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Purification to Homogeneity and Properties of Mannosidase II from Mung Bean Seedlings†

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Received August 9, 1989; Revised Manuscript Received October 25, 1989

ABSTRACT: Mannosidase II was purified from mung bean seedlings to apparent homogeneity by using a combination of techniques including DEAE-cellulose and hydroxyapatite chromatography, gel filtration, lectin affinity chromatography, and preparative gel electrophoresis. The release of radioactive mannose from GlcNAc[^{3}H]Man_5GlcNAc was linear with time and protein concentration with the purified protein, did not show any metal ion requirement, and had a pH optimum of 6.0. The purified enzyme showed a single band on SDS gels that migrated with the M_r 125K standard. The enzyme was very active on GlcNAcMan_5GlcNAc but had no activity toward Man_5GlcNAc, Man_9GlcNAc, Glc_3Man_9GlcNAc, or other high-mannose oligosaccharides. It did show slight activity toward Man_3GlcNAc. The first product of the reaction of enzyme with GlcNAcMan_5GlcNAc, i.e., GlcNAcMan_4GlcNAc, was isolated by gel filtration and subjected to digestion with endoglucosaminidase H to determine which mannose residue had been removed. This GlcNAcMan_4GlcNAc was about 60% susceptible to Endo H indicating that the mannosidase II preferred to remove the α1,6-linked mannose first, but 40% of the time removed the α1,3-linked mannose first. The final product of the reaction, GlcNAcMan_3GlcNAc, was characterized by gel filtration and various enzymatic digestions. Mannosidase II was very strongly inhibited by swainsonine and less strongly by 1,4-dideoxy-1,4-imino-D-mannitol. It was not inhibited by deoxymannojirimycin.

The biosynthesis of the oligosaccharide chains of the N-linked glycoproteins of plant cells, like those of animal cells, involves two series of reactions. The first series of reactions gives rise to the common intermediate that is the precursor for all of the N-linked oligosaccharides. In these reactions, the individual sugars, GlcNAc, mannose, and glucose, are added sequentially to the lipid carrier, dolichyl-P, to form the precursor lipid-linked oligosaccharide, $Glc_3Man_9(GlcNAc)_2$-PP-dolichol, which then serves as the donor of oligosaccharide to certain asparagine residues on the protein (Struck & Lennarz, 1980; Elbein, 1979; Staneloni & Leloir, 1982). Oligosaccharide transfer is a cotranslational event and occurs in the endoplasmic reticulum of the cell while the protein is still being synthesized on membrane-bound polysomes (Kiely et al., 1976; Lingappa et al., 1978; Rothman & Lodish, 1977).

Once the oligosaccharide has been transferred to protein, a series of reactions occur that result in modifications of the oligosaccharide chains to give rise to the various types of N-linked structures (Hunt et al., 1978; Hubbard & Ivatt, 1981). These reactions, which are referred to as processing reactions, are fairly well documented in animal cells, and many of the enzymes have been highly purified, but they are not nearly as well understood in plant cells (Elbein, 1988). The initial reactions of this processing pathway begin in the endoplasmic reticulum (ER), probably while the protein is still being synthesized. Two membrane-bound glucosidases, called glucosidase I and glucosidase II, remove the three glucoses to give a $Man_9(GlcNAc)_2$-protein. Glucosidase I removes the outermost α1,2-linked glucose (Grinna & Robbins, 1979; Hettkamp et al., 1984; Shailubhai et al., 1987) while glucosidase II removes the next two α1,3-linked glucoses (Michael & Kornfeld, 1980; Burns & Touster, 1982; Martiniuk et al., 1985; Brada & Dubach, 1984). The resulting $Man_9(GlcNAc)_2$ structure may remain as a high-mannose type, or it may be further modified in the ER and in the Golgi. There are several recently described enzymes found in animal cells that can act on these N-linked glycoproteins, but their exact role or function in the processing pathway is unclear at this time. For example, an α-mannosidase has been demonstrated in the ER that can remove a single or several of the α1,2-linked mannose residues to give $Man_{6-8}(GlcNAc)_2$-glycoproteins (Bischoff et al., 1986). There is also an endomannosidase in the Golgi of some animal cells that cleaves a glucosyl-α1,3-mannose from the $Glc_1Man_9(GlcNAc)_2$-protein to give a $Man_8(GlcNAc)_2$-protein (Lubas & Spiro, 1988). Once the protein enters the cis-Golgi as the $Man_{8-9}(GlcNAc)_2$-protein, it can be trimmed by

†This study was supported by grants from the National Institutes of Health (NIH DK 21800) and from the Robert A. Welch Foundation.
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0006-2960/90/0429-2168$02.50/0

mannosidase I which can remove all of the α1,2-linked mannose residues (Forsee & Schutzbach, 1983; Tulsiani & Touster, 1988; Tulsiani et al., 1982; Tabas & Kornfeld, 1979). The resulting $Man_5(GlcNAc)_2$-protein is then the substrate for GlcNAc transferase I which adds a GlcNAc, from UDP-GlcNAc, to the 3-mannose branch (Harpaz & Schachter, 1980; Oppenheimer & Hill, 1981). This GlcNAc transferase I is probably in the medial Golgi. The addition of GlcNAc to the oligosaccharide is probably the signal for another mannosidase, called mannosidase II, to remove the α1,3- and α1,6-linked mannoses from the 6-mannose branch to give a $GlcNAcMan_3(GlcNAc)_2$-protein (Tabas & Kornfeld, 1978; Tulsiani et al., 1982). This reaction has been reported to be present in the Golgi apparatus, but to be distributed throughout this organelle in rat liver (Novikoff et al., 1983). Further reactions in the processing pathway involve the addition of other sugars to form various complex types of oligosaccharides.

In plants, much less is known about the processing of the N-linked glycoproteins, but studies in suspension-cultured cells or in cotyledons (Hori & Elbein, 1983; Bollini et al., 1983) suggested that modifications of the oligosaccharide chains do occur in vivo. Several of the processing glycosidases have now been purified from plants, including glucosidase I (Szumilo et al., 1986a) and mannosidase I (Szumilo et al., 1986b). In the present paper, we describe the purification to apparent homogeneity of mannosidase II from mung bean seedlings and the properties of this enzyme. The presence of this enzyme in plants shows that these cells also trim high-mannose chains to precursor oligosaccharides that can become either hybrid or "complex" structures. In animal cells, mannosidase II may play a pivotal role in oligosaccharide processing, but whether this is also true in plants remains to be established.

