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A Retaining Endo-β-Mannosidase from a Dicot Plant, Cabbage

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An endo-β-mannosidase [EC 3.2.1.152, glycoside hydrolase family 2], which hydrolyzes the Manβ1-4GlcNAc linkage of *N*-glycans in an endo-manner, has been found in plant tissues [Ishimizu, T., Sasaki, A., Okutani, S., Maeda, M., Yamagishi, M., and Hase, S. (2004) *J. Biol. Chem*. 279, 38555–38562]. So far, this glycosidase has been purified only from a monocot plant, a lily. Here, an endo-β-mannosidase was purified from a dicot plant, cabbage (*Brassica oleracea*), and characterized. The cabbage endo-β-mannosidase consists of four polypeptides. These four polypeptides are encoded by a single gene, whose nucleotide sequence is homologous to those of the lily and Arabidopsis endo-β-mannosidase genes. ^{1}H NMR analysis of the stereochemistry of the hydrolysis of pyridylaminated (PA) Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc showed that the cabbage endo-β-mannosidase is a retaining glycoside hydrolase, as are other glycoside hydrolase family 2 enzymes. The enzymatic characteristics, including substrate specificity, of the cabbage enzyme are very similar to those of the lily enzyme. These endo-β-mannosidases specifically act on Man$_n$Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (*n* = 0 to 2). These results suggest that the endo-β-mannosidase is present in at least the angiosperms, and has common roles, such as the degradation of *N*-glycans.

Key words: anomer proton, endo-β-mannosidase, mannosidase, *N*-glycan, plant.

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; PA-, pyridylamino-; *p*NP, *p*-nitrophenyl. The structures and abbreviations for the sugar chains are listed in Fig. 1.

Several glycoside hydrolases are involved in the processing or degradation of *N*-glycans. As a hydrolase acting on the Manβ1-4GlcNAc linkage of *N*-glycans, exo-type β-mannosidase [EC 3.2.1.25] has been identified in mammals, and well studied in the context of β-mannosidosis (*1*, *2*). Endo-β-mannosidase [EC 3.2.1.152], which hydrolyzes the Manβ1-4GlcNAc linkage of *N*-glycans in an endo-manner, was recently found in a plant (*3*–*6*). Purification of this enzyme from lily flowers was accomplished, and it was characterized (*4*, *5*). This endoglycosidase does not hydrolyze *p*-nitrophenyl β-mannoside, which is a good substrate for β-mannosidase, or β-mannohexaose, which is a good substrate for β-mannanase [EC 3.2.1.78] (*7*, *8*). Thus, endo-β-mannosidase can be distinguished from other β-mannosidases due to its substrate specificity (*3*, *5*). Endo-β-mannosidase acts on high-mannose type *N*-glycans not bearing the Manα1-3Manβ linkage, such as (Man)$_n$Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (*n* = 0 to 2) (*3*, *5*). These *N*-glycans are generated through the action of enzymes like the α-mannosidase from jack bean, which prefers to hydrolyze the Manα1-3Manβ linkage (*9*–*11*). On the basis of its substrate specificity, endo-β-mannosidase appears to be involved in trimming *N*-glycans to GlcNAcβ1-4GlcNAc, which is found in plant glycoproteins (*12*, *13*). The optimum pH of this enzyme is 5.0 (*4*, *5*), and therefore it is postulated that the enzyme is localized in an acidic organelle such as vacuoles. The primary structure of endo-β-mannosidase has been elucidated (*4*, *5*). The lily endo-β-mannosidase consists of at least three polypeptides, which are translated from a single gene encoding a protein comprising 952 amino acid residues (*5*). This protein is modified through post-translational proteolysis to generate the three polypeptides. Sequences homologous to that of the endo-β-mannosidase have been found only in plant genomes, indicating that this endoglycosidase is a plant-specific enzyme (*4*, *5*). Endo-β-mannosidase is classified into glycoside hydrolase family 2 of the GH-A clan (*14*, *15*). In addition, endo-β-mannosidase has transglycosylation activity, and has been used for the formation of the β-mannosyl linkage, which is difficult to control chemically (*16*).

The substrate specificity, biochemical features, and primary structure of an endo-β-mannosidase have been elucidated, however, this enzyme has been purified only from the flowers of a lily (a monocot plant), so far. Here, we attempted to purify this enzyme from cabbage (a dicot plant) in order to determine the similarities and differences between endo-β-mannosidases from different sources. The purified enzyme from the dicot plant was characterized and compared with the enzyme from the monocot plant. In addition, we determined the stereochemical course of hydrolysis of a substrate by this endo-β-mannosidase.

MATERIALS AND METHODS

Materials—Fresh market cabbage (*Brassica oleracea*) leaves were used as the enzyme source. The pyridylaminated (PA) sugar chains listed in Fig. 1 were prepared as reported previously (*11*, *17*). Materials were obtained from the following suppliers: DEAE-Sephacel, Octyl Sepharose CL-4B, and a Superdex 200 column (1.6 × 60 cm) from

