Plant Physiol. (1986) 81, 383–389
0032-0889/86/81/0383/07/$01.00/0

Purification and Properties of a Glycoprotein Processing α-Mannosidase from Mung Bean Seedlings[1]

Received for publication December 9, 1985 and in revised form January 23, 1986

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ABSTRACT

The microsomal fraction of mung bean seedlings contains mannosidase activities capable of hydrolyzing [^{3}H]mannose from the [^{3}H]$Man_9GlcNAc$ as well as for releasing mannose from *p*-nitrophenyl-α-D-mannopyranoside. The glycoprotein processing mannosidase was solubilized from the microsomes with 1.5% Triton X-100 and was purified 130-fold by conventional methods and also by affinity chromatography on mannan-Sepharose and mannosamine-Sepharose. The final enzyme preparation contained a trace of aryl-mannosidase, but this activity was inhibited by swainsonine whereas the processing enzyme was not. The pH optimum for the processing enzyme was 5.5 to 6.0, and activity was optimum in the presence of 0.1% Triton X-100. The enzyme was inhibited by ethylenediaminetetraacetate while Ca^{2+} was the most effective cation for reversing this inhibition. Mn^{2+} was considerably less effective than Ca^{2+} and Mg^{2+} was without effect. The processing mannosidase was inhibited by α1,2- and α1,3-linked mannose oligosaccharides (50% inhibition at 3 millimolar), whereas free mannose and α1,6-linked mannose oligosaccharides were ineffective. Mannosamine was also an inhibitor of this enzyme. The aryl-mannosidase and the processing mannosidase could also be distinguished by their susceptibility to various processing inhibitors. The aryl-mannosidase was inhibited by swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol but not by deoxymannojirimycin or other inhibitors, while the processing mannosidase was only inhibited by deoxymannojirimycin. The processing mannosidase was incubated for long periods with [^{3}H]$Man_9GlcNAc$ and the products were identified by gel filtration. Even after a 24 hour incubation, the only two radioactive products were $Man_5GlcNAc$ and free mannose. Thus, this enzyme appears to be similar to the animal processing enzyme, mannosidase I, and is apparently a specific α1,2-mannosidase.

In animal cells, the oligosaccharide chains of the N-linked glycoproteins are biosynthesized by the sequential addition of the sugars, GlcNAc, mannose, and glucose from their sugar nucleotide derivatives to a lipid carrier to form the lipid-linked oligosaccharide, $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol (4, 5, 21). This oligosaccharide is then transferred to protein where it undergoes a number of modifications or processing reactions as the protein migrates from its initial site of synthesis in the RER through the various Golgi stacks (14, 15). Thus, the three glucose residues are probably removed in the ER; the terminal α1,2-linked glucose by glucosidase I and the next two α1,3-linked glucoses by glucosidase II (14, 15). These reactions lead to the formation of a $Man_9(GlcNAc)_2$ structure which may either result in high-mannose glycoproteins, or this $Man_9(GlcNAc)_2$ oligosaccharide may be acted upon by various α-mannosidases to produce hybrid or complex structures. Thus, an α-mannosidase in the ER (2), and one/or several α-mannosidases in the Golgi apparatus, called mannosidase IA/B (9, 17, 26) remove all of the α1,2-linked mannoses to give a $Man_5(GlcNAc)_2$-protein. This oligosaccharide is the acceptor for a GlcNAc transferase, called GlcNAc transferase I, that adds a GlcNAc to the mannose that is linked α1,3 to the β-linked mannose (1, 11, 18). The resulting GlcNAc-$Man_5(GlcNAc)_2$-protein is the substrate for a second Golgi mannosidase, called mannosidase II, that removes the α1,3- and α1,6-linked mannoses to give a GlcNAc-$Man_3(GlcNAc)_2$-protein (25, 27, 28). This oligosaccharide contains the core region of the complex types of oligosaccharides and can be elongated by the addition of GlcNAc, galactose, sialic acid and fucose, as well as by other substitutions (14).

The biosynthesis of the N-linked oligosaccharides of plant glycoproteins appears to involve the same types of lipid-linked saccharide intermediates, and several laboratories have shown the presence or formation of a $Glc_3Man_9(GlcNAc)_2$-pyrophosphoryl-dolichol (12, 16, 22), and transfer of this oligosaccharide to protein (21). Furthermore, although plants do not contain typical types of complex oligosaccharides, they do contain modified structures having L-fucose and D-xylose that must result from processing reactions (3, 13). In fact, in cultured soybean cells, pulse-chase studies showed the migration of [^{3}H]mannose-label from the $Glc_3Man_9(GlcNAc)_2$-protein to a $Man_9(GlcNAc)_2$-protein and eventually to a $Man_7(GlcNAc)_2$-protein (13). Nevertheless, not very much information is available on processing reactions in plants. Recently, a glucosidase I that removes the terminal α1,2-linked glucose from the $Glc_3Man_9(GlcNAc)_2$ was solubilized and purified from the membrane fraction of mung bean seedlings (24). During that purification, evidence for the presence of glucosidase II was also obtained.

Recently, microsomal and cytosolic α-mannosidase activities were demonstrated in mung bean hypocotyls, and these activities were found to hydrolyze the α1,2-linked mannose residues from the $Man_5(GlcNAc)_2$ substrate. The α1,2mannosidase activities were distinguished from previously reported aryl-mannosidase since they were optimally active in the presence of Ca^{2+} between 5.5 and 6.0, were inhibited by Zn^{2+}, and had essentially no activity on *p*-nitrophenyl-α-mannopyranoside (8). Thus, it is likely that this enzyme is a glycoprotein processing mannosidase. In the present report, we describe the solubilization and purification of a glycoprotein processing α-mannosidase from mung beans and the properties of this enzyme. The enzyme was purified about 130-fold from microsomes using the natural biosynthetic intermediate, $Man_9GlcNAc$, as substrate. The purified enzyme

[1] Supported by grants from the National Institutes of Health (AM 21800), the National Science Foundation (PCM 8300733), and the Robert A. Welch Foundation. A preliminary report on this work was presented at the VIII International Symposium on Glycoconjugates, Houston, TX, September 1985.

cleaved this oligosaccharide to the $Man_5GlcNAc$ product indicating that the enzyme was an α1,2-mannosidase.

