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A Novel α1,2-L-Fucosidase Acting on Xyloglucan Oligosaccharides is Associated with Endo-β-Mannosidase*

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Endo-β-mannosidase, which hydrolyses the Manβ1-4GlcNAc linkage of *N*-glycans in an endo-manner, was discovered in plants. During the course of the purification of the enzyme from lily flowers, we found a higher molecular mass form of the enzyme (designated as EBM II). EBM II was purified by column chromatography to homogeneity and its molecular composition revealed EBM II to be comprised of endo-β-mannosidase and an associated protein. The cDNA of this associated protein encodes a protein with slight homology to the fucosidase domain of bifidus AfcA. EBM II has α1,2-L-fucosidase activity and acts on a fucosylated xyloglucan nonasaccharide. The amino acid sequence of this associated protein has no similarity to known plant α-L-fucosidases. These results show that EBM II is a novel α1,2-L-fucosidase and a protein complex containing endo-β-mannosidase.

Key words: endo-β-mannosidase, fucosidase, plant, protein complex, xyloglucan.

Abbreviations: BGA, blood group A; FL, fucosyllactose; LEA, Lewis[a]; LNFP, lacto-*N*-fucopentaose; PA-, pyridylaminated.

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Endo-β-mannosidase (EC 3.2.1.152) is a plant-specific glycoside hydrolase that acts on *N*-glycans (*1*, *2*). It cleaves the Manβ1-4GlcNAc linkage in *N*-glycans in an endo-manner to produce mannooligosaccharides and chitobiose. Because endo-β-mannosidase has not been found in species other than plants, the degradation mechanism of *N*-glycans of plants appears to be different from those of other species. This endoglycosidase hydrolyses high-mannose type *N*-glycans lacking the Manα1-3Manβ linkage (*1*, *3–5*). Because these sugar chains are generated by the action of a plant α-mannosidase that preferentially hydrolyses the Manα1-3Manβ linkage, substrate specificity of endo-β-mannosidase is complementary to that of plant α-mannosidase (*6*). Therefore, it is considered that these two glycosidases act cooperatively on *N*-glycans to degrade them to mannooligosaccharides and chitobiose. This notion is consistent with the observation that these two enzymes reside in the vacuole (*7*), one of the roles of which is degradation of biomolecules. Alternatively, these glycosidases may hydrolyse *N*-glycans on a glycoprotein such as S-RNase, which has chitobiose as a major *N*-glycan (*6*, *8*). To elucidate the functions of endo-β-mannosidase further, structural and functional analyses are underway in our laboratory. During the course of the purification of endo-β-mannosidase, we found a higher molecular mass protein that has endo-β-mannosidase activity (designated as EBM II). In this present study, we purified EBM II from lily flowers and analysed its structure. These results reveal that EBM II consists of endo-β-mannosidase and an associated protein that has slight homology to the fucosidase domain of the bifidus AfcA protein (*9*), and that EBM II has both endo-β-mannosidase activity and α1,2-L-fucosidase (EC 3.2.1.63) activity.

*Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB326211, for the cDNA of the lily α1,2-L-fucosidase.

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MATERIALS AND METHODS

Materials—Lily (*Lilium longiflorum* Thumb. cv. Hinomoto) flowers were used as an enzyme source. DEAE-Sephacel, Sephacryl S-200, Octyl Sepharose CL-4B and a Superdex 200 column (1.6×60 cm) were obtained from GE Healthcare Biosciences. Bio-gel P2 was obtained from Bio-Rad Laboratories. The Shodex Asahipak NH2-P column (0.46×5 cm) was purchased from Showa Denko. The *p*-nitrophenyl α-fucopyranoside was purchased from Sigma-Aldrich. α1,2-L-Fucosidase from *Corynebacterium* was purchased from Takara-Bio. All other chemicals used were of the highest grades available commercially.

Preparation of Pyridylaminated Sugar Chains—Pyridylaminated (PA)-derivatives of *N*-glycans listed in Table 1 were prepared as reported previously (*1*, *3*). 2′-Fucosyllactose (2′FL) (Fucα1-2Galβ1-4Glc) was from Sigma-Aldrich. Lacto-*N*-fucopentaose I (LNFP I) (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), LNFP III (Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc), blood group A (BGA) [GalNAcα1-3(Fucα1-2)Gal], blood group A tetrasaccharide (BGAT) [GalNAcα1-3(Fucα1-2)Galβ1-4Glc], Lewis[a] (LEA) [Galβ1-3(Fucα1-4)GlcNAc], and 3-fucosyllactose (3FL) [Galβ1-4(Fucα1-3)Glc] were purchased from Dextra Laboratories. These sugar chains were pyridylaminated at their reducing ends with 2-aminopyridine using the method of Hase (*10*).

