Endo-\(\alpha\)-Mannosidase, A Plant Enzyme Acting on \(N\)-Glycan: Purification, Molecular Cloning, and Characterization

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SUMMARY

Endo-β-mannosidase is a novel endoglycosidase which hydrolyzes the \( \text{Man}\beta 1-4\text{GlcNAc} \) linkage in the trimannosyl core structure of \( N \)-glycans. This enzyme was partially purified and characterized in a previous report [Sasaki, A., Yamagishi, M., Mega, T., Norioka, S., Natsuka, S., and Hase, S. (1999) \( J. \text{Biochem.} \) 125, 363-367]. Here we report the purification and molecular cloning of endo-β-mannosidase. The purified enzyme gave a single band on native-PAGE and three bands on SDS-PAGE with molecular masses of 42, 31, and 28 kDa. Amino acid sequence information from these three polypeptides allowed the cloning of a homologous gene, \textit{AtEBM}, from \textit{Arabidopsis thaliana}. \textit{AtEBM} was engineered for expression in \textit{Escherichia coli} and the recombinant protein comprised a single polypeptide chain with a molecular mass of 112 kDa corresponding to sum of molecular masses of three polypeptides of the lily enzyme. The recombinant protein hydrolyzed pyridylamino derivatives (PA-) of \( \text{Man}\alpha 1-6\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc} \) into \( \text{Man}\alpha 1-6\text{Man} \) and \( \text{GlcNAc}\beta 1-4\text{GlcNAc}-\text{PA} \), showing that \textit{AtEBM} is an endo-β-mannosidase. \textit{AtEBM} hydrolyzed \( \text{Man}_n\text{Man}\alpha 1-6\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}-\text{PA} \) (\( n=0\sim2 \)) but not PA-sugar chains containing \( \text{Man}\beta 1-3\text{Man} \) or \( \text{Xyl}\beta 1-2\text{Man} \) as for the lily endo-β-mannosidase. \textit{AtEBM} belonged to the clan GH-A of glycosyl hydrolases. Site-directed mutagenesis experiments revealed that two glutamic acid residues (Glu464 and Glu549) conserved in this clan were critical for enzyme activity. Amino acid sequence of \textit{AtEBM} has distinct differences from those of the bacterial, fungal, and animal exo-type \( \beta \)-mannosidases. Indeed, \textit{AtEBM}-like genes are only found in plants, indicating that endo-β-mannosidase is a plant specific enzyme. The role of this enzyme in processing and/or degradation of \( N \)-glycan will be discussed.
INTRODUCTION

Endo-\(\beta\)-mannosidase is a novel endoglycosidase that hydrolyzes a \(\text{Man}\beta_1-4\text{GlcNAc}\) linkage in the trimannosyl core structure of \(N\)-glycans (1). Along with endo-\(\beta\)-\(N\)-acetylglucosaminidase and peptide \(N\)-glycanase, it is classified as an endo-type hydrolase that acts close to the reducing end of \(N\)-glycans attached to proteins. Discovery of this enzyme was based on structural analysis of the \(N\)-glycans of Japanese pear S-RNases which has chitobiose as the major \(N\)-glycan (2, 3). This enzyme activity has been identified in the crude extract from lily flowers using a pyridylaminated (PA-) \(\text{Man}\alpha_1-6\text{Man}\beta_1-4\text{GlcNAc}\beta_1-4\text{GlcNAc}\) as substrate (1). The partially purified enzyme hydrolyzed \(\text{Man}_n\text{Man}\alpha_1-6\text{Man}\beta_1-4\text{GlcNAc}\beta_1-4\text{GlcNAc-PA}\) (\(n=0-2\)) to \(\text{Man}_n\text{Man}\alpha_1-6\text{Man}\) and \(\text{GlcNAc}\beta_1-4\text{GlcNAc-PA}\), confirming that this enzyme was an endoglycosidase. It does not hydrolyze \(\beta\)-mannohexasaccharide, and is therefore different from \(\beta\)-mannanase (4). It also differs from \(\beta\)-mannosidases because endo-\(\beta\)-mannosidase does not hydrolyze \(p\)-nitrophenyl \(\beta\)-mannoside (\(p\text{NP} \beta\text{-Man}\)) (1, 5). Substrates containing \(\text{Man}\alpha_1-3\text{Man}\) and/or \(\text{Xyl}\beta_1-2\text{Man}\) structures were not hydrolyzed with the partially purified enzyme. The substrate specificity of endo-\(\beta\)-mannosidase complements that of Jack bean \(\alpha\)-mannosidase, which displays a preference for a \(\text{Man}\alpha_1-3\text{Man}\) linkage. Therefore the \(N\)-linked chitobiose in S-RNase (3) is probably produced by consecutive digestion of high-mannose type \(N\)-glycans with \(\alpha\)-mannosidase and endo-\(\beta\)-mannosidase. We are interested both in the function of endo-\(\beta\)-mannosidase in the plant cell and also the enzyme mechanism of this unique substrate specificity.