Experimental Procedures

Materials. [2-^{3}H]Mannose (925 Ci/mmol) was obtained from Pathfinders Labs, St Louis, MO. Endo-β-*N*-acetylglucosaminidase H was purchased from Health Research Labs, Albany, NY. Pronase was from Calbiochem, hydroxyapatite (Bio-Gel HT) and Bio-Gel P-4 were from Bio-Rad, and DE-52 was from Whatman Chemical Separations, Ltd. Concanavalin A–Sepharose (10 mg of Con A/mL of gel), jack bean β-*N*-acetylglucosaminidase, dithiothreitol, *p*-nitrophenyl α-mannopyranoside, Triton X-100, and Sephacryl S-300 were obtained from Sigma Chemical Co. Deoxymannojirimycin was kindly provided by Drs. G. Legler and E. Bause, Universitat Koln (Legler & Julich, 1984). 1,4-Dideoxy-1,4-imino-D-mannitol was a generous gift from Dr. George Fleet, Oxford University (Fleet et al., 1984), and "Glc-swainsonine" (2-episwainsonine) was kindly provided by Drs. Barry Sharpless and Curtis Adams, MIT (Adams et al., 1986). Castanospermine and 6-epicastanospermine were isolated from the seeds of *Castanospermum australe* (Hohenschutz et al., 1980; Molyneux et al., 1986). Swainsonine was isolated from various species of *Astragalus* as previously described (Molyneux & James, 1982; Davis et al., 1984). Dr. Clint Ballou, University of California, Berkeley, provided the following samples: α1,6-mannotriose, α1,2-mannotriose, and the tetrasaccharide Man-α1,3Man-α1,2Man-α1,2Man. [^{3}H]Mannose-labeled $GlcNAcMan_5GlcNAc$ was prepared by incubating influenza virus infected MDCK cells in the presence of deoxymannojirimycin and labeling the glycoproteins with [2-^{3}H]mannose. The resulting $Man_9(GlcNAc)_2$ structure was incubated with the plant mannosidase I and then with the plant GlcNAc transferase I to produce the desired oligosaccharide (Szumilo & Elbein, 1985). GlcNAc transferase I was prepared from mung bean microsomes and partially purified as described (Szumilo et al., 1986c). The enzyme was assayed as described.

Preparation of Membrane Fraction from Mung Bean Seedlings. Mung beans were soaked in tap water overnight at 25 °C and were spread on moist cotton and kept in the dark for 2–3 days to allow germination. The seedlings were picked by hand and kept in ice. One kilogram of seedlings was blended in 500 mL of extraction buffer [50 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose, 0.5 mM dithiothreitol, 1 mM EDTA, and 0.5% poly(vinylpyrrolidone)] in a Waring blender for 10 s, three times. The resulting suspension was filtered through eight layers of cheesecloth, and the filtrate was centrifuged at 3000*g* for 10 min to remove whole cells. The supernatant liquid from this centrifugation was then centrifuged at 100000*g* for 45 min to isolate the microsomal fraction.

Solubilization of Mannosidase Activity. The membrane fraction was washed with 50 mM HEPES buffer, pH 7.4, containing 0.1% Triton X-100 and was then centrifuged at 100000*g* for 45 min. The resulting pellet was suspended in the solubilization buffer (50 mM HEPES buffer containing 5% glycerol and 1.5% Triton X-100) and homogenized for 15 min in a Dounce homogenizer. The suspension was subjected to ultracentrifugation as before, and the supernatant liquid, containing the mannosidase activity, was removed and saved. The residue was reextracted with the same solubilization buffer, and after centrifugation, this supernatant liquid was pooled with the first supernatant fraction.

Assay Procedure for Mannosidase II Activity. Mannosidase II was assayed by using [^{3}H]mannose-labeled $GlcNAcMan_5GlcNAc$ as the substrate. This oligosaccharide was prepared from [^{3}H]mannose-labeled $Man_9GlcNAc$ in the following way. MDCK cells were infected with influenza virus and placed in the presence of deoxymannojirimycin (25 μg/mL) to inhibit mannosidase I. After 1 h, [2-^{3}H]mannose (25 μCi/mL of growth medium) was added to label the glycoproteins and the cells were allowed to incubate in the presence of isotope and inhibitor for about 40 h in order to produce mature virus. The labeled virus was isolated from the medium by ultracentrifugation, and the purified virus was digested with Pronase to produce glycopeptides. These glycopeptides were isolated on columns of Bio-Gel P-4, digested with endo-β-*N*-acetylglucosaminidase H, and rechromatographed on the column. Most of the radioactivity migrated in the area expected for a $Man_9GlcNAc$, although smaller amounts of $Man_8GlcNAc$ and $Man_7GlcNAc$ were also seen. Each of the high-mannose peaks were pooled and rechromatographed on a long calibrated column of Bio-Gel P-4. The high-mannose peaks were incubated with the partially purified mannosidase I from mung beans as previously described (Szumilo et al., 1986b) in order to produce the radiolabeled $Man_5GlcNAc$. This oligosaccharide was isolated by chromatography on a long (1.5 × 150 cm) column of Bio-Gel P-4. The $Man_5GlcNAc$ was then incubated with a partially purified preparation of the plant GlcNAc transferase I in the presence of unlabeled 5 mM UDP-GlcNAc, 5 mM Mn^{2+}, 1 mM AMP, and 25 μg/mL swainsonine (to inhibit any contaminating mannosidase II) (Szumilo et al., 1986c). This incubation resulted in the formation of $GlcNAcMan_5GlcNAc$, which was isolated and purified on the long columns of Bio-Gel P-4.

The reaction mixture for the assay of mannosidase II contained 100 mM MES buffer, pH 6.0, 0.1% Triton X-100, 3500 cpm of $GlcNAcMan_5GlcNAc$, and various amounts of enzyme, all in a final volume of 0.2 mL. The incubations were for 15–60 min, and the reactions were stopped by the addition of 2.5% phosphotungstic acid and 5% trichloroacetic acid. The

Table I: Purification Procedure for Mannosidase II

step	volume (mL)	total protein (mg)	total activity (units)[a]	specific activity (units/mg)	yield (%)
microsomes	420	10395	3659	0.35	100
solubilized enzyme	600	6212	3696	0.59	101
first DEAE-cellulose	620	846	3310	3.9	90
second DEAE-cellulose	260	337	2056	6.1	56
hydroxyapatite	66	34	1857	53	50
concanavalin A–Sepharose	8.5	4	593	126	16
Sephacryl S-300	8.0	0.75	329	438	9

[a] One unit of mannosidase II is defined as the amount of enzyme that catalyzes the release of 1000 cpm of mannose from the $GlcNAcMan_5GlcNAc$ substrate in 60 min.

removal of mannose from the $GlcNAcMan_5GlcNAc$ was assayed by gel filtration on columns of Bio-Gel P-4 and also by assay on columns of concanavalin A–Sepharose as previously described (Szumilo & Elbein, 1985).