The xyloglucan fragments, XXFG [Xylα1-6Glcβ1-4(Xylα1-6)Glcβ1-4(Fucα1-2Galβ1-2Xylα1-6)Glcβ1-4Glc]

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Table 1. **Structures and abbreviations of sugar chains used in the present study.**

Abbreviation	Structure
M1	Manβ1-4GlcNAcβ1-4GlcNAc
M2B	Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc
M2A	Manβ1-4GlcNAcβ1-4GlcNAc Manα1–3
M3B	Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1–3
M3C	Manα1–3 Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc
M4B	Manα1–6 Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1–3
M5A	Manα1–6 Manα1–3 Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1–3
M9A	Manα1-2Manα1–6 Manα1-2Manα1–3 Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-2Manα1-2Manα1–3
M2F	Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc 3 | Fucα1
M2FX	Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc 2 3 | | Xylβ1 Fucα1
AG1.2	GlcNAcβ1-2Manα1–6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1–3
2'FL	Fucα1-2Galβ1-4Glc
3FL	Galβ1-4Glc 3 | Fucα1
LNFRI	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
BGA	GalNAcα1-3Gal 2 | Fucα1
BGAT	GalNAcα1-3Galβ1-4Glc 2 | Fucα1
LEA	Galβ1-3GlcNAc 4 | Fucα1

(continued)

Table 1. **Continued.**

Abbreviation	Structure
XXFG	Fucα1 | 2 Galβ1 | 2 Xylα1 Xylα1 Xylα1 | | | 6 6 6 Glcβ1-4 Glcβ1-4 Glcβ1-4 Glcβ
XLFG	Fucα1 | 2 Galβ1 Galβ1 | | 2 2 Xylα1 Xylα1 Xylα1 | | | 6 6 6 Glcβ1-4 Glcβ1-4 Glcβ1-4 Glcβ

and XLFG [Xylα1-6Glcβ1-4(Galβ1-2Xylα1-6)Glcβ1-4(Fucα1-2Galβ1-2Xylα1-6)Glcα1-4Glc] were prepared from mung bean hypocotyls according to the method of Kato and Matsuda (*11*) with modifications. The frozen hypocotyls (500 g) were homogenized in 1.5 l of 50 mM Tris–HCl buffer, pH 7.6, using a Waring blender. The insoluble materials collected by centrifugation (30,000*g* for 10 min) were extracted with 400 ml of 4% potassium hydroxide for 20 h at ambient temperature under anaerobic conditions. The pellet obtained by centrifugation (30,000*g* for 10 min) was further extracted with 200 ml of 24% potassium hydroxide as described earlier. The supernatant after centrifugation (30,000*g* for 10 min) was neutralized with acetic acid. It was dialysed against 5 l of distilled water twice and 5 l of 10 mM potassium phosphate buffer, pH 6.0. This solution was applied to a DEAE-Sephacel column (2.8 × 10 cm) and the cell wall polysaccharides (250 mg) in the unbound fractions were collected. The polysaccharides were then digested with 2.5 mg of cellulase from *Trichoderma viride* (Wako Pure Chemicals) at 40°C for 72 h. The digest was applied to a Bio-gel P2 column (1.7 × 160 cm), and phenol–sulfuric acid reaction positive-fractions collected. The resulting oligosaccharides were tagged with 2-aminopyridine as described above. The fluorescent-labelled xyloglucan fragments were separated on an NH2P column equilibrated in 0.3% (v/v) acetic acid in 93% acetonitrile adjusted to pH 7.0 with ammonia at a flow rate of 0.6 ml/min. Elution was performed with a linear gradient of acetonitrile from 93% to 20% for 40 min. Sugar chains were detected by their fluorescence using excitation and emission wavelengths between 310 and 380 nm, respectively. Each fragment was further purified on a Cosmocil 5C18P column equilibrated in 0.1 M of ammonium acetate buffer, pH 4.0, in 0.025% of 1-butanol at a flow rate of 1.5 ml/min. Elution was performed with a linear gradient of 1-butanol from 0.025% to 0.5% for 55 min. The structures of XXFG-PA and XLFG-PA were confirmed by MALDI-TOF-MS. The observed $[M+H]^+$ values of the purified XXFG-PA

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and XLFG-PA were 1149.2 (calculated molecular mass: 1149.5 Da) and 1611.3 (1611.6 Da), respectively.

Enzyme Assay—Endo-β-mannosidase activity was measured as described previously (*1*, *3*). α-Fucosidase activity was measured using several PA-sugar chains as substrates. The enzyme and 12.5 μM of the substrate in 0.16 M sodium phosphate buffer, pH 5.5, were incubated at 37°C for 20 min. The enzyme reaction was terminated by heating at 100°C for 3 min. The products were separated by size-fractionation HPLC and quantified using their fluorescence. Size-fractionation HPLC was performed on a Shodex Asahipak NH2P column at a flow rate of 0.6 ml/min with isocratic elution. A Shimadzu LC-9A pump equipped with a Hitachi fluorescence detector L-7485 was used. The eluents used were 0.3% (v/v) acetic acid adjusted to pH 7.0 with aqueous ammonia in a mixture from 67 : 33 to 83 : 17 (v/v) acetonitrile : water. The elution conditions for xyloglucan oligosaccharides are described earlier.

Purification of EBM II from Lily Flowers—All purification procedures were performed at 4°C.

Step 1. Preparation of a crude enzyme solution

Lily flower buds without anthers (2,000 g) frozen in liquid nitrogen were powdered with a Waring blender. The powder was suspended in 6,000 ml of 0.1 M sodium phosphate buffer, pH 6.0 and homogenized with stirring for 2 h. The homogenate was centrifuged at 28,000*g* for 30 min, and the supernatant (7,400 ml) was used as a crude enzyme solution.

