Purification and Properties of a Golgi-Derived (α1,2)-Mannosidase-I from Baculovirus-Infected Lepidopteran Insect Cells (IPLB-SF21AE) with Preferential Activity toward Mannose$_6$-*N*-Acetylglucosamine$_2$[†]

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ABSTRACT: Because the availability and subcellular distribution of processing mannosidases in cells play such powerful roles in determining ultimate structures of glycoconjugates, we desired to identify, characterize, and investigate possible regulation of mannosidases in infected and noninfected lepidopteran insect cells. Since our previous observations that a mannosidase activity that converted $Man_6GlcNAc_2$ to $Man_5GlcNAc_2$ was enhanced in virus-infected cells, thus providing the necessary intermediate for further processing to complex-type oligosaccharides, we attempted purification of this enzyme. A mannosidase was isolated and purified from membranes, operationally defined as Golgi, of recombinant baculovirus-infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. The molecular mass of this protein was approximately 63 kDa. Assays performed by measuring the conversion of NaB^3H_4-reduced $Man_6GlcNAc_2$-ol to $Man_5GlcNAc$-[^{3}H]$GlcNAc_2$-ol demonstrated that the mannosidase activity was dependent on the presence of divalent cations, which was optimal for Ca^{2+} at pH 6.0. Inclusion of 1-deoxymannojirimycin resulted in 50% inhibition at a concentration of 20 μM, whereas swainsonine did not show such inhibition. No activity was observed with *p*-nitrophenyl α-D-mannoside (4 mM) as a substrate. The preferred reduced oligosaccharide substrate was $Man_6GlcNAc_2$-ol, with lower activities obtained with $Man_9GlcNAc_2$-ol, $Man_8GlcNAc_2$-ol, and $Man_7GlcNAc_2$-ol. With $Man_6GlcNAc_2$-ol as substrate, products smaller than reduced $Man_5GlcNAc_2$-ol were not observed. Mannose was also liberated from the glycoprotein, ovalbumin. These properties are consistent with an enzyme classification as a type I (α1,2)-Man_6-mannosidase.

In order for Asn-linked glycoprotein processing to provide complex-type glycans, the $Glc_3Man_9GlcNAc_2$[1] that is transferred from dolichol pyrophosphate onto relevant Asn residues in proteins must be trimmed by glucosidases and mannosidases. These steps occur in both the ER and the Golgi apparatus. Processing of protein-linked $Glc_3Man_9GlcNAc_2$ oligosaccharide first requires removal of Glc, steps that occur in the rough ER (Shailubhai et al., 1991). Two membrane-bound α-glucosidases that catalyze this phase of the trimming process, namely, glucosidase I (Hettkamp et al., 1984; Shailubhai et al., 1987) and glucosidase II (Burns & Touster, 1982), have been described.

A variety of processing mannosidases exist. Mannosidases with specificity for (α1,2)-linked Man residues are normally found in the ER and the cis- and medial-compartments of the Golgi. Several such enzyme activities have been identified, and several of the enzymes have been purified and characterized [for a recent review, see Moremen et al. (1994)]. One member of this enzyme family (rat liver ER mannosidase) has been shown in *in vivo* experiments to catalyze cleavage of a specific (α1,2)-linked Man residue from $Man_9GlcNAc_2$, yielding $Man_8GlcNAc_2$, prior to transport of this intermediate to the Golgi complex (Bischoff et al., 1986). This reaction was also shown to occur in the cases of Sindbis viral glycoproteins B and PE2 in the rough ER of chick embryo fibroblasts (Hakimi & Atkinson, 1982). The yeast (α1,2)-mannosidase has been most clearly demonstrated to remove a single specific Man residue from the $Man_9GlcNAc_2$ oligosaccharide of yeast mannoproteins (Jelinek-Kelley et al., 1985; Jelinek-Kelley & Herscovics, 1988; Ziegler & Trimble, 1991). Other mammalian Ca^{2+}-dependent (α1,2)-mannosidases of ER origin isolated from rabbit (Forsee & Schutzbach, 1981), calf (Schweden et al., 1986) and pig (Schweden & Bause, 1989) livers have been described. While these enzymes were shown to cleave $Man_9GlcNAc$ to $Man_5GlcNAc$, the calf and pig liver enzymes, and perhaps the rabbit liver enzyme, catalyzed efficient removal of only three of the four (α1,2)-linked Man residues from $Man_9GlcNAc_2$, resulting in $Man_6GlcNAc_2$. However, structural modification of the chitobiosyl core was shown to influence the extent of hydrolysis to either the Man_6 or the Man_5-containing product (Bause et al., 1992). These investigations suggest that in certain cases processing of $Man_9GlcNAc_2$ by ER and early-acting Golgi-specific (α1,2)-mannosidases may proceed only to $Man_6GlcNAc_2$, and that the final product formed depends upon the subtle nature of the substrate presented to the enzyme and the types of mannosidases available. *In vivo* studies corroborate these findings. Specifically, the ER-resident protein hydroxymethylglutaryl-CoA reductase is trimmed only to $Man_6GlcNAc_2$−protein (Bischoff et al., 1990). Further, in MOPC 315 murine plastocytoma cells, newly synthesized immu-