Assay for Arylmannosidase Activity. The arylmannosidase was assayed by using *p*-nitrophenyl α-mannoside as the substrate. The assay mixture contained 50 mM sodium acetate buffer, pH 4.5, and 5 mM *p*-nitrophenyl α-mannoside in a final volume of 0.2 mL. The incubation was usually for 1 h at 37 °C, and the reaction was stopped by the addition of 2 mL of 0.4 M glycine buffer, pH 10.0. The amount of *p*-nitrophenol released was measured by its absorbance at 410 nm.

Polyacrylamide Gel Electrophoresis. The preparative polyacrylamide gel electrophoresis was done at 4 °C in tube gels containing 7% acrylamide according to the method of Orr (1972), using a TEA (triethanolamine)–TES [*N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid] buffer system. The pH of the stacking and resolving gels was maintained at 5.8 and 6.8, respectively. The tube gel apparatus and the buffers were cooled before use. The sample of mannosidase II (2 mL) was made to 20% with respect to sucrose and applied to 12 tube gels. During the electrophoresis, the current was maintained at 2 mA/tube until the bromophenol dye band had entered the resolving gel, and then the current was raised to 5 mA/tube and maintained at that level. Enzyme was located by reaction with substrate as described under Results.

SDS gel electrophoresis was done according to the method of Laemmli (1970) in 10% gels. Gels were stained for protein with 0.05% Coomassie Blue in 10% acetic acid containing 20% 2-propanol and destained in a mixture of 10% 2-propanol and 10% acetic acid. When purified enzyme was used, the gels were silver stained as described (Switzer et al., 1979).

Buffers. The various buffers used in these studies were as follows: Buffer A contained 10 mM HEPES, pH 7.1, 0.1% Triton X-100, 10% glycerol, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 0.2 mM dithiothreitol (DTT). Buffer B contained 10 mM sodium phosphate, pH 7.0, 0.1% Triton X-100, 10% glycerol, 0.05 mM PMSF, and 0.2 mM DTT. Buffer C contained 10 mM HEPES, pH 7.1, 0.1% Triton X-100, 10% glycerol, 0.5 M NaCl, 0.5 mM PMSF, 0.2 mM DTT, 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 2 mM $CaCl_2$.

Enzymatic Digestions. For β-*N*-acetylglucosaminidase treatment, the oligosaccharides were placed in 50 mM citrate buffer, pH 5.0, and 40 milliunits of enzyme was added. Incubations were done overnight under a toluene atmosphere. For α-mannosidase treatment, the oligosaccharides were incubated in 50 mM citrate buffer, pH 4.5, containing 250 milliunits of enzyme and 1 mM $ZnCl_2$ for 24 h under a toluene atmosphere. In both cases, the release of labeled monosaccharide, or the change in migration of the oligosaccharide, was monitored by chromatography on columns of Bio-Gel P-4.

For endo-β-*N*-acetylglucosaminidase H digestions, the protein samples were denatured in 0.5% SDS at 100 °C for 5 min. The samples were then diluted 10 times with 50 mM citrate buffer, pH 6.0, in order to dilute the SDS concentration to 0.05%, and 10 milliunits of Endo H was added. Incubations were for 24 h at 37 °C under a toluene atmosphere.

Other Methods. Protein concentration was measured by a Coomassie Blue procedure (Bradford, 1976). Oligosaccharides were separated on 1.5 × 150 cm columns of Bio-Gel P-4 (200–400 mesh) that had been calibrated with various oligosaccharide standards, including $Glc_3Man_9GlcNAc$, $Man_9GlcNAc$, $Man_5GlcNAc$, $GlcNAcMan_5GlcNAc$, $GlcNAcMan_3GlcNAc$, and $Man_3GlcNAc$. Columns were equilibrated and run in 0.35% acetic acid at room temperature.

The molecular weight of mannosidase II was determined by gel filtration on Sephacryl S-300, and also by SDS–polyacrylamide gel electrophoresis. A number of molecular weight standards were run on the column, including apoferritin (M_r 443 000), β-amylase (M_r 200 000), alcohol dehydrogenase (M_r 150 000), bovine serum albumin (M_r 66 000), and carbonic anhydrase (M_r 29 000).

Results

(I) Purification of Mannosidase II. (A) DEAE-cellulose Chromatography. The solubilized enzyme (600 mL), prepared from 200 g of mung bean microsomes (this amount of microsomes came from 24 lb of mung bean seedlings), was loaded onto a 4 × 50 cm column of DEAE-cellulose (Whatman DE-52) that had been equilibrated with buffer A. The columns were washed with 500 mL of equilibration buffer and eluted with 1 L of a linear gradient of 0–0.4 M NaCl in buffer A. With this gradient, the enzyme was eluted at about 150–200 mM NaCl as shown in Figure 1 (top). This step gave about a 7-fold increase in specific activity of mannosidase II with only a 10% loss in total activity (see Table I). Active fractions were pooled (about 620 mL) and dialyzed overnight against buffer A. The dialyzed enzyme solution was then applied to a 2.5 × 50 cm column of DE-52 that had been equilibrated with buffer A. The column was washed with 200 mL of buffer A and then eluted with 800 mL of a linear gradient from 0 to 0.25 M NaCl in buffer A. As shown in Figure 1 (middle profile), the mannosidase II eluted in a symmetrical peak at about 100–125 mM NaCl. Active fractions were pooled (about 400 mL), concentrated to about 260 mL, and dialyzed overnight in buffer B.

