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Purification and characterization of β-xylosidase that is active for plant complex type *N*-glycans from tomato (*Solanum lycopersicum*): removal of core α1-3 mannosyl residue is prerequisite for hydrolysis of β1-2 xylosyl residue

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Abstract In this study, we purified and characterized the β-xylosidase involved in the turnover of plant complex type *N*-glycans to homogeneity from mature red tomatoes. Purified β-xylosidase (β-Xyl'ase Le-1) gave a single band with molecular masses of 67 kDa on SDS-PAGE under a reducing condition and 60 kDa on gelfiltration, indicating that β-Xyl'ase Le-1 has a monomeric structure in plant cells. The *N*-terminal amino acid could not be identified owing to a chemical modification. When pyridylaminated (PA-) *N*-glycans were used as substrates, β-Xyl'ase Le-1 showed optimum activity at about pH 5 at 40 °C, suggesting that the enzyme functions in a rather acidic circumstance such as in the vacuole or cell wall. β-Xyl'ase Le-1 hydrolyzed the β1-2 xylosyl residue from $Man_1Xyl_1GlcNAc_2$-PA, $Man_1Xyl_1Fuc_1GlcNAc_2$-PA, and $Man_2Xyl_1Fuc_1GlcNAc_2$-PA, but not that from $Man_3Xyl_1GlcNAc_2$-PA or $Man_3Xyl_1Fuc_1GlcNAc_2$-PA, indicating that the α1-3 arm mannosyl residue exerts significant steric hindrance for the access of β-Xyl'ase Le-1 to the xylosyl residue, whereas the α1-3 fucosyl residue exerts little effect. These results suggest that the release of the β1-2 xylosyl residue by β-Xyl'ase Le-1 occurs at least after the removal the α-1,3-mannosyl residue in the core trimannosyl unit.

Keywords β-xylosidase · Plant *N*-glycan · Fruit ripening · *Solanum lycopersicum*

Abbreviations

β-Xyl'ase-Le	β-xylosidase from *Solanum lycopersicum*
α-Man'ase	α-mannosidase
β-GlcNAc'ase	β-*N*-acetylglucosaminidase
PA-	pyridylamino
RP-HPLC	reverse-phase HPLC
Man	D-mannose
Xyl	D-xylose
Fuc	L-fucose
GlcNAc	*N*-acetyl-D-glucosamine
MX	Xylβ1-2Manβ1-4GlcNAcβ1-4GlcNAc-PA
MFX	Xylβ1-2Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA
M2FX	Manα1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA
M3FX	Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA
GN2M3FX	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc-PA

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Introduction

One of the structural features of plant *N*-glycosylation is the occurrence of the β1-2-xylosyl residue linked to β-mannosyl and the α1-3-fucoyl residue linked to the terminal GlcNAc residues of the core pentasaccharide. For plant *N*-glycoproteins, secreted glycoproteins, such as cell surface glycoproteins or pollen allergens [1–8], frequently carry complex type *N*-glycans having a Lewis a epitope, whereas storage glycoproteins in seeds carry truncated xylose/fucose-containing type (pauci-mannose-type) glycans. Strasser *et al.* reported that a complete suppression of the β-1, 2-xylosyl- and α-1,3-fucosyl-transferase genes in *Arabidopsis thaliana* gives no significant morphological phenotype nor any deleterious effect, suggesting that the formation of complex type *N*-glycans is unnecessary for plant growth, at least, under standard culture conditions [9]. On the other hand, however, it has recently been reported that suppression of α-mannosidase (α-Man'ase) and β-*N*-acetylglucosaminidase (GlcNAc' ase) genes could delay fruit maturation, suggesting that the turnover of *N*-glycoproteins is associated with fruit ripening [10]. Until now, it has remained unclear whether the rate of degradation of complex type *N*-glycoproteins can regulate fruit ripening or softening. To elucidate the physiological significance of the turnover of complex type *N*-glycoproteins involved in plant growth or fruit ripening, it is necessary to construct a transgenic plant in which the expression of the β-xylosidase and α-fucosidase genes, in addition to the α-Man'ase and GlcNAc' ase genes, is suppressed. Although some plant β-xylosidase genes responsible for the degradation of the hemicellulose component in cell walls have been identified [11, 12], it has not been confirmed whether their products are associated with the turnover of plant *N*-glycans.

As for plant β-xylosidase (β-Xyl'ase) involved in the degradation of plant *N*-glycans, Tezuka *et al.* characterized, for the first time, β-Xyl'ase activity using enzyme partially purified from the culture broth of sycamore cells [13], and Peyer *et al.* purified the enzyme from potatoes to homogeneity and characterized some enzymatic properties [14]. In our previous studies [15–17], we have already characterized ENGase, PNGase, and α-Man'ase from tomatoes, and identified the genes encoding these enzymes. Furthermore, we revealed the structural features of *N*-glycans of glycoproteins and free *N*-glycans expressed in tomatoes [18, 19], these results well agreed with those of some previous reports [20–24]. In this report, therefore, as a first step in constructing a transgenic tomato plant that lacks β-xylosidase (β-Xyl'ase) and α-fucosidase (α-Fuc'ase) activities, we purified and characterized β-Xyl'ase (β-Xyl'ase Le-1) from tomatoes. β-Xyl'ase Le-1 could hydrolyze the β1-2-xylosyl linkage in $Man_2Xyl_1Fuc_1GlcNAc_2$ (M2FX), $Man_1Xyl_1Fuc_1GlcNAc_2$ (MFX), and $Man_1Xyl_1GlcNAc_2$ (MX) but not that in $Man_3Xyl_1Fuc_1GlcNAc_2$ (M3FX), indicating that the release of the α1-3-linked mannosyl residue in the trimannosyl core is a prerequisite for the action of β-Xyl'ase Le-1.

