gal'ase Gb-1.* Unless stated otherwise, protein purification steps were carried out at 4 °C. During the purification steps, *p*NP-β-D-Gal was mainly used as the substrate for detecting the β-Gal'ase activity, and (Galβ1-4)2GN2M3 was used to confirm the β-Gal'ase activity toward *N*-glycan. The protein concentration was determined by measuring the absorbance at 280 nm with bovine serum albumin (BSA) as the standard.

*Step 1. Preparation of crude β-Gal'ase Gb-1.* Mature seeds of *G. biloba* (2.8 kg) were homogenized in acetone and the resulting defatted powder (1.5 kg) was

suspended in 5 L of 50 mM Tris–HCl buffer (pH 7.9) containing 0.5 M NaCl and left at 4 °C overnight. The mixture was then squeezed through two layers of gauze and centrifuged at 8000 × *g* for 30 min. The supernatant was saturated with 100% ammonium sulfate. The precipitate was collected after centrifugation and dialyzed against deionized water, and the resulting supernatant was used as a crude soluble β-Gal'ase Gb fraction.

*Step 2. DEAE-cellulose column chromatography (Stepwise method).* The crude enzyme dialyzed against 20 mM Tris–HCl buffer (pH 8.0) was loaded onto a DEAE cellulose column (4 × 48 cm) previously equilibrated with the same buffer. After washing the column with the same buffer, proteins bound to the resin were eluted by addition of the same buffer containing 0.1, 0.2, 0.3, and 0.5 NaCl in a stepwise manner. The β-Gal'ase Gb fractions eluted in the 20 mM Tris–HCl buffer (pH 7.9) containing 0.1 M NaCl were pooled and 100% saturated with ammonium sulfate.

*Step 3. DEAE-cellulose column chromatography (Linear gradient method).* The precipitate obtained in Step 2 was dialyzed against 20 mM Tris–HCl buffer (pH 8.0) and was loaded again onto a DEAE cellulose column (4 × 48 cm) previously equilibrated with the same buffer. After washing the column with the same buffer, the absorbed enzyme was eluted with a linear gradient (0–0.3 M) of NaCl in the same buffer (Fig. 1-I). The β-Gal'ase Gb active fractions were pooled and saturated with 100% ammonium sulfate.

*Step 4. QAE-Toyopearl column chromatography.* The precipitate obtained in Step 3 was dissolved in the same buffer (20 mM Tris–HCl, pH 8.0) and loaded onto the QAE-Toyopearl column previously equilibrated with the same buffer. After washing the column with the same buffer, the adsorbed protein was eluted with a linear gradient (0–0.3 M) of NaCl in the same buffer. As shown in Fig. 1-II, two β-Gal'ase Gb activities (F-1 and F-2) against *p*NP-β-D-Gal were separated, but only the former fraction (F-1) showed significant activity against the complex-type PA-sugar chain, (Galβ1-4)2GN2M3. Hence, F-1 was used for further purification of a β-Gal'ase Gb-1 that is active against β-galactosylated *N*-glycans.

*Step 5. Butyl-Toyopearl column chromatography (hydrophobic interaction chromatography).* The precipitate obtained in Step 4 was dissolved in 10 mM Tris–HCl buffer, pH 8.0, and dialyzed against the same buffer. Solid ammonium sulfate was added to obtain a final concentration of 1.5 M. The resulting enzyme solution was loaded onto the Butyl-Toyopearl column (2.5 × 45 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 gradient (1.5–0 M) of ammonium sulfate in the same buffer. The β-Gal'ase Gb-1 active fractions (horizontal bar in Fig. 1-III) were collected and saturated with ammonium sulfate.

*Step 6. QA-825 HPLC.* *Ginkgo* β-Gal'ase Gb-1 was further purified with a Jasco 880-PU HPLC apparatus equipped with a Shodex IEC QA-825 column (0.8 × 7.5 cm). The β-Gal'ase Gb-1 fraction obtained in Step 5 was dialyzed against 20 mM Tris–HCl buffer (pH 8.0) and applied to an anion exchange column (QA-825) previously equilibrated with the same buffer. The absorbed proteins were eluted by a linear gradient of NaCl from 0 to 0.3 M in the same buffer at a flow rate of 1.0 mL/min. The β-Gal'ase Gb-1 fractions (horizontal bar in Fig. 1-IV) were pooled and saturated with ammonium sulfate.

*Step 7. Hydroxyapatite HPLC.* The precipitate obtained in Step 6 was dissolved in 10 mM sodium phosphate buffer, pH 6.8, and applied to the hydroxyapatite column (CHT5-I, 1.0 × 6.4 cm) previously equilibrated with the same buffer. After washing the column, bound proteins were eluted using a linear gradient of sodium phosphate buffer (pH 6.8) from 10 to 500 mM. The β-Gal'ase Gb-1 active fractions (horizontal bar in Fig. 1-V) were pooled and concentrated using a Corning® Spin-X® UF 10 MW cut-off membrane filter at 3,000 rpm for 1 h.