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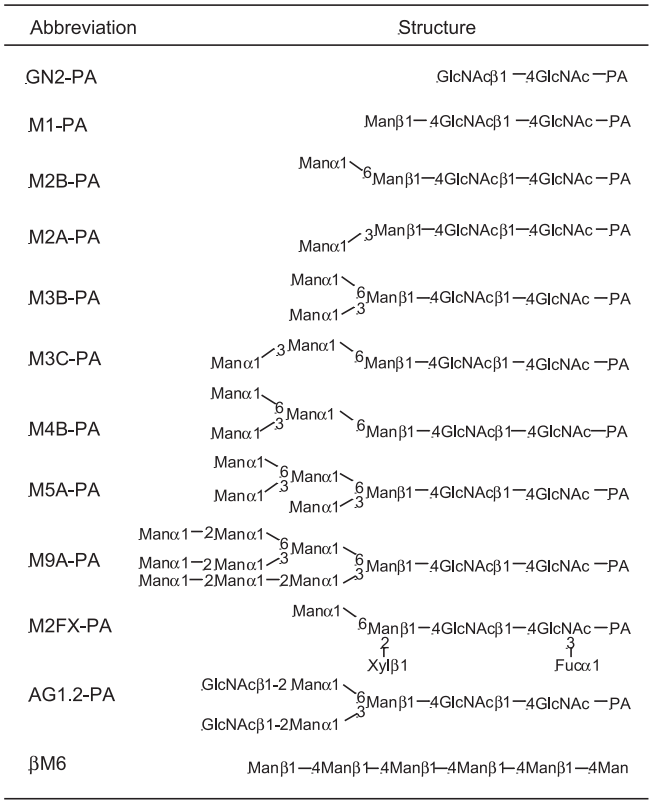

Fig. 1. **Sugar chain structures and abbreviations used in this study.**

Amersham Biosciences (Piscataway, NJ); hydroxyapatite from Seikagaku Kogyo (Tokyo, Japan); an HA 1000 hydroxyapatite column (0.75 × 7.5 cm) from Tosoh (Tokyo, Japan); a Poros HS column (0.46 × 10 cm) from Applied Biosystems (Foster City, CA); a Shodex Asahipak NH2-P column (0.46 × 10 cm) from Showa Denko (Tokyo, Japan); a CarboPac PA-1 column (0.2 × 25 cm) from Dionex (Sunnyvale, CA); a Cosmosil 5C18-P column (0.46 × 15 cm) from Nacarai Tesque (Kyoto, Japan); β1-4mannohexaose (βM6) from Biocon Japan (Nagoya, Japan); Diaflo and Amicon ultra membranes from Millipore (Bedford, MA); Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man, Manα1-6(Manα1-3)Man from Funakoshi (Tokyo, Japan); and Manα1-6Man, Manα1-3Man from Sigma (St. Louis, MO).

Determination of Endo-β-Mannosidase Activity—Endo-β-mannosidase activity was assayed as described previously (*3*) with minor modifications. The reaction mixture, comprising 12.5 μM M2B-PA, 156 mM ammonium acetate buffer, pH 5.0, and the enzyme solution, in a total volume of 16 μl, was incubated at 37°C for 20 min. The product, GN2-PA, was separated by size-fractionation or reversed-phase high performance liquid chromatography (HPLC) and then quantified using fluorescence. One unit of enzyme activity was defined as the amount of enzyme that released 1 nmol of GN2-PA from M2B-PA per minute under the conditions used. For investigation of the pH dependence of the enzyme, the initial hydrolysis rates were measured as described above, but in a 156 mM citrate-phosphate buffer with a pH range of 2.0 to 8.0. The substrate specificity of the purified enzyme was investigated using the sugar chains listed in Fig. 1 (at the concentration of 31 μM) as substrates. The K_m and V_{max} values of the enzyme for M2B-PA were determined by assaying with various concentrations of M2B-PA (0.38, 0.56, 0.75, and 0.94 mM) and 0.11 unit/ml of the enzyme. The values were calculated from a Lineweaver-Burk plot (*18*).

HPLC—HPLC was carried out using a Shimadzu LC-9A apparatus equipped with a Hitachi fluorescence detector, L-7485. Size-fractionation HPLC was performed on a Shodex Asahipak NH2-P column. Enzyme assay products were eluted with 0.3% (v/v) acetic acid in a 80:20 (v/v) mixture of acetonitrile:water adjusted to pH 7.0 with ammonium solution, at a flow rate of 0.8 ml/min, and monitored with a fluorescence spectrophotometer (excitation, 310 nm; emission, 380 nm). Reversed-phase HPLC was performed on a Cosmosil 5C18-P column. Two eluents, A (0.1 M ammonium acetate, pH 4.0) and B (0.1 M ammonium acetate, pH 4.0, with 0.5% 1-butanol), were used. After a sample had been injected into the column equilibrated with the eluent ratio of A:B = 95:5 (v/v), a linear gradient (to the eluent ratio of A:B = 0:100 v/v) was applied over 55 min, at a flow rate of 1.5 ml/min. Elution was monitored as to fluorescence (excitation, 315 nm; emission, 400 nm). High pH anion-exchange chromatography (HPAEC) was performed as described previously (*3*). The enzyme products were analyzed on a CarboPac PA-1 column connected to a pulsed amperometric detector (Dionex DX-500) at a flow rate of 1.0 ml/min. Two eluents, C (100 mM sodium hydroxide) and D (100 mM sodium hydroxide, 100 mM sodium acetate) in aqueous solution, were used. The column was equilibrated with Eluent C. After injecting a sample, the column was eluted with Eluent C for the initial 3 min, and then the proportion of Eluent D was increased linearly to 15% in 20 min, and then to 37% in 7 min.

Purification of Endo-β-Mannosidase from Cabbage Leaves—All operations were performed at 4°C. The protein concentration was determined from the absorption at 280 nm using bovine serum albumin as a standard. Buffer E was a 10 mM sodium phosphate buffer, pH 6.0.