MATERIALS AND METHODS

Materials. [2-^{3}H]Mannose (25 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, hydroxylapatite (Biogel HT) and Biogel P-4 were from Biorad, and DE-52 was from Whatman Chemical Separation, LTD. Concanavalin A-Sepharose (10 mg ConA/ml gel), yeast mannan, mannosamine, DTT, *p*-nitrophenyl-α-mannopyranoside, Triton X-100, and Sephacryl S-300 were obtained from Sigma Chemical Co. The following oligosaccharides were generously donated by Dr. Clint Ballou, University of California: α1,6-mannotriose, α1,2-mannotriose, Manα1,3Manα1,2Manα1,2Man. Deoxymannojirimycin was kindly supplied by Drs. G. Legler and E. Bause, University of Koln (7, 10) and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) was a gift from Drs. L. Fellows and S. Evans (6). Swainsonine was isolated from *Swainsona canescens* or *Astragalus sp.* (5) and castanospermine was from *Castanospermum australe* (23). Mannan was attached to epoxy-activated Sepharose 6B, and D-mannosamine to AH-Sepharose 4B according to the manufacturer's information (Pharmacia Fine Chemicals). The ^{3}H-mannose-labeled $Man_9GlcNAc$ was prepared by incubating influenza virus-infected MDCK cells in [2-^{3}H]mannose in the presence of deoxymannojirimycin. The glycoproteins were digested with pronase and the glycopeptides were isolated and treated further as described (23).

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 to 3 d for germination. The seedlings were picked by hand and kept in ice. One kg of seedlings were blended in 500 ml of extraction buffer (50 mM Hepes buffer [pH 7.4], containing 0.25 M sucrose, 0.5 mM DTT, 1 mM EDTA, and 0.5% PVP) for 10 s (3×) in a Waring Blendor. The resulting suspension was passed through eight layers of cheesecloth and the filtrate was centrifuged at 3,000*g* for 10 min to remove whole cells and large particles. The supernatant liquid from this centrifugation was then centrifuged at 100,000*g* for 45 min to isolate the membrane fraction.

Solubilization of Mannosidase Activity. The membrane pellet was washed with 50 mM Hepes buffer (pH 7.4), containing 0.1% Triton X-100, and was then centrifuged at 100,000*g* for 45 min. The resulting pellet was resuspended in solubilization buffer (50 mM Hepes buffer [pH 7.4], 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 solubilized mannosidase activity, was removed and saved. The residue was reextracted with the same solubilization buffer and, after centrifugation, the supernatant liquid was pooled with the first supernatant.

Purification of the Glycoprotein Processing Mannosidase I. The solubilized material (80–100 ml) was applied to a 2 × 20 cm column of DE-52 which had been equilibrated with 10 mM Hepes buffer (pH 7.4), containing 10% glycerol, 0.25% Triton X-100, 0.5 mM DTT. The column was washed first with the above buffer, and then mannosidase activity was eluted with 200 ml of a linear gradient of 0 to 500 mM NaCl in the same buffer. The mannosidase activity emerged at about 70 to 120 mM NaCl (Fig. 1). Two peaks of aryl-mannosidase activity also eluted from the column and one of these peaks coincided with the processing mannosidase activity. The most active fractions of processing mannosidase (45–60) were pooled and used for further purification.

The pooled fractions from the above step were concentrated to about 8 ml on an Amicon filtration apparatus, and were then dialyzed overnight against 10 mM Hepes buffer containing 10% glycerol, 0.25% Triton X-100, and 0.5 mM DTT. The dialyzed fraction was applied to a 1 × 10 cm column of DE-52, and after washing with buffer, the column was eluted with 200 ml of a 0 to 250 mM NaCl solution. Again the aryl-mannosidase and processing mannosidase activities eluted together, prior to the large protein peak.

The active fractions from this DE-52 column were pooled and concentrated to about 2 ml on the Amicon filtration apparatus. This enzyme fraction was applied to a 1 × 120 cm column of Sephacryl S-300 that had been equilibrated with 25 mM Hepes buffer (pH 7.4) containing 10% glycerol, 0.25% Triton X-100, and 0.5 mM DTT. The enzyme was eluted with the same buffer. The peak of aryl-mannosidase activity emerged from the column first followed by the peak of processing mannosidase. However, there was considerable overlap in these two peaks.

The peak of processing mannosidase activity from the Sephacryl column was pooled and concentrated to about 2 ml and was applied directly to a column containing 1.5 ml of the mannan-Sepharose gel in a Pasteur pipette. The column was washed with buffer and then the enzyme was eluted with 50 ml of a linear gradient of 0 to 100 mM NaCl in the 10 mM Hepes buffer (pH 7.4) containing 10% glycerol, 0.25% Triton X-100, and 0.5 mM DTT. The processing mannosidase eluted from the column first, followed by the aryl-mannosidase.

The fractions from the mannan-Sepharose column containing processing mannosidase activity were pooled and concentrated to about 1 or 2 ml, and dialyzed overnight against the 10 mM Hepes buffer (pH 7.4) containing 10% glycerol, 0.25% Triton X-100, and 0.5 mM DTT. This enzyme fraction was applied to a small column of mannosamine-Sepharose (2 ml of gel), also contained in a Pasteur pipette. After washing the column with buffer, the processing mannosidase was eluted with 25 mM NaCl in buffer (Fig. 2).