*Step 8. Gel filtration through TSK-Gel G3000SWXL.* Final purification of β-Gal'ase Gb-1 was carried out using a Jasco 880-PU HPLC apparatus with a Jasco Intelligent UV/VIS detector (870 UV) and the TSK-Gel G3000SWXL column (0.78 × 60 cm). The concentrated enzyme obtained in Step 7 was dialyzed against 100 mM Na-phosphate buffer (pH 6.8) containing 100 mM Na-sulfate, and loaded onto the column previously equilibrated with the same buffer at a flow rate of 0.5 mL/min (Fig. 2-I).

*SDS-PAGE and blotting of purified β-Gal'ase Gb-1.* SDS-PAGE was carried out according to the method described by Laemmli et al.[37] on 12.5% acrylamide gel in 0.1 M Tris–glycine buffer using a slab gel apparatus under reducing condition with 1% 2-methanol or non-reducing condition. Precision Plus Protein™ Standards (Bio-Rad) were used as the marker proteins for molecular mass determination (250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa). The proteins on the gel were transferred to PVDF membranes (Sequi-Blot™; Bio-Rad; CA, USA) for 30 min at 120 mA on a Horize-blot system (AE-6675 P/N; Atto Co.) using 0.1 M Tris–glycine containing 5% 2-methanol (pH 8.8) as the transfer buffer. The membrane was stained with Coomassie Brilliant Blue R-250, and used for the analysis of N-terminal amino acid sequence.

*Effect of pH and temperature on the activity of β-Gal'ase Gb-1.* The optimum pH against *p*NP-β-D-Gal and (Galβ1-4)2GN2M3 was determined by incubating the enzyme in varying buffers ranging from