Materials and methods

Materials

Mature red tomatoes were collected from the farm of the Research Institute, Kagome Co., Ltd., (Tochigi, Japan). The Cosmosil 5C18-AR column (0.6×25 cm) used was from Nakalai Tesque, Kyoto, Japan. The Shodex IEC SP-825 column and Shodex PH column were purchased from Showa Denko (Tokyo, Japan). DEAE cellulose and Q-Sepharose were purchased from Amersham Pharmacia Biotech AB (Upsala Sweden). Butyl-Toyopearl was purchased from Tosoh (Tokyo, Japan). *p*-Nitrophenyl β-D-xylopyranoside (*p*NP-β-Xyl) was from Sigma Co. (St. Louis, USA). Authentic PA-sugar chains (GN2M3FX, M3FX, M2FX, MFX, and MX) were prepared as described in our previous papers [7, 25, 26].

Assay system for β-xylosidase (β-Xyl'ase) activity during enzyme purification

β-Xylosidase activity was assayed using *p*NP-β-Xyl as a synthetic substrate during enzyme purification. The enzyme solution (10-40 μl) was added to 100 μl of *p*NP- β-Xyl (1 mM as a final concentration) in 0.1 M MES buffer, pH 6.0. After incubation at 37 °C for 3 h, the reaction was stopped by adding 400 μl of 0.1 M Na_2CO_3. The amount of *p*-nitrophenol released was monitored 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 minute at 37 °C.

Assay system of β-xylosidase β-Xyl'ase) for pyridylaminated N-glycans

A reaction mixture (10 μl) containing about 20 pmol of PA-sugar chain ($Man_1Xyl_1Fuc_1GlcNAc_2$-PA, MFX) in 0.15 M sodium acetate buffer (pH 4.5), was incubated at 37 °C for 24 h. The enzymatic reaction was stopped by heating in boiling water for 3 min. After centrifugation, an aliquot (10 μl) of the reaction mixture was dissolved in 0.02 % TFA (90 μl), and analyzed with a Jasco 880-PU HPLC apparatus with a Jasco Intelligent spectrofluorometer and a Cosmosil 5C18-AR column (0.6 x 25 cm). The 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 at 310 nm, emission at 380 nm).

Purification of tomato β-Xyl'ase During purification, *p*NP-β-Xyl was used as the substrate in measuring β-xylosidase activity. Protein concentration was measured by measuring the absorbance at 280 nm with bovine serum albumin (BSA) as the standard protein.

Step 1. Preparation of crude extract Mature red tomatoes (20 kg) were cut into small pieces and homogenized through a twice-folded gauze. The homogenate was centrifuged at 8,000 rpm for 30 min. The supernatant was dialyzed overnight against 20 mM Tris–HCl buffer, pH 7.9 (10 L). After dialysis, the dialyzate was centrifuged, and the resulting supernatant was used as the crude soluble β-xylosidase (β-Xyl'ase) fraction.

Step 2. Fractionation of β-Xyl'ase activity using DEAE cellulose The crude enzyme solution dialyzed against 20 mM Tris–HCl buffer (pH 7.9) was mixed with DEAE cellulose (1.5 L wet volume) and left to stand at 4 °C overnight. After washing the cellulose with the same buffer (5 L), bound proteins were eluted with 20 mM Tris–HCl buffer (pH 7.9) containing 0.5 M NaCl. β-Xyl'ase activity was predominantly recovered in the run-through fraction, and the run-through fraction was 100 % saturated with ammonium sulfate.

Step 3. Q-Sepharose column chromatography The precipitate obtained in step 2 was dissolved in a minimum volume of 20 mM Tris–HCl buffer, pH 8.5, dialyzed against the same buffer, and then loaded onto a Q-Sepharose column (2.5×30 cm) previously equilibrated with the same buffer. The absorbed β-Xyl'ase activity was eluted using a linear gradient of NaCl from 0 to 0.2 M in the same buffer (Fig. 1-I). The β-Xyl'ase active fractions indicated by a horizontal bar were collected and saturated with ammonium sulfate.

Step 4. Butyl-Toyopearl column chromatography The precipitate obtained in step 3 was dissolved in 20 mM Tris–HCl buffer, pH 8.0, and dialyzed against the same buffer. Solid ammonium sulfate was added to a final concentration of 1.5 M. The resulting enzyme solution was loaded onto the Butyl-Toyopearl column (2.8×33 cm) previously equilibrated with 10 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 − 0.0 M) of ammonium sulfate in the same buffer (Fig. 1-II). The β-Xyl'ase active fraction indicated by a horizontal bar was pooled and concentrated using an Vivaspin 20 (10,000MWCOPES) membrane filter at 8,000 rpm for 30 min.