Step 2. Ammonium sulfate precipitation

The precipitate formed with ammonium sulfate at 35% saturation was dissolved in 10 mM sodium phosphate buffer, pH 6.0, and then dialyzed against the same buffer. The supernatant (155 ml) obtained by centrifugation at 28,000*g* for 30 min was used in the next purification step.

Step 3. DEAE-Sephacel chromatography

The supernatant was loaded onto a DEAE-Sephacel column (2.7 × 45 cm) equilibrated in 10 mM sodium phosphate buffer, pH 6.0 and the column washed with the same buffer. The enzyme activity was eluted with a linear gradient of sodium chloride from 0 to 0.5 M. The endo-β-mannosidase fraction (540 ml) was concentrated to 4.0 ml using an Amicon YM-30 membrane.

Step 4. Sephacryl S-200 gel filtration

The concentrated fraction from step 3 was loaded onto a Sephacryl S-200 gel filtration column (1.7 × 194 cm) equilibrated in 10 mM sodium phosphate buffer, pH 6.0, containing 0.1 M sodium chloride. The endo-β-mannosidase was eluted with the same buffer. The fraction containing endo-β-mannosidase activity (17 ml) was collected and buffer exchanged into 10 mM sodium phosphate buffer, pH 6.0, containing 0.7 M ammonium sulfate. The sample was concentrated to 2.0 ml using an Amicon Ultra YM-10.

Step 5. Octyl sepharose CL-4B chromatography

The concentrated fraction from step 4 was applied to an Octyl Sepharose CL-4B column (1.2 × 8.5 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.0, containing 0.7 M ammonium sulfate. The column was washed with the same buffer, and then the proteins eluted with a linear gradient of ammonium sulfate (0.7 to 0 M). The fractions with endo-β-mannosidase activity (140 ml) were collected and buffer exchanged to 10 mM sodium phosphate buffer, pH 6.0 containing 0.1 M sodium chloride. The fractions were then concentrated to 4.0 ml using an Amicon YM-30 membrane.

Step 6. Superdex 200 gel filtration

The concentrated fraction from step 5 was loaded onto two tandemly connected Superdex 200 gel filtration columns using a Waters 515 HPLC pump at a flow rate of 1 ml/min. The columns were equilibrated in 10 mM sodium phosphate buffer, pH 6.0 containing 0.1 M sodium chloride. The endo-β-mannosidase was eluted with the same buffer. The fraction containing endo-β-mannosidase activity (9.0 ml) was desalted and concentrated to 1.4 ml using an Amicon Ultra YM-10, and stored on ice.

Native-PAGE—Native-PAGE was performed using a 5.0% acrylamide continuous slab gel according to the method of Davis (*12*). Proteins were stained with Coomassie brilliant blue R-250. The gel was sliced at 2 mm intervals, and proteins in each slice were extracted in 0.5 M sodium phosphate buffer, pH 6.0, using a pellet mixer. Endo-β-mannosidase activity of each extract was measured.

Amino Acid Sequence Analyses—The purified enzyme was electrophoresed (SDS-PAGE) and the separated polypeptides were electrotransferred onto a polyvinylidene difluoride membrane as described previously (*13*). The *N*-terminal amino acid sequences of the polypeptides were determined by Edman degradation using a Procise 494 HT (Applied Biosystems). For internal amino acid sequence analysis, the 46 kDa-polypeptides from a SDS-PAGE gel were digested with lysylendopeptidase overnight at 35°C. The digested peptides were separated by C18 reversed-phase HPLC. They were then sequenced by Edman degradation.

Molecular Cloning of a cDNA Encoding Endo-β-mannosidase-associated Protein from Lily—Total RNA from young lily leaves was prepared as described previously (*14*). The mRNA was purified from the total RNA using a PolyATract mRNA isolation system (Promega). Double-stranded cDNA was synthesized from the mRNA using Thermoscript reverse transcriptase (Invitrogen). This cDNA was used as a template for PCR and rapid amplification of cDNA ends (RACE).

The FASTA program found some plant cDNAs and ESTs homologous to the amino acid sequences of the 46 kDa-polypeptide. These are from *Oryza sativa* (Os10g0339600), *Arabidopsis thaliana* (At4g34260), *Zea mays* (CO455210/CO521678/DR830859/DV531895) and *Glycine max* (BF009125/BG043019/BI786518/BU544194/BU765720/CA785205). Degenerate primers (F1, GCNYTNTGGCCNATGGGNGG; F2, CACCTGGCCNATGGGNGGNTGG;