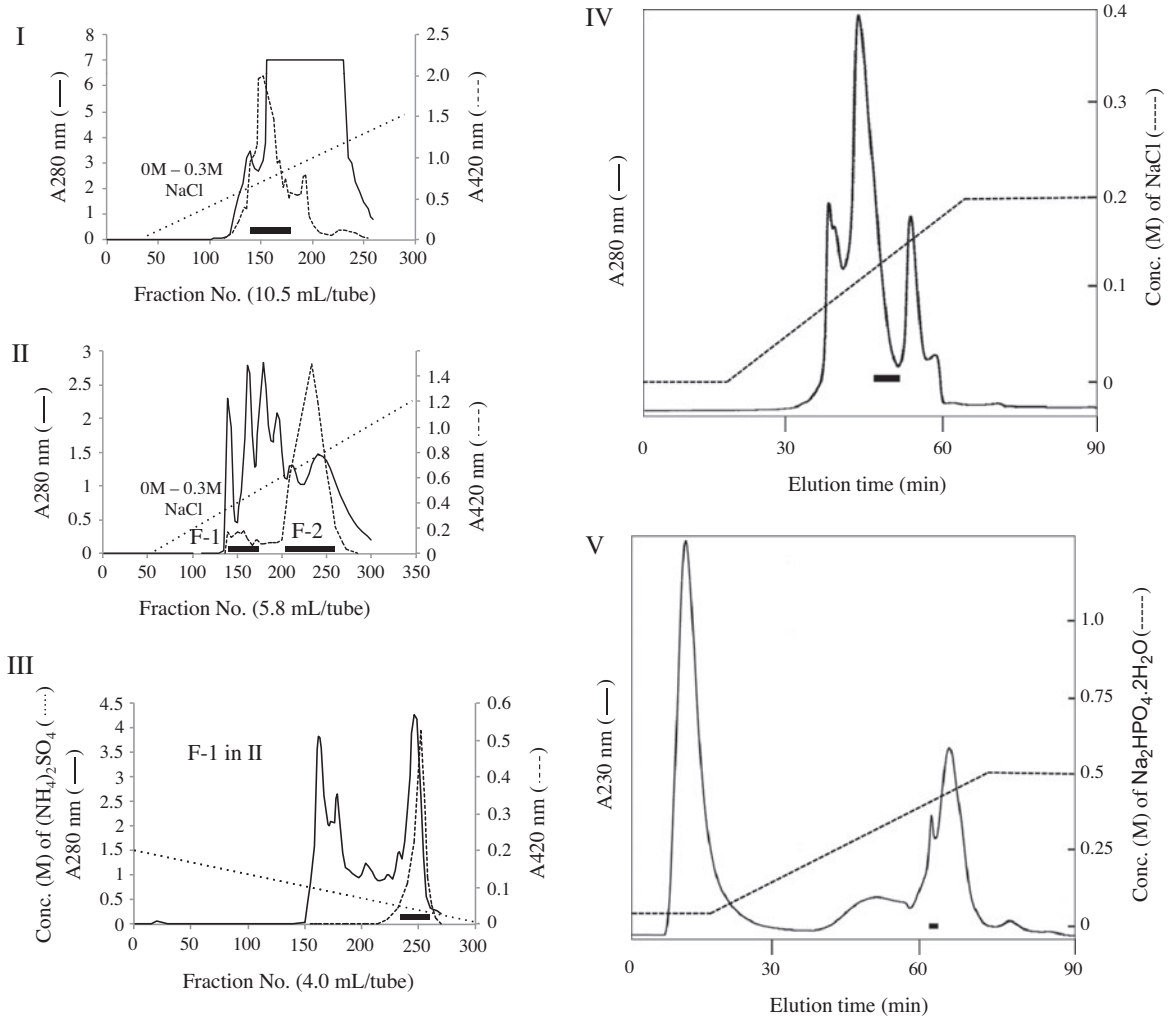


Fig. 1. Purification Profiles of β-Gal'ase Gb-1.

Note: I, DEAE Cellulose chromatography. Proteins were eluted by linear gradient of NaCl from 0 to 0.3 M in the 20 mM Tris–HCl buffer (pH 8.0). The protein fractions indicated by horizontal bar were pooled for further steps. II, QAE-Toyopearl chromatography. Proteins were pooled by linear gradient of NaCl from 0 to 0.3 M in the 20 mM Tris–HCl buffer (pH 8.0). Although two major enzyme fractions were obtained, the first fractions, indicated by horizontal bar, were used in this study based on significant activity towards the animal complex type *N*-glycan (Galβ1-4) 2GN2M3-PA). III, Butyl-Toyopearl chromatography. Proteins were eluted by linear gradient of ammonium sulfate from 1.5 to 0 M in the same buffer. The protein fractions indicated by horizontal bar were pooled for further steps. IV, QA-825 HPLC. Proteins were eluted by linear gradient of NaCl from 0 to 0.2 M in the 20 mM Tris–HCl buffer (pH 8.0). V, Hydroxyapatite HPLC. Proteins were eluted by linear gradient of Na-phosphate buffer from 0.01 to 0.5 M. Active Protein fractions were pooled for final purification as indicated by horizontal bar.



pH 2.0 to 8.0 (pH 3.0 for glycine–HCl, 3.5–6.0 for citrate-NaOH, 6.0–7.0 for MES-NaOH, and 8.0 for Tris–HCl) at 37 °C for 30 min for *p*NP-β-D-Gal and 2 h for (Galβ1-4)2GN2M3. The effects of temperature were examined at 8 different temperatures (20, 30, 37, 40, 50, 60, 70, and 80 °C) using both *p*NP-β-D-Gal and Gal(β1-4)2GN2M3.

*Effects of divalent metal ions on the activity of β-Gal'ase Gb-1.* The effects of metal ions on the activity of *β-Gal'ase Gb-1* were examined using $FeCl_2$, $MnCl_2$, $CoCl_2$, $CaCl_2$, $ZnCl_2$, $CuSO_4$, $MgCl_2$, and EDTA at a final concentration of 5 mM. After pre-incubation of various metal ions with purified *β-Gal'ase Gb-1* (in glycine–HCl buffer, pH 3.5), the reaction mixture was further incubated with *p*NP-β-D-Gal.

*Substrate specificity of β-Gal'ase Gb-1.* The substrate specificity of purified β-Gal'ase Gb-1was analyzed using authentic PA-sugar chains: (Galβ1-4) 2GN2M3, (Galβ1-3)2GalN2GN2M3, and (Galβ1-3) 2GN2M3FX. PA-sugar chains (approximately 50 pmol) were incubated with purified β-Gal'ase Gb-1 in 0.1 M Na-acetate buffer (pH 5.0) at 37 °C for 2 h (10 μL of total reaction mixture). The PA-sugar chains incubated with the purified β-Gal'ase Gb-1 were analyzed by SF-HPLC using a Shodex Asahipak NH2P-50-4E column (0.46 × 25 cm). The PA-sugar chain mixture was eluted by increasing the water content in water–acetonitrile from 20 to 80% linearly over 55 min at a flow rate of 0.7 mL/min. PA-sugar chains were detected with a Jasco FP-920 Intelligent Fluorescence detector (excitation at 310 nm, emission at 380 nm).