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[1] Abbreviations: Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; GlcNAc-T1, *N*-acetylglucosaminyltransferase I; dMJ, 1-deoxymannojirimycin; Nph-α-D-Man, *p*-nitrophenyl α-D-mannoside; ER, endoplasmic reticulum; AcMNPV, wild-type *Autographa californica* nuclear polyhedrosis virus; ConA, concanavalin A; MOI, multiplicity of infection; pi, post-infection; HPAEC-PAD, high-performance liquid chromatography with pulsed amperometric detection; $DodSO_4$/PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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noglobulin A α-chains possessed Man_8-, Man_7-, and $Man_6GlcNAc_2$, but not $Man_5GlcNAc_2$, when transport of the proteins from the ER to the Golgi complex was blocked (Hickman et al., 1984).

The Golgi apparatus in other mammalian cells also contains (α1,2)-mannosidases. Two rat liver Golgi-associated α-mannosidases (IA and IB) have been purified (Tabas & Kornfeld, 1979; Tulsiani & Touster, 1988), which cleave $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$. These steps provide the substrate for GlcNAc-TI, a necessary occurrence in allowing further oligosaccharide processing to complex and hybrid glycans. Additionally, a Ca^{2+}-independent Golgi-derived endo-α-mannosidase has been found that liberates from $Glc_{1-3}Man_9GlcNAc_2$ the di-, tri-, and tetrasaccharides, $Glc_{1-3}Man$, consequently providing $Man_8GlcNAc_2$ (Lubas & Spiro, 1988; Moore & Spiro, 1990). In this case, the $Man_8GlcNAc_2$ is a different isomer than that produced by the yeast- and rat liver ER (α1,2)-mannosidases. Finally, a rat brain microsomal α-mannosidase has been identified with activity toward (α1,2)-, (α1,3)-, and (α1,6)-linked Man residues, yielding the core glycan Man(α1,3)[Man(α1,6)]-Man(β1,4)GlcNAc(β1,4)GlcNAc, which is an alternate substrate for GlcNAc-TI (Tulsiani & Touster, 1985). There also appears to be a functionally significant subcellular distribution of (α1,2)-mannosidases. When secretion of IgA was inhibited in MOPC 315 cells with monensin, which blocks within the Golgi complex, $Man_6GlcNAc_2$−IgA accumulated, with the glycan containing one (α1,2)-linked Man residue. From this, it was postulated that the conversion of $Man_6GlcNAc_2$−IgA to $Man_5GlcNAc_2$−IgA occurred in the *trans*-Golgi, catalyzed by a specific (α1,2)-mannosidase (Hickman et al., 1984). These results point to processes that lead to trimming of $Glc_3Man_9GlcNAc_2$ to $Man_6GlcNAc_2$, and suggest that formation of $Man_5GlcNAc_2$ requires special consideration.

We have shown previously that a Man_6-mannosidase activity exists in lepidopteran insect cells (IPLB-SF-21AE) that appears to be enhanced as a result of infection of these cells with baculovirus (Davidson et al., 1991). Since the presence of such an enzyme would provide a product with the ability to utilize the GlcNAc-TI shown to be present in such types of cells (Altmann et al., 1993; Velardo et al., 1993), thereby accommodating a pathway for assembly of complex- and hybrid-type glycans on proteins, it became important to isolate and characterize the properties of this putative enzyme prior to additional studies of its regulation. Our success in this effort is the subject of this communication.