In this paper, we describe the purification of endo-\(\beta\)-mannosidase from lily flowers. Partial amino acid sequence data from the purified protein enabled us to clone the corresponding gene from Arabidopsis thaliana. The gene was engineered for expression in Escherichia coli, and the recombinant protein was found to possess endo-\(\beta\)-mannosidase
activity. The sequence of the enzyme was characterized and the substrate specificity of the enzyme was investigated. Based on these results, its possible function in vivo is discussed.

EXPERIMENTAL PROCEDURES

Materials

Flower buds of the lily (Lilium longiflorum Thunb. cv. Hinomoto) were used as an enzyme source. PA-sugar chains listed in Table I were prepared as reported previously (6, 7). pNP α-Man and pNP β-Man were purchased from Nacalai Tesque (Kyoto, Japan), and Manα1-6Man was from Sigma (St. Louis, MO). DEAE-Sephacl, a Superdex 200 column (1.6 ¥ 60 cm), and a Mono Q HR 5/5 column (0.5 ¥ 5 cm) were purchased from Amersham Biosciences (Piscataway, NJ). The HA 1000 hydroxyapatite column (0.75 ¥ 7.5 cm) was from Tosoh (Tokyo, Japan), and the Poros HS column (0.46 ¥ 10 cm) from Applied Biosystems (Foster City, CA). The Shodex Asahipak NH2-P columns (0.46 ¥ 7 cm) were from Showa Denko (Tokyo, Japan). The Inertsil ODS-3 column (0.46 ¥ 25 cm) was from GL Sciences (Tokyo, Japan), and the CarboPac PA-1 column (0.2 ¥ 25 cm) was from Dionex (Sunnyvale, CA). Diaflo membranes (XM-50 and YM-10) were from Millipore (Bedford, MA). The BCA protein assay reagent kit was from Pierce (Rockford, IL). Jack bean α-mannosidase and almond peptide N-glycanase were purchased from Seikagaku Kogyo (Tokyo, Japan). The anti-lily endo-α-mannosidase antibody was raised by immunizing a rabbit with the purified lily endo-α-mannosidase.

Assay of endo-α-mannosidase activity

Endo-α-mannosidase activity was measured as described previously (1). Briefly, the enzyme
and 12.5 mM of M2B-PA in 16 mL of 0.16 M ammonium acetate buffer, pH 5.0, were incubated at 37 °C for 30 min. The chitobiose-PA generated was quantified by size-fractionation HPLC. The pH dependence and stability of the enzyme activities were measured using M2B-PA as a substrate with 0.2 M sodium citrate phosphate buffer, pH 3.0 - 8.0. One unit of enzyme activity was defined as the amount of enzyme that released 1 nmol of PA-chitobiose from 12.5 mM of M2B-PA per minute under the conditions used.

**High performance liquid chromatography**

PA-sugar chains were analyzed by size-fraction and reversed-phase HPLC with the following elution conditions. Size-fractionation HPLC was performed on an NH2-P column at the flow rate of 0.6 mL/min by isocratic elution. The eluent used was 3 % (v/v) acetic acid in a 400:85 (v/v) mixture of acetonitrile:water adjusted to pH 7.3 with triethylamine. Reversed-phase HPLC was performed on an Inertsil ODS-3 column at the flow rate of 1.5 mL/min. The eluents used were 0.1 M ammonium acetate, pH 6.0 (Buffer A), and 0.1 M ammonium acetate, pH 6.0, containing 1.0 % 1-butanol (Buffer B). The column was equilibrated in Buffer A containing 3 % Buffer B. After injecting a sample, the proportion of Buffer B was increased linearly to 50 % in 45 min. A Beckman model 332 chromatograph equipped with a Hitachi model 650-10M fluorescence spectrophotometer was used. PA-derivatives were detected by their fluorescence using an excitation and an emission wavelength of 310 nm and 380 nm, respectively. All HPLC procedures were carried out at 25 °C. For high pH anion-exchange chromatography, sugar chains were analyzed on a CarboPac PA-1 column equipped with a Dionex DX-500 system at the flow rate of 1.0 mL/min using isocratic elution in 0.1 M sodium hydroxide. The elution of sugar chains was monitored with a pulsed amperometric detector.
Purification of endo-β-mannosidase from lily flowers

All purification procedures were carried out below 4 °C. The amount of protein was determined using absorbance at 280 nm or a BCA protein assay reagent kit with bovine serum albumin as a standard.

**Step 1. Preparation of a crude enzyme solution:** Flower buds of lily (1,200 g) frozen with liquid nitrogen were powdered with a Waring blender. The powder was suspended in 4,800 mL of 0.1 M sodium phosphate buffer, pH 6.0, and homogenized with a Polytron homogenizer (10,000 rpm, 3 min). The homogenate was centrifuged at 28,000 $\times$ g for 30 min, and the supernatant 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 obtained by centrifugation at 46,000 $\times$ g for 30 min was used in the next purification step.