Assay Procedure for Processing Mannosidase. Incubation mixtures for the determination of the processing mannosidase contained 120 mM Mes buffer (pH 6.0), 0.1% Triton X-100, 5 mM $CaCl_2$, 5000 cpm of [^{3}H]mannose-labeled $Man_9GlcNAc$, and various amounts of the enzyme preparation, all in a final volume of 0.2 ml. The reactions were initiated by the addition of enzyme and the incubations were done for 15 to 30 min. At the end of this incubation, the reactions were deproteinized in one of two ways: If the Concanavalin A-Sepharose binding assay was to be used, the reactions were deproteinized by the addition of TCA to a final concentration of 10% and phosphotungstic acid to a final concentration of 2.5%. After cooling, the precipitate was removed by centrifugation and the supernatant liquid was applied to the column. If the paper chromatographic assay was to be used to measure the release of [^{3}H]mannose, the reaction mixtures were deproteinized by the addition of ethanol to a final concentration of 75%. After removal of the precipitate, the supernatant liquid was concentrated to dryness and spotted on Whatman 3MM paper and run in butanol:pyridine:H_2O (6:4:3).

The use of Concanavalin A-Sepharose to assay the release of glucose or mannose from these high-mannose oligosaccharides has been described previously (20, 23, 24). Briefly, small columns of Concanavalin A-Sepharose (0.25 ml of resin) were prepared in Pasteur pipettes and washed with 5 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl, and then with 5 ml of acetate buffer (pH 5.5) containing 2 mM $MnCl_2$ and 2 mM $CaCl_2$. The deproteinized supernatant liquid was applied to the column, and the released monosaccharides were washed through the column with two 1 ml washes of buffer. More than 95% of the radioactivity from a control incubation with enzyme and [^{3}H] mannose emerged from the column in these two washes. Control

incubations were done with heat-killed enzyme and ^{3}H-Man$_9$GlcNAc to be certain that all of this radioactivity bound to the column. This assay compared well to the paper chromatographic assay or to a gel filtration assay for this enzyme.

Assay of Aryl-Mannosidase Activity. Incubation mixtures for the determination of aryl-mannosidase activity contained 80 mM acetate buffer (pH 4.5), 0.1% Triton X-100, 5 mM $ZnCl_2$, 2 mM *p*-nitrophenyl-α-D-mannopyranoside, and various amounts of the enzyme preparation, all in a final volume of 0.2 ml. Incubations were for 30 min to 1 h at 37°C. The reaction was stopped by the addition of 2.5 ml of 0.4 M glycine buffer (pH 10.4). The mixture was centrifuged if necessary, and the amount of liberated *p*-nitrophenol was determined at 410 nm in a Beckman spectrophotometer.

RESULTS

Purification of the Processing Mannosidase I. Crude extracts of mung bean seedlings were found to contain α-mannosidase activity capable of hydrolyzing *p*-nitrophenyl-α-D-mannopyranoside, and also activity capable of removing mannose units from a [^{3}H]mannose-labeled Man$_9$GlcNAc substrate. Since aryl-mannosidases such as jack bean α-mannosidase will also cleave mannose residues from the Man$_9$GlcNAc, it was important to purify these enzymes to determine whether this plant does contain the processing mannosidase analogous to animal cell mannosidase I. The processing mannosidase from mung beans was first purified by chromatography on DE-52 (Fig. 1). When this column was eluted with a linear gradient of NaCl, a sharp peak of mannosidase activity emerged in fractions 45 to 60 that had good activity on the Man$_9$GlcNAc substrate. This peak also contained aryl-mannosidase activity, but some of the aryl-mannosidase was eluted in an earlier peak. This procedure gave a 3-fold purification over the solubilized extract, with a recovery of 55% (Table I). After a second separation on DE-52, the enzyme was chromatographed on Sephacryl S-300, and was then separated by affinity chromatography on mannan-Sepharose (Fig. 2). This column separated the processing mannosidase, which emerged first, from the aryl-mannosidase. However, there was a slight overlap between the two peaks of activity, so that the processing mannosidase still contained traces of aryl-mannosidase activity. This small amount of aryl-mannosidase was not a problem since it could be inhibited with swainsonine (see below), whereas the processing enzyme is not affected by swainsonine. The final step in the purification was another affinity chromatography step on a mannosamine-Sepharose column as described in "Materials and Methods."

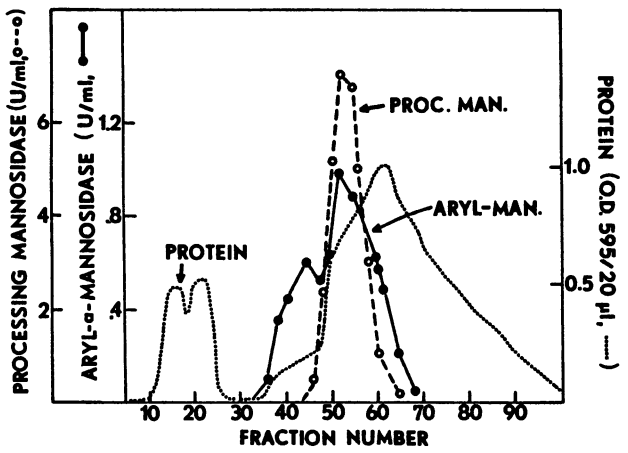

FIG. 1. Chromatography of mannosidase I on DE-52. Eighty ml of solubilized enzyme were applied to a 1.8 × 20 cm column of DE-52 that had been equilibrated with 10 mM Hepes buffer containing 10% glycerol, 0.25% Triton X-100, and 0.5 mM DTT. The column was washed with this buffer and the enzymic activity was then eluted with 200 ml of a linear gradient of 0 to 500 mM NaCl in the same buffer. Fractions (3.5 ml) were collected and examined for aryl-mannosidase (●——●), and processing mannosidase (O– – –O) activities as described in "Materials and Methods." Protein (· · · ·) was determined using the Biorad reagent. The processing mannosidase eluted between 70 and 130 mM NaCl.