(B) Hydroxyapatite Chromatography. A 2.5 × 12 cm column of hydroxyapatite was prepared and equilibrated with buffer B. The dialyzed enzyme fractions from DE-52 were applied to the column, and the column was washed with buffer B until protein was no longer detected in the effluent. The bound proteins were eluted with 500 mL of a linear gradient of 10–300 mM potassium phosphate in buffer B. As shown in the lower profile of Figure 1, the mannosidase II activity was eluted at about 220–250 mM potassium phosphate and was clearly separated from a large peak of aryl-α-mannosidase

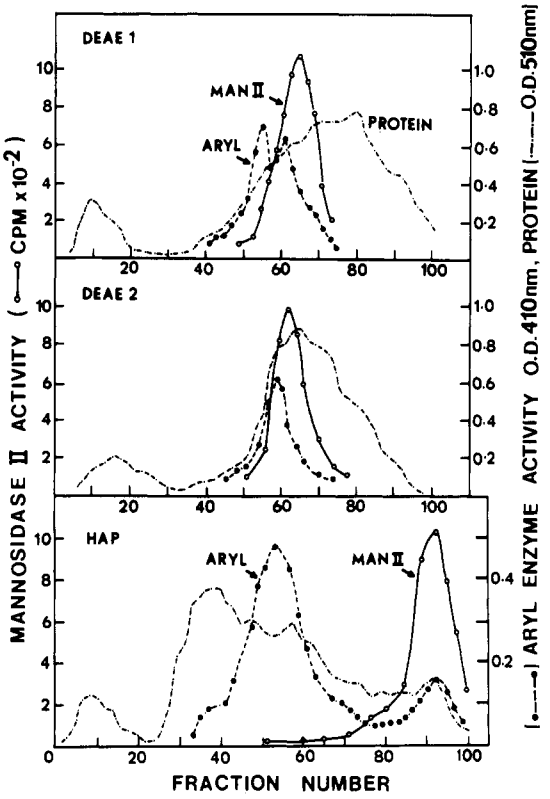

FIGURE 1: Purification procedures for mannosidase II. The upper profile shows the chromatography of the enzyme on the first DEAE-cellulose column. The arrow marked aryl (dashed line, closed circles) shows the elution positions for two peaks of α-mannosidase activity, while the arrow marked Man II (solid line, open circles) shows the elution profile of the processing mannosidase II. The middle profile shows the elution positions of the same enzymes after a second chromatography on another DEAE-cellulose column. The lowest profile demonstrates the almost complete separation of aryl-mannosidase (assayed with *p*-nitrophenyl α-mannoside) from mannosidase II on columns of hydroxyapatite. Details of these column procedures are given in the text.

that had eluted with the mannosidase II on the DE-52 columns. It should be noted, however, that all of the aryl-mannosidase activity could not be removed from the mannosidase II. As will be shown later in this paper, mannosidase II is able to release mannose from *p*-nitrophenyl α-mannoside (or from 4-methylumbelliferyl α-mannoside). The active fractions from the hydroxyapatite column (fractions 83–100) were pooled, dialyzed overnight in buffer C, and concentrated on an Amicon ultrafiltration apparatus to 6 mL.

(*C*) *Concanavalin A–Sepharose Column Chromatography.* About 3 mL of concanavalin A–Sepharose was washed with 50 mL of buffer C and was then gently mixed with 6 mL of enzyme from the hydroxyapatite step. The mixture was placed on a platform rocker and gently rocked for about 3 h in the cold. The suspension was then transferred to a small column (1.5 × 8 cm), and was washed with at least 6 column volumes of buffer C. Four milliliters of 0.3 M methyl α-mannoside, in buffer C, was then applied to the column, and the column was stoppered and left overnight in the cold room. The next day the column was opened and the eluate was collected in fractions. More of the 0.3 M methyl α-mannoside was applied to the column, and the eluate was collected in fractions. Fractions containing the enzyme activity were pooled (8.5 mL) and dialyzed overnight in buffer A.

(*D*) *Sephacryl S-300 Chromatography.* The dialyzed and concentrated enzyme eluted from concanavalin A was applied to a 2 × 150 cm column of Sephacryl S-300, equilibrated in buffer A. After application of the enzyme, the column was eluted with buffer A. The mannosidase II activity was eluted at 255–265 mL, while the void volume of the column was 205 mL.

As summarized in Table I, at this stage of purification, the 1200-fold purified protein had a specific activity of 438 units/mg and showed one major and some minor bands on SDS gels. This enzyme was further purified to apparent homogeneity by preparative gel electrophoresis on native gels as indicated below. It was not possible to determine the specific activity of this final enzyme preparation, but it did show only a single band on SDS gels.

(*E*) *Preparative Gel Electrophoresis.* The mannosidase II from the Sephacryl S-300 column was concentrated with a Centricon 30 to about 2 mL and was then subjected to polyacrylamide gel electrophoresis on nondenaturing gels as described under Experimental Procedure. At the end of the electrophoresis, the enzyme was located by reaction with the fluorescent substrate 4-methylumbelliferyl α-mannoside. After reaction, the gel was immediately washed with deionized water, put into 0.4 M glycine buffer, pH 10, and examined under a UV light. A fluorescent band with an R_f of 2.2 cm was detected. A second gel was cut into 0.2-cm pieces starting at the origin, and each section was incubated with [^{3}H]-mannose-labeled GlcNAcMan$_5$GlcNAc to measure processing activity. The position of the processing mannosidase II activity exactly matched that of the fluorescent band (data not shown). With the electrophoretic mobility of the active band as a marker, the mannosidase band was cut out from the remaining gels, and the gel pieces were pooled. The processing activity was eluted by mashing the gels in buffer A and allowing them to stand overnight in that buffer.

By the use of these procedures, the mannosidase II was purified more than 1200-fold from mung bean microsomes with a recovery of about 9%. These data are presented in Table I. Since the enzyme binds to columns of concanavalin A and can be eluted with methyl α-mannoside (but not methyl α-glucoside), it is likely that it is a glycoprotein with mannose-containing oligosaccharides. Since O-linked mannose has not been demonstrated in higher plants, it is likely to be an N-linked glycoprotein with high-mannose chains. However, treatment of this protein with Endo H did not result in a shift in the migration of the protein on SDS gels, suggesting that either the N-linked oligosaccharide(s) is resistant to this enzyme or that the mannose is present in other forms. Mannosidase IA purified from rat liver Golgi membranes bound to concanavalin A but was found to be resistant to Endo H (Tulsiani & Touster, 1988). A number of plant N-linked oligosaccharides have also been found to be resistant to digestion by Endo H (Faye et al., 1986), but the exact reason for this resistance is unknown.