EXPERIMENTAL PROCEDURES

Materials. The oligosaccharides $Man_3GlcNAc_2$, $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$ were obtained from Oxford GlycoSystems (Rosedale, NY). These all have structures derived from mammalian glycoprotein processing and, according to the supplier, are of one (>90%) isomeric structure, with the exception of $Man_7GlcNAc_2$. Each oligosaccharide was reduced with NaB^3H_4 (American Radiolabeled Chemicals, Inc., St. Louis, MO) using the materials and procedures in the Glycan radiolabeling kit from Oxford GlycoSystems for the GlycoMap 1000 instrument. Calculated specific radioactivities were in the range of 0.8−2.5 Ci/mmol for the reduced oligosaccharides. Each radiolabeled reduced oligosaccharide displayed a single radioactivity peak from chromatography on Bio-Gel P-4 (Oxford GlycoSystems, GlycoMap 1000) and on HPAEC-PAD (Dionex, Sunnyvale, CA). Swainsonine, dMJ, *p*-nitrophenyl-α-D-mannoside, ConA−Sepharose, cellulose phosphate, yeast hexokinase, rabbit muscle pyruvate kinase, rabbit muscle lactate dehydrogenase, ATP, phosphoenolpyruvate, NADH, and the protein standard mixture (14 000−70 000 Da) used for $DodSO_4$/PAGE analyses were purchased from the Sigma Chemical Co. (St. Louis, MO). DEAE-Cellulose (DE52) was purchased from Whatman (Maidstone, England), and hydroxyapatite was from Bio-Rad (Hercules, CA).

Insect Cell Infection. IPLB-SF-21AE cells were maintained in 1-L spinner flasks at 26 °C in culture in Excell 400 media (JRH Biosciences, Lexena, KS). The cells were split 1:3 (v:v) with media every 2−3 days. Cells (1.5×10^6 cells/mL) were infected with wild-type AcMNPV at a MOI of approximately 2−4, and the insect cell Golgi-rich fraction was isolated at 72 h, pi.

Preparation of Golgi Membranes. The procedure previously described (Velardo et al., 1993) was employed with the following modification. In the final step, involving centrifugation in sucrose, the concentration of sucrose in the lower layer was changed from 1.25 to 1.0 M, and the top layer containing the membranes to be fractionated contained 0.5 instead of 0.32 M sucrose.

Mannosidase Assays. The standard assay with $[^3H]Man_6GlcNAc_2$-ol was conducted as follows. Except as noted for substrate specificity studies, the assay mixture contained (20 μL total volume) 100 mM NaOAc, pH 6.0, 5 mM $CaCl_2$/5 mM $MgCl_2$, and 8000−10 000 dpm of $[^3H]Man_6GlcNAc_2$-ol substrate. After addition of enzyme and incubation at 37 °C for appropriate times, the assay tube was placed in a boiling water bath for 3 min, cooled, and centrifuged to remove any particulate material. The $[^3H]Man_6GlcNAc_2$-ol and $[^3H]Man_5GlcNAc_2$-ol in the supernatant fluid were then separated by HPAEC-PAD on a Dionex liquid chromatography unit equipped with two tandomly linked CarboPac PA-1 columns. Using solutions of 200 mM NaOH (A), 1 mM NaOH (B), and 1 M sodium acetate in 200 mM NaOH (C), the columns were equilibrated and eluted, over a 20 min period, with 47% A/50% B/3% C (v:v:v). Next, a linear gradient was applied (up to 40 min) with a limit solution of 25% A/50% B/25% C (v:v:v). This latter solution was continued for times up to 60 min. A flow rate of 0.8 mL/min was maintained, and 0.5-min fractions were collected. Radioactivity was determined by liquid scintillation counting using Scintiverse cocktail.

Assay with Nph-α-D-Man. The assay mixture contained 4 mM substrate, 100 mM NaOAc, pH 6.0, 10 mM $CaCl_2$, and enzyme in a total volume of