Using the outlined purification procedure, the plant mannosidase I was purified about 130-fold with a recovery of about 13%. The purification procedure and the recovery data are summarized in Table I. At the final stage of purification, the enzyme was somewhat labile and could only be kept in ice for a few d as long as the buffer contained 10% glycerol. However, at the DE-52 stage of purification, the enzyme was quite stable in buffer containing 10% glycerol and could be kept for at least 2 weeks in an ice bucket. We usually found it convenient to store the DE-52 enzyme in an ice bucket, and to purify it further just before use. The release of [^{3}H]mannose from the Man$_9$GlcNAc was linear with time of incubation up to about 20 min using either the DE-52 enzyme or the more purified enzyme fractions. It was also linear with respect to the amount of protein added up to about 100 µg of the DE-52 enzyme preparation or about 10 µg of the more purified enzyme fraction (data not shown).

Effect of pH and Detergent on the Mannosidases. The processing mannosidase was compared to the aryl-mannosidase in terms of its pH optima (Fig. 3). In this experiment, the DE-52 enzyme preparation was used, and this figure shows that these two enzyme activities were clearly distinguishable on the basis of their pH optima. Thus, the processing mannosidase gave a fairly sharp pH optimum at 5.5 to 6.0, and the activity was markedly diminished at higher and lower pH values. On the other hand, the aryl-mannosidase had a pH optimum at 4.0 or below, and activity rapidly declined as the pH was raised. Thus, at pH 6.0, the aryl-mannosidase activity was 35 to 40% below the activity at pH 4.0. The processing mannosidase could also be distinguished from the aryl-mannosidase on the basis of its stimulation by Triton X-100 (Fig. 4). In this case, the two activities were examined in the microsomal enzyme fraction, and activity was tested at various concentrations of Triton X-100. It can be seen that the addition of this detergent to incubation mixtures of aryl-mannosidase with the *p*-nitrophenyl-α-D-mannoside had essentially no effect on the reaction, although there might have been slight inhibition at the higher detergent concentrations. On the other hand, the processing mannosidase activity was greatly stimulated by the addition of Triton X-100 to incubation mixtures. Thus, activity increased with increasing detergent concentrations up to about 0.1% and then activity leveled off at higher concentrations. This detergent was kept in all of the buffers used to purify the enzyme, usually at 0.25%.

Requirement of Mannosidase I for Ca^{2+}. The activity of the partially purified enzyme was inhibited by the addition of EDTA to the incubations (Fig. 5). Thus, at 4 mM EDTA, the activity of the processing mannosidase was inhibited 60 to 75%. However, this inhibition by EDTA could be overcome by adding various amounts of Ca^{2+} to the incubation mixtures. For example, as shown in the figure, at an EDTA concentration of 2.5 mM, increasing amounts of Ca^{2+} restored the activity not only back to the normal level but well beyond. At 5 mM Ca^{2+} in the presence of 2.5 mM EDTA, the enzymic activity was at least 50% over that of control activity observed in the absence of Ca^{2+} and EDTA. Mn^{2+} could also overcome the EDTA inhibition, but it was not nearly as effective as Ca^{2+}, and only brought the activity back to the control levels. Mg^{2+} had only a small stimulatory effect. A variety of other divalent cations were tested both in the presence and absence of EDTA, Fe^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} were all stimulatory to some extent but all of them were much less effective than Ca^{2+} (data not shown). The effect of cation con-

Table I. *Purification Procedure for Mannosidase I*

One unit of mannosidase is defined as that amount of enzyme that catalyzes the release of 1000 cpm of [^{3}H]mannose from the $Man_9GlcNAc$ substrate in 60 min.

Step	Protein		Activity		Purification	Yield
	Amount	Yield	Total	Spec. Act		
	mg	%	*units*	*units/mg*	*fold*	%
1. Solubilized extract	403	100	1088	2.7	1	100
2. DE-52 (first)	73	18	606	8.3	3	55
3. DE-52 (second)	9	2.2	448	49.8	18.7	45
4. Sephacryl S-300	5.4	1.3	410	76	28.1	37
5. Mannan-Sepharose	1.4	0.34	284	203	75.1	27
6. Mannosamine-Sepharose	0.6	0.15	212	353	131	19

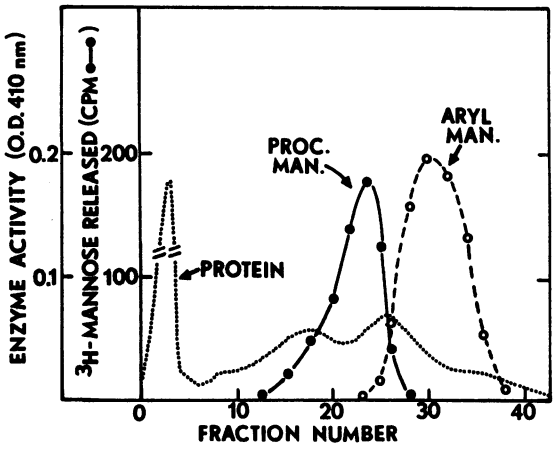

FIG. 2. Purification of mannosidase I on mannan-Sepharose columns. Two ml of enzyme from the Sephacryl S-300 column were applied to a column containing 1.5 ml of gel of mannan-Sepharose contained in a Pasteur pipette. The column was washed with buffer, and the enzyme was eluted with 50 ml of a linear gradient of 0 to 100 mM NaCl in the 10 mM Hepes buffer (pH 7.4) containing 10% glycerol, 0.25% Triton X-100, and 0.5 mM DTT. Fractions were collected and assayed for aryl-mannosidase (O– – –O) and processing mannosidase (●——●) activities as described in the text. Fractions were also tested for protein (· · · ·).