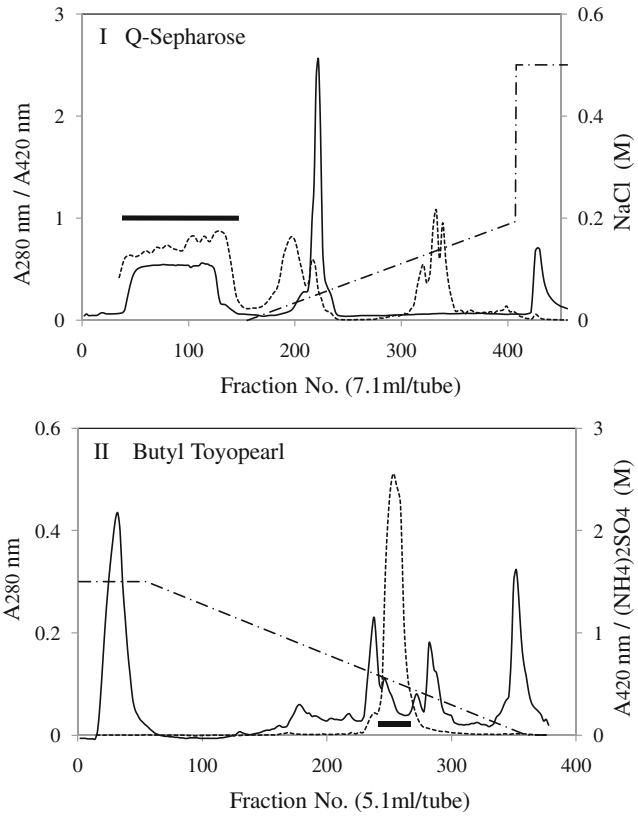


Fig. 1 Purification Profiles of β-Xyl Le-1 (Q-Sepharose and Butyl-Toyopearl chromatographies). I, Q-Sepharose chromatography. Proteins were eluted using a linear gradient of NaCl from 0 M to 0.3 M. Solid line indicates the protein elution (280 nm) and the dotted line indicates the β-xylosidase activity (420 nm). Although several enzyme active fractions were eluted using the linear gradient of NaCl, the run-through fraction, indicated by a horizontal bar, was used in this study. II, Butyl-Toyopearl chromatography. Proteins were eluted using a linear gradient (1.5-0 M) of ammonium sulfate in the buffer

Step 5. SP-HPLC Tomato β-Xyl'ase was further purified with a Jasco 880-PU HPLC apparatus equipped with a Shodex ICE SP-825 column (0.8 x 7.5 cm). The concentrated enzyme obtained in step 4 was dialyzed against 20 mM MES buffer (pH 6.0), and applied to the cation exchange column equilibrated with the same buffer. After washing the column with the same MES buffer, the column-bound enzyme was 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 (Fig. 2-I). β-Xyl'ase activity was recovered in the run-through fraction, and the active fraction was pooled and concentrated using an Amicon Centriprep-30 membrane filter at 3,000 rpm for 1 h.

Step 6. Phenyl HPLC The concentrated β-Xyl'ase activity obtained in step 5 was dialyzed against 20 mM Tris–HCl buffer, pH 8.0, and solid ammonium sulfate was added to a final concentration of 1.5 M. The enzyme

solution was loaded into a Shodex ICE PH-814 column (0.8 x 7.5 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 − 0.0 M) of ammonium sulfate in the same buffer (Fig. 2-II). The β-Xyl'ase active fraction indicated by a horizontal bar was pooled and concentrated using an Vivaspin 20 (10,000MWCOPES) membrane filter at 8,000 rpm for 30 min.

Step 7. Gelfiltration through Superdex S-200 column Tomato β-Xyl'ase obtained by Phenyl HPLC (Step 6) was finally purified by gelfiltration HPLC using a Superdex S-200 (1.6 x 120 cm) (Fig. 3-I). The concentrated enzyme obtained in step 5 was dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 0.2 M Na_2SO_4, and the dialyzate was loaded onto the column previously equilibrated with the same buffer. The column was developed at a flow rate of 0.7 ml/min.