## Results and discussion

*Purification of β-Gal'ase Gb-1*

A summary of the purification of β-Gal'ase Gb-1 is shown in Table 1. The β-Gal'ase Gb-1 was purified up

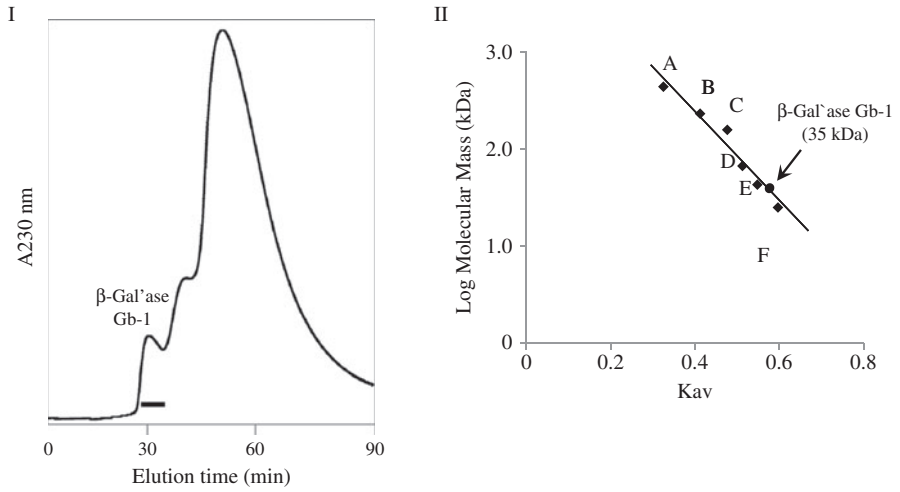


Fig. 2. Gel-Filtration and SDS-PAGE of β-Gal'ase Gb-1.

Note: I, Gel filtration profile on a TSK-Gel G3000SWXL column (0.78 × 60 cm). The column was developed with 0.1 M Na-phosphate buffer (pH 6.8) containing 0.1 M $Na_2SO_4$ at a flow rate of 0.5 mL/min. II. Estimation of molecular mass of β-Gal'ase Gb-1 by gel filtration. The marker proteins used were as follows: (A) ferritin (440 kDa); (B) catalase (232 kDa); (C) aldolase (158 kDa); (D) BSA (67 kDa); (E) ovalbumin (43 kDa); (F) chymotripsinogen A (25 kDa). ♦, marker proteins; ●, β-Gal'ase Gb-1.



to approximately 270-fold. Although the yield of purified enzyme seems to be very low (0.17%), this low yield might result from the overestimated protein amount at early stages (crude extract and DEAE chromatography) because of large contamination of non-protein components bearing ultraviolet absorption. In fact, twice of DEAE chromatography was very effective to exclude non-protein components for the following column chromatography. Elution profiles for each purification step of β-Gal'ase Gb-1 are shown in Fig. 1. In Step 2 (QAE-Toyopearl), as shown in Fig. 1-II, two activities against *p*NP-β-D-Gal were separated, but only the former fraction (F-1) showed significant activity against the pyridylaminated *N*-glycan, (Galβ1-4)2GN2M3. In this study, we used F-1 for the subsequent purification steps, and further purification and characterization of β-Gal'ase in F-2 will be described elsewhere. After the hydroxyapatite chromatography (Fig. 1-V), the β-Gal'ase was finally purified using gel filtration with a TSK G3000SWXL column (Fig. 2-I). The purified β-Gal'ase Gb-1 showed hydrolytic activities against both *p*NP-β-D-Gal and (Galβ1-4)2GN2M3.

*Molecular mass and subunits*

The apparent native molecular size of β-Gal'ase Gb-1 as estimated by gel filtration using a TSK Gel G3000SWXL column was about 35 kDa (Fig. 2-I and -II). In contrast, when the purity of β-Gal'ase Gb-1 was checked by SDS-PAGE on a 12.5% gel under non-reducing and reducing conditions, the purified β-Gal'ase Gb-1 gave a single band with a corresponding molecular mass of approximately 32 kDa under the non-reducing condition (Fig. 3, L1) and a single band with a corresponding molecular mass of 16 kDa under the reducing condition (Fig. 3, L2). These results suggest that the native protein may function as a homodimeric structure *in vivo*.

*Effect of pH and temperature*

When β-Gal'ase Gb-1 was incubated with (Galβ1-4)2GN2M3 or *p*NP-β-D-Gal as substrates at various pHs, the maximum activity was obtained at pH 5.0 for (Galβ1-4)2GN2M3 as a pseudo-natural substrate, but at pH 3.5 for *p*NP-β-D-Gal as a synthetic substrate (Fig. 4-I and -II). However, the activity markedly decreased at near-neutral pH, and only approximately 15% of full enzyme activity was observed at pH 8.0. Thus, β-Gal'ase Gb-1 may reside in the acidic region of plant cells, either in the cell wall or in vacuoles. β-Gal'ases isolated from other plant sources, such as carambola fruit,[18] mung bean,[38] mango fruit,[20] and kidney bean,[39] were also reported to be optimally active