Step 1. Preparation of a crude enzyme solution: Cabbage leaves (7.8 kg) were homogenized with a food processor and then 200 ml of 1.0 M sodium phosphate buffer, pH 6.0, was added to the homogenate. After stirring for 20 min, the sample was centrifuged at 28,000 × *g* for 20 min, and the supernatant (6,200 ml) was used as the crude extract.

Step 2. Ammonium sulfate precipitation: The crude extract was brought to 65% ammonium sulfate saturation, and then the mixture was centrifuged at 28,000 × *g* for 30 min. The pellet was suspended in Buffer E, dialyzed against the same buffer, and then centrifuged at 28,000 × *g* for 20 min. The supernatant (640 ml) was used in the next purification step.

Step 3. DEAE-Sephacel chromatography: The dialyzed enzyme solution was applied to a DEAE-Sephacel column (5.6 × 35 cm) equilibrated with Buffer E. The column was washed with the same buffer, and then the proteins were eluted with a linear gradient of sodium chloride, from 0 to 500 mM. The fractions containing endo-β-mannosidase activity (905 ml) were collected and concentrated to 5.0 ml using an Amicon YM-30 membrane.

Step 4. Superdex 200 gel filtration: The active pool from step 3 was loaded onto two tandemly connected Superdex 200 gel filtration columns equilibrated with Buffer E containing 0.1 M sodium chloride. The endo-β-mannosidase fractions (22 ml) were eluted with the same solution at a flow rate of 1.0 ml/min, and concentrated to 2 ml with an Amicon YM-30 membrane.

Step 5. Hydroxyapatite chromatography: The concentrated Superdex 200 fraction was placed on a hydroxyapatite column (2.0 × 17 cm) that had been equilibrated with Buffer E. The column was then washed with the same buffer, and the enzyme was eluted with a linear gradient of sodium phosphate, from 10 to 500 mM. The fractions containing endo-β-mannosidase activity (128 ml) were concentrated using an Amicon YM-30 membrane, and the buffer was changed to Buffer E containing 0.86 M ammonium sulfate (1 ml).

Step 6. Octyl Sepharose CL-4B chromatography: The pooled fractions were applied to an Octyl Sepharose CL-4B column (1.2 × 8.5 cm) equilibrated with Buffer E containing 0.86 M ammonium sulfate. After washing with the same buffer, the adsorbed materials were eluted with a linear gradient of ammonium sulfate, from 0.86 to 0 M. The fractions containing the enzyme activity (30.5 ml) were collected and concentrated to 600 μl with an Amicon Ultra YM-10 membrane.

Step 7. Poros HS chromatography: A Poros HS column was equilibrated with 50 mM sodium acetate, pH 4.0, and the concentrated enzyme fraction from step 6 was applied to it. The column was then washed with the same buffer, at a flow rate of 1.0 ml/min. The enzyme fraction was eluted with a linear gradient of sodium chloride, from 0 to 1.0 M, in 60 min. The fractions containing the enzyme activity (3.0 ml) were concentrated to 600 μl with an Amicon Ultra YM-10 membrane.

Step 8. HA 1000 (hydroxyapatite) chromatography: An HA 1000 column was equilibrated with Buffer E. After injecting the sample, the column was washed with the same buffer for 20 min at a flow rate of 0.5 ml/min. Elution was carried out with a linear gradient of sodium phosphate (10 to 500 mM) in 80 min. The enzyme fractions (4.0 ml) were pooled and concentrated to 500 μl with an Amicon ultra YM-10 membrane.

Step 9. HA 1000 rechromatography: This chromatography was performed as described above. The endo-β-mannosidase fractions (8.0 ml) were pooled and concentrated to 400 μl with an Amicon ultra YM-10 membrane, and then stored at 4°C.

Gel Electrophoresis—Native-PAGE was performed on a 5.0% polyacrylamide gel, pH 7.5, according to the method of Davis (*19*). SDS-PAGE was performed on a 10% polyacrylamide gel according to the method of Laemmli (*20*) under reducing conditions with 2-mercaptoethanol. Tricine SDS-PAGE was carried out on a 16.5% polyacrylamide gel by the method of Schägger and Jagow (*21*). Proteins on the gels were stained with Coomassie Brilliant Blue R-250.

Amino Acid Sequence Analyses—The purified endo-β-mannosidase was subjected to SDS-PAGE, and then proteins in the gel were electrotransferred to a polyvinylidene difluoride membrane and visualized with Coomassie Brilliant Blue R-250, using the method of Hirano and Watanabe (*22*). Protein bands were excised and analyzed for N-terminal amino acid sequences.

Cloning of the Cabbage Endo-β-Mannosidase cDNA—Total RNA was prepared from cabbage leaves as described previously (*23*). mRNA was isolated from total RNA using a PolyATtract mRNA isolation system (Promega, Madison, WI). cDNA was transcribed from the mRNA using Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA). The initial PCR reaction was carried out using KOD plus DNA polymerase (Toyobo, Tokyo, Japan) with the following primers: F1, 5′-GCCNAAYGGNATGGGNAARC-3′ and R1, 5′-GTDATCCARAAYTCYTGCCANAC-3′. These primers were derived from nucleotide sequences conserved in the Arabidopsis and lily endo-β-mannosidases (*4*, *5*). The PCR product (369 bp) was subcloned into the pGEM-T easy vector and then sequenced. On the basis of the nucleotide sequence of this PCR product, two primers: F2, 5′-GAACATGATCCGTTGCTGGGG-3′ and R2, 5′-CACTCTCAACCTTACGGAATCC-3′, were prepared for the rapid amplification of cDNA ends (RACE). The 5′- and 3′-RACE reactions were performed using KOD plus DNA polymerase and cabbage leaf cDNA to which 5′- and 3′-adaptor oligonucleotides (Generacer kit, Invitrogen) had been ligated. Primers R2 and F2 were used for the 5′- and 3′-RACE reactions, respectively. Prominent amplified fragments derived through the 5′-RACE (1,181 bp) and 3′-RACE (1,797 bp) reactions were subcloned into the pGEM-T easy vector, and then their nucleotide sequences were determined.

