The β-xylanase, which is active against plant complex type N-glycans, was purified to homogeneity from Ginkgo biloba seeds. The N-terminal amino acid sequence, G-S-A-A-G-N-R-, of the Ginkgo β-xylanase (β-Xylase Gb) was consistent with the deduced internal amino acid sequence of an Arabidopsis β-xylanase (AtBXL1). β-Xylase Gb hydrolyzed the β-1,2 xylosyl residue from Xylβ1-2Manβ1-4GlcNAcβ1-4GlcNAc-PA and Xylβ1-2Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA, but not that from Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA.

Key words: β-xylanase activity; plant N-glycan; N-glycan turnover; Ginkgo biloba

In a previous study, 1) we purified and characterized a tomato β-xylanase (β-Xylase Le1) that released a xylosyl residue from truncated type plant N-glycans. β-Xylase Le1, a monomeric enzyme, had a molecular weight of about 60 kDa and exhibited optimal activity at about pH 5.0 for fluorescence-labeled N-glycan Xylβ1-2Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (MFX), which indicates that this tomato glycosidase functions in acidic environments, such as the vacuole or cell wall. 1) Furthermore, we found that β-Xylase Le1 hydrolyzed β-1,2 xylosyl residues from Xylβ1-2Manβ1-4GlcNAcβ1-4GlcNAc-PA (MX), Xylβ1-2Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (MFX), and Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (M3FX), but not that from Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (M3FX), suggesting the possibility that the α-1,3-mannosyl residue causes serious steric hindrance of the action of β-Xylase Le1, but we could not identify the amino acid terminal sequence of β-Xylase Le1, perhaps due to a chemical modification in the N-terminus. To elucidate the physiological significance of the turnover of complex type N-glycoproteins involved in plant growth and fruit ripening, it is prerequisite to construct a transgenic plant in which expression of the β-xylanase and α-fucosidase genes, in addition to the α-Man‘ase and GlcNAc’ase genes, is suppressed. Although some plant β-xylanase genes responsible for the degradation of the hemicellulose component in cell walls have been identified, 2–5) it has not been determined whether their products are associated with the turnover of plant N-glycans. In this study, we purified a β-xylanase from another plant material, Ginkgo biloba seeds, to gain information on the partial amino acid sequences of the enzyme involved in the turnover of plant complex type N-glycans. Since we have confirmed that storage glycoproteins in Ginkgo biloba seeds carry plant complex type N-glycans exclusively, 6,7) suggesting that β-xylanase associated with N-glycan turnover occurs, we used Ginkgo seeds as starting materials.

Crude enzyme was extracted from acetone-defatted powder (908 g) of Ginkgo biloba seeds (collected in Inazawa, Aichi Prefecture, Japan) using 50 mM Tris–HCl buffer (pH 7.9), containing 0.2 M NaCl. Through all the purification steps, the activity of β-xylanase was measured using pNP-β-xylopyranoside (pNP-β-Xyl) as synthetic substrate and Xylβ1-2Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA (MFX). 1) The enzyme solution (20 μL) was added to 40 μL of pNP-β-Xyl (final concentration 5 mM) in 0.1 M Na-acetate buffer (pH 4.0), and the reaction mixture was incubated at 37 °C for 3 h. The amount of p-nitrophenol released was quantified by measuring the absorbance at 420 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min at 37 °C. When a fluorescence-labeled oligosaccharide was used as pseudo-natural substrate, the reaction mixture (30 μL), containing about 27 pmol of M3FX in 0.15 M sodium acetate buffer (pH 4.0), was incubated at 37 °C for 14 h. The products obtained were analyzed with a Jasco 880-PU HPLC apparatus with a Jasco Intelligent spectrofluorometer (Jasco, Tokyo) and a Cosmosil SC18-AR column (0.6 × 25 cm, Nacalai Tesque, Kyoto, Japan). PA-sugar chains were eluted and separated by increasing the acetonitrile concentration in 0.02% TFA.
linearly from 0 to 10% for 30 min at a flow rate of 1.0 mL/min. PA-sugar chains were detected with a Jasco FP-920 Intelligent Fluorescence detector (excitation 310 nm, emission 380 nm). \(^1\) Ginkgo \(\beta\)-xylosidase was purified by a combination of ion-exchange chromatography (Q-Sepharose and Shodex QAE column (Showa Denko, Tokyo)), hydrophobic interaction chromatography (Butyl-Toyopearl and Shodex Phenyl column (Showa Denko, Tokyo)), gel filtration by the Superdex S-200 column (Showa Denko, Tokyo, Japan), and gel filtration by the purification procedure used in our previous study. \(^1\)