**Step 3. DEAE-Sephacel chromatography:** The supernatant (160 mL) was placed on a DEAE-Sephacel column (4.6 $\times$ 55 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.0, and the column was 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 (470 mL) was concentrated to 6.5 mL with an Amicon XM-50 membrane.

**Step 4. Superdex 200 gel filtration:** The concentrated fraction in step 3 was loaded onto two tandemly connected Superdex 200 gel filtration columns using a fast protein liquid chromatography (FPLC) system at a flow rate of 1 mL/min. The columns were equilibrated with 20 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 was collected and concentrated to 5 mL with an Amicon XM-50 membrane.

Step 5. Hydroxyapatite chromatography: A hydroxyapatite HA 1000 column connected with an FPLC system was equilibrated with 10 mM sodium phosphate buffer, pH 6.0, at a flow rate of 1 mL/min. After injection of the concentrated enzyme solution, the column was washed with the same buffer for 10 min. The endo-β-mannosidase was eluted with a linear gradient of sodium phosphate (10 to 500 mM) in 50 min. The fractions with endo-β-mannosidase activity were desalted and concentrated to 1.3 mL with an Amicon YM-10 membrane.

Step 6. Mono Q chromatography: The enzyme fraction obtained in step 5 was applied to a Mono Q HR 5/5 column equilibrated with 10 mM sodium phosphate buffer, pH 6.0, at a flow rate of 1 mL/min. The column was washed with the same buffer for 10 min. The endo-β-mannosidase was eluted with a linear gradient of sodium chloride from 0 to 0.4 M in 60 min. The fraction containing endo-β-mannosidase was desalted and concentrated to 2 mL with an Amicon YM-10 membrane.

Step 7. Poros HS chromatography: The concentrated fraction obtained in step 6 was applied to a Poros HS column equilibrated with 50 mM sodium acetate buffer, pH 4.0, at a flow rate of 1 mL/min. The column was washed with the same buffer for 10 min, and the enzyme was eluted with a linear gradient of sodium chloride from 0 to 1 M in 60 min. The endo-β-mannosidase fraction was pooled, concentrated to 2 mL with an Amicon YM-10 membrane, and stored on ice.

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE was performed on a 10 % polyacrylamide gel, pH 7.5, according to the method of Davis (8). SDS-PAGE was carried out by the method of Laemmli (9) under reducing
conditions with 2-mercaptoethanol. Proteins were stained with Coomassie Brilliant Blue R-250.

**Amino acid sequence analyses**

The purified lily enzyme was analyzed by SDS-PAGE, and the three polypeptide subunits were electrotransferred onto a polyvinylidene difluoride membrane using the method of Hirano and Watanabe (10). Protein bands were excised and submitted for N-terminal amino acid sequencing. Edman degradation sequencing was performed by APRO Life Science Institute (Naruto, Japan). For internal amino acid sequence analyses, protein bands on an SDS-PAGE gel were excised, and they were digested with trypsin at pH 8.0 for 20 h at 35 °C. Peptides were separated by reversed-phase HPLC and their elution was monitored at 210 nm. Purified peptides were submitted for amino acid sequencing.

**Molecular cloning of the Arabidopsis endo-β-mannosidase gene, AtEBM**

The amino acid sequence obtained from the purified lily endo-β-mannosidase was screened against the protein database using the TFASTA program (11). A candidate gene for endo-β-mannosidase (registered as AY045934 and AC000106 for DDBJ/EMBL/GenBank database or At1g09010 for a transcribed unit in the Arabidopsis genome database) was identified. The candidate gene was amplified from *A. thaliana* cDNA using the following primers: F1, 5’-GCGCCATATGGCGGAGATCGGAAG-3’; R1, 5’-GCGCGCTCAGTCACAAACACCTAAAACC-3’. The PCR primers were derived from the putative N- and C-terminal amino acid sequences of the AY045934/AC000106/At1g09010 gene product. We also incorporated *Nde*I and *Bpu*1102I restriction sites into the F1 and R1 primers, respectively (underlined). KOD DNA
polymerase (Toyobo, Osaka, Japan) was used according to the manufacturer’s instructions. The cloned gene was named \textit{AtEBM}. \textit{A. thaliana} shoot cDNA (kindly donated from Dr. T. Kakimoto of Osaka University) was used as a template for the PCR. PCR cycling was: 94 °C (1 min); 30 cycles of 94 °C (0.25 min), 52 °C (0.5 min), 70 °C (3 min); 70 °C (10 min). The amplified DNA fragment was cloned into pBluescript vector (Stratagene, La Jolla, CA) and then sequenced on an ABI PRISM 377 DNA Sequencer using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) for the labeling of the sequencing reactions.