Stereochemistry of Hydrolysis of the Glycosidic Linkage by the Purified Enzyme—The stereochemistry of hydrolysis was determined using proton nuclear magnetic resonance (^{1}H NMR). The reaction mixture consisted of 3 mM M2B-PA, 49 milliunits of the purified enzyme, and 17 mM sodium phosphate buffer, pH 4.6, in a total volume of 30 μl. The substrate and buffer materials were dissolved in 99.9% D_2O and repeatedly lyophilized prior to use. ^{1}H NMR spectra were recorded at 10 min intervals on a JEOL LA-500 spectrometer at 37°C, with 64 scans.

RESULTS AND DISCUSSION

Purification of the Cabbage Endo-β-Mannosidase—Endo-β-mannosidase was purified from cabbage leaves by DEAE-Sephacel, Superdex 200, Hydroxyapatite, Octyl Sepharose, Poros HS and HA 1000 column chromatography (Fig. 2). Two peaks of endo-β-mannosidase activity were detected on the DEAE-Sephacel chromatography (Fig. 2A). Both peaks were collected and applied to the next column because they appeared to exist in equilibrium (data not shown). This phenomenon was also observed for the lily extract and will be discussed elsewhere. The major peak from the Superdex 200 gel filtration column (Fig. 2B) was collected and purified further (Fig. 2, C–G). From 7.8 kg of cabbage leaves, 23 μg of the enzyme was obtained, with 11,000-fold purification (Table 1). Native PAGE analysis of the purified enzyme gave a single band that migrated with the enzyme activity (Fig. 3A). SDS-PAGE and Tricine SDS-PAGE detected four polypeptides with molecular masses of 42, 34, 28 and 10 kDa (Fig. 3, B–C), indicating that the cabbage endo-β-mannosidase is