(*II*) *Homogeneity and Stability of Mannosidase II.* The mannosidase II that was obtained from the preparative gel electrophoresis appeared to be essentially homogeneous when examined by SDS gel electrophoresis as shown in Figure 2. A single protein band with an apparent molecular weight of 125K was observed. The purified enzyme was stable for several days when stored in 10% glycerol in HEPES buffer, pH 7.2, at 0–4 °C. The enzyme could also be kept in the frozen state in 20% glycerol in buffer A for at least 2 months without significant loss of activity. However, the mannosidase II lost most of its activity within 1 week when stored in buffers below pH 6.0.

(*III*) *Properties of the Purified Mannosidase II.* The release of mannose from the [^{3}H]mannose-labeled GlcNAcMan$_5$GlcNAc by the purified mannosidase II was

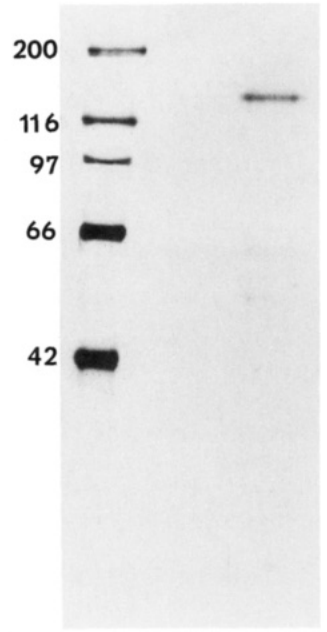

FIGURE 2: SDS gel electrophoresis of mannosidase II. The mannosidase II was eluted from native gels, and 2 μg of this preparation was loaded on 10% polyacrylamide gels. Proteins were stained with silver as described. (Left lane) Molecular weight marker standards were from the Bio-Rad kit as follows: myosin, 200K; *Escherichia coli* β-galactosidase, 116K; rabbit muscle phosphorylase *b*, 97K; bovine serum albumin, 66K; ovalbumin, 42K.

fairly linear with both time and protein concentration as shown in Figures 3 and 4. Figure 3 shows the assay of the enzyme on columns of Bio-Gel P-4 and the peak of increasing radioactivity in the mannose area (fractions 181–191) as a function of time of incubation. The sum of radioactivity in the mannose area was fairly proportional to the time of incubation. The figure also shows that the migration of the oligosaccharide is shifted with time from a GlcNAcMan$_5$GlcNAc (NM_5) to a GlcNAcMan$_3$GlcNAc with the loss of 2 mannose residues. In fact, the ratio of radioactivity in the two peaks seen after 16 h of incubation (lowest profile) was approximately 3:2 (peak NM_3:M). Figure 4 shows that the release of radioactive mannose was also proportional to the amount of enzyme added to the incubation mixtures, up to about 20 μg of protein. In this case, the release of mannose was determined by the concanavalin A–Sepharose assay method, but this method and the gel filtration method gave essentially the same results.

A number of metal ions were tested, at various concentrations, to determine whether any of these would stimulate the enzymatic activity. These included Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. The enzyme did not show any requirement for any of these metals, and the activity was not affected by the addition of various amounts of EDTA or EGTA to the incubation mixtures. However, $CuCl_2$ was quite inhibitory and inhibited the activity more than 75% at 0.2 mM concentrations.

The effect of pH of the incubation mixture on the release of mannose from GlcNAc-Man$_5$GlcNAc was examined over the range pH 4.5–7.5 by using acetate, MES, and HEPES buffers as shown in Figure 5. The enzyme showed optimum activity at about pH 6.0, and the activity rapidly decreased as the pH was lowered below about 5.7 or raised above 6.3. Since detergent is necessary to solubilize the enzyme from the microsomes, we examined the effect of detergent on the activity of mannosidase II. We first used Bio-Beads as previously described (Holloway, 1973) to remove as much of the detergent as possible and then examined the effect of adding various

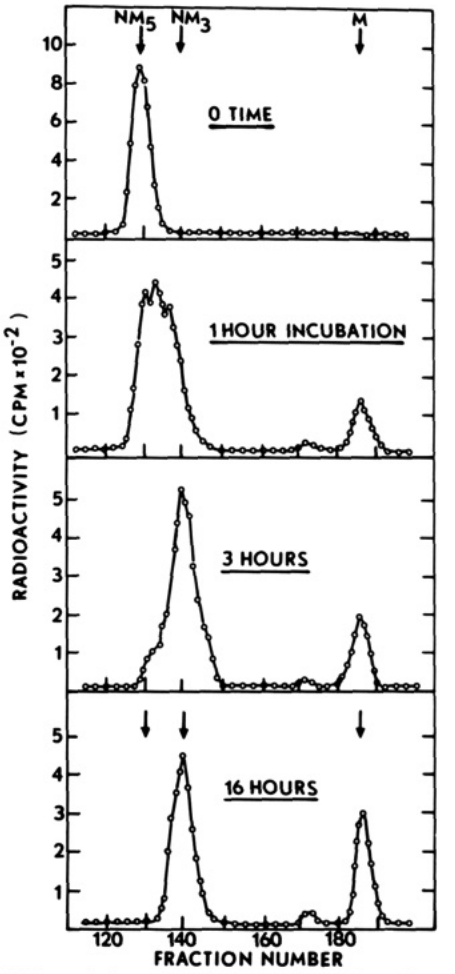

FIGURE 3: Effect of time of incubation on activity of mannosidase II. Incubation mixtures, containing [^{3}H]mannose-labeled GlcNAcMan$_5$GlcNAc, were prepared as described in the text. The assay mixtures were incubated for various times as indicated in the figure. Aliquots from each assay tube were subjected to gel filtration on a calibrated column of Bio-Gel P-4 as shown.