\textbf{Expression of AtEBM in E. coli}

The PCR product was subcloned into the \textit{NdeI} and \textit{Bpu1102I} sites of a bacterial expression vector pET15-b (Novagen, Madison, WI). The resulting plasmid, AtEBMpET15, was transformed into \textit{Escherichia coli} RosettaBlue cells (Novagen). Cells were grown in 500 mL of Luria-Bertani medium containing 50 µg/mL carbenicilin at 16 °C to an \textit{OD}_{600} of 0.5. The gene was expressed with induction at 16°C 12 h with 1 mM isopropyl-1-thio-\textit{D}-galactopyranoside. The cells were harvested by centrifugation (3,000 \( \times \) g for 10 min at 4 °C). Cell pellets were resuspended and disrupted in 10 mL of 20 mM sodium acetate buffer, pH 6.0, containing 2 \( \mu \)g/mL of aprotinin, 5 \( \mu \)g/mL of leupeptin, 0.7 \( \mu \)g/mL of pepstatin, 1 mg/mL of lysozyme, and 25 U/mL of benzonase (Novagen) for 2 h on ice. Resuspended cells were subjected to three complete cycles of freeze-thawing. The extract was then centrifuged at 5,500 \( x \) g for 10 min at 4 °C. The resultant supernatant was dialyzed against 20 mM sodium acetate, pH 6.0, containing 2 \( \mu \)g/mL of aprotinin, 5 \( \mu \)g/mL of leupeptin, 0.7 \( \mu \)g/mL of pepstatin. The sample was then applied to a DEAE-Sephacel column (2.6 \( \times \) 42 cm) equilibrated in 20 mM sodium phosphate, pH 6.5. The column was washed with the same buffer, and the recombinant protein was eluted with a linear gradient of sodium chloride from
0 to 1 M. The endo-β-mannosidase fraction was collected and used as an enzyme solution.

**Site-directed mutagenesis**

Site-directed mutagenesis of Glu464 and Glu549 of AtEBM was performed by overlap extension PCR according to the method Higuchi *et al.* (12). The AtEBMpET15 construct was used as a template DNA for mutagenic PCR reaction. For construction E464ApET15 (Glu 464 in AtEBM replaced Ala), the PCR fragments obtained with the oligonucleotides E464A sense/R2 (E464A sense, 5’-GTGGGTGGAAATGCACAAGTTCCG-3’; R2, 5’-GTACATGGACTGGCTCTGCAGCGG-3’) and F1/E464A antisense (E464A antisense, 5’-GGCGGAACTTGTGCATTTCACCCAC-3’) were used as a template for another PCR reaction with F1 and R2 as primers. The resulting fragment was subcloned in *Bam*HI/*Sac*I sites of AtEBMpET15b. The E549ApET15 (Glu 549 in AtEBM replaced Ala) was constructed as described above using E549A sense and E549A antisense primers (E549A sense, 5’-TTCAATCCGGCGGTCCGCTCGGT-3’; E549A, antisense, 5’-AACCGAGCCCGACCGCGGATTGAAC-3’). The resultant mutants were expressed in *E. coli* and an enzyme solution for each mutant was prepared as described above.

**Western Blotting**

Proteins were separated by SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was reacted with the first antibody (raised in rabbit against the endo-β-mannosidase purified from lily flowers) and then with the second antibody (alkaline phosphatase-conjugated antibody raised in goat against rabbit IgG; ICN Biomedicals, Irvine, CA). Positive bands were visualized with 5-bromo-4-chloro-3-indolylphoaphate and nitro blue tetrazolium.
RESULTS

Purification of the lily endo-β-mannosidase

Endo-β-mannosidase was purified from lily flowers using a seven-step protocol described in “Experimental procedures” (Fig. 1). The results of the purification are summarized in Table II. Two peaks displaying endo-β-mannosidase activity were detected after DEAE-Sephacel chromatography (third step) and gel filtration chromatography (fourth step), indicating that at least two forms of endo-β-mannosidase exist in lily flowers (Fig. 1A, B). The first peak from the anion exchange column and the second peak from the gel filtration column were used for purification. Characterization of the remaining peaks will be described elsewhere. Poros HS chromatography (last step) was effective in removing residual contaminating proteins although the yield of enzyme activity was relatively low in this step. From 1.2 kg of lily flowers, 36 mg of the purified enzyme was obtained. The purified protein gave a single band on native-PAGE and a single peak by gel filtration chromatography, which coincided with the enzyme activity (Fig. 2A, B). Assuming that the protein is roughly globular, the native molecular mass was estimated to be ~78 kDa by gel filtration chromatography (Fig. 2B). SDS-PAGE of the purified enzyme showed that endo-β-mannosidase is comprised of three polypeptides with molecular masses of 28, 31, and 42 kDa (Fig. 2C). The combined molecular mass of these three bands is 101 kDa. The three bands were also detected under non-reducing conditions on SDS-PAGE (data not shown), indicating that these peptides did not bound covalently among them by a disulfide bond.

