Endo-β-mannosidase, a Plant Enzyme Acting on N-Glycan

PURIFICATION, MOLECULAR CLONING, AND CHARACTERIZATION*

Received for publication, June 21, 2004
Published, JBC Papers in Press, July 7, 2004, DOI 10.1074/jbc.M406886200

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Endo-β-mannosidase is a novel endoglycosidase that hydrolyzes the Manβ1-4GlcNAc 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., Natsume, S., and Hase, S. (1999) J. Biochem. 125, 365–367). Here we report the purification and molecular cloning of endo-β-mannosidase. The enzyme purified from lily flowers 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, AtEBM, from Arabidopsis thaliana. AtEBM was engineered for expression in Escherichia coli, and the recombinant protein comprised a single polypeptide chain with a molecular mass of 112 kDa corresponding to the sum of molecular masses of three polypeptides of the lily enzyme. The recombinant protein hydrolyzed pyridylaminylated Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc into Manα1-6Man and GlcNAcβ1-4GlcNAc-PA, showing that AtEBM is an endo-β-mannosidase. AtEBM hydrolyzed Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (n = 0–2) but not PA-sugar chains containing Manα1-3Manβ or Xyloseβ1-2Manβ as for the lily endo-β-mannosidase. AtEBM belonged to the clan GH-A of glycosyl hydrolases. Site-directed mutagenesis experiments revealed that two glutamic acid residues (Glu-464 and Glu-549) conserved in this clan were critical for enzymatic activity. The amino acid sequence of AtEBM has distinct differences from those of the bacterial, fungal, and animal exo-type β-mannosidases. Indeed, AtEBM-like genes are only found in plants, indicating that endo-β-mannosidase is a plant-specific enzyme. The role of this enzyme in the processing and/or degradation of N-glycan will be discussed.

* This work was supported in part in the 21st Century COE program (Creation of Integrated EcoChemistry), the Protein 3000 Program, and the Japan Health Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: PA, pyridylamino; GlcNAc, N-acetyl-D-glucosamine; Man, α-mannose; pNP, N-p-nitrophenoxy, HPLC, high performance liquid chromatography.

Endo-β-mannosidase is a novel endoglycosidase that hydrolyzes the Manβ1-4GlcNAc linkage in the trimannosyl core structure of N-glycans (1). Along with endo-β-N-acetylglucosaminidase and peptide N-glycanase, it is classified as an endo-type hydrolase that acts close to the reducing end of the N-glycans attached to proteins. Discovery of this enzyme was based on a structural analysis of the N-glycans of Japanese pear S-RNases, which has chitobiase as the major N-glycan (2, 3). This enzyme activity has been identified in the crude extract from lily flowers using a pyridylamino (PA)1 Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc as the substrate (1). The partially purified enzyme hydrolyzed Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (n = 0–2) to Manα1-6Man and GlcNAcβ1-4GlcNAcβ1-4GlcNAc-PA, confirming that this enzyme was an endoglycosidase. It does not hydrolyze β-mannohexa-αcharrase and is therefore different from β-mannanase (4). It also differs from β-mannosidases, because endo-β-mannosidase does not hydrolyze p-nitrophenyl-β-mannoside (pNP β-Man) (1, 5). Substrates containing Manα1-3Manβ and/or Xyloseβ1-2Manβ structures were not hydrolyzed with the partially purified enzyme. The substrate specificity of endo-β-mannosidase complements that of the jack bean α-mannosidase, which displays a preference for a Manα1-3Manβ linkage. Therefore the N-linked chitobiase in S-RNase (3) is probably produced by consecutive digestion of high mannose-type N-glycans with α-mannosidase and endo-β-mannosidase. We are interested both in the function of endo-β-mannosidase in the plant cell and also the enzyme mechanism of this unique substrate specificity.

In this paper, we describe the purification of endo-β-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-β-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. Hinamoto) 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. DEAE-Sephadex, 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. 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 Ashishpak 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. Jack bean α-mannosidase and almond peptide
N-glycans were purchased from Seikagaku Kogyo (Tokyo, Japan). The anti-lily endo-β-mannosidase antibody was raised by immunizing a rabbit with the purified lily endo-β-mannosidase.