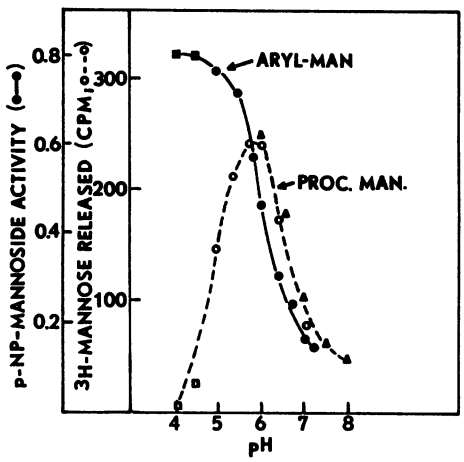

FIG. 3. Effect of pH on aryl-mannosidase and processing mannosidase activities. Incubation mixtures were as described in the text and contained the *p*-nitrophenyl-α-D-mannopyranoside for the aryl-mannosidase or the [^{3}H]$Man_9GlcNAc$ substrate for the processing mannosidase. The pH of the incubations were adjusted using either acetate (■——■), Mes (●– – –●), or Hepes (△——△) buffers of different pH values. In these experiments 45 μg of the DE-52 enzyme were used in each incubation. The release of [^{3}H]mannose or the liberation of *p*-nitrophenol was measured as described in the text.

centration in the absence of EDTA was also examined using the microsomal enzyme preparation. In this case, the activity for releasing [^{3}H]mannose from the ^{3}H-$Man_9GlcNAc$ was stimulated by the addition of Ca^{2+}, with optimal activity being observed at about 5 mM cation concentration. On the other hand, in these experiments, Mn^{2+} and Mg^{2+} were inhibitory (data not shown). We assume that these divalent cations are inhibitory because they compete with endogenous Ca^{2+} for the enzyme and therefore high concentrations can displace the Ca^{2+} and inhibit activity. The microsomal enzyme preparation did not show an absolute requirement for Ca^{2+} and the activity was only stimulated about 20 or 30% at 5 mM Ca^{2+}. Nevertheless, these studies do indicate that this enzyme requires Ca^{2+}. Forsee (8) earlier showed that mung bean mannosidase that cleaved mannose units from the $Man_5(GlcNAc)_2$ substrate was a Ca^{2+}-requiring enzyme.

Inhibition of Mannosidase Activity by Various Mannose Oligosaccharides. To obtain some information about the specificity of the processing mannosidase in terms of glycosidic linkage preference, several different mannose oligosaccharides were tested as inhibitors of the processing mannosidase in its action on the [^{3}H]$Man_9GlcNAc$ (Fig. 6). In this experiment, various amounts of each of the mannose oligosaccharides were added to the incubation mixtures along with [^{3}H]$Man_9GlcNAc$ and other reaction components, and the reactions were initiated by the addition of enzyme. In this case, the release of [^{3}H]mannose was measured by the paper chromatographic assay rather than the Concanavalin A-Sepharose assay in order to avoid the possibility that these mannose oligosaccharides might prevent the binding of [^{3}H]$Man_9GlcNAc$ to the Concanavalin A. It can be seen from the figure that none of the mannose oligosaccharides was a particularly good inhibitor, but those oligosaccharides having a terminal α1,2- or α1,3-linked mannose were equally effective as inhibitors and showed 50% inhibition at about 3 mM concentrations. Mannosamine was also an inhibitor and was about as effective as the above oligosaccharides. On the other hand, free mannose or the α1,6-linked mannose trisaccharide were not inhibitory, even at 3 mM concentrations. These studies would suggest that this plant processing enzyme could hydrolyze both α1,2-linkages and α1,3-linkages. However, characterization studies on the nature of the products formed (see below) indicate that this enzyme only releases α1,2-linked mannoses from the $Man_9GlcNAc$.

Effect of Processing Inhibitors on Mannosidase I and Aryl-Mannosidase. A number of compounds have been shown to be *in vivo* inhibitors of glycoprotein processing by virtue of the fact that they inhibit the glucosidases or mannosidases that are involved in removing these sugars. The effects of these inhibitors on the processing mannosidase I and the aryl-mannosidase were compared. In these experiments, shown in Figure 7, A and B,

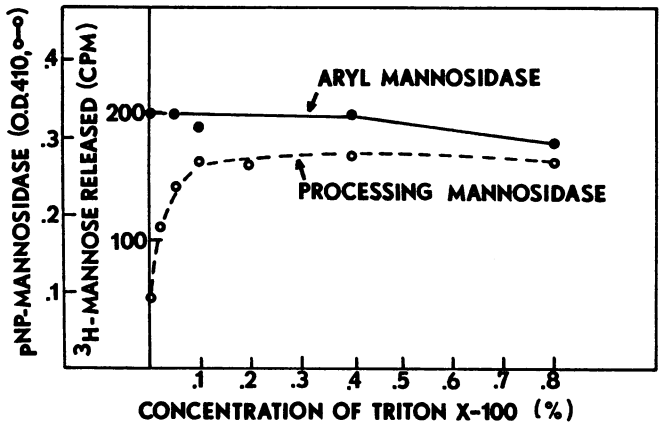

FIG. 4. Effect of Triton X-100 concentration on aryl-mannosidase and processing mannosidase activities. In these experiments, the microsomal enzyme was used to examine the effect of detergent concentration. Microsomes (50 μg) were incubated in a standard incubation mixture with either *p*-nitrophenyl-α-D-mannopyranoside or [^{3}H]Man$_9$GlcNAc in the presence of various amounts of Triton X-100 as indicated in the figure. After 15 min of incubation, the release of [^{3}H]mannose or *p*-nitrophenol was measured as described.