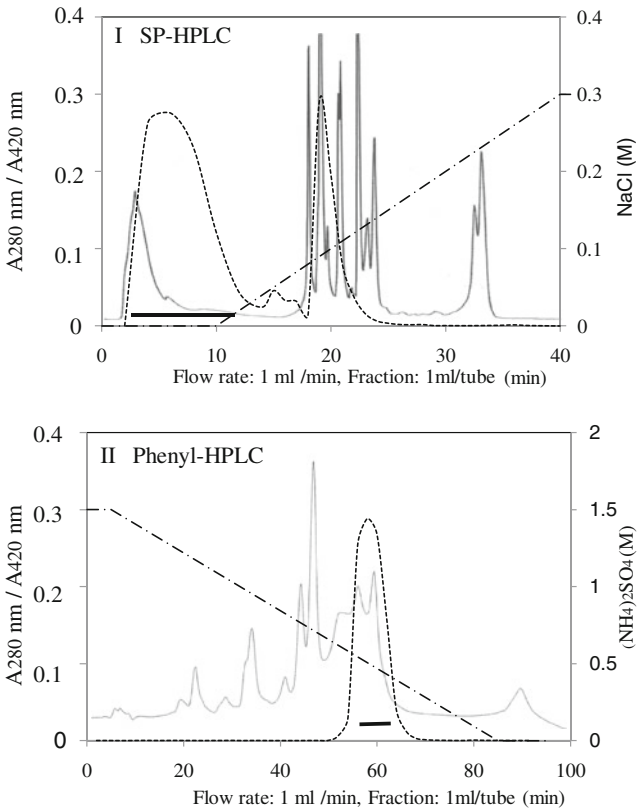


Fig. 2 Purification Profiles of β-Xyl Le-1 (SP-HPLC and Phenyl-HPLC). I, SP-HPLC. Solid line indicates the protein elution (280 nm) and the dotted line indicates the β-xylosidase activity (420 nm). Although several enzyme active fractions were eluted using the linear gradient of NaCl, the run-through fraction, indicated by a horizontal bar, was used in this study, because a significant activity for PA-oligosaccharides (MFX) was confirmed. The elution was collected at a rate of 1 ml/tube each. II, Phenyl-HPLC. Proteins were eluted using a linear gradient (1.5-0 M) of ammonium sulfate in the buffer. The elution was collected at a rate of 1 ml /tube each

SDS-PAGE and blotting of purified tomato β-Xyl'ase SDS-PAGE was carried out as described by Laemmli and Favre [27] on 10 % acrylamide gel in 0.1 M Tris-glycine buffer using a slab gel apparatus. The gel was stained with Coomassie Brilliant Blue R-250. The marker proteins used for molecular mass determination were Precision Plus Protein™ Standards (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa, and 10 kDa) (Bio-Rad). The proteins on the gel were transferred to a PVDF membrane for 30 min on Horize-blot (AE-6675 P/N, Atto, Japan) using 0.1 M Tris-glycine containing 5 % 2-methanol (pH 8.8) as the transfer buffer. The PDVF membrane (Sequi-Blot™, Bio-Rad) was then dried and used in the analysis of the N-terminal amino acid sequence.

Effects of pH and temperature on β-Xyl'ase activity The optimum pH of tomato β-Xyl'ase was determined using MFX (20 pmol) and *p*NP-β-Xyl (1 mM) as the substrates at various pHs (3.0–8.0) after incubation at 40 °C for 10 h for *p*NP-β-Xyl (100 μl of total reaction mixture) and for 24 h for MFX (10 μl of total reaction mixture), respectively. The buffers used in this experiment were 0.1 M glycine-HCl (pH 3.0), 0.1 M sodium acetate (pHs 4.0 -5.0), 0.1 M MES (pH 6.0), and 0.1 M HEPES (pHs 7.0–8.0). The effects of temperature were examined at various temperatures (20, 30, 37, 40, 50, 60, 70, and 80 °C) using MFX and *p*NP-β-Xyl as the substrates after incubation for 3 h for *p*NP-β-Xyl and for 24 h for MFX, respectively.

Effects of divalent metal ions on β-Xyl'ase activity The effects of metal ions on tomato β-Xyl'ase activity were examined using Fe^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and EDTA at a concentration of 5 mM. After preincubating various metal ions and inhibitors with purified tomato β-xylosidase in 0.1 M sodium acetate buffer (pH 4.0 for *p*NP-β-Xyl, pH 5.0 for MFX), the reaction mixtures (10 μl of total reaction mixture for MFX, 100 μl for *p*NP-β-Xyl) were further incubated with MFX (20 pmol) or *p*NP-β-Xyl (2 mM) as the substrates at 37 °C (3 h for *p*NP-β-Xyl, 24 h for MFX, respectively).

Substrate specificity of tomato β-Xyl'ase The substrate specificity of purified tomato β-Xyl'ase was analyzed using authentic PA-sugar chains: GN2M3FX, M3FX, M2FX, MFX, and MX. PA-sugar chains (20 pmol) were incubated with purified tomato β-Xyl'ase in 0.1 M Na-acetate buffer (pH 5.0) at 37 °C for 24 h (10 μl of total reaction mixture). The PA-sugar chains produced by the purified β-Xyl'ase were analyzed by RP-HPLC on a Cosmosil 5C-18 AR column (0. 6×25 cm).