**Assay of Endo-β-mannosidase Activity**

Endo-β-mannosidase activity was measured as described previously (1). Briefly, the enzyme and 12.5 μM M2B-PA in 16 μl of 0.16 M ammonium acetate buffer, pH 5.0, were incubated at 37 °C for 30 min. The chitoiose-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 0.2M sodium citrate phosphate buffer, pH 6.0, and homogenized with a Polytron homogenizer (2).

**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 YM-10 membrane.

**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 the lily (1200 g) were homogenized with a Polytron homogenizer (10,000 rpm, 3 min). The homogenate was centrifuged at 28,000 × 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 × 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-Sephael column (4.6 × 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 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 a fast protein liquid chromatography system was equilibrated with 10 mM sodium phosphate buffer, pH 6.0, at a flow rate of 1 ml/min. After an 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 (10–500 mM) within 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 over 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 over 60 min. The endo-β-mannosidase fraction was pooled, concentrated to 2 ml with an Amicon YM-10 membrane, and stored on ice.

**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, 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 data base using the TFASTA program (11). A candidate gene for endo-β-mannosidase (registered as At045934 and AC000106 for DBJ/EMBL/GenBank™ data base 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'-GGCGCA-TATGGCGGAGATCGGGAAG-3'; R1, 5'-GGCGCGCTCAGCTCAACCAGC-AAAACACTAACC3'. The PCR primers were derived from the putative N- and C-terminal amino acid sequences of the At045934/
AC009016/At1g09010 gene product. We also incorporated NdeI and Bpu1102I 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 AtEBM. A. thaliana shoot cDNA (kindly donated from Dr. T. Kakimoto of Osaka University, Osaka, Japan) 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), and 72 °C (3 min), and 72 °C (30 min). The amplified DNA fragment was cloned into pBluescript vector (Stratagene) 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.

Expression of AtEBM in E. coli

The PCR product was subcloned into the NdeI and Bpu1102I sites of a bacterial expression vector pET15-b (Novagen, Madison, WI). The resulting plasmid, AteBMPetT15, was transformed into E. coli RosettaBlue cells (Novagen). Cells were grown in 500 ml of Luria-Bertani medium containing 50 µg/ml carbenicillin at 16 °C to an A600 of 0.5. The gene was expressed with an induction at 16 °C for 16 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were harvested by centrifugation (3000 × 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 µg/ml aprotinin, 5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 1 mg/ml lysozyme, and 25 units/ml benzonase (Novagen) for 2 h on ice. Resuspended cells were subjected to three complete cycles of freeze-thawing. The extract was then centrifuged at 5500 × g for 10 min at 4 °C. The resultant supernatant was dialyzed against 20 mM sodium acetate, pH 6.0, containing 2 µg/ml aprotinin, 5 µg/ml leupeptin, and 0.7 µg/ml pepstatin. The sample was then applied to a DEAE-Sephaloc column (2.6 × 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 Glu-464 and Glu-549 of AtEBM was performed by overlap extension PCR according to the method Higuchi et al. (12). The AteBMPetT15 construct was used as a template DNA for mutagenic PCR reaction. For the construction of E464ApetT15 (Glu464 in AtEBM replaced Ala), the PCR fragments obtained with the oligonucleotides E464A sense/R2 (E464A sense, 5′-TGGGTGGGAAATGCAC-AAGTTCCGCC-3′), R2, 5′-GTACATGGACTGCTCGTCCAGG and F1 (E464A antisense, 5′-GGCGAGATTTGCA-TTTCCACCAAC-3′) were used as templates for another PCR reaction with F1 and R2 as primers. The resulting fragment was subcloned in BamHII/SacI sites of AteBMPetT15b. The E549ApetT15 (Glu549 in AtEBM replaced Ala), the PCR fragments obtained with the oligonucleotides E549A sense/E464A antisense primers (E549A sense, 5′-TTCATATCCGG-GGTGCGCTCGTGG-3′) and F1/E464A antisense (E464A antisense, 5′-AAGTGACCGCG-GTTCCGGAGTGAAC-3′) were used as templates for another PCR reaction with F1 and R2 as primers. The resulting fragment was subcloned in BamHII/Sacl sites of AteBMPetT15b. The E549ApetT15 (Glu549 in AtEBM replaced Ala) was constructed as described above using E549A sense and E549A antisense primers (E549A sense, 5′-TTCATATCCGG-GGTGCGCTCGTGG-3′) and E549A antisense (E549A antisense, 5′-AAGTGACCGCG-GTTCCGGAGTGAAC-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-indolylphosphate 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 under “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-Sephaloc chromatography (step 3) and gel filtration chromatography (step 4), indicating that at least two forms of endo-β-mannosidase exist in lily flowers (Fig. 1, A and B). The first peak from the anion exchange column and the second peak from the gel filtration column were used for purification.