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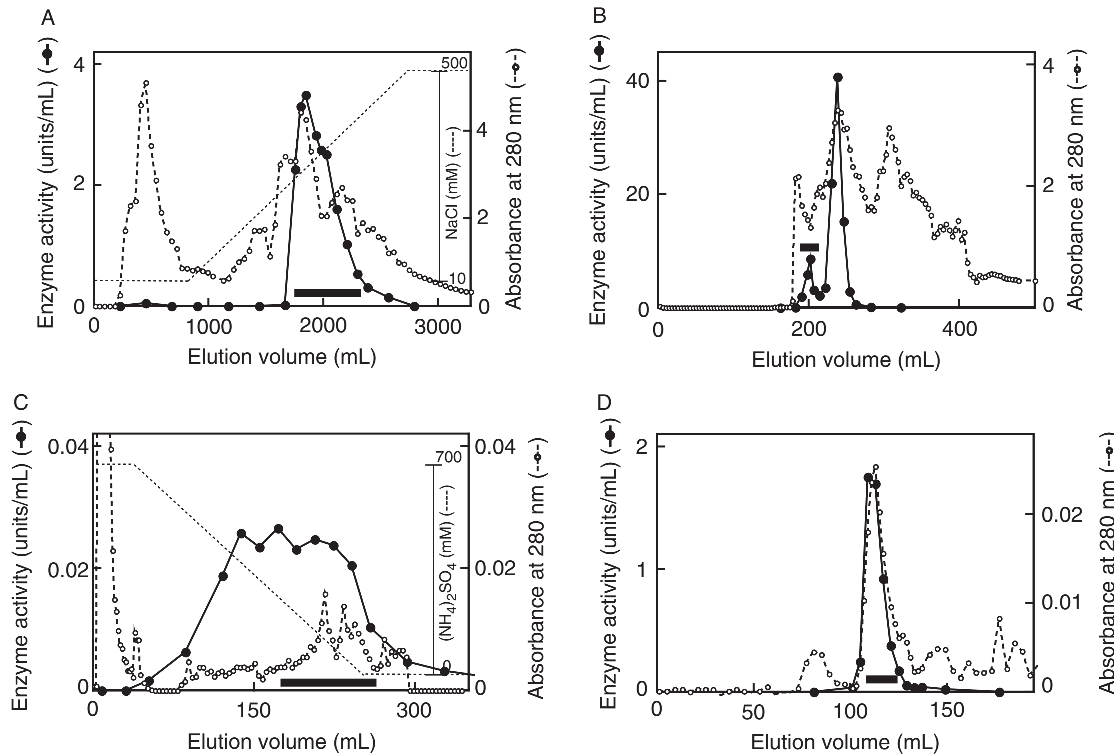

Fig. 1. **Purification of EBM II from lily flowers.** Endo-β-mannosidase was assayed using M2B-PA as a substrate. (A) DEAE-Sephacel chromatography of the proteins precipitated by 35% ammonium sulfate. (B) Sephacryl S-200 gel filtration of the pooled fractions indicated by the horizontal bar in A. (C) Octyl Sehpharose CL-4B chromatography of the pooled fractions indicated by the horizontal bar in B. (D) Superdex 200 gel filtration of the pooled fractions indicated by the horizontal bar in C.

F3, CACCAAYSANGAYACNHTNTGGACNGG; R1, CCRA ARTTNGCRTCDATYTGRAANGG; and R2, CRTCDATY TGRAANGGNGGRTGNGC) were designed based on the conserved amino acid sequences: ALWPMGGAW for F1 and F2; $N^{D}/E/_{H}DT^{L}/_{I}$WTG for F3; AHPPFQIDANFG for R1 and R2. The PCR reaction was performed using KOD plus DNA polymerase (Toyobo). The first PCR employing primers F1 and R1 was performed using a program of 30 cycles of 98°C for 15 s, 53°C for 30 s and 68°C for 60 s. The amplified products were used as template DNA for a second PCR. The same program was utilized for the second PCR using primers F2 and R2. The PCR fragment (719 bp) was subcloned into the pENTR vector (Invitrogen) and then sequenced. On the basis of the nucleotide sequence of this PCR product, two primers (R3, CTTG CAGTCTTACTATCTGGGG and R4, CCAAATAACCTC CACGACCTTC) were prepared for the next PCR. Another PCR using primers F3 and R3 was performed using a program of 30 cycles at 98°C for 15 s, 48°C for 30 s, and 68°C for 90 s. The amplified products were used in the next PCR. This PCR employing primers F3 and R4 was performed using a program of 30 cycles at 98°C for 15 s, 51°C for 30 s and 68°C for 90 s. The amplified product (1,487 bp) was subcloned into the pENTR vector and then sequenced. For RACE of the cDNA, the following four primers (F4, CAACATGGAAAATGTCTTGTTGGGC; F5, CACCGCCTTCGGGAAGCTGAACATGC; R5, CCAAGTG GTTGGTAAACATCTGATGG; and R6, CACCGCAGCTA AGGATGCTTCTGCATAC) were synthesized on the basis of the nucleotide sequence of the amplified PCR fragments. The 3′-RACE reaction using primers F4 and F5 was performed using a Generacer kit (Invitrogen) according to the manufacturer's instructions. The amplified fragment (655 bp) was subcloned into the pENTR vector, and its nucleotide sequence then determined. The 5′-RACE reaction using primers R5 and R6 was also performed with the same kit. The prominent fragment (447 bp) was subcloned into the pENTR vector and then sequenced.