Molecular cloning of the Arabidopsis gene, AtEBM, encoding homologous amino acid
sequence to that of the purified lily endo-$\beta$-mannosidase

The purified lily enzyme was composed of three polypeptides (Fig. 2C). $N$-terminal amino acid sequences of the 28, 31, 42 kDa polypeptides were determined as EYHQTHELSI, GKQVLDGWLAARSTELETGVQ, and ETEPSQYLDGTRVYIQGSMWEGFA, respectively. Internal amino acid sequences were: VFIRGGNWILSDGLL and FHADMNFNMIR for the 28 kDa polypeptide, DVATQYVEGWDW for the 31 kDa polypeptide, and GTGSGVAFFLHF for the 42 kDa polypeptide. We searched for genes encoding protein homologous to these amino acid sequences using TFASTA program. Several candidates were retrieved from the database. We decided to focus our attention on the Arabidopsis gene, AY045934/AC000106/At1g09010. At1g09010 was registered as a cDNA of 3280 bp containing a 1944 bp open reading frame (ORF) encoding a 74 kDa polypeptide with 647 amino acid residues. However, the PCR product amplified by primers constructed from genomic sequence around At1g09010 (AC000106) gave a 2835 bp ORF encoding a 107 kDa polypeptide with 944 amino acid residues (Fig. 3). This cloned gene was named AtEBM in this study. The putative amino acid sequence of AtEBM was homologous to all the peptide sequences derived from the three polypeptides of the lily enzyme. Furthermore, the molecular mass of the putative AtEBM protein (107 kDa) corresponded to the sum of the three polypeptides of the lily enzyme (101 kDa). The data strongly suggested that AtEBM encodes an endo-$\beta$-mannosidase.

Comparison of the amino acid sequence of AtEBM with those of other $\beta$-mannosidases

Sequence analysis revealed that AtEBM belongs to the glycosyl hydrolase family 2 (13). The amino acid sequence of the purified lily endo-$\beta$-mannosidase and AtEBM were aligned with those of $\beta$-mannosidases from mammal (Bos taurus) (14), fungi (Aspergillus aculeatus)
(15), bacteria (*Cellulomonas fimi*) (16), and thermophilic bacteria (*Thermotoga neapolitana*) (17) (Fig. 3). AtEBM had 17 to 18% identity with mammalian β-mannosidases associated with β-mannosidosis (14, 18, 19, 20), 13 to 14% identity with fungal β-mannosidases involved in mannan degradation (15, 21), 16 to 20% identity with bacterial β-mannosidases (16, 22), and 20 to 21% identity with thermophilic bacterial β-mannosidases (17). The most homologous gene (73% identity) was from *Gossypium hirsutum* (AY187062), but a functional characterization of the encoded protein has not yet been performed. The conserved amino acid residues between endo-β-mannosidase and β-mannosidases (Fig. 3) appear to be important for the hydrolysis of Man[β]1-4GlcNAc. Among them, Glu549 of AtEBM is predicted to be a catalytic nucleophile because the corresponding Glu residue of *C. fimi* β-mannosidase was identified as an essential nucleophile in a chemical modification experiment (23). AtEBM is classified as a GH-A hydrolase (24) which are proposed to have a (β/α)8 barrel structure and a general acid catalytic mechanism with retention of anomeric configuration. The catalytic proton donor residue has been identified in some hydrolases within the clan GH-A (25), and corresponds to Glu464 of AtEBM. The amino acid sequence of endo-β-mannanase (26) showed some homology to the sequence around these two Glu residues in endo-β-mannosidase (data not shown). The phylogenetic tree based on the full amino acid sequences of β-mannosidases was constructed using a neighbor-joining method (27) (Fig. 4). AtEBM clustered with the *G. hirsutum* gene and was clearly separated from other β-mannosidases.

**Endo-β-mannosidase activity of recombinant AtEBM and its mutants**

To determine whether AtEBM possesses endo-β-mannosidase activity, we engineered the *AtEBM* gene for heterologous expression in *E. coli*. *E. coli* cells are suitable for host cells of
expression of endo-β-mannosidase gene because *E. coli* cells do not have any endo-β-mannosidase activity. The PCR fragment encoding the protein consisted of 944 amino acid residues was subcloned into an expression vector (pET15b) to give AtEBMpET15. A soluble polypeptide with a molecular mass of 112 kDa was newly expressed in *E. coli* (Fig. 5, lane 2). This newly expressed protein cross-reacted with the anti-lily endo-β-mannosidase antibody (Fig. 5, lane 6), although this antibody non-specifically interacted with some *E. coli* proteins (Fig. 5, lane 5~8). Comparison between the hydrolysis activity against M2B-PA for the soluble fractions of cells transformed with AtEBMpET15 and that of mock cells (68 mU/g protein and <0.01 mU/g protein, respectively) showed that recombinant AtEBM hydrolyzed M2B-PA to GN2-PA. The soluble fraction of mock cells did not change the exogenous substrate, M2B-PA, showing that contaminating activity in mock cells was not affected the results. Two mutants, in which Glu464 and Glu549 of AtEBM were substituted to alanine residues, were constructed (E464ApET15 and E549ApET15, respectively) and expressed in *E. coli*. Glu464 and Glu549 are the proposed catalytic proton donor and nucleophile, respectively. Expression level of both mutants, E464A and E549A, were similar to that of AtEBM (Fig. 5, lane 3, 4, 7, and 8), but they did not show any enzyme activity (<0.01 mU/g protein). These results showed that Glu464 and Glu549 in AtEBM were critical for enzyme activity.