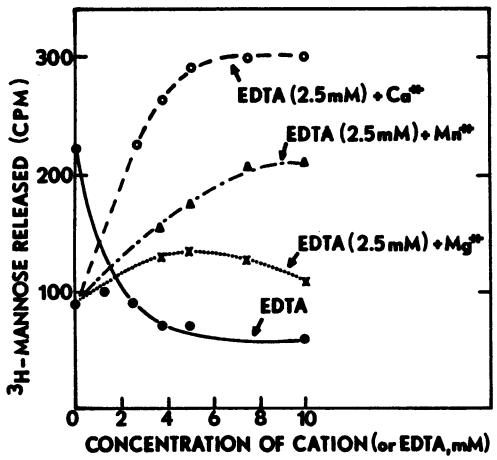

FIG. 5. Effect of EDTA and divalent cations on the processing mannosidase. Incubation mixtures were prepared as described in the text and contained the [^{3}H]Man$_9$GlcNAc. Some of the incubations were done in the presence of various amounts of EDTA (●——●). In other incubation mixtures, 2.5 mM EDTA was added along with increasing amounts of either Ca^{2+} (O– – –O), Mn^{2+} (Δ·—·Δ) or Mg^{2+} (×···×). The reactions were initiated by the addition of 53 μg of DE-52 enzyme and incubations were for 15 min. The liberation of [^{3}H]mannose was measured as described.

the DE-52 enzyme fraction was used, and various amounts of each of the processing inhibitors were added to the incubation mixtures containing either the *p*-nitrophenyl-α-D-mannopyranoside or the [^{3}H]Man$_9$GlcNAc. In the case of the experiments involving the aryl-mannosidase, the release of *p*-nitrophenol was measured at 410 nm, whereas when the Man$_9$GlcNAc was used as substrate, the release of [^{3}H]mannose was determined by the paper chromatographic assay. Figure 7A shows that swainsonine was a potent inhibitor of the aryl-mannosidase activity and gave 50% inhibition at about 0.2 μM. On the other hand, the data in Figure 7B show that this alkaloid had no effect on the plant processing mannosidase I, even at concentrations of 60 μM. Swainsonine has previously been shown to be an inhibitor of mannosidase II in animal cells and to cause the formation of hybrid types of oligosaccharides (5, 27). The fact that swainsonine acts on the aryl-mannosidase but not on the plant processing

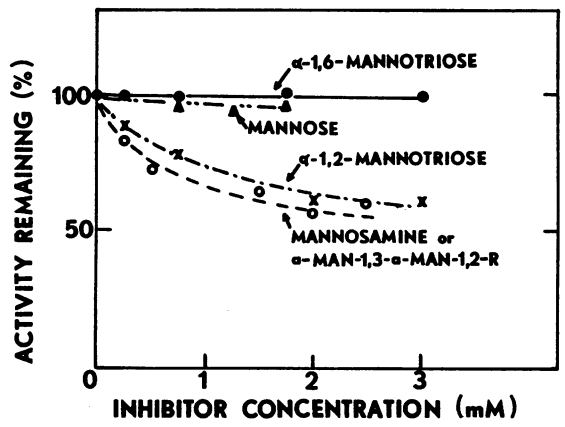

FIG. 6. Effect of mannose oligosaccharides on the liberation of [^{3}H] mannose from the [^{3}H]Man$_9$GlcNAc. Incubation mixtures contained 20 mM Mes buffer (pH 6.0), 0.1% Triton X-100, 10 μg swainsonine, 5000 cpm of [^{3}H]Man$_9$GlcNAc, and various amounts of the mannose oligosaccharides as indicated in the figure. Thus, the following compounds were tested: Manα1,3Manα1,2-R (O– – –O), mannosamine (O– – –O), α1,2-mannotriose (×·—·×), mannose (Δ·—·Δ), α1,6-mannotriose (●——●). Following the addition of these oligosaccharides to the incubation mixtures, the reactions were initiated by the addition of 35 μg of the Sephacryl S-300 enzyme fraction. After an incubation of 1 h, the liberation of [^{3}H]mannose was determined by the paper chromatographic assay.

mannosidase not only demonstrates that these are distinct enzymes, but also gives us a way to inhibit the aryl-mannosidase without affecting the processing activity.

Figure 7A also shows that the aryl-mannosidase was inhibited by 1,4-dideoxy-1,4-imino-D-mannitol (DIM), but again the processing mannosidase was resistant to this compound. Thus, 50% inhibition of the aryl-mannosidase was seen at about 1 μM, but no effect on the processing mannosidase occurred even at 60 μM (Fig. 7B). DIM has also been shown to be a competitive inhibitor of jack bean α-mannosidase, but in animal cells it inhibits glycoprotein processing and gives rise to Man$_9$(GlcNAc)$_2$ structures. Thus, in animal cells this compound appears to inhibit mannosidase I (19). On the other hand, deoxymannojirimycin inhibited the processing mannosidase (Fig. 7B), but did not inhibit the aryl-mannosidase (Fig. 7A). However, the deoxymannojirimycin was a much poorer inhibitor of the plant processing enzyme than it appears to be of the animal enzyme (7, 10). Several other processing inhibitors have been reported to inhibit the processing glucosidases of animal cells. These inhibitors, castanospermine (24), deoxynojirimycin (20), and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (6) were tested on both enzymes to determine whether they had any effect. Figure 7, A and B, shows that castanospermine (CAST), deoxynojirimycin (DNM), and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) had no effect on either enzyme.