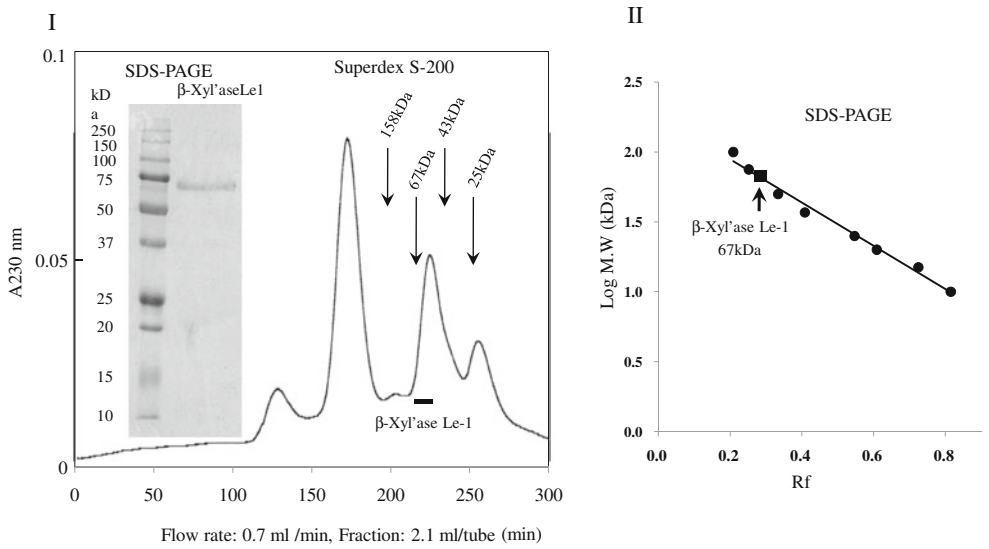


Fig. 3 Gelfiltration and SDS-PAGE. I, Gelfiltration profile on a Superdex S-200 column (1.6 x 120 cm). The column was developed with 20 mM Tris–HCl buffer (pH 8.0), containing 0.2 M Na_2SO_4 at a flow rate of 0.7 ml/min. The elution was collected at a rate of 2.1 ml/tube each. The marker proteins used were as follows: aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDal), and chymotrypsinogen (25 kDa). The purity of tomato β-xylosidase was checked by SDS-PAGE using 10 % acrylamide, in which the protein was stained with Coomassie brilliant blue. II, Determination of molecular mass by SDS-PAGE

Results and discussion

Purification of tomato β-xylosidase

A summary of the purification of β-xylosidase is shown in Table 1. The tomato β-xylosidase (β-Xyl'ase Le-1) was purified to homogeneity about 4,500-fold. When the tomato β-xylosidase was fractionated using DEAE cellulose, both non-absorbed fraction and absorbed fractions eluted with 0.5 M NaCl contained β-xylosidase activity. However, the enzyme activity was predominantly recovered from the non-absorbed fraction. Hence, we used the non-absorbed fraction in further purification of β-xylosidase. The enzyme active fraction was further purified with a Q-Sepharose column (Fig. 1-I), followed by Butyl Toyopearl hydrophobic interaction chromatography (Fig. 1-II), SP-HPLC (Fig. 2-I), and Phenyl HPLC (Fig. 2-II). In the second anion-exchange (Q-Sepharose) chromatography, as shown in Fig.1-I, although the predominant β-xylosidase activity was eluted in the run-through fraction, some activities were eluted as a result of the increase in NaCl concentration, and the predominant enzyme fraction was used for the following purification step. After the hydrophobic interaction chromatography (Fig. 1-II), in which only one enzyme active fraction was obtained and the apparent total activity and yield increased (Table 1), the enzyme assay was repeated, but almost the same results were obtained. At this time, it is unclear what factors increased the total activity or yield; however, some inhibitors for β-xylosidase might be removed by Butyl-Toyopearl chromatography. The β-xylosidase obtained by hydrophobic interaction chromatography was further purified by cation-exchange chromatography (SP-HPLC), as shown in Fig. 2-I. In this cation-exchange chromatography, the predominant activity was recovered in the run-through fraction, but other enzyme activities were eluted as a result of the increase in NaCl

Table 1 Summary of purification of β-Xyl'ase Le-1 from mature red tomatoes

Purification step	Total protein (mg)	Total activity (munit)	Specific activity (munit/mg)	Yield (%)	Purification fold
Crude enzyme	25,758	6,270	0.24	100	1.0
DEAE Cellulose	1,274	1,120	0.88	17.9	3.7
Q-Sepharose	521	530	1.0	8.5	4.6
Butyl Toyopearl	21.1	840	40	13.4	165
SP-HPLC	2.66	520	200	8.3	813
Phenyl-HPLC	0.66	190	280	3.0	1,200
Superdex 200-HPLC	0.11	120	1100	1.9	4,545

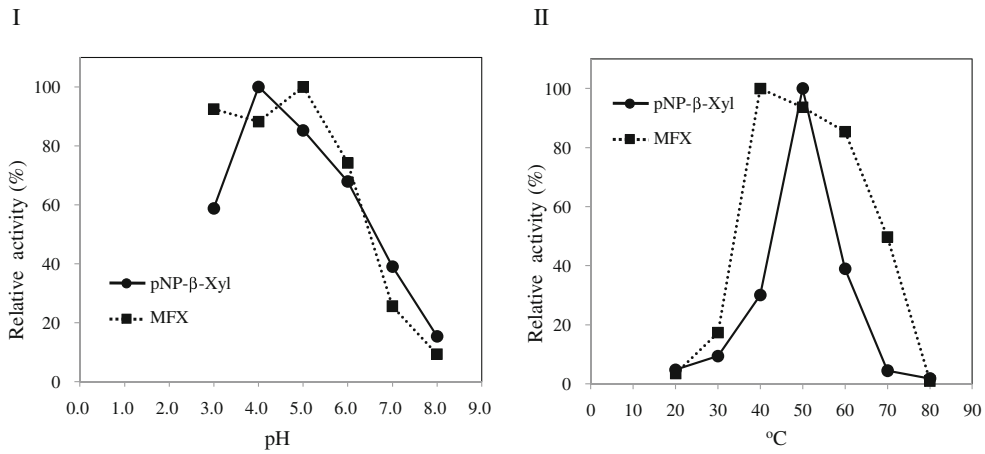


Fig. 4 Effects of pH and temperature on β-Xyl'ase Le-1 Activity. I. Effect of pH. The enzyme activities at pH 4.0 for *p*NP-β-Xyl or at pH 5.0 for MFX were taken to be 100 %. The incubation conditions were described in Materials and Methods. Each activity for MFX was calculated on the basis of the peak area of the remaining substrate (MFX) and the product (MF). II. Effect of temperature. The enzyme activities at 40 °C for MFX or at 50 °C for *p*NP-β-Xyl were taken to be 100 %. The incubation conditions were described in Materials and Methods

concentration. In this study, we used the run-through fraction for the final purification step because of a predominant activity. Finally, the β-xylosidase was purified to homogeneity by gelfiltration with a Superdex S-200 column (Fig. 3-I). The enzyme activities were assayed using aliquots from every other tube and the active fractions (tube numbers from 71 to 75) were collected as the purified β-Xyl'ase Le-1. During these purification steps, several β-xylosidase activities were separated by anion and cation ion-exchange chromatography, indicating two possibilities that several different molecules of β-xylosidase with different surface electrical charges are expressed or the same enzyme molecule was recovered from the different fractions due to some non-specific interaction with other protein components expressed in tomatoes.