RESULTS

Purification of EBM II—During the course of purification of endo-β-mannosidase from lily flowers (*1*), we detected two peaks containing endo-β-mannosidase activity by Sephacryl S-200 gel filtration (Fig. 1B). The lily endo-β-mannosidase has been purified from the major peak previously (*1*). In this study, an enzyme, EBM II in the minor peak eluted earlier than the major one has been further purified from lily flowers by ammonium sulfate precipitation, DEAE-Sephacel, Sephacryl S-200, Octyl-Sepharose, and Superdex 200 (Fig. 1). The results of the purification are summarized in Table 2. From 2.0 kg of lily flowers, 280 μg of purified EBM II were obtained. Substrate specificity of lily EBM II toward *N*-glycans was very similar to that of lily endo-β-mannosidase (Table 3). Optimum pH, pH stability and stability to heat of lily EBM II were also very similar to those of lily endo-β-mannosidase (*1*, *4*). The purified enzyme gave a single band

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Table 2. **Summary of the purification of EBM II from lily flowers.**

Step	Procedure	Activity (units)	Protein (mg)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
1	Crude enzyme	9,200	300,000	100	0.031	1
2	Ammonium sulfate precipitation (0–35%)	3,800	11,000	41	0.35	11
3	DEAE-Sephacel	780	1,800	8.5	0.43	14
4	Sephacryl S-200	80	33	0.87	2.4	77
5	Octyl Sepharose CL-4B	22	1.2	0.24	18	580
6	Superdex 200	10.4	0.28	0.085	37	1200

Table 3. **Substrate specificity of purified EBM II and endo-β-mannosidase.**

Substrate	Relative hydrolysis rate (%)	
	EBM II	Endo-β-mannosidase
M2B-PA	100	100
M3C-PA	42	48
M4B-PA	34	42
M1-PA	9	4
M2A-PA	ND	ND
M3B-PA	ND	ND
M5A-PA	ND	ND
M9A-PA	ND	ND
AG1.2-PA	ND	ND

Relative hydrolysis rates to that of M2B-PA are shown. ND indicates not detectable.

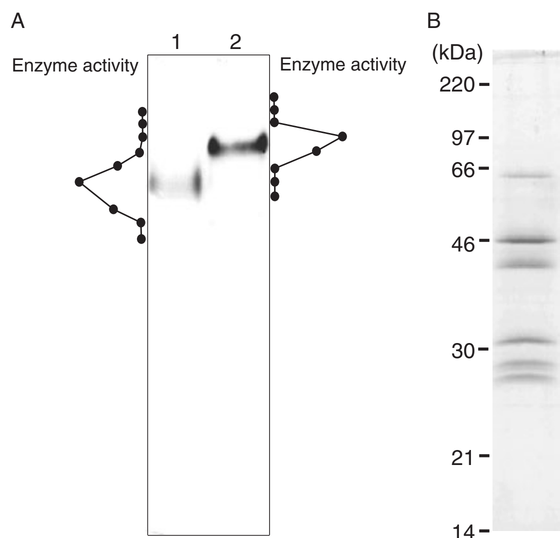

Fig. 2. **PAGE analysis of the purified enzyme.** (A) Native-PAGE of the purified enzyme. The gel was sliced at 2 mm intervals and the endo-β-mannosidase activity of proteins extracted from each slice was measured. Lane 1, endo-β-mannosidase; lane 2, EBM II. (B) SDS-PAGE (12% polyacrylamide) of the purified enzyme. The protein was stained with Coomassie brilliant blue R-250. The positions of molecular mass standards (kDa) are indicated on the left of the panel.

bearing enzyme activity on native-PAGE (Fig. 2A). The mobility of EBM II on native-PAGE was slower than that of endo-β-mannosidase (Fig. 2A), indicating a larger molecular mass. SDS-PAGE of the purified protein showed that EBM II was comprised of six polypeptides with molecular masses of 65, 46, 42, 31, 28 and 27 kDa (Fig. 2B).

EBM II Consists of Endo-β-mannosidase and Associated Protein—The *N*-terminal amino acid sequences of the 65-, 46-, 42-, 31-, 28- and 27-kDa polypeptides were determined to be EVLISTAERVKSFKVDEDPS, EVLIS, ETEDP, GKQVL, EYHQT and YHQTH, respectively. The sequences of the 42-, 31- and 28-kDa polypeptides correspond to three polypeptides, which comprise endo-β-mannosidase (*1*). Based on the sequence the 27-kDa polypeptide is presumed to be derived from the 28-kDa polypeptide. The *N*-terminal amino acid sequences of the 65- and 46-kDa polypeptides correspond with each other, showing that these two polypeptides are translated from the same gene. These analyses show that EBM II is comprised of endo-β-mannosidase and associated protein and that the associated protein does not influence the characteristics of the endo-β-mannosidase activity.

Molecular Cloning of the Endo-β-mannosidase-associated Protein—To analyze the functions of endo-β-mannosidase-associated protein, we cloned its cDNA. The 46-kDa polypeptide was excised from a polyacrylamide gel and then subject to lysylendopeptidase digestion. The amino acid sequences of two isolated peptides were determined to be VNYEASGWVAHQVSD and IARDGTIMEWAQNFE. A search of the FASTA database using these sequences as queries identified genes encoding functionally unknown proteins from *O. sativa* (Os10g0339600) and *A. thaliana* (At4g34260). A database search against these two sequences also found several ESTs from *Z. mays* and *G. max*. Using several primers whose design was based on conserved sequences within these molecules, the cDNA encoding the endo-β-mannosidase-associated protein was amplified from the lily leaf cDNA by PCR and RACE techniques. The nucleotide sequence of the cloned cDNA was deposited in the DDBJ/EMBL/GenBank databases under the accession number AB326211. The amino acid sequence deduced from the nucleotide sequence of the cloned cDNA contained all the amino acid sequences determined from the endo-β-mannosidase-associated protein. The cDNA encodes a protein consisting of 854 amino acid residues with the *N*-terminus of the endo-β-mannosidase-associated protein located at position 379 (Fig. 3A). The lily endo-β-mannosidase-associated protein and its plant homologues have 51 to 64% sequence identity to each other. They also have low sequence identity to the fucosidase domain of AfcA protein from *Bifidobacterium bifidum* (22 to 23%) (*9*) and α-L-fucosidase from