Table 1. Summary of purification of β-Gal'ase Gb-1.

| Purification step | Total protein (mg) | Total activity (unit)[a] | Specific activity (unit/mg) | Recovery (%) | Purification fold[b] |
|---|---|---|---|---|---|
| Crude enzyme | 30338 | 20443.4 | 0.67 | 100 | 1 |
| DEAE cellulose (Batch method) | 11536.8 | 10127.2 | 0.88 | 49.5 | 1.3 |
| DEAE cellulose (Linear gradient) | 1222.2 | 4689.1 | 3.84 | 22.9 | 5.7 |
| QAE-Toyopearl | 175.45 | 959.7 | 5.47 | 4.7 | 8.1 |
| Butyl-Toyopearl | 23.3 | 226.5 | 9.72 | 1.1 | 14.4 |
| QA-825 HPLC | 6.85 | 102.8 | 15 | 0.5 | 22.3 |
| Hydroxyapatite HPLC | 1.4 | 37 | 26.5 | 0.18 | 39.3 |
| Gel filtration HPLC | 0.2 | 36 | 182.5 | 0.17 | 270.8 |

[a]Enzyme activity was determined using *p*NP-β-D-galactopyranoside. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min at 37 °C.

[b]Fold purification was calculated with respect to the specific activity of the crude enzyme.

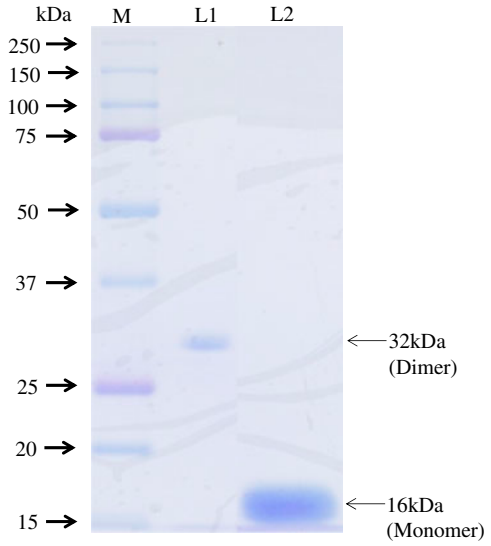


Fig. 3. SDS-PAGE of purified β-Gal'ase Gb-1.

Note: Protein subunits were separated by SDS-PAGE using 12.5% polyacrylamide gel and stained with Coomassie brilliant blue R-250. M, Marker proteins (precision plus protein™ standard, Bio-Rad, 250–15 kDa). L1, β-Gal'ase Gb-1 with 2-mercaptoethanol. L2, β-Gal'ase Gb-1 without 2-mercaptoethanol.

at acidic pH. Optimum activity for both *p*NP-β-D-Gal and (Galβ1-4)2GN2M3 was obtained at approximately 60 °C (data not shown). This optimum temperature is consistent with previously reported values for mung bean and kidney bean β-Gal'ases.[38,39])

*Effects of divalent metal ions*

The effects of various metal ions on β-Gal'ase activity were studied by incubating the purified enzyme with the synthetic (*p*NP-β-D-Gal) substrate in the presence of metal ions at a final concentration of 5.0 mM. $Fe^{2+}$ and $Cu^{2+}$ significantly inhibited β-Gal'ase Gb-1 activity (17% inhibition for $Fe^{2+}$ and 43% inhibition for $Cu^{2+}$), while other divalent metal ions ($Mg^{2+}$, $Co^{2+}$, $Ca^{2+}$, $Zn^{2+}$, and $Mn^{2+}$) and EDTA showed no significant effect on the hydrolytic activity against *p*NP-β-D-Gal (101% for $Mn^{2+}$, 98% for $Co^{2+}$, 102% for $Ca^{2+}$, 96% for $Zn^{2+}$, 96% for $Mg^{2+}$, and 103% for EDTA). These results indicated that this *Ginkgo* β-Gal'ase is not a metalloenzyme, although many plant α-mannosidases that are involved in the processing or degradation of *N*-glycans require $Ca^{2+}$ or $Zn^{2+}$ ions.