Molecular mass and N-terminal amino acid sequence

Gelfiltration using the Superdex S-200 column revealed that the molecular mass of the native state was about 60 kDa (Fig. 3-I). When the purity of β-xylosidase was checked by SDS-PAGE on 10 % gel under a reducing condition, as shown in Fig. 3-I, the purified β-xylosidase (β-Xyl'ase Le-1) gave a single band with a corresponding molecular mass of 67 kDa (Fig 3-II). These results suggest that β-Xyl'ase Le-1 functions as a monomeric structure in tomato cells. Since the molecular mass of purified potato β-xylosidase is about 40 kDa [14], the gene of β-Xyl'ase Le 1 must be different from the putative tomato homologue corresponding to the potato enzyme gene. The *N*-terminal amino acid sequence of β-Xyl'ase Le-1 was analyzed using an electroblotted enzyme on a PVDF membrane, but no

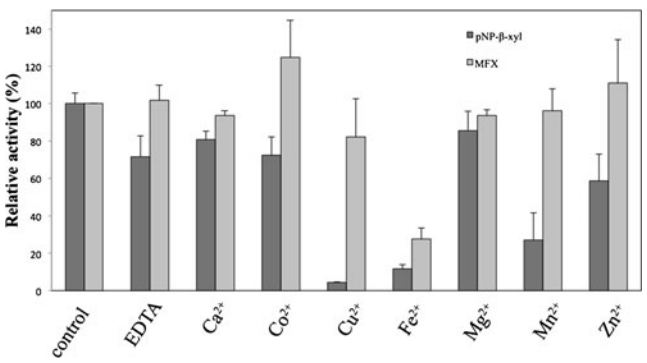


Fig. 5 Effects of divalent metal ions and EDTA. For the calculation of the percent relative activity, the control sample activity was taken to be 100 %. The incubation conditions were described in Materials and Methods. The black bars are the enzyme activities for *p*NP-β-Xyl and the gray bars are those for MFX. The incubation conditions were described in Materials and Methods

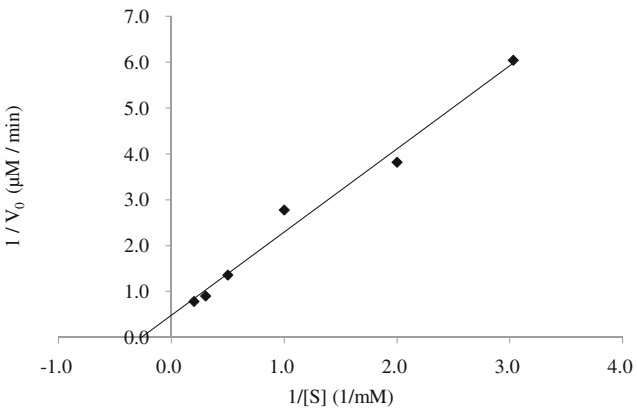


Fig. 6 Kinetic analysis of β-Xyl'ase Le-1 activity for *p*NP-β-Xyl. The purified β-Xyl'ase Le-1 (0.107 munit) was incubated with *p*NP-β-Xyl of various concentrations in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C for 60 min (total volume of the reaction mixture, 100 μl). Kinetic parameters were estimated from the Lineweaver-Burk plot

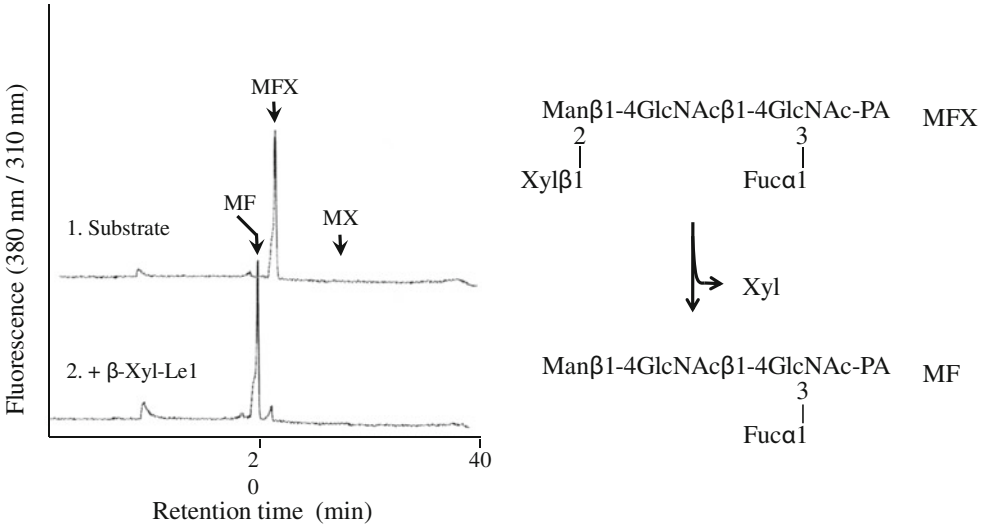


Fig. 7 RP-HPLC profile of MFX treated with β-Xyl'ase Le-1. MFX ($Man_1Xyl_1Fuc_1GlcNAc_2$-PA) was incubated with β-Xyl Le-1 in 0.2 M MES buffer (pH 6.0) at 37 °C for 24 h. 1, Substrate; 2, treated with β-Xyl Le-1