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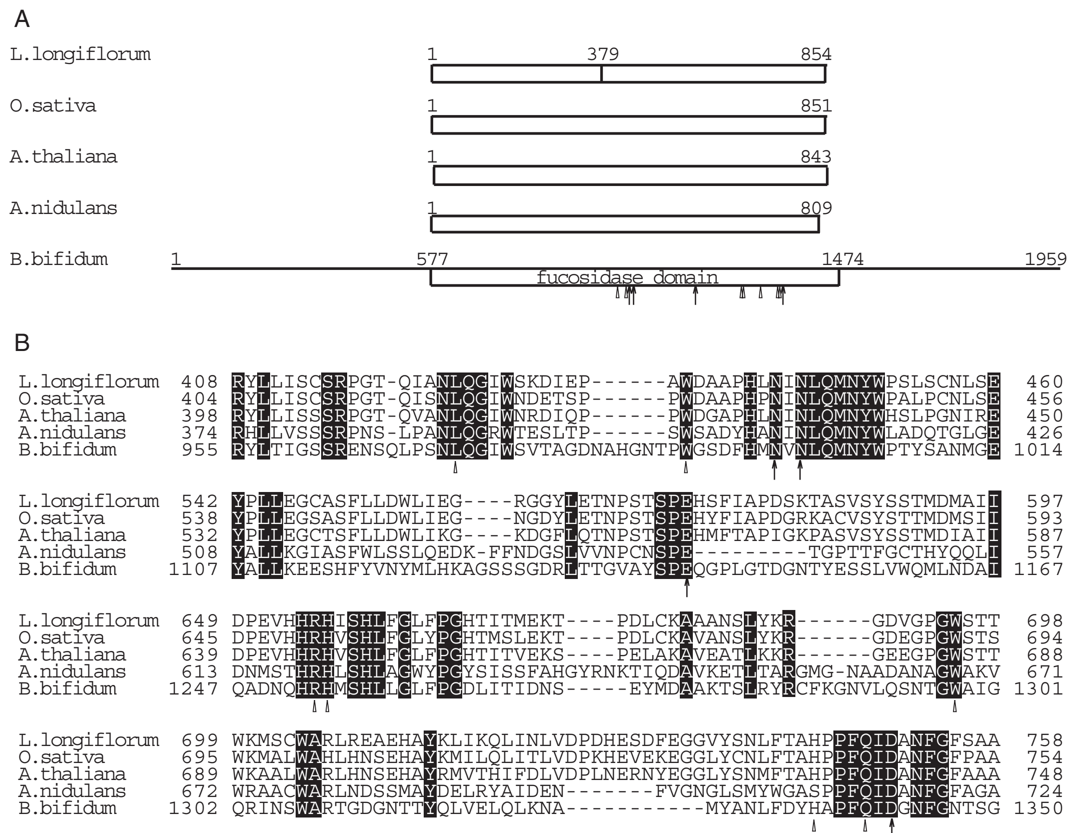

Fig. 3. **Structural analysis of the lily endo-β-mannosidase-associated protein (α1,2-fucosidase).** (A) Schematic representation of the lily endo-β-mannosidase-associated protein, *B. bifidum* α1,2-L-fucosidase, *A. nidulans* α1,2-L-fucosidase, and their homologues from *A. thaliana* and *O. sativa*. Numbering starts at a probable initiation codon of each sequence and the lengths are indicated at the right. The positions of the amino acid residues that are involved in the catalytic reaction and substrate binding are shown by arrows and triangles, respectively. The position of the *N*-terminus of the lily endo-β-mannosidase-associated protein (Glu379) is shown by a vertical bar. (B) Multiple alignment of the amino acid sequences of the lily endo-β-mannosidase-associated protein and its homologues. Alignment was created using the ClustalW program. The conserved amino acid residues are highlighted in black. Introduced gaps are indicated by hyphens. The *L. longiflorum*, *L. longiflorum* (lily) endo-β-mannosidase-associated protein (AB326211); *O. sativa*, *Oryza sativa* putative α1,2-L-fucosidase (AK066931, Os10g0339600), *A. thaliana*, *Arabidopsis thaliana* putative α1,2-L-fucosidase (AY125494, At4g34260), *A. nidulans*, *Aspergillus nidulans* α1,2-L-fucosidase (ABF50892), *B. bifidum*, *Bifidobacterium bifidum* AfcA (AY303700).

Aspergillus nidulans (24 to 26%) (*15*) (Fig. 3). These enzymes are classified to the glycoside hydrolase family 95. All the amino acid residues of the fucosidase domain of AfcA critical for catalysis and substrate binding (*16*) are conserved in the lily endo-β-mannosidase-associate protein, suggesting that the protein has α1,2-L-fucosidase activity.