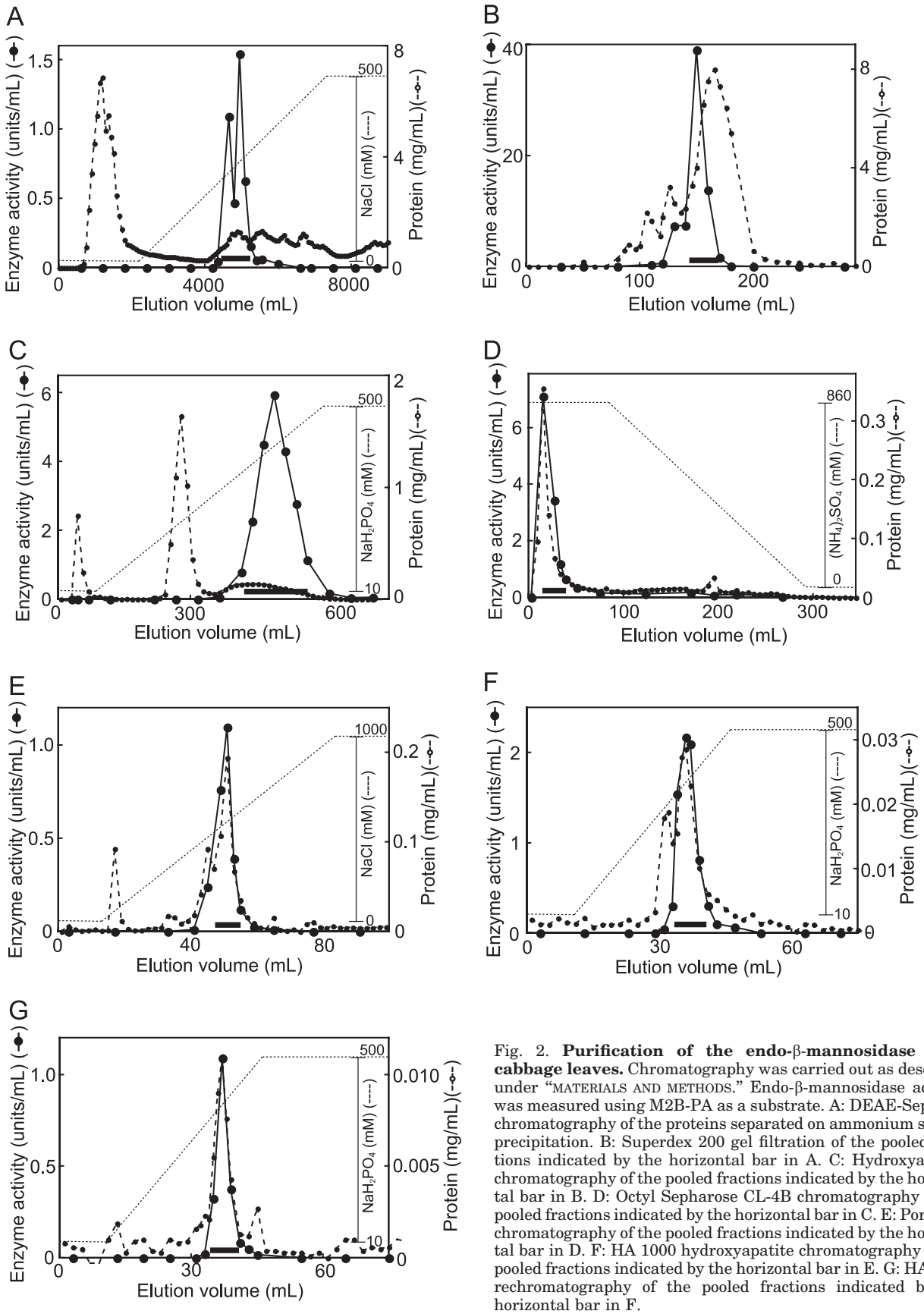

Fig. 2. **Purification of the endo-β-mannosidase from cabbage leaves.** Chromatography was carried out as described under "MATERIALS AND METHODS." Endo-β-mannosidase activity was measured using M2B-PA as a substrate. A: DEAE-Sephacel chromatography of the proteins separated on ammonium sulfate precipitation. B: Superdex 200 gel filtration of the pooled fractions indicated by the horizontal bar in A. C: Hydroxyapatite chromatography of the pooled fractions indicated by the horizontal bar in B. D: Octyl Sepharose CL-4B chromatography of the pooled fractions indicated by the horizontal bar in C. E: Poros HS chromatography of the pooled fractions indicated by the horizontal bar in D. F: HA 1000 hydroxyapatite chromatography of the pooled fractions indicated by the horizontal bar in E. G: HA 1000 rechromatography of the pooled fractions indicated by the horizontal bar in F.

Table 1. **Summary of the purification of the endo-β-mannosidase from cabbage leaves.**

	Step	Activity (units)	Protein (mg)	Recovery (%)	Specific activity (units/mg)	Purification (-fold)
1	Crude enzyme	1,800	110,000	100	0.016	1
2	Ammonium sulfate precipitation	1,400	17,000	78	0.082	5
3	DEAE-Sephacel	740	1,200	41	0.62	39
4	Superdex 200	470	140	26	3.4	210
5	Hydroxyapatite	190	14	11	14	880
6	Octyl Sepharose	110	4.9	6.1	22	1,400
7	Poros HS	23	0.89	1.2	26	1,600
8	HA1000	4.5	0.13	0.25	35	2,200
9	HA1000	4.1	0.023	0.23	180	11,000

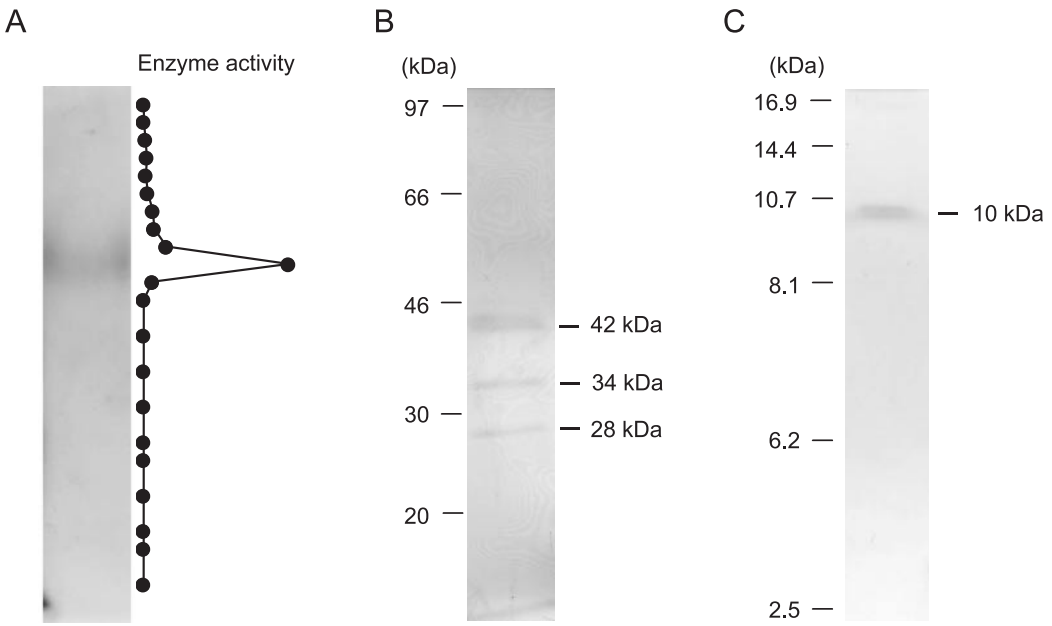

Fig. 3. **PAGE of the purified cabbage endo-β-mannosidase.** A: Native-PAGE of the purified enzyme. The gel was sliced at 2 mm intervals, and the enzyme activity was measured using M2B-PA as a substrate for proteins extracted from each slice. B: SDS-PAGE (10% polyacrylamide) of the purified enzyme. C: Tricine SDS-PAGE (16.5% polyacrylamide) of the purified enzyme. The proteins were stained with Coomassie Brilliant Blue R-250. The positions of molecular mass standards are indicated on the left of the panels.

composed of four polypeptides. The sum of the molecular masses of these polypeptides is 114 kDa.