*N-terminal amino acid sequence and* $K_m$ *and* $V_{max}$ *for pNP-β-D-Gal*

After blotting the β-Gal'ase Gb-1 (32 and 16 kDa) from polyacrylamide gel onto a PVDF membrane, N-terminal amino acid sequences were analyzed by protein sequencer (SHIMADZU PPSQ-31). The *N*-terminal amino acid sequences of β-Gal'ase Gb-1 in both the reducing and non-reducing condition were identified as H-K-A-N-X-V-T-V-A-F-V-M-T-Q-H-, suggesting that β-Gal'ase Gb-1 may consist of two identical subunits. The N-terminal sequence has no significant homology with other plant β-galactosidases purified and characterized to date, but BLAST search in NCBI network

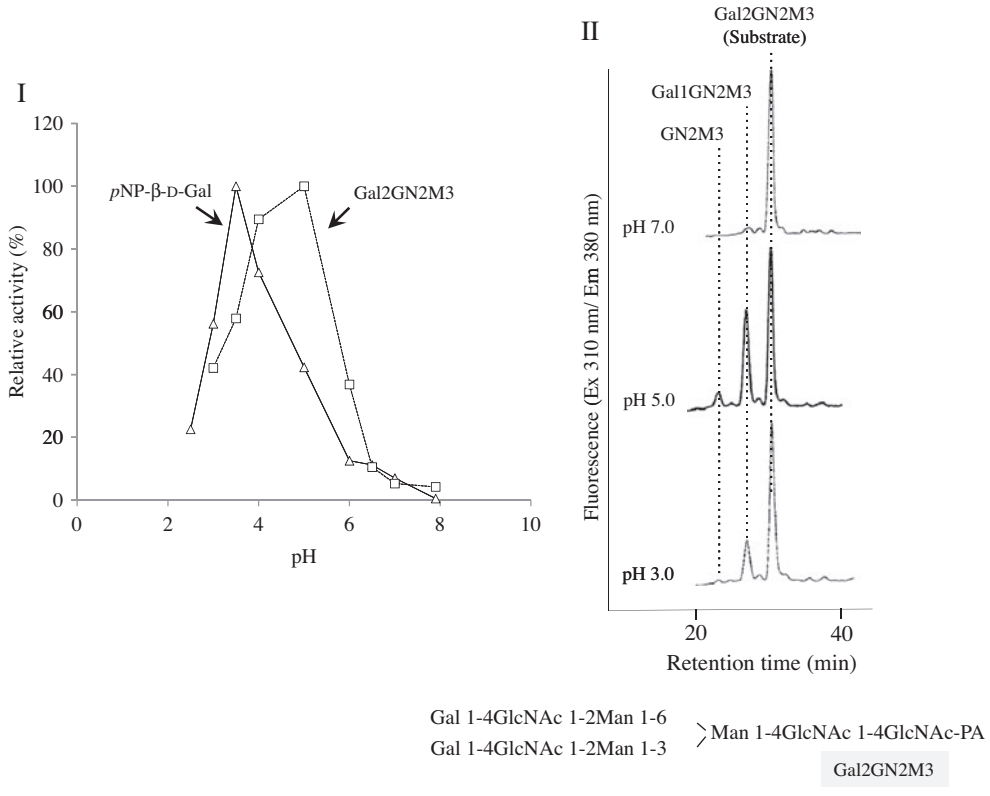


Fig. 4. Effect of pH on β-Gal'ase Gb-1 activity.

Note: The enzyme activities at pH 3.5 for *p*NP-β-D-Gal or at pH 5.0 for the animal complex-type *N*-glycan, (Galβ1-4)2GN2M3, were taken to be 100%. β-Gal'ase Gb-1 was incubated with *p*NP-β-D-Gal or with Gal2GN2M3-PA as substrates in buffers of different pHs (pH 2.5–7.9) at 37 °C for 2 h. I, When the PA-sugar chain was used as a pseudo-substrate, the products were analyzed with SF-HPLC. When *p*NP-β-D-Gal was used as a synthetic substrate, activity was calculated by measuring the concentration of released *p*-nitrophenol at 420 nm. △, *p*NP-β-D-Gal; □, complex-type *N*-glycan, (Galβ1-4)2GN2M3. II, SF-HPLC profiles of products by β-Gal'ase Gb-1 digestion of (Galβ1-4)2GN2M3 at pH 3.0, 5.0, and 7.0.

found that this N-terminal sequence has significant identity (80%) with the internal amino acid sequence of precursor of putative β-galactosidase from *Aspergillus flavus* NRRL3357 (Sequence ID:B8NKI4.2). If the precursor protein is proteolyzed or maturated by an endopeptidase in the fungus, the molecular mass of the putative fungal β-Gal'ase may become similar to that of β-Gal'ase Gb-1. The $K_m$ and $V_{max}$ values of β-Gal'ase Gb-1 for *p*NP-β-D-Gal were 1.72 mM and 4.21 μM/min/mg protein, respectively (Fig. 5).