Characterization of the Reaction Product. In order to determine how many mannose residues were removed from the Man$_9$GlcNAc by the partially-purified processing mannosidase, the [^{3}H]Man$_9$GlcNAc was incubated with enzyme for various time periods, and the products of the reaction were identified by gel filtration on columns of Biogel P-4. Swainsonine was included in all of these reaction mixtures at 10 μg/ml to inhibit aryl-mannosidase. In separate experiments, this amount of swainsonine was shown to be sufficient to block the liberation of *p*-nitrophenol from the *p*-nitrophenyl-α-D-mannopyranoside. The profiles obtained on the Biogel P-4 columns at various times of incubation are shown in Figure 8. The upper profile is that of the control (*i.e.* 0 time) incubation, or of an incubation of [^{3}H] Man$_9$GlcNAc with heat-inactivated enzyme. As expected, all of

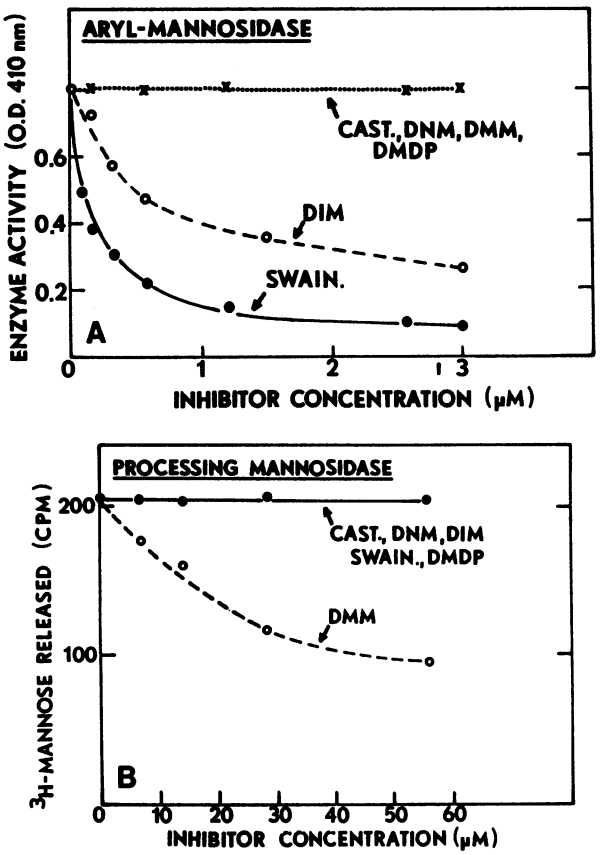

FIG. 7. Effect of various processing inhibitors on the activity of the aryl-mannosidase and the processing mannosidase I. Figure 7, A and B, compares the effects of various processing inhibitors on the activity of the aryl-mannosidase (A) and the activity of the glycoprotein processing mannosidase (B). A, Incubations were as described in the text with *p*-nitrophenyl-α-D-mannopyranoside as substrate and various amounts of the processing inhibitors as follows: swainsonine (SWAIN, ●—●), 1,4-dideoxy-1,4-imino-D-mannitol (DIM, O---O): castanospermine (CAST), deoxynojirimycin (DNM), deoxymannojirimycin (DMM), 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP) all shown by (×····×). In these experiments, the DE-52 enzyme (23 μg of protein) was preincubated with the processing inhibitors and the reaction was started by the addition of substrate. Incubations were for 15 min, and the *p*-nitrophenol liberated was measured at 410 nm in alkali. B, Incubations were as described with [^{3}H]Man$_9$GlcNAc to measure the processing mannosidase. Again, 23 μg of DE-52 enzyme was preincubated for 5 min with the processing inhibitors, and the reactions were started by the addition of Man$_9$GlcNAc. After 15 min incubation, the release of [^{3}H]mannose was measured by the paper chromatographic assay.

the radioactivity emerged in a sharp peak corresponding to the Man$_9$GlcNAc standard. On the other hand, after 4 h of incubation, a number of radioactive peaks were detected. The major peak corresponded to a Man$_8$GlcNAc standard, while smaller peaks emerged in the Man$_7$GlcNAc and Man$_6$GlcNAc areas. There was also a significant peak of radioactivity corresponding to free mannose. At 12 h of incubation, there was significantly more liberation of free mannose, and a greater distribution of the radioactivity in the smaller-sized oligosaccharides. Thus, the Man$_8$GlcNAc peak (seen at 4 h) decreased in amount and there was a significant increase in the amount of radioactivity in Man$_7$GlcNAc and Man$_6$GlcNAc. In addition, a significant peak of Man$_5$GlcNAc was observed. The lower profile shows that by 24 h of incubation, only two radioactive peaks were detected, corresponding to Man$_5$GlcNAc and free mannose. These data strongly support the hypothesis that this enzyme specifically removes α1,2-linked mannoses from the Man$_9$GlcNAc and is therefore analogous to the animal mannosidase I.