Primary Structure of the Cabbage Endo-β-Mannosidase—The N-terminal amino acid sequences of the four polypeptides of the purified cabbage endo-β-mannosidase were found to be KDSDPSVYL, TLLDSGWLA, TENVLIPARG and GSDPGVAF for the 42, 34, 28 and 10 kDa polypeptides, respectively. Meanwhile, a cDNA exhibiting homology to the Arabidopsis and lily endo-β-mannosidase cDNAs (*4*, *5*) was amplified from cabbage leaf mRNA as described under "MATERIALS AND METHODS," and its nucleotide sequence was determined. The putative amino acid sequence, deduced from the nucleotide sequence of the cloned cDNA, contains all the amino acid sequences identified in the purified protein (Fig. 4A), indicating that the cDNA encodes the cabbage endo-β-mannosidase. The cDNA encodes a protein consisting of 946 amino acid residues with a predicted molecular mass of 107 kDa, and the four polypeptides of the enzyme are encoded by this single gene. The order of the four polypeptide chains in the primary structure of the cabbage enzyme is shown in Fig. 4B. Recent tricine SDS-PAGE analysis of the lily endo-β-mannosidase revealed that it also consisted of four polypeptides (data not shown). It contained an 8-kDa polypeptide corresponding to the cabbage 10-kDa polypeptide as well as the three polypeptides (42, 31, and 28 kDa) previously reported (*4*). The proteolysis sites in the cabbage enzyme, shown by inverted triangles in Fig. 4A, are similar to those in the lily enzyme. These sites may be located on the molecular surface. The sum of the molecular masses of the four polypeptides (114 kDa) is larger than the predicted molecular mass of the enzyme (107 kDa), indicating that some post-translational modifications occur in the cabbage endo-β-mannosidase. The molecular mass of the 34-kDa polypeptide is larger than that calculated from the amino acid sequence (29 kDa), therefore a modification may occur in this polypeptide. Within this polypeptide, four potential *N*-glycosylation sites are located. Two of these sites (Asn-110 and Asn-239) are conserved in plant endo-β-mannosidases (Fig. 4A). Further analysis is needed for structural elucidation of the post-translational modification of endo-β-mannosidase. A database search for sequences homologous to that of the cabbage enzyme revealed the *Arabidopsis* and lily endo-β-mannosidases, and putative glycosyl hydrolases from cotton, poplar, medicago, and rice (Fig. 4A). The sequence identities among these enzymes are 68 to 88%. The conserved amino acid residues are dispersed throughout the sequences. These putative glycosyl hydrolases appear to be endo-β-mannosidases. Two