The characterization of the remaining peaks will be described elsewhere. Poros HS chromatography (step 7) 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 µg 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. 2, A and 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 were not bound covalently by a disulfide bond.

Molecular Cloning of the Arabidopsis Gene, AtEBM, Encoding Homologous Amino Acid Sequence to That of the Purified Lily Endo-β-mannosidase—The purified lily enzyme was composed of three polypeptides (Fig. 2C). N-terminal amino acid sequences of the 28-, 31-, and 42-kDa polypeptides were determined as EYHQTHELSEI, GKVKLDSGWLAARSTELELTGVQ, and ETEDPSQYLGDTRVY1QGSMWEGFA, respectively. Internal amino acid sequences were VFIRGGNWIDGLL and FHADMNFNMIR for the 28-kDa polypeptide, DVAQYQYEGWDW for the 31-kDa polypeptide, and GTSGVYAFFLHF for the 42-kDa polypeptide. We searched for genes encoding proteins homologous to these amino acid sequences using TFASTA program. Several candidates were retrieved from the data base. 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 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 open reading frame 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 of 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-β-mannosidase.

Comparison of the Amino Acid Sequence of AtEBM with Those of Other β-Mannosidases—Sequence analysis revealed that AtEBM belongs to the glycosyl hydrolase family 2 (13). The amino acid sequence of the purified lily endo-β-mannosidase and AtEBM were aligned with those of β-mannosidases from mammal (Bos taurus) (14), fungi (Aspergillus aculeatus) (15), bacteria (Cellulomonas fimii) (16), and thermophilic bacteria (Thermotoga neapolitana) (17) (Fig. 3). AtEBM had 17–18% identity with mammalian β-mannosidases associated with β-mannosidosis (14, 18–20), 13–14% identity with fungal β-mannosidases associated with mannan degradation (15, 21), 16–20% identity with bacterial β-mannosidases (16, 22), and 20–21% 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 Manβ1-4GlcNAc. Among them, Gly-549 of AtEBM is predicted to be a catalytic nucleophile, because the corresponding Gly
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), and these hydrolases are proposed to have an (α/β)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 Glu-464 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 het-

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**TABLE II**

Summary of the purification of endo-β-mannosidase from lily flowers

| Step | Procedure               | Activity | Protein | Recovery | Specific activity | Purification |
|------|-------------------------|----------|---------|----------|------------------|--------------|
|      |                         | units    | mg      | %        | units/mg         | fold         |
| 1    | Crude enzyme            | 5600     | 26000   | 100      | 0.22             | 1            |
| 2    | Ammonium sulfate precipitation | 3200 | 870     | 56       | 3.6              | 17           |
| 3    | DEAE-Sephacel           | 800      | 80      | 14       | 10               | 46           |
| 4    | Superdex 200            | 280      | 3.7     | 4.9      | 75               | 340          |
| 5    | Hydroxyapatite          | 170      | 1.1     | 3.0      | 160              | 720          |
| 6    | Mono Q                  | 110      | 0.46    | 1.9      | 230              | 1100         |
| 7    | Poros HS                | 9.2      | 0.036   | 0.16     | 280              | 1200         |
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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, γ-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.