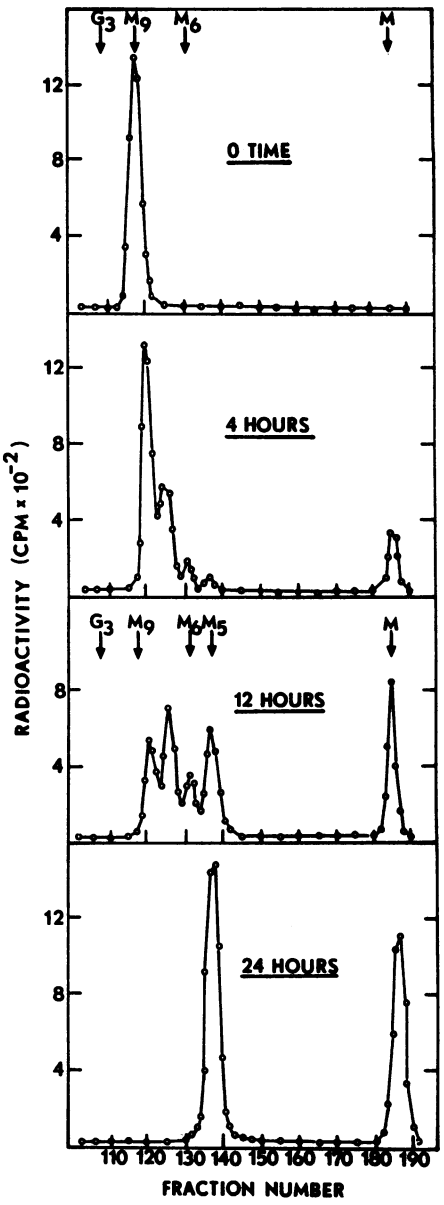

FIG. 8. Characterization of the product of the processing mannosidase I. Large scale incubation mixtures were prepared containing 10,000 cpm of [^{3}H]Man$_9$GlcNAc, 10 μg of swainsonine, 35 μg of the mannan-Sepharose enzyme fraction, and the usual buffer mixture. The incubations were from 0 time to 24 h as shown in the various profiles presented in the figure. At the end of the incubation, the mixture was deproteinized with alcohol (added to 75%), and the supernatant liquid was concentrated to a small volume and applied to a column of Biogel P-4 (1.5 × 200 cm). The column was eluted with 2% acetic acid and the various fractions were analyzed for radioactivity. The arrows indicate the positions of various standards as follows: G_3, Glc$_3$Man$_9$GlcNAc; M_9, Man$_9$GlcNAc; M_6, Man$_6$GlcNAc, M_5, Man$_5$GlcNAc, M, mannose. The upper profile shows the 0 time incubation (*i.e.* alcohol added immediately after enzyme), the next profile is after 4 h of incubation, the next to the bottom profile is after 12 h of incubation, and the lowest profile is after 24 h of incubation.

DISCUSSION

Earlier studies with suspension-cultured soybean cells indicated that trimming or processing of the oligosaccharide chains of the asparagine-linked glycoproteins occurred *in vivo* (13). Thus, when soybean cells were pulse-labeled with [2-^{3}H]mannose, the initial Glc$_3$Man$_9$(GlcNAc)$_2$-peptide was trimmed or shortened during the chase to Man$_7$(GlcNAc)$_2$-peptides to

$Man_9(GlcNAc)_2$-peptides. However, when the chase was done in the presence of the glucosidase I inhibitor, castanospermine, no removal of glucose residues occurred, although several mannose residues could still be removed. Thus, that study presented evidence for the *in vivo* processing of the oligosaccharide chains of the N-linked glycoproteins in plants, and suggested that the first enzyme, since it was inhibited by castanospermine, might be analogous to glucosidase I of animal cells. The glucosidase I was purified from mung bean seedlings and was shown to catalyze the removal of the terminal α1,2-linked glucose from the $Glc_3Man_9GlcNAc$ (24). This enzyme was also quite sensitive to inhibition by castanospermine, indicating that it was the site of inhibition of processing in the soybean system. During the course of purification of the glucosidase I, another neutral glucosidase that removed the two α1,3-linked glucoses from the $Glc_2Man_9GlcNAc$ was separated on DEAE-cellulose. Presumably, this enzyme is the glucosidase II.

Recently, Forsee (8) demonstrated the presence of α-mannosidase activities in the microsomal and cytosolic fractions of mung bean hypocotyls that removed the α1,2-linked mannose units from a $Man_5(GlcNAc)_2$ oligosaccharide. In spite of the fact that the $Man_5(GlcNAc)_2$ is not a naturally occurring intermediate in the processing pathway, it seemed likely that that enzyme was the processing mannosidase analogous to mannosidase I of animal cells. We have solubilized this enzyme from the microsomal fraction of mung bean seedlings and have purified it about 130-fold. The purified enzyme fraction still contained a trace of arylmannosidase, but this activity could be completely inhibited by adding swainsonine to the incubation mixtures. When this enzyme was incubated for 24 h with the $[^3H]Man_9GlcNAc$ in the presence of swainsonine, the only two products observed were the $Man_5GlcNAc$ and free mannose. Thus, this enzyme appears to be a specific α-1,2-mannosidase that is analogous to the enzyme described by Forsee (8), and also analogous to the animal cell mannosidase I activities (9, 17, 26). Also similar to those other reports (8, 9) is the fact that the purified enzyme had a pH optimum of 5.5 to 6.0, and was stimulated by Ca^{2+}.

The finding that mung beans contain mannosidase I activity, in addition to glucosidase I and glucosidase II, strongly indicates that processing of N-linked glycoproteins can proceed at least as far as the $Man_5(GlcNAc)_2$-protein stage. However, since some plant glycoproteins have been reported to have $Xyl\text{-}Man_3(GlcNAc)_2$-structures, it seems likely that processing can go beyond the $Man_5(GlcNAc)_2$ step. It will be interesting to determine whether a GlcNAc residue must be added to this structure in order to trim additional mannose residues as occurs in animal cells, or whether the xylose and/or fucose can serve as signals for other processing reactions. In that regard it should be mentioned that studies from Chrispeels' laboratory (3, 29) have shown that GlcNAc is transiently attached to the high-mannose oligosaccharides. This GlcNAc could be the signal for an enzyme similar to the animal cell mannosidase II to remove several more mannose units. It will also be of considerable importance to determine whether the terminal xylose and fucose units of these N-linked glycoproteins play any role in recognition reactions, or participate in other aspects of glycoprotein function.

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