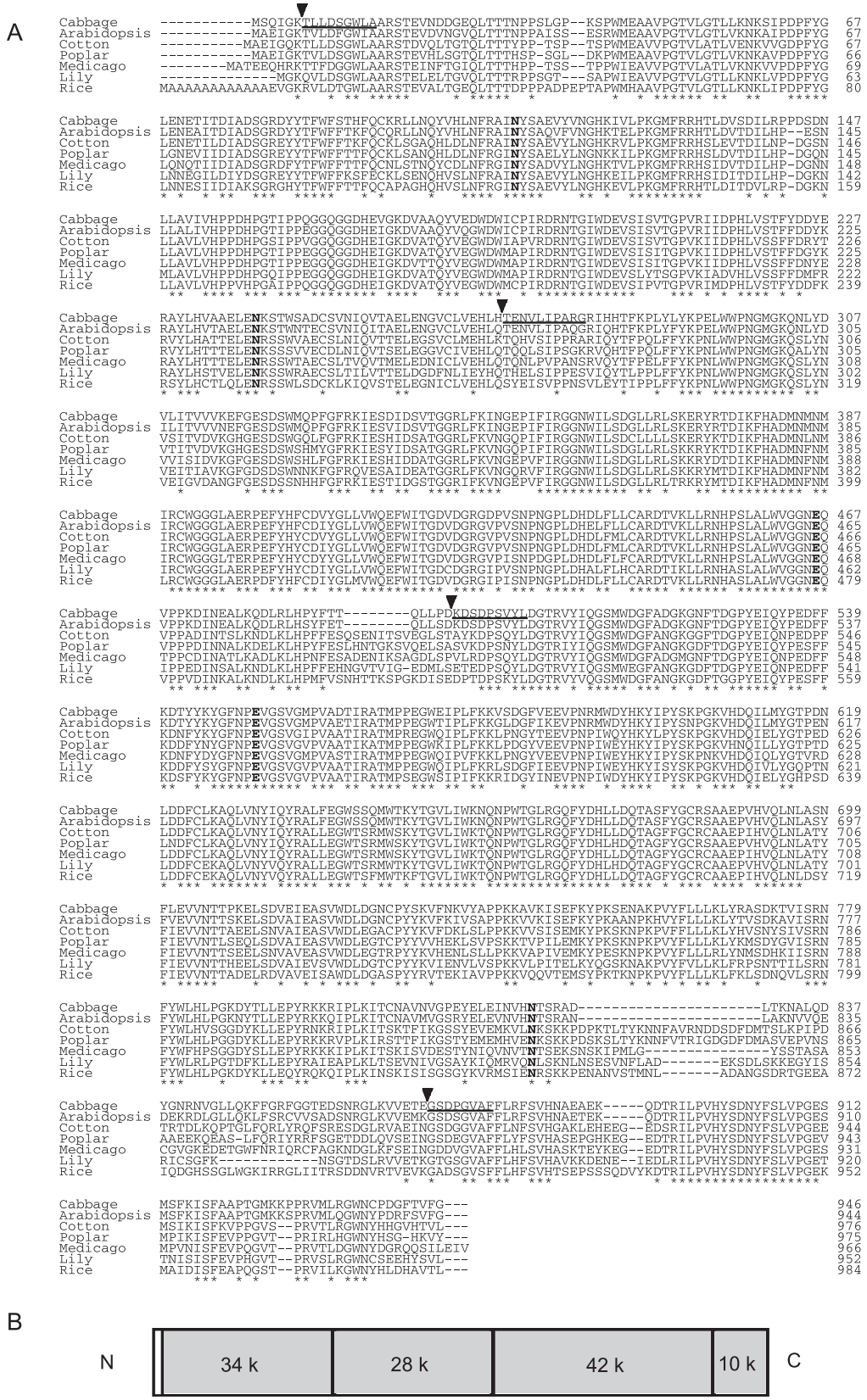

Fig. 4. **Primary structure of the cabbage endo-β-mannosidase.** A: Amino acid sequence alignment of the cabbage endo-β-mannosidase, other plant endo-β-mannosidases, and endo-β-mannosidase-like proteins. The sequences (with database references) are: Cabbage, *Brassica oleracea* endo-β-mannosidase (AB249904); Arabidopsis, *Arabidopsis thaliana* endo-β-mannosidase (AB122060); Cotton, *Gossypium hirsutum* putative glycosyl hydrolase (AY187062); Poplar, *Populus trichocarpa* putative glycosyl hydrolase (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html); Medicago, *Medicago truncatula* putative glycosyl hydrolase (AC135319); Lily, *Lilium longiflorum* endo-β-mannosidase (AB185918); and Rice, *Oryza sativa* putative glycosyl hydrolase (AK119412/AC107085). Amino acid sequences were aligned using the ClustalW program. Gaps are indicated by dashes. The conserved amino acid residues are indicated by asterisks. Amino acid sequences confirmed by N-terminal sequencing are underlined. Two catalytically important glutamic acid residues and three conserved potential *N*-glycosylation asparagine residues are shown in bold. Putative proteolytic sites are shown by inverted triangles. B: The order of the four polypeptides of the cabbage endo-β-mannosidase in its primary structure. The size of each polypeptide was determined on SDS-PAGE or tricine SDS-PAGE, shown in Fig. 2.

Table 2. **Substrate specificity of the purified cabbage endo-β-mannosidase.**

Substrate	Hydrolysis rate
M2B-PA	100
M3C-PA	34
M4B-PA	40
M1-PA	2.6
M2A-PA	ND
M3B-PA	ND
M5A-PA	ND
M9A-PA	ND
AG1.2-PA	ND
M2FX-PA	ND
*p*NP α-Man	ND
*p*NP β-Man	ND
βM6	ND

The value obtained with M2B-PA was taken as 100. ND, not detectable.

Table 3. **Comparison of the characteristics of the endo-β-mannosidases from monocot and dicot plants.**

	Dicot (cabbage)	Monocot (lily)[a]
Substrate	$(Man)_n$Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (n = 0 to 2)	$(Man)_n$Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (n = 0 to 2)
Optimum pH	4.5	5.0
pH stability	Relatively stable at pH 3.0 to 6.0	Relatively stable at pH 3.0 to 6.0
K_m value for M2B-PA	0.54 mM	1.2 mM
Components[b]	10 kDa, 28 kDa, 34 kDa, 42 kDa	8 kDa, 28 kDa, 31 kDa, 42 kDa

[a]The characteristics of the lily enzyme were cited from Sasaki *et al.* (2005) (*5*). [b]The molecular mass of each component of the endo-β-mannosidases was estimated from the SDS-PAGE results.

catalytically important glutamic acid residues, identified on site-directed mutagenesis of the Arabidopsis endo-β-mannosidase (*4*), are also conserved. Endo-β-mannosidase-like sequences have not been found in any species other than plant ones.

Characterization of the Cabbage Endo-β-Mannosidase—The substrate specificity of the cabbage endo-β-mannosidase was investigated using PA-sugar chains. As shown in Table 2, among the sugar chains tested, the best substrate for the enzyme was M2B-PA, although the enzyme hydrolyzed M3C-PA and M4B-PA as well. When M2B-PA was used as a substrate, the enzyme produced Manα1-6Man and GlcNAcβ1-4GlcNAc-PA in a molar ratio of 1.0: 1.1 (data not shown). This indicated that the hydrolysis occurred in an endo-manner. The enzyme showed limited activity toward M1-PA, but none toward M2A-PA, M3B-PA, M5A-PA, M9A-PA, or AG1.2-PA, all of which possess the Manα1-3Manβ structure. It is concluded that the cabbage endo-β-mannosidase hydrolyzes the Manβ1-4GlcNAc linkage of *N*-glycans not bearing the Manα1-3Man structure, in an endo-manner. The cabbage enzyme did not hydrolyze *p*NP β-Man, which is a good substrate for β-mannosidase, or βM6, which is a good substrate for β-mannosidase and β-mannanase. This substrate specificity is similar to that of the lily enzyme (*5*).

The cabbage endo-β-mannosidase showed maximum activity at pH 4.5. The enzyme was relatively stable over the pH range of 3.0 to 6.0. The K_m and V_{max} values for M2B-PA were determined to be 0.54 mM and 12 μmol/min·mg, respectively, from a Lineweaver-Burk plot.