**Characterization of AtEBM**

The substrate specificity of the recombinant AtEBM was studied using PA-sugar chains. The endo-type hydrolase activity of the partially purified recombinant AtEBM was confirmed by observing hydrolysis of Manβ1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA to Manβ1-6Man and GlcNAcβ1-4GlcNAc-PA at the molar ratio of 1.0:1.1. It also hydrolyzed M3C-PA in an
endo-type manner to Man\(1-3\)Man\(1-6\)Man and GlcNac\(1-4\)GlcNac-PA (data not shown). The relative hydrolysis rates against PA-sugar chains are summarized in Table III. M2B-PA was the best substrate among the PA-sugar chains studied. M3C-PA and M4B-PA were hydrolyzed to GN2-PA, and M1-PA underwent slight hydrolysis. But the enzyme did not hydrolyze PA-sugar chains containing the Man\(1-3\)Man\(1-6\)Man and GlcNac\(1-4\)GlcNac structure such as M2A-PA, M3B-PA, M5A-PA, M9A-PA, and Bi-PA. As with the lily enzyme, M2X-PA containing a Xyl\(1-2\)Man\(1-3\)Man\(1-6\)Man structure was not hydrolyzed by AtEBM (1).

The optimal pH of the partially purified AtEBM was 5.0 (Fig. 6A). The enzyme activity was retained after incubation for 2 h at pH values between 4 to 6 (Fig. 6B), but was unstable below pH 3 and above pH 7. As with the lily enzyme, the enzyme activity of recombinant AtEBM was not perturbed by the addition of 10 mM EDTA or 2 mM Ca\(^{2+}\) (1).

**DISCUSSION**

Endo-\(\beta\)-mannosidase from lily flowers was purified to homogeneity for the first time. The molecular mass of the lily enzyme estimated from gel filtration chromatography (78 kDa) (Fig. 2B) did not correspond to the apparent molecular mass by SDS-PAGE (101 kDa) (Fig. 2C). This discrepancy may be caused by interaction of the enzyme with the dextran resin of the superdex gel leading to a delay in the elution of the protein from the column. Similar results have been reported for other glycosyl hydrolases (28, 29, 30).

The lily endo-\(\beta\)-mannosidase is composed of three polypeptides (28, 31, 42 kDa) (Fig. 2C). All the amino acid sequence data from the lily enzyme aligned with the putative amino acid sequence of *AtEBM* (Fig. 3). Hence the three polypeptides of the lily enzyme may be encoded by a single gene homologous to *AtEBM*. Indeed, partial nucleotide sequence of a
cDNA clone of the lily enzyme encodes all three polypeptides (unpublished data). Even when the proteins were extracted from lily flowers in the presence of a protease inhibitor cocktail, the purified enzyme consisted of three polypeptides, concluding that these three polypeptides are not artificially generated during purification procedures (data not shown). The lily endo-β-mannosidase may be translated as a single polypeptide chain, which is then immediately subject to proteolysis to generate the three polypeptides. Recombinant AtEBM from *E. coli* consisted of a single polypeptide that possessed similar characteristics to the lily endo-β-mannosidase activity, suggesting that posttranslational proteolysis of the lily enzyme may not be essential for catalytic activity. The conclusion of this topic must await expression of the lily endo-β-mannosidase gene in a heterologous system.

Amino acid sequence alignment of AtEBM and homologous proteins revealed that AtEBM belonged to the clan GH-A glycosyl hydrolase (24) (Fig. 3). Enzyme activity of AtEBM mutated at Glu464 or Glu549 was not detected in this study, clearly showing that these two Glu residues of AtEBM are critical for enzyme activity. These two Glu residues are completely conserved in the clan GH-A, suggesting that endo-β-mannosidase has a same catalytic mechanism as other glycosyl hydrolases in the clan GH-A.

Endo-β-mannosidase appears to be plant specific because this activity was not detected in mammalian cells (rat liver, mouse liver, quail liver, salmon egg, and zebrafish egg) (our unpublished results), and because highly homologous sequences to endo-β-mannosidase are not found in other than the plant genome sequences. All the homologous genes from species other than plant were exo-type β-mannosidase (Fig. 3). The only highly homologous gene, *G. hirsutum* (cotton) gene (AY187062), presumably encodes an endo-β-mannosidase.