EBM II has α1,2-L-Fucosidase Activity—The best substrate for the bifidus α1,2-L-fucosidase is 2′FL (*9*). The lily EBM II was therefore reacted with 2′FL to investigate its α1,2-L-fucosidase activity. This reaction produced a new peak at 5.8 min on a size-fractionation HPLC (Fig. 4A). The retention time of this peak corresponded to that of a α1,2-L-fucosidase (from *Corynebacterium*) digest of 2′FL, thus showing that EBM II has α1,2-L-fucosidase activity. The optimum pH for α1,2-L-fucoside activity was 5.5. The endo-β-mannosidase showed no α1,2-L-fucosidase activity (Table 4), and thus the α1,2-L-fucosidase activity is derived from the

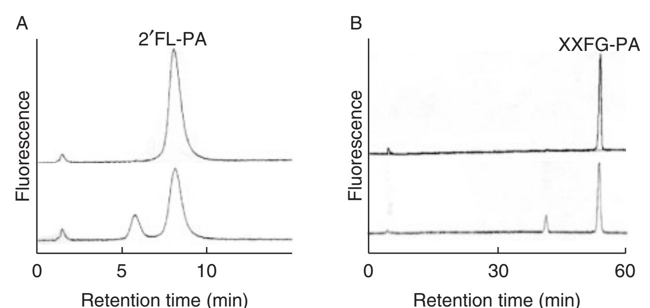

Fig. 4. **α1,2-Fucosidase activity of lily EBM II.** Fucosidase activity was assayed using size-fractionation HPLC. (A) Pyridylaminated 2′-fucosyllactose, 2′FL-PA, was incubated with the purified lily EBM II (lower panel) or denatured enzyme (upper panel) at 37°C for 20 min. (B) Pyridylaminated xyloglucan nonasaccharide, XXFG-PA, was incubated with the purified enzyme (lower panel) or denatured enzyme (upper panel) as described earlier.

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Table 4. **Substrate specificity for fucosidase activity of the purified EBM II and endo-β-mannosidase.**

Substrate	Relative hydrolysis rate (%)	
	EBM II	Endo-β-mannosidase
2′FL-PA	100	ND
LNFP I-PA	6.7	ND
XXFG-PA	66	ND
XLFG-PA	40	ND
3FL-PA	ND	ND
BGA-PA	ND	ND
BGAT-PA	ND	ND
LEA-PA	ND	ND
M2FX-PA	ND	ND
M2F-PA	ND	ND
PNP-αFuc	ND	ND

Relative hydrolysis rates to that of 2′FL-PA are shown. ND indicates not detectable.

endo-β-mannosidase-associated protein. EBM II was then incubated with the pyridylaminated xyloglucan oligosaccharide, XXFG-PA, which is a known plant sugar chain that contains an α1,2-fucoside linkage. A new peak at 45 min appeared that corresponded to the peak obtained from a *Corynebacterium* α1,2-L-fucosidase-digest of XXFG-PA (Fig. 4B). This shows that EBM II has α1,2-L-fucosidase activity against the xyloglucan nonasaccharide. The substrate specificity of α-L-fucosidase activity was studied using pyridylaminated sugar chains. The relative hydrolysis rates are summarized in Table 4. The enzyme hydrolyzed LNFP I although its hydrolysis rate was much slower than that observed against 2′FL. It also acted on a xyloglucan decasaccharide, XLFG, to similar extent as to 2′FL and XXFG. These sugar chains bear α1,2-fucoside linkages at their non-reducing ends. The enzyme did not act against sugar chains bearing a α1,2-fucoside linkage at positions other than the non-reducing ends such as BGA and BGAT. Furthermore, no activity could be observed against sugar chains bearing α1,3- or α1,4-fucoside linkages, such as 3FL, LEA, M2F and M2FX. The enzyme also did not hydrolyze *p*-nitrophenyl α-fucopyranoside. Therefore, these results show that EBM II hydrolyses the α1,2-fucoside linkage at non-reducing ends of sugar chains including those of xyloglucan oligosaccharides.

DISCUSSION

With regard to plant α-L-fucosidases, two enzymes bearing different substrate specificities from almond emulsin were first characterized in the 1970's (*17*). One was an α1,3/4-L-fucosidase and the other was an α1,2-L-fucosidase which hydrolysed 2′FL. Purification of these enzymes has been attempted by some laboratories and recently, Zeleny *et al*. (*18*) accomplished the purification of an α1,3/4-L-fucosidase to homogeneity from almonds and identified its corresponding Arabidopsis cDNA (At1g67830). This gene, however, has previously been claimed to encode an α1,2-L-fucosidase (AtFXG1) active against xyloglucan (*19*). Zeleny *et al*. (*18*), however, reported that the AtFXG1 did not exhibit α1,2-L-fucosidase activity. Molecular cloning of a gene for pea α1,2-L-fucosidase that acts on xyloglucan has been reported (*20*); however, this report was also denied afterward (*21*). Another *Arabidopsis* α1,2-L-fucosidase (AtFUC1, At2g28100) has been also identified, however, this gene product did not hydrolyze fucosylated xyloglucan oligosaccharide (*19*). Thus, the gene encoding the plant α1,2-L-fucosidase that acts on xyloglucan has not been unequivocally identified so far. Thus, this is the first report of the molecular cloning of the cDNA encoding a plant α1,2-L-fucosidase that acts on fucosylated xyloglucan. The amino acid sequence of the lily α1,2-L-fucosidase has no similarity to known plant α-L-fucosidases (*18*, *19*).