amino acid was identified probably owing to chemical modifications. Itai *et al.* have identified two tomato β-xylosidase genes (LeXYL1 and LeXYL2) and confirmed the enzyme activity for *p*NP-β-Xyl of the gene-products [11]. They also found that these two β-xylosidase genes were relatively highly expressed during fruit development, suggesting that these enzymes are involved in the degradation of hemicellulose components. At this time, however, it is not clear whether the β-Xyl'ase Le-1 gene corresponds to one of these tomato β-xylosidase genes or to other unknown genes.

Effects of pH and temperature on β-Xyl'ase Le-1 activity

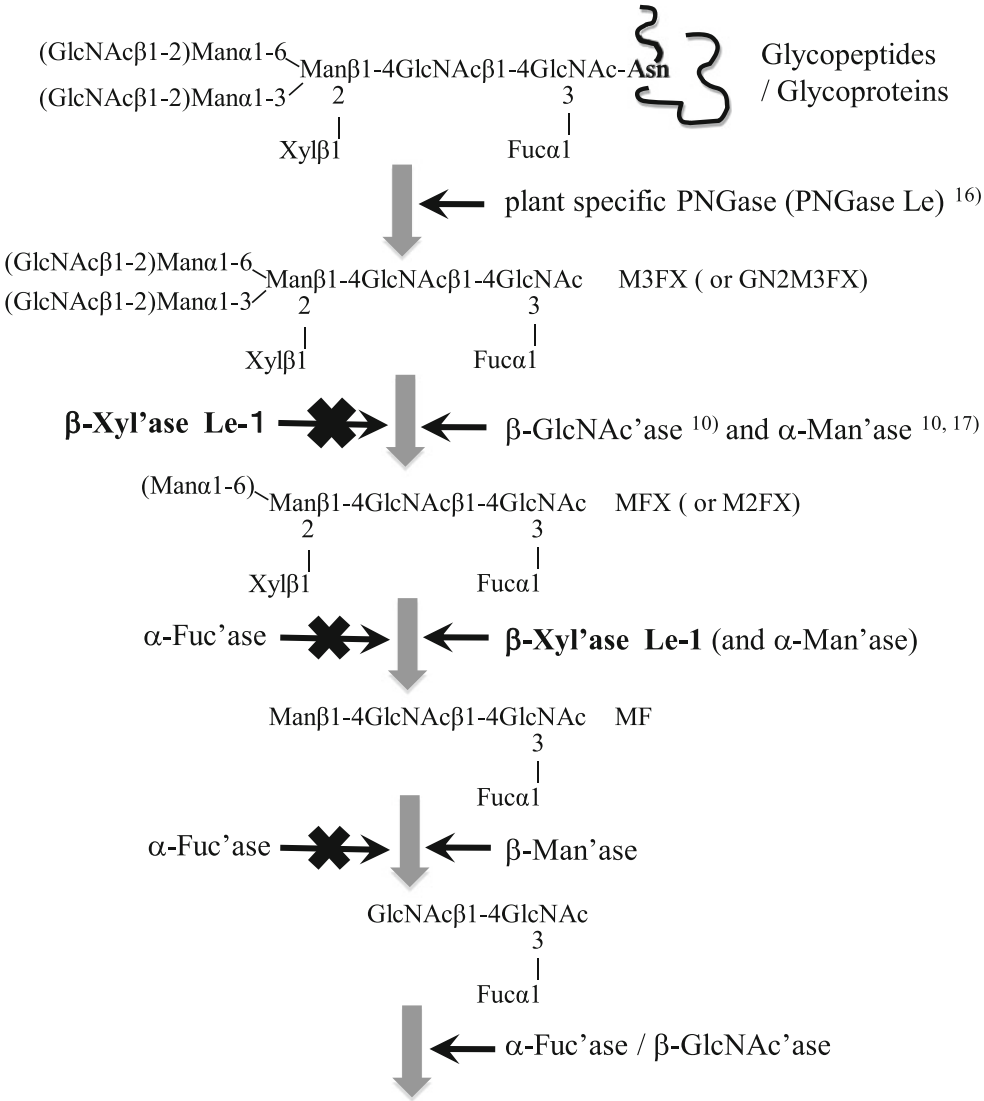
When β-Xyl'ase Le-1 was incubated with $Man_1Xyl_1Fuc_1GlcNAc_2$-PA (MFX) or *p*NP-β-Xyl as substrates at various pHs, the maximum activity was obtained at pH 5.0 for MFX as a pseudo-natural substrate, but at pH 4.0 for *p*NP-β-Xyl as a synthetic substrate (Fig. 4-I). Although β-xylosidase activity markedly decreased in the neutral-pH region, less than 20 % of the full enzyme activity was observed at pH 8.0. Thus, β-Xyl'ase Le-1 might reside in the slightly acidic region of plant