The optimal pH and pH stability of the enzyme suggested that it operates in an acidic organelle such as the vacuole or a secretory vesicle. S-RNase, a candidate substrate for
endo-β-mannosidase, is a secretory protein. The N-glycan of S-RNase may be subject to hydrolysis by endo-β-mannosidase in one of the organelles or secretory vesicles that the protein passes through during secretion. Any potential signal sequences that target the protein for organelle were not found in the sequence of AtEBM. The precise location of the endo-β-mannosidase within the cell remains to be determined.

Both the lily enzyme and that subsequently identified from *A. thaliana* (AtEBM) catalyzed the hydrolysis of the Manβ1-4GlcNAc linkage in the trimannosyl core structure of the N-linked sugar chains. But the endo-β-mannosidase does not hydrolyze the Manβ1-4GlcNAc linkage in oligosaccharides containing Manα1-3Manβ. Therefore this enzyme has complementary substrate specificity to that of Jack bean α-mannosidase which hydrolyzes Manβ1-3Manβ faster than Manα1-6Manβ (7, 31, 32). *In vivo*, the trimannosyl core structure may be hydrolyzed initially by α-mannosidase, and then endo-β-mannosidase may act on the resulting Manβ1-6Manβ1-4GlcNAcβ1-4GlcNAc structure. Judging from the substrate specificities of endo-β-mannosidase, along with plant α-mannosidase, both enzymes are presumably involved in N-glycan processing and/or degradation in plant cells. In mammalian cells, lysosomal α-mannosidase which specifically hydrolyzes Manβ1–6Man has been found (33). This hydrolase can compensate for the deficiency of an endo-β-mannosidase in terms of processing and/or degradation of the trimannosyl core structure.

Identification of the *A. thaliana* gene encoding endo-β-mannosidase has enabled us to analyze the function of this enzyme. A gene knockout study in *A. thaliana* will provide clues to the function of endo-β-mannosidase *in vivo*.

REFERENCES


The nucleotide sequence reported for *AtEBM* has been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases with accession number AB122060.

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The abbreviations used are: FPLC, fast protein liquid chromatography; GlcNAc, *N*-acetyl-d-glucosamine; Man, d-mannose; PA-, pyridylamino-. The structures and abbreviations for the sugar chains are listed in Table I.
FIGURE LEGENDS

Fig. 1  **Purification of endo-β-mannosidase from lily flowers**

Chromatography was carried out as described in “Experimental Procedures”. The fractions indicated by the *bars* were collected. Endo-β-mannosidase activity was measured with M2B-PA as a substrate. A: DEAE-Sephacel chromatography. B: Superdex 200 gel filtration of the pooled fraction indicated by the *bar* in A. C: Hydroxyapatite chromatography of the pooled fraction indicated by the *bar* in B. D: Mono Q chromatography of the pooled fraction in C. E: Poros HS chromatography of the pooled fraction in D.

Fig. 2  **Native-PAGE and gel filtration of the purified enzyme, and SDS-PAGE of proteins in each purification step**

A: Native-PAGE of the purified enzyme. The enzyme activity of gel slices was measured using M2B-PA as a substrate. B: Superdex 200 gel filtration of the purified enzyme. The *arrows* indicate the elution positions of standard proteins; a, α-globulin (160 kDa); b, bovine serum albumin (66 kDa); c, ovalbumin (45 kDa); d, cytochrome c (12 kDa). C: SDS-PAGE was carried out under the reducing conditions with 2-mercaptoethanol. Lane 1, the proteins after superdex 200 gel filtration; lane 2, the proteins after hydroxyapatite chromatography; lane 3, the proteins after Mono Q chromatography; lane 4, the proteins after Poros HS chromatography. The positions of molecular mass standards (kDa) are indicated on the left of the panel.

Fig. 3  **Amino acid sequence alignment of endo-β-mannosidases and exo-type β-mannosidases**
A. thaliana, Arabidopsis thaliana endo-\(\beta\)-mannosidase (AB122060); G. hirsutum, Gossypium hirsutum putative glycosyl hydrolase (AY187062); B. taurus, Bos taurus \(\beta\)-mannosidase (U17432); A. aculeatus, Aspergillus aculeatus \(\beta\)-mannosidase (AB015509); C. fimi, Cellulomonas fimi \(\beta\)-mannosidase (AF126472); T. neapolitana, Thermotoga neapolitana \(\beta\)-mannosidase (AY033395). Sequences were aligned using the CLUSTALW program (34). Gaps are marked by dashes. The conserved amino acid residues in all the \(\beta\)-mannosidases aligned are in bold. The proposed catalytic nucleophile (Glu559 of AtEBM) and proton donor (Glu464 of AtEBM) are marked by asterisks.