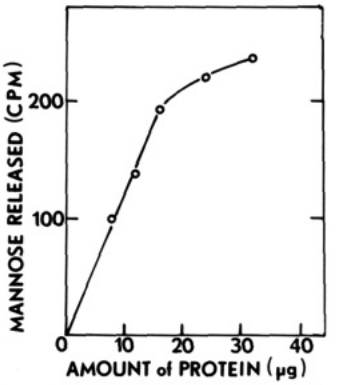

FIGURE 4: Effect of amount of enzyme on the formation of GlcNAcMan$_3$GlcNAc. Incubation mixtures were prepared as described in the text but contained various amounts of the enzyme fraction from the hydroxyapatite column. The release of [^{3}H]mannose was measured according to the concanavalin A binding assay.

amounts of Triton X-100. This method is reported to reduce the Triton X-100 concentration to below 0.005%, which is well below the CMC for this detergent (0.015%). The enzyme retained good activity after the removal of detergent (1906 cpm of mannose released in the standard assay) but was stimulated about 25%–30% by the addition of Triton X-100. Thus, when 0.01% detergent was added to the enzyme, 2304 cpm of mannose were released, and this increased to 2490 cpm

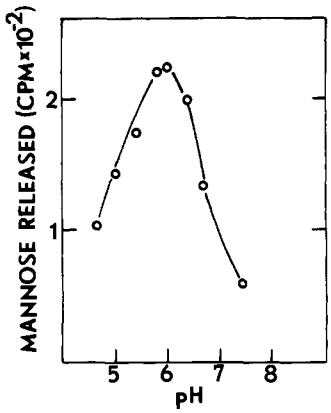

FIGURE 5: Effect of pH of the assay mixture on the activity of mannosidase II. The enzyme from the Sephacryl S-300 column (5 μg of protein) was used in these experiments. Assay mixtures were as described in the text, but the pH of the reaction mixtures was varied as indicated. The following buffers were used: acetate from pH 4.5 to 5.5; MES from pH 5.5 to 6.5; HEPES from pH 6.5 to 7.5. The [^{3}H]mannose released was measured according to the concanavalin A binding assay.

Table II: Substrate Specificity of Mannosidase II

substrate[a]	[^{3}H]Man released (cpm)	substrate[a]	[^{3}H]Man released (cpm)
$GlcNAcMan_5GlcNAc$	2039	$Man_9GlcNAc$	66
$Man_3GlcNAc$	268	$Glc_3Man_9GlcNAc$	36
$Man_5GlcNAc$	29		

[a] Assay mixtures were as described in the text except that the substrate was altered as indicated. In each case, the same amount of radioactivity was used. Since all of the oligosaccharides were derived from the same source, the specific activity of the labeled mannose residues should be similar.

of mannose released by the addition of 0.05% Triton X-100. Little or no additional stimulation of enzyme activity occurred at concentrations of detergent up to 1% (data not shown).

(IV) Substrate Specificity of Mannosidase II. The substrate specificity of the purified mannosidase II was examined by testing the ability of the enzyme to release mannose from a variety of [^{3}H]mannose-labeled oligosaccharides. The results of this experiment are shown in Table II. It can be seen that mannose was not released from any of the high-mannose oligosaccharides such as $Man_5GlcNAc$ or $Man_9GlcNAc$ nor was it released from the $Glc_3Man_9GlcNAc$. However, $GlcNAcMan_5GlcNAc$ was an excellent substrate and was rapidly hydrolyzed to $GlcNAcMan_3GlcNAc$ (see below for characterization). Interestingly, a small amount of activity was observed with $Man_3GlcNAc$, but the significance of this activity is not known at this stage.

Since mannosidase II catalyzes the removal of both the α1,3-linked and the α1,6-linked mannose residues, it was of interest to try and determine whether the enzyme removed all of one of the linkages (for example, all of the α1,6-linked mannose) before catalyzing the removal of the other linkage. Thus the $GlcNAcMan_5(GlcNAc)_2$-peptide was incubated for fairly short times with mannosidase II, and the products were separated on a long column of Bio-Gel P-4. As seen in Figure 6 (profile 3) at fairly short times (i.e., 15 min), the major peak is still the $GlcNAcMan_5(GlcNAc)_2$-peptide, but increasing amounts of $GlcNAcMan_4(GlcNAc)_2$-peptide are seen. The shoulder of $GlcNAcMan_4(GlcNAc)_2$-peptide (Figure 6, profile 3, shaded area) was pooled separately from the $GlcNAcMan_5(GlcNAc)_2$-peptide area and was rerun on the Bio-Gel P-4 column as seen in Figure 6 (profile 4). This second chromatography gave a fairly symmetrical and mostly homogeneous peak of $GlcNAcMan_4(GlcNAc)_2$-peptide. This $GlcNAcMan_4(GlcNAc)_2$-peptide was then treated with Endo H to determine whether it was susceptible to this enzyme. If mannosidase II had removed the α1,3-linked mannose from the α1,6 branch, the glycopeptide would become resistant to Endo H [$GlcNAcMan_5(GlcNAc)_2$-peptide is susceptible to Endo H as seen in Figure 6, profiles 1 and 2]. However, if mannosidase II removed the α1,6-linked mannose first, then the resulting $GlcNAcMan_4(GlcNAc)_2$-peptide should be susceptible to this enzyme. The lowest panel in Figure 6