The α1,2-L-fucosidase activity in the crude extracts of almond emulsin, pea epicotyles, germinating nasturtium seeds and azuki bean epicotyls has been reported to date (*17*, *22–24*). Substrate specificities of these enzymes are such that they hydrolyse terminal α1,2-fucoside linkages including xyloglucan oligosaccharides but do not hydrolyse *p*-nitrophenyl α-fucopyranoside. These specificities correspond to those of the lily α1,2-L-fucosidase identified in this study, suggesting that the α1,2-L-fucosidases in previous reports are homologous proteins to the lily enzyme.

This α1,2-fucosidase is shown to associate with endo-β-mannosidase of lily flowers. This association was found during gel filtration chromatography purification of endo-β-mannosidase from lily flowers (Fig. 1B). This phenomenon was also observed in cabbage leaves (*5*). At least a part of endo-β-mannosidase is associated with α1,2-L-fucosidase under these conditions, although how part of endo-β-mannosidase makes a complex in nature remains to be investigated. The substrates for endo-β-mannosidase (*N*-glycans) are different from those of α1,2-L-fucosidase (fucosylated xyloglucan). Therefore, this raises the question as to the significance of the association between these glycoside hydrolases. Endo-β-mannosidase is located in the vacuole (although it may also be present elsewhere within the cell) (*7*), where many degradation enzymes exist. Therefore, it needs to be protected from vacuolar proteolysis in some way. An example of such a mechanism is seen with four hydrolases (β-galactosidase, α-neuraminidase, *N*-acetylaminogalacto-6-sulfate sufatase and cathepsin A), which form a complex in the mammalian lysosomes to protect each other from lysosomal proteases (*25*). The association between endo-β-mannosidase and α1,2-L-fucosidase may therefore be in order to enhance the stability of each other.

The *N*-terminal amino acid sequence of the lily α1,2-L-fucosidase is EVLIS, with the *N*-terminal residue being Glu379 of a precursor protein encoded by its cDNA (Fig. 3A). The X-ray crystal structures of the fucosidase domain of bifidus AfcA, which has homology to the lily enzyme, showed that this fucosidase domain consists of a *N*-terminal β region, a helical linker region, a central helical barrel domain and a *C*-terminal β region (*16*). All the amino acid residues involved in the catalytic reaction and substrate binding in this α1,2-L-fucosidase are concentrated in the central helical barrel domain (*16*). The mature lily α1,2-L-fucosidase contains the region equivalent to the central helical barrel domain

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(Fig. 3) that is consistent with the fact that EBM II has α1,2-L-fucosidase activity. However, the *N*-terminal β region of the fucosidase domain of AfcA is necessary for α1,2-L-fucosidase activity (*9*). It will be interesting to know whether the lily endo-β-mannosidase-associated protein has α1,2-L-fucosidase activity in isolation or whether it exhibits the activity only after it interacts with endo-β-mannosidase.

The lily α1,2-fucosidase was identified as a major 46 kDa-protein and a minor 65 kDa-protein (Fig. 2B). Because the calculated molecular mass of a protein consisting of Glu379 to Ile854 is 54 kDa, the 46 kDa-protein may be a result of proteolysis during maturation. The 65 kDa-protein is likely to be post-translationally modified but the details of this modification remain to be elucidated at present.

Signal peptide and known sorting signals for intracellular localization were not detected in the amino acid sequence of the lily α1,2-L-fucosidase. Given that many exoglycosidases are found in the vacuolar compartment, the lily α1,2-L-fucosidase may also locate to the vacuole and have a role in the degradation of oligosaccharides. Localization of endo-β-mannosidase to the vacuole (*7*) and the acidic optimum pH of the lily α1,2-L-fucosidase support this idea. Alternatively, the enzyme may locate to the cell wall given that an azuki bean α1,2-L-fucosidase was extracted from cell walls with 1 M sodium chloride (*24*). This idea is also consistent with the results of this study because many enzymes localized at cell walls have their optimum pH in the acidic region. In this case, as Hoson *et al*. (*24*) suggested, α1,2-L-fucosidase may work as a trigger of degradation of xyloglucans which have lower molecular mass (>50 kDa).

A good substrate for α1,2-L-fucosidase, xyloglucan nonasaccharide, XXFG, has been shown to inhibit auxin-induced elongation of pea stems at nanomolar concentrations (*26*, *27*) and to promote cell elongation of peeled stem segments at higher concentration (*28*). It also stimulates glucan synthase activity and increases the viability of carrot protoplasts (*29*). In contrast, the oligosaccharide produced upon digestion of XXFG by α1,2-L-fucosidase, XXLG, does not inhibit auxin-induced elongation at nanomolar levels (*30*), showing that the terminal fucosyl residue of XXFG is essential for inhibition of auxin-induced growth. The octasaccharide, XXLG, has been reported to be a signaling molecule that modulates α-fucosidase activity (*31*). Thus, the α1,2-L-fucosidase identified here may participate in regulating auxin-induced elongation by controlling the level of the xyloglucan elicitors. *In vivo* functional analysis of the α1,2-L-fucosidase and its target oligosaccharides can now be investigated using mutants of the gene encoding the α1,2-L-fucosidase.

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