Fig. 4  Neighbor-joining phylogenetic tree of amino acid sequences of endo-\(\beta\)-mannosidases and \(\beta\)-mannosidases
Sequences used are from Capra hircus (U46067), Homo sapiens (U60337), Mus musculus (AF306557), Aspergillus niger (AJ251874), Thermobifida fusca (AJ489440), Thermotoga maritima (AE001806), in addition to the sequences used in Fig. 3. The bar represents the number of nucleotide substitutions.

Fig. 5  SDS-PAGE and western blotting of AtEBM and its mutants expressed in E.coli
Lysates of E. coli cells transformed with pET15 (lane 1, 5), AtEBMpET15 (lane 2, 6), E464ApET15 (lane 3, 7), and E549ApET15 (lane 4, 8) or were subjected to SDS-PAGE (10 % acrylamide). Proteins were stained by coomassie brilliant blue R-250 (lane 1–4). Proteins were transferred onto polyvinilydene difluoride membrane and immunostained with anti-endo-\(\beta\)-mannosidase antibody (lane 5–8). The arrow in the left side of the gel shows the position of the newly expressed protein bands in lane 2–4. The positions of molecular mass standards (kDa) are indicated on the left of the panel.
Fig. 6  **pH dependence and pH stability of AtEBM**

Enzyme activity was measured using M2B-PA as a substrate. The buffer used was 0.2 M sodium citrate phosphate buffer (pH 3.0 to 8.0).  
A: pH dependence of the enzyme. The value obtained with pH 5.0 was taken as 100 %.  
B: Stability of the enzyme after pre-incubation for 2 h at 37 °C with each buffer. The value obtained with pH 6.0 was taken as 100 %.
Table I. Sugar chain structures and abbreviations used in the present study.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN2-PA</td>
<td>GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M1-PA</td>
<td>Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M2B-PA</td>
<td>Man(^\text{1})−6Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M2A-PA</td>
<td>3Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M3B-PA</td>
<td>6Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M3C-PA</td>
<td>3Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M4B-PA</td>
<td>6Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M5A-PA</td>
<td>3Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M9A-PA</td>
<td>6Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td>M2X-PA</td>
<td>2Xyl(^\text{1})</td>
</tr>
<tr>
<td>Bi-PA</td>
<td>GlcNAc(^\text{1})−2Man(^\text{1})</td>
</tr>
<tr>
<td></td>
<td>6Man(^\text{1})−4GlcNAc(^\text{1})−4GlcNAc−PA</td>
</tr>
<tr>
<td></td>
<td>GlcNAc(^\text{1})−2Man(^\text{1})</td>
</tr>
</tbody>
</table>

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Table II. Summary of the purification of endo-\(\beta\)-mannosidase from lily flowers

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Activity (U)</th>
<th>Protein (mg)</th>
<th>Recovery (%)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude enzyme</td>
<td>5600</td>
<td>26000</td>
<td>100</td>
<td>0.22</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium Sulfate Precipitation</td>
<td>3200</td>
<td>870</td>
<td>56</td>
<td>3.6</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>DEAE-Sephacel</td>
<td>800</td>
<td>80</td>
<td>14</td>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>Superdex 200</td>
<td>280</td>
<td>3.7</td>
<td>4.9</td>
<td>75</td>
<td>340</td>
</tr>
<tr>
<td>5</td>
<td>Hydroxyapatite</td>
<td>170</td>
<td>1.1</td>
<td>3.0</td>
<td>160</td>
<td>720</td>
</tr>
<tr>
<td>6</td>
<td>Mono Q</td>
<td>110</td>
<td>0.46</td>
<td>1.9</td>
<td>230</td>
<td>1100</td>
</tr>
<tr>
<td>7</td>
<td>Poros HS</td>
<td>9.2</td>
<td>0.036</td>
<td>0.16</td>
<td>260</td>
<td>1200</td>
</tr>
</tbody>
</table>
Table III. Substrate specificity of the purified endo-[\beta]-mannosidase. Relative hydrolysis rates to that of M2B-PA are shown.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis rate (%)</th>
</tr>
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<tbody>
<tr>
<td>M2B-PA</td>
<td>100</td>
</tr>
<tr>
<td>M3C-PA</td>
<td>37</td>
</tr>
<tr>
<td>M4B-PA</td>
<td>6.6</td>
</tr>
<tr>
<td>M1-PA</td>
<td>2.3</td>
</tr>
<tr>
<td>M2A-PA</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>M2X-PA</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>M3B-PA</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>M5A-PA</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>M9A-PA</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Bi-PA</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Endo-\(\beta\)-mannosidase, a plant enzyme acting on N-glycan: Purification, molecular cloning, and characterization

Takeshi Ishimizu, Akiko Sasaki, Satoshi Okutani, Mami Maeda, Mai Yamagishi and Sumihiro Hase

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