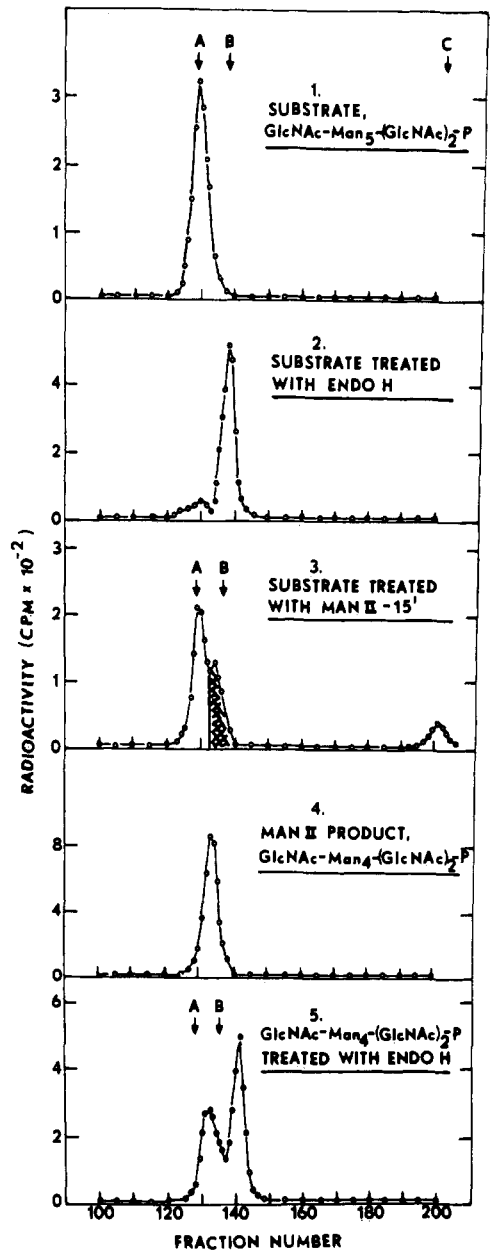

FIGURE 6: Identification of the position of the first mannose released by mannosidase II. The substrate of the reaction, i.e., $GlcNAcMan_5(GlcNAc)_2$-peptide (upper profile, 1), was completely susceptible to the action of Endo H as shown in the second profile (2). The $GlcNAcMan_5(GlcNAc)_2$-peptide was treated for short times (i.e., 15 min) with purified mannosidase II in order to isolate $GlcNAcMan_4(GlcNAc)_2$-peptide as shown in the third profile (3). The shaded area was pooled and rechromatographed on the column to isolate pure $GlcNAcMan_4(GlcNAc)_2$-peptide as shown in profile 4. This purified $GlcNAcMan_4(GlcNAc)_2$-peptide was then treated exhaustively with Endo H to determine its susceptibility to this enzyme. The products of Endo H treatment were separated on the same Bio-Gel P-4 column as shown in the lowest profile (5). The arrows indicate the migration position of standards as follows: A = $GlcNAcMan_5(GlcNAc)_2$-peptide; B = $GlcNAcMan_5GlcNAc$; C = mannose.

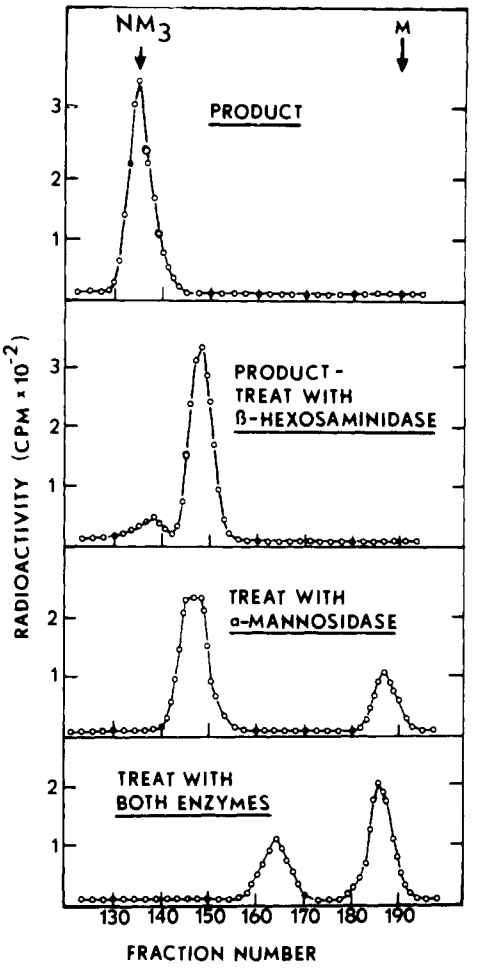

FIGURE 7: Characterization of the products formed by mannosidase II. The [^{3}H]mannose-labeled substrate, GlcNAcMan$_5$GlcNAc, was incubated overnight with purified mannosidase II as described in the text. The reaction mixture was then subjected to chromatography on a 1.5 × 150 cm column of Bio-Gel P-4. Fractions corresponding to the standard GlcNAcMan$_3$GlcNAc were pooled, concentrated, and rechromatographed on Bio-Gel as shown in the top profile. Part of this oligosaccharide was treated with β-hexosaminidase, and the products were chromatographed on the column as indicated in the second profile. Another part of the oligosaccharide was treated with α-mannosidase, and the profile of these products is shown in the third profile. Finally, an aliquot of the oligosaccharide was treated with both β-hexosaminidase and α-mannosidase, and these products were run on the Bio-Gel P-4 column as indicated in the lowest profile. Standards shown by the arrows are GlcNAcMan$_3$GlcNAc (NM_3) and mannose (M).

indicates that about 60% of the GlcNAcMan$_4$(GlcNAc)$_2$-peptide is susceptible to digestion by Endo H and 40% is resistant, indicating that this glycopeptide was a mixture of two species: 40% retaining the α1,6-linked mannose and 60% the α1,3-linked mannose. These results indicate that the enzyme can remove either mannose first but apparently prefers the α1,6-linked mannose.

(*V*) *Characterization of the Oligosaccharide Product.* The products formed upon incubation of the purified mannosidase II with [^{3}H]mannose-labeled GlcNAcMan$_5$GlcNAc were subjected to gel filtration on standardized columns of Bio-Gel P-4, and to treatments with various glycosidases in order to characterize them. The results of this experiment are presented in Figure 7. The upper profile shows the oligosaccharide product from Figure 3 (lowest profile) resulting from a 16-h incubation with mannosidase II rechromatographed on the Bio-Gel P-4 column. It can be seen that the oligosaccharide emerged in a fairly symmetrical peak in the same area as a GlcNAcMan$_3$GlcNAc standard. When this oligosaccharide

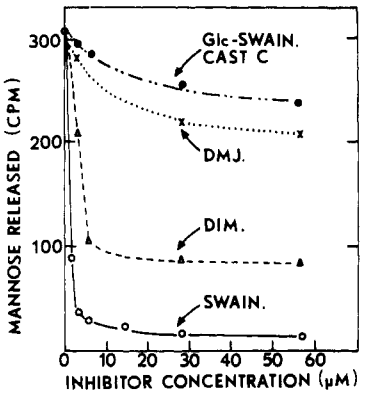

FIGURE 8: Effect of various processing inhibitors on the activity of mannosidase II. The purified mannosidase II was preincubated for 5 min with various amounts of each of the inhibitors as indicated in the figure, and then the various reaction ingredients (GlcNAcMan$_5$GlcNAc, MES buffer, Triton X-100) were added and enzyme activity was measured by the concanavalin A binding assay.

was treated with β-*N*-acetylhexosaminidase and rechromatographed on the same column, there was a shift in the position of the major peak of radioactivity that was consistent with the loss of 1 GlcNAc residue. As expected, no radioactivity was released from this oligosaccharide. On the other hand, treatment of the oligosaccharide with α-mannosidase also caused a shift in the position of the oligosaccharide to a slower migrating (i.e., smaller sized) position, but in this case there was the release of radioactivity in the mannose area of the column. The ratio of radioactivity remaining in the oligosaccharide to that in the mannose area was about 2.2:1 (1704 cpm:754 cpm). Finally, as shown by the lowest profile, when the oligosaccharide was treated with both β-*N*-acetylhexosaminidase and α-mannosidase, the larger oligosaccharide completely disappeared and was replaced by a large peak of radioactivity in the mannose area (1185 cpm) and a new peak of radioactivity corresponding to a ManGlcNAc standard (797 cpm). Although the ratio of the radioactivity in the mannose to ManGlcNAc is less than the expected 2:1, the combined results of the characterization shown here indicate that the product of mannosidase II action is GlcNAcManα1,3-(Manα1,6)Manβ GlcNAc.