*Substrate specificity of β-Gal'ase Gb-1*

The substrate specificity of β-Gal'ase Gb-1 was analyzed with various complex-type PA-sugar chains carrying β1-4 or β1-3 galactosyl residues at their non-reducing ends. As shown in Fig. 6, β-Gal'ase Gb-1 preferentially removed β1-4 galactosyl residues in animal complex-type *N*-glycan, (Galβ1-4)2GN2M3, whereas it removed β1-3 galactosyl residues in the insect complex-type structure, (Galβ1-3)2GalN2GN2M3, and plant complex-type structure,

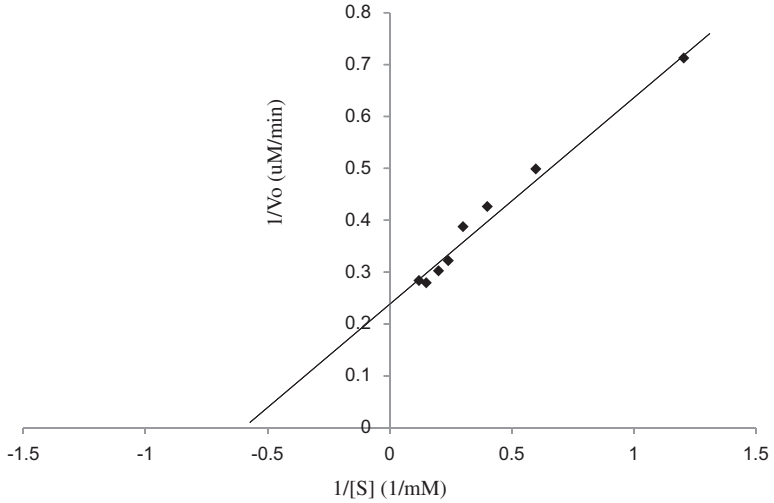


Fig. 5. Kinetic analysis of β-Gal'ase Gb-1 activity for *p*NP-β-D-Gal.

Note: The purified β-Gal'ase Gb-1 (0.75 μg) was incubated with *p*NP-β-D-Gal of various concentrations in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C for 30 min (total volume of the reaction mixture, 100 μL). Kinetic parameters were estimated from the Lineweaver–Burk plot.

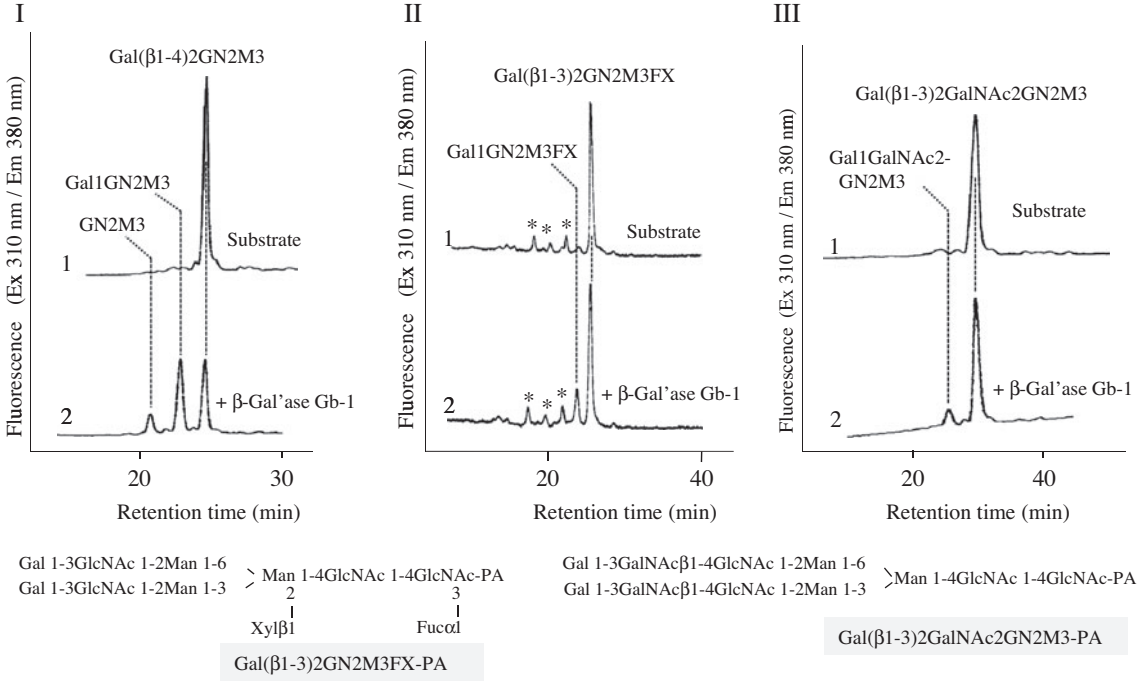


Fig. 6. Substrate specificities of β-Gal'ase Gb-1.