The optimum pH and pH stability of endo-β-mannosidase support the idea that this enzyme is located in an acidic organelle such as vacuoles. Recent analysis of the Arabidopsis vacuole proteome revealed that this enzyme is localized at least in vacuoles (*24*), where biomolecules are degraded into their components in a plant cell. Although jack bean α-mannosidase–like enzyme and peptide: *N*-glycanase (PNGase) are not reported in cabbage, these plant enzymes acting on *N*-glycans exhibit optimum pHs in the acidic regions (*25–27*), indicating that they are also localized in vacuoles. Therefore, endo-β-mannosidase may be involved in the degradation of *N*-glycans in vacuoles in conjunction with jack bean α-mannosidase-like enzyme and PNGase.

The similarities in characteristics (Table 3) and primary structures (Fig. 4) between the dicot (cabbage) and monocot (lily) endo-β-mannosidases, along with the existence of homologous sequences in other monocot and dicot species (Fig. 4), suggest that this enzyme is common to the angiosperms. In various angiosperm species, endo-β-mannosidase may have common roles, such as degradation of *N*-glycans.

Stereochemistry of Hydrolysis of M2B-PA by the Cabbage Endo-β-Mannosidase—To determine whether the hydrolysis of a substrate by endo-β-mannosidase occurs *via* a mechanism of retention or inversion of the anomeric configuration, the hydrolysis of M2B-PA was monitored by means of ^{1}H NMR. This is important for classification of this enzyme because the stereochemistry of hydrolysis by glycoside hydrolases reflects their tertiary structures (*28*). Endo-β-mannosidase produced Manα1-6Man and GlcNAcβ1-4GlcNAc-PA as products (Fig. 5A). Figure 5B shows the signals from the anomeric protons of the mannose moieties in the substrate M2B-PA (Ha, Hb) and the product Manα1-6Man (Ha′ and Hb′) after 32 min reaction. These signals were assigned on the basis of the chemical shifts of these protons reported in previous papers (*29*, *30*). The signals at δ 4.96 and 4.78 ppm are due to the anomeric protons of M2B-PA, Ha and Hb, respectively. After the enzyme reaction had started, the signals of the anomeric protons of Manα1-6Man, Ha′α (δ 4.95), Ha′β (δ 4.94), Hb′α (δ 5.21), and Hb′β (δ 4.93), appeared. Assuming that the proportions of M2B-PA, Manα1-6Manα, and Manα1-6Manβ are (100 − α − β)%, α%, and β%, the proportion of the peak area at δ 5.21 and that of the multiple peaks around δ 4.95 are taken to be α and 100 + β. The α/β anomer contents of Manα1-6Man can be calculated from this relationship. The time course of the α/β anomeric contents of Manα1-6Man is shown in Fig. 5C. At reaction time 12 min, the α/β ratio was 35:65. As the reaction proceeded, the α/β ratio gradually changed to 70:30, corresponding to the natural α/β ratio of D-mannose (66:34) (*31*). This indicated that the initial product was Manα1-6Manβ and that the hydrolysis of the substrate by endo-β-mannosidase proceeded with retention of the anomeric configuration. This is in line with other members of glycoside hydrolase family 2

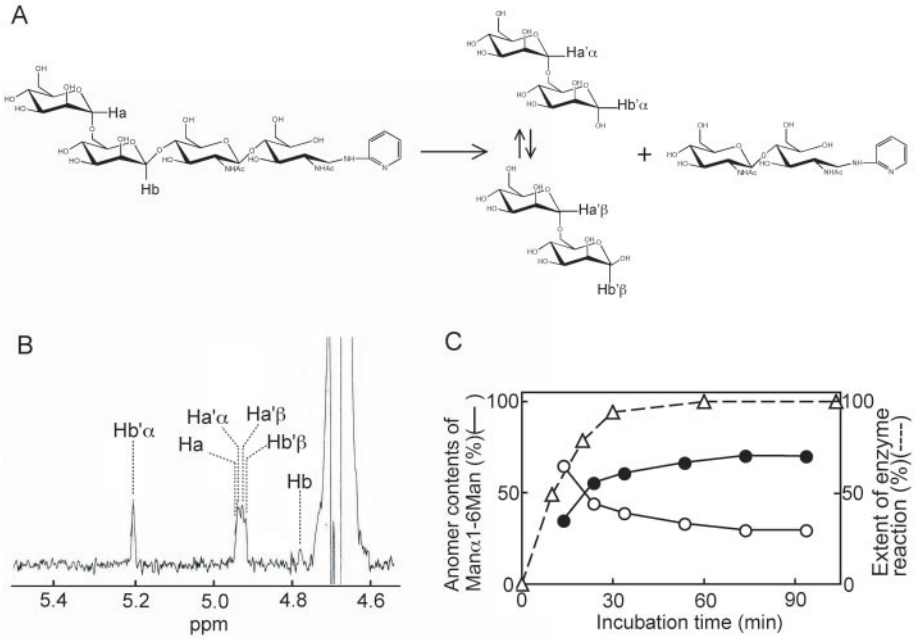

Fig. 5. **Stereochemistry of the hydrolysis of M2B-PA by the cabbage endo-β-mannosidase.** A: Reaction scheme. B: ^{1}H NMR spectra obtained after a 32 min reaction time. The region containing the signals of the anomeric protons (4.6 to 5.5 ppm) is shown. The signals due to the anomeric protons of the mannose moieties in the substrate and product are indicated. C: A time course showing the relative contents of the α (closed circles) and β (open circles) anomers of Manα1-6Man, and the extent of the reaction (triangles).

of the GH-A clan, which are also believed to hydrolyze glycosyl linkages while retaining their anomeric configurations.

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