Table 2 Substrate specificity of β-Xyl'ase Le-1

Substrate		Relative activity (%) [a]
Manβ1-4GlcNAcβ1-4GlcNAc-PA 2 Xylβ1	MX	110
Manβ1-4GlcNAcβ1-4GlcNAc-PA 2 3 Xylβ1 Fucα1	MFX	100
Manα1-6 Manβ1-4GlcNAcβ1-4GlcNAc-PA 2 3 Xylβ1 Fucα1	M2FX	100
Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA 2 Xylβ1	M3X	ND [b]
Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA 2 3 Xylβ1 Fucα1	M3FX	ND [b]
GlcNAcβ1-2Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc-PA 2 3 Xylβ1 Fucα1	GN2M3FX	ND [b]

a) Enzyme activity for MFX was taken as 100 %. b) ND, not detected

Scheme 1 Proposed degradation pathway of complex type *N*-glycans in tomatoes



cells probably in either the cell wall or the vacuole, and this optimum pH for the synthetic substrate is very similar to those of the sycamore enzyme (pH 4.0) and potato enzyme (pH 4.0–4.5) that could act on plant *N*-glycans. Optimum activity was obtained at about 40 °C for MFX and at about 50 °C for *p*NP-β-Xyl (Fig. 4-II).

Effects of divalent metal ions and inhibitors on β-Xyl'ase Le-1 activity

When β-Xyl'ase Le-1 was incubated with MFX, no metal ions increased its activity, but most of them decreased its activity when a synthetic substrate was used (Fig. 5). Cu^{2+}, Fe^{2+}, and Mn^{2+} significantly inhibited the activity of β-Xyl'ase Le-1 for *p*NP-β-Xyl, but Mn^{2+} had no harmful effect on the activity for the *N*-glycan substrate, suggesting that the inhibition modes of metal ions on the catalytic mechanism may differ between a synthetic substrate and a pseudo-natural oligosaccharide substrate. Although EDTA inhibited about 30 % of the hydrolytic activity for *p*NP-β-Xyl, it showed no inhibitory activity for MFX, indicating that β-Xyl'ase Le-1 requires no metal ions for the full enzyme activity involved in the turnover of complex type *N*-glycans in tomatoes.

Kinetic parameters (Km and Vmax)

The Km and Vmax values for the *p*NP-β-Xyl of β-Xyl'ase Le-1 were 3.7 mM and 2.1 μM/min, respectively (Fig. 6).

Substrate specificity of β-Xyl Le-1

The substrate specificity of β-Xyl'ase Le-1 was analyzed with various plant complex type PA-sugar chains. As shown in Fig. 7 and Table 2, β-Xyl'ase Le-1 could completely hydrolyze the β1-2-xylosyl linkage in $Man_2Xyl_1Fuc_1GlcNAc_2$-PA (M2FX), $Man_1Xyl_1Fuc_1GlcNAc_2$-PA (MFX), and $Man_1Xyl_1GlcNAc_2$-PA (MX), but not that in $Man_3Xyl_1Fuc_1GlcNAc_2$-PA (M3FX) or $GlcNAc_2Man_3$

$Xyl_1Fuc_1GlcNAc_2$-PA (GN2M3FX) that carry the α1-3-mannosyl residue, suggesting that the α-1,3-mannosyl residue exerts serious steric hindrance for the action of β-Xyl'ase Le-1, but that the α1-6-mannosyl residue in the trimannosyl core structure exerts little effect. This specificity of β-Xyl'ase Le-1 was basically similar to those of other β-xylosidases purified from plants [13, 14] and snail [28]. The presence of the core α1-3-fucosyl residue in *N*-glycan substrates did not abrogate the activity, and the removal of the residue slightly increased the activity (MFX,100 %; MX,110 %). Hence, β-Xyl'ase Le-1 can be used, in addition to structural analysis, for the preparation of axylosyl and fucosylated glycans for the immunological analysis of antigenic plant *N*-glycans.

Considering the substrate specificities of β-Xyl'ase Le-1 together with those of other plant β-xylosidases reported to date, a degradation pathway of the plant complex type *N*-glycans in tomatoes can be postulated (Scheme 1). The plant complex *N*-glycans linked to glycopeptides/glycoproteins may be released first by the action of a plant-specific acidic peptide:*N*-glycanase (PNGase) such as PNGase Le [16] in the vacuole or cell wall. The resulting free *N*-glycans, prior to the action of β-xylosidase, must be digested by β-GlcNAc'ase [10] and/or α-Man'ase [10, 17], providing M2FX or MFX structures. The resulting M2FX or MFX may be digested by β-Xyl'ase Le-1, producing M2F or MF structures. In our preliminary experiment, we detected no significant α-fucosidase activity for MFX and MF in the crude extract of tomatoes or cultured rice cells, although α-fucosidase active for the Lewis a epitope (Galβ1-3(Fucα1-4)GlcNAcβ1-) in plant complex type *N*-glycans was detected (data not shown). The latter α-fucosidase activity should correspond to the recombinant *Arabidopsis* α-fucosidase that is active for the Lewis a unit, but not for $Man_3Fuc_1GlcNAc_2$-PA (M3F) [29]. It may be reasonable to assume, therefore, that the hydrolysis of the core α1-3- fucosyl linkage occurs after removing the β1-2-xylosyl and β1-4-mannosyl residues.

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