Note: The substrate specificities of β-Gal'ase Gb-1 were determined using authentic PA-sugar chains (~100 pmol). I, (Galβ1-4)2GN2M3 (animal complex-type structure). II, (Galβ1-3)2GN2M3 (plant complex-type structure). III, (Galβ1-3)2GalN2GN2M3 (insect complex-type structure). These β-galactose-containing *N*-glycans were incubated with β-Gal'ase Gb-1 (2.6 μg) in 0.5 M citrate buffer, pH 5.0, at 37 °C for 6 h. The resulting products were analyzed using SF-HPLC with a Shodex Asahipak NH2P-50-4E column (0.46 × 25 cm). Several peaks marked with asterisks were not *N*-glycans, but rather contaminative fluorescence substances.

(Galβ1-3)2GN2M3FX. Although this substrate specificity is similar to that of Jack bean β-Gal'ase,[40] the activity of β-Gal'ase Gb-1 against β1-3 galactosyl residues was relatively higher than that of the Jack bean enzyme (relative activity of Jack bean enzyme against Galβ1-4GlcNAc and Galβ1-3GlcNAc is 100:1.3[40]). At present, native substrates for β-Gal'ase Gb-1 remain to be identified, but the hydrolytic activity toward the Galβ1-3GlcNAc unit suggests that the plant *N*-glycans bearing the Lewis a epitope are candidate native substrates. Furthermore, it is noteworthy that de-fucosylation of the Lewis a epitope in the plant complex-type *N*-glycan is a prerequisite for β-Gal'ase Gb-1 activity to remove the β1-3 galactosyl residue, suggesting that de-fucosylation of the Lewis a epitope must be the first step for degradation of plant complex-type *N*-glycans bearing a Lewis a epitope. Sekimata et al. purified a radish β-Gal'ase that specifically hydrolyzes β1-3 and β1-6 galactosyl residues in arabinogalactan.[41] Kotake et al. identified the gene of the radish β-Gal'ase and analyzed its substrate specificity in detail.[14] The radish β-Gal'ase showed substantial activity toward β1-3 or β1-6 galacto-oligosaccharides but not Galβ1-3GlcNAc or Galβ1-3GalNAc,[14] indicting that this radish β-Gal'ase may not be involved in the turnover of plant complex-type *N*-glycans, and the physiological functions *in situ* of the radish enzyme and the β-Gal'ase Gb-1 are different from each other. β-Gal'ase Gb-1 has the hydrolytic activity against both the Galβ1-4GlcNAc and the Galβ1-3GlcNAc units, but it is obscure at the present time whether this Ginkgo enzyme has the same ability to hydrolyze β1-3 galacto-oligosaccharide. To clarify whether β-Gal'ase Gb-1 is involved in degradation both of hemicellulose components and plant complex-type *N*-glycan, detail kinetic analysis of β-Gal'ase Gb-1 with plant complex-type *N*-glycans and β1-3 galacto-oligosaccharide as substrates is required in the next step.

## Conclusion

To date, hundreds of genes encoding plant β-Gal'ase have been identified, and expression analyses of some of these genes have provided insight into the molecular mechanism of the cell wall construction or degradation involved in plant development or fruit maturation. On the other hand, the plant β-Gal'ase involved in the turnover of *N*-glycoproteins or *N*-glycans remains to be characterized. Therefore, in this study, we focused on the purification and characterization of a plant β-Gal'ase that is active against *N*-glycans bearing β1-3 galactosyl residues as a first step to understand the physiological significance of plant complex-type *N*-glycans for plant development or differentiation. Purified β-Gal'ase Gb-1, which has a homodimeric structure consisting of a 16 kDa subunit, showed substantial activity toward β1-4 galactosyl residue in animal complex-type *N*-glycan and modest activity toward β1-3 galactosyl residue in plant complex-type *N*-glycans with optimum pH at 5.0, suggesting an involvement of this β-Gal'ase Gb-1 in the turnover of *N*-glycoproteins, *N*-glycopeptides, or free *N*-glycans. This substrate specificity is very similar to that of Jack bean β-Gal'ase, although the N-terminal amino acid sequence of β-Gal'ase Gb-1 shows very little homology with other plant β-Gal'ases reported to date. To confirm the ubiquitous occurrences of an orthologous gene of β-Gal'ase Gb-1 in other higher plants requires molecular identification of the gene based on information of internal amino acid sequences, and these studies are currently in progress. Furthermore, given the molecular masses (around 100 kDa in average) of some plant GH35 family β-Gal'ase consisting of about 800 up to 1,000 amino acid residues, it may be possible to assume that the β-Gal'ase Gb-1 (16 kDa) purified in this study may be the smallest GH35 family β-Gal'ase, suggesting that this *Ginkgo* enzyme may be catalytic domain of GH35 family β-Gal'ase and the carbohydrate binding module (CBM) may be localized in the N-terminal region of other GH35 family β-Gal'ases.

## Author contribution

Y.K. shared responsibility for the writing of the manuscript with M.Z.R and M.M. All authors were responsible for the study concept and design. M.Z.R and M.M carried it out. All authors contributed to the critical revision of the manuscript.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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