Ginkgo \(\beta\)-xylosidase (\(\beta\)-Xyl'ase Gb) was purified about 160-fold to homogeneity (total units, 2.2 mU; specific activity, 37.9 mU/mg), as shown in Fig. 1A. Regarding the purification of \(\beta\)-xylosidase from tomato fruits, several different \(\beta\)-xylosidase activities were found during the purification steps, but only one significant level of \(\beta\)-xylosidase activity was found during purification steps described above. The molecular weight of the purified \(\beta\)-Xyl'ase Gb was estimated to be about 62 kDa on SDS–PAGE on a 15% gel under a reducing condition, and 61 kDa in the native state by gel filtration using Superdex S-200. These results suggest that \(\beta\)-Xyl'ase Gb functions as a monomeric protein, and that its molecular weight is comparable to that of tomato \(\beta\)-Xyl'ase Le1 \(^1\) (61 kDa by gel filtration). The maximum level of activity was obtained at pH 5.0 for MFX as pseudo-natural substrate, and at pH 3.5 for \(\beta\)-Xyl'ase Gb and tomato \(\beta\)-xylosidase (\(\beta\)-Xyl'ase Le1) \(^1\) possessed hydrolytic activity for \(p\)-nitrophenyl \(\alpha\)-L-arabinofuranoside (\(p\)NP-\(\alpha\)-Ara). \(p\)NP-\(\alpha\)-Ara (10 mM) was incubated with Ginkgo enzyme (0.1 mU) and \(\beta\)-Xyl'ase Le1 (0.1 mU) in 25 \(\mu\)L of 0.1 M Glycine-HCl buffer (pH 4.0) at 37 °C for 15 min. When aryl glycoside substrates were used, both \(\beta\)-Xyl'ase Gb and \(\beta\)-Xyl'ase Le1 showed optimal activity for \(p\)NP-\(\alpha\)-Ara rather than for \(p\)NP-\(\alpha\)-Xyl (\(\alpha\)-L-arabinofuranoside activity, 100%; \(\beta\)-xylosidase activity, 17.3% for the Ginkgo enzyme and 9.0% for the tomato enzyme). These substrate specificities for the synthetic substrates were very similar to those of the barely \(\beta\)-Xyl'ases (\(\alpha\)-Ara'ase, \(\alpha\)-ARA-1) \(^2\), the Arabidopsis \(\beta\)-Xyl'ase (XYL1) \(^5\) equivalent to AtBXL1 \(^4\), and radish \(\alpha\)-L-arabinofuranosidase/\(\beta\)-d-xylosidase. \(^10\)

To identify the gene of plant \(\beta\)-xylosidase involved in the turnover of plant complex type \(N\)-glycans and \(N\)-glycoproteins, it is necessary to obtain information on the partial amino acid sequences of \(\beta\)-Xyl'ase expressed in plant cells. Since the N-terminal sequence of the purified \(\beta\)-Xyl'ase Gb was found to be G-S-A-A-G-N-R-, we performed a homology search using the BLAST program (NCBI, GenBank). This N-terminal sequence almost completely coincided with part of the deduced amino acid sequence of one of the Arabidopsis \(\beta\)-Xyl'ases, AtBXL1 \(^4\), \(^5\), Minic et al. \(^5\) purified two \(\beta\)-xylosidases (XYL1 and XYL4) and one \(\alpha\)-arabinofuranosidase (ARA1) from stem tissues of Arabidopsis thaliana, and found that all of them showed both \(\alpha\)-L-arabinofuranosidase and \(\beta\)-xylosidase activities. According to the deduced amino acid sequence of Arabidopsis \(\beta\)-xylosidase (XYL1, or AtBXL1) cDNA, \(^4\), \(^5\) XYL1 consists of 702 amino acids and its molecular weight