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 the hydrolysis of Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA to Manα1-6Man and GlcNAcβ1-4GlcNAc-PA at the molar ratio of 1:0:1. It also hydrolyzed M3C-PA in an endo-type manner to Manα1-3Manα1-6Man and GlcNAcβ1-4GlcNAc-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 Manα1-3Manβ structures such as M2A-PA, M3B-PA, M5A-PA, M9A-PA, and Bi-PA. As with the lily enzyme, M2X-PA containing a Xyloseβ1-2Manβ 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 and 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 Ca2+ (1).

DISCUSSION

Endo-β-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 an 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 (28–30).

The lily endo-β-mannosidase is composed of three polypeptides (28, 31, and 42 kDa) (Fig. 2C). All of 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, the partial nucleotide sequence of a cDNA clone of the lily enzyme encodes all three polypeptides.2 Even when the proteins were extracted from lily flowers in the presence of a protease inhibitor mixture, the purified enzyme consisted of three polypeptides, leading us to conclude that these three polypeptides are not artificially generated during the 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 the expression of the lily endo-β-mannosidase gene in a heterologous system.

An 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 Glu-464 or Glu-549 was not detected in this study,

2 T. Ishimizu and S. Hase, unpublished data.
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-\(\beta\)-mannosidase has the same catalytic mechanism as other glycosyl hydrolases in the clan GH-A.

\*Endo-\(\beta\)-mannosidase appears to be plant-specific, because

**FIG. 3.** Amino acid sequence alignment of endo-\(\beta\)-mannosidases and exo-type \(\beta\)-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 (AY15508); C. fimi, Cellulomonas fimi \(\beta\)-mannosidase (AF126472); T. neapolitana, Thermotoga neapolitana \(\beta\)-mannosidase (AF166398). 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 (Glu-559 of AtEBM) and proton donor (Glu-464 of AtEBM) are marked by asterisks.
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Fig. 4. Neighbor-joining phylogenetic tree of amino acid sequences of endo-β-mannosidases and β-mannosidases. Sequences used are from Capra hircus (U46067), Homo sapiens (U60337), Mus musculus (AF306557), A. 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 (lanes 1 and 5), AtEBMpET15 (lanes 2 and 6), E464ApET15 (lanes 3 and 7), and E549ApET15 (lanes 4 and 8) or were subjected to SDS-PAGE (10% acrylamide). Proteins were stained by Coomassie Blue R-250 (%). The positions of molecular mass standards (kDa) are indicated on the left side of the gel shows the position of the newly expressed protein bands in lanes 2–4. The arrow in the left side of the gel shows the position of the newly expressed protein bands in lanes 5–8. The positions of molecular mass standards (kDa) are indicated on the left of the panel.

Table III
Substrate specificity of the purified endo-β-mannosidase

| Substrate | Hydrolysis rate (%) |
|-----------|---------------------|
| M2B-PA    | 100                 |
| M3c-PA    | 37                  |
| M4B-PA    | 6.6                 |
| M1-PA     | 2.3                 |
| M2A-PA    | <0.01               |
| M2X-PA    | <0.01               |
| M3B-PA    | <0.01               |
| M8A-PA    | <0.01               |
| M9A-PA    | <0.01               |
| Bi-PA     | <0.01               |

Relative hydrolysis rates to that of M2B-PA are shown.

this activity was not detected in mammalian cells (rat liver, mouse liver, quail liver, salmon egg, and zebrafish egg), and because sequences highly homologous to endo-β-mannosidase are not found in other genomes than the plant genome sequen. All of the homologous genes from species other than plant were exo-type β-mannosidase (Fig. 3). The only highly homologous gene, the 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 an 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 the enzyme 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 a complementary substrate specificity to that of jack bean α-mannosidase, which hydrolyzes Manα1-3Manβ 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 Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc 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 Manα1-6Manβ, 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 knock-out study in A. thaliana will provide clues to the function of endo-β-mannosidase in vivo.

S. Hase, unpublished results.
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Endo-β-mannosidase, a Plant Enzyme Acting on N-Glycan: PURIFICATION, MOLECULAR CLONING, AND CHARACTERIZATION
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J. Biol. Chem. 2004, 279:38555-38562.
doi: 10.1074/jbc.M406886200 originally published online July 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406886200

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