(*VI*) *Effect of Inhibitors on Mannosidase II.* We tested the effects of various glycoprotein processing inhibitors on the purified mannosidase II as shown in Figure 8. It can be seen from this figure that swainsonine (SWAIN) [(1*S*,2*R*,8*R*,8a*R*)-trihydroxyindolizidine] was a very potent inhibitor of the plant mannosidase II and gave 50% inhibition at less than 1 μM concentrations. In fact, depending on the enzyme preparation being used, 50% inhibition occurs at about 10–25 nM. The plant mannosidase II is also inhibited to some extent by 1,4-dideoxy-1,4-imino-D-mannitol (DIM), but this is a much weaker inhibitor than is swainsonine. It is not clear whether this compound also inhibits the animal mannosidase II, but the N-linked oligosaccharide products formed by cells in the presence of this inhibitor suggest that the major site is mannosidase I with secondary inhibition of mannosidase II (Palamarczyk et al., 1985). Other inhibitors, such as deoxymannojirimycin (DMJ), 6-epicastanospermine (Cast C), and 2-episwainsonine ("Glc-Swain") were either very weak inhibitors or essentially ineffective.

Several mannose-containing oligosaccharides were also tested as inhibitors of mannosidase II. A trisaccharide of mannose residues linked in α1,2-linkages was not an inhibitor of the enzyme even at 2 mM concentrations, whereas an α1,6-linked trisaccharide of mannose did inhibit to some extent

but required millimolar concentrations (i.e., 40% inhibition at 1.5 mM) for inhibition (data not shown). A tetrasaccharide of mannose with the nonreducing mannose linked in α1,3-linkage to an α1,2-linked trisaccharide of mannose was also tested and found to be inactive in inhibiting mannosidase II.

Discussion

Mannosidase II appears to be a key enzyme in the processing of N-linked oligosaccharides in animal cells (Schachter et al., 1983). Thus, the removal of the two mannose residues from the α1,6 branch of the $GlcNAcMan_5(GlcNAc)_2$-protein by mannosidase II differentiates the hybrid types of structures from the complex types of chains. That this step is essential for the formation of complex types of oligosaccharides has been shown by using the plant alkaloid swainsonine, a potent inhibitor of α-mannosidases (Dorling et al., 1980; Kang & Elbein, 1983) which specifically inhibits the processing mannosidase II but not mannosidase I (Tulsiani et al., 1982). When various animal cell lines are incubated in the presence of swainsonine for appropriate periods of time, all of their N-linked glycoproteins have hybrid types of oligosaccharides rather than the usual complex chains (Elbein, 1987).

In the present paper, the purification and characterization of mannosidase II from mung bean seedlings are described. Although plants are known to produce a variety of N-linked glycoproteins that have either high-mannose or modified types of oligosaccharides (Lis & Sharon, 1981; Lehle & Tanner, 1983), until recently, relatively little was known about the processing of the oligosaccharide chains of these glycoproteins (Elbein, 1988). Early radiolabeling and pulse–chase studies of the N-linked glycoproteins of plant tissue cultures (Hori et al., 1982) and of cotyledons (Bollini et al., 1983) indicated that the oligosaccharide chains were altered in size and sugar content during the chase. Further in vivo studies indicated that these reactions occurred mostly in the Golgi apparatus (Chrispeels, 1985). Other recent studies in plant systems have involved the isolation and purification of several of these processing glycosidases (Szumilo et al., 1986a,b) and glycosyltransferases (Johnson & Chrispeels, 1987; Szumilo et al., 1987).

The modified oligosaccharides of plant cells differ in several interesting ways from those of animal cells, and these differences may be critical in regulation of the pathway, or in the function of specific glycoproteins. Thus, plant glycoproteins show the following differences from animal N-linked glycoproteins: (1) the plant oligosaccharides lack sialic acid; (2) the plant modified structures have a core fucose that is linked α1,3 to the internal GlcNAc, whereas the animal cell oligosaccharides have an internal α1,6-linked fucose; and (3) many plant N-linked glycoproteins have a xylose residue linked β1,2 to the β-linked mannose of the core trisaccharide (Lis & Sharon, 1981; Takahashi et al., 1986; Sturm et al., 1987). In vitro biosynthetic studies incubating Golgi fractions with a variety of glycopeptide acceptors have indicated that the addition of fucose and xylose occurs at the same time as, or sometime after, the action of mannosidase II (Johnson & Chrispeels, 1987). That is, in these studies, $GlcNAcMan_5(GlcNAc)_2$-peptide, $GlcNAcMan_3(GlcNAc)_2$-peptide, and $GlcNAc_2Man_3(GlcNAc)_2$-peptide were all fairly good acceptors of fucose, whereas $GlcNAcMan_5(GlcNAc)_2$-peptide was not a good acceptor of xylose but the other glycopeptides were. Thus a critical step in the processing may be the removal of the two mannose residues by mannosidase II.

The purified mannosidase II described here is similar in most respects to the animal mannosidase II reported earlier. Thus the plant enzyme also has a subunit molecular weight of about 125K, has a pH optimum of about 6.1, and hydrolyzes *p*-nitrophenyl α-D-mannoside as well as the α1,3- and α1,6-linked mannose units of $GlcNAcMan_5GlcNAc$. In addition, the plant mannosidase II, like the animal enzyme, is very susceptible to inhibition by the plant alkaloid swainsonine but is not inhibited by deoxymannojirimycin. The plant enzyme is also inhibited by 1,4-dideoxy-1,4-imino-D-mannitol, but this inhibitor has not been tested directly on the animal mannosidase II. Whether mannosidase II undergoes any type of regulation in the plant cell, or in the animal cell, is not known at this time. Future studies will attempt to localize this enzyme within the plant cell and to determine its mechanism of biosynthesis and targeting.

Registry No. Mannosidase II, 82047-77-6; swainsonine, 72741-87-8; 1,4-dideoxy-1,4-imino-D-mannitol, 95189-02-9.

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