\[\text{Fig. 1. SDS–PAGE and Gel Filtration of Purified } \beta\text{-Xyl'ase Gb.}
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A. The purity of tomato \(\beta\)-xylosidase was checked by SDS–PAGE using 10% acrylamide under the reduced condition, in which the protein was stained with Coomassie Brilliant Blue. \(\beta\)-Mer and M mean \(\beta\)-mercaptoethanol and molecular marker, respectively. The marker proteins used for molecular mass determination were Precision Plus Protein™ Standards (Bio-Rad). B. Determination of molecular masses by gel filtration with a Superdex S-200 column (1.6 × 120 cm). The column was developed with 20 mM Tris–HCl buffer (pH 7.9) containing 0.1 M NaCl at a flow rate of 0.7 mL/min. The marker proteins used were as follows: aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa).
is much larger than that of the purified *Arabidopsis* enzyme (64 kDa). Although the N-terminal amino acid sequence of XYL1 could not be identified, Minic *et al.* predicted their N-terminal amino acid sequence to be L(31)-R-P-L-F-A based on information on the N-terminal amino acid sequence of one of the barley/C12-xylosidases and the position of the putative signal sequence in the deduced amino acid sequence of XYL1. Considering the predicted N-terminus and molecular weight of the native enzyme, Minic *et al.* assumed that about 140 amino acid residues from 631 to 774 in the C-terminal region are cleaved off. Amino acid sequence, G-T-A-A-G-N-R- (Fig. 3A), which corresponds to the N-terminal amino acid sequence of native β-Xyl’ase Gb (G-S-A-A-G-N-R-), is located at 198(G) to 204(R) in the deduced amino acid sequence of XYL1. As judged by these observations, the processing (maturation) mechanisms of *Arabidopsis* β-Xyl’ase and β-Xyl’ase Gb due to endogenous endopeptidase are different from each other. We found that the deduced amino acid sequence of XYL1 (AtBXL1) shared a high level of homology with that of one of the putative genes of the tomato/C12-xylosidases (*Solyc11g044910*) (Fig. 3B). Furthermore, we identified recently the N-terminal amino acid sequence of the newly purified native tomato β-Xyl’ase Le1 as Q-G-G-G-G-, although the intensities of the signals observed in Edman-degradation analysis were weak perhaps due to pyroglutamylation of the N-terminus Gln residue of many of the native tomato enzymes. The N-terminal amino acid sequence identified was found in the deduced amino acid sequence of *Solyc11g044910*, and the position corresponded to the

![Fig. 2. Substrate Specificity of β-Xyl’ase Gb against PA-Sugar Chains.](image)

Each of the PA-sugar chains (20 pmol) was incubated with β-Xyl’ase Gb (correctly α-Ara’ase/β-Xyl’ase Gb) in 0.2 M MES buffer (pH 5.0) at 37°C for 14 h. The products obtained by β-Xyl’ase Gb digestion were analyzed by RP-HPLC, as described in our previous report.1) A, Relative activity against several xylosylated N-glycans. B, Structures of substrates.

![Fig. 3. Partial Amino Acid Sequences of *Arabidopsis* and Ginkgo β-Xyl’ase and the Phylogenic Tree of *Arabidopsis* and Tomato β-Xylosidases.](image)

A, Partial amino acid sequence of *Arabidopsis* β-xylosidase (XYL1 or AtBXL1) and the N-terminal amino acid sequence of β-Xyl’ase Gb (correctly α-Ara’ase/β-Xyl’ase Gb). B, Phylogenic tree of *Arabidopsis* and tomato β-xylosidases.
internal amino acid sequence, 198(G) to 204(R), of AtBXL1. To confirm that the product of Solyc11g044910 has the same enzymatic properties as β-Xyl’ase Le1, we are currently constructing the expression system of Solyc11g044910 in yeast and insect cells. The enzymatic properties of recombinant tomato β-Xyl’ase will be described elsewhere.

Based on the substrate specificity and the N-terminal amino acid sequence of β-Xyl’ase Gb, it can be considered to be an α-L-arabinofuranosidase/β-D-xylosidase that belongs to glycosyl hydrolase family 3. It is well known that the α-L-arabinofuranosidase/β-D-xylosidases (barley α-Ara’ase/β-Xyl’ases, the Arabidopsis β-Xyl’ases (AtBXL14), and radish α-Ara’ase/β-Xyl’ases10) are associated with turnover of arabinogalactan proteins, arabinan, and arabinoxylan. At this time, it is not clear whether these α-L-arabinofuranosidase/β-D-xylosidases hydrolyze the β-1,2-xylosyl residue in plant complex type N-glycans, or are involved in the turnover of N-glycoproteins. It is plausible to assume, however, that β-Xyl’ase Gb (correctly α-Ara’ase/β-Xyl’ase Gb), as well as other plant α-Ara’ase/β-Xyl’ases (barley, Arabidopsis, and radish enzymes1,2,4,10), play important roles in the turnover of cell-wall components. To determine the degradation mechanism of plant β-xylosylated glycoconjugates, it is significant to determine whether the α-Ara’ase/β-Xyl’ase ubiquitously distributed in plants has a dual function involved in the turnover of both cell-wall components and N-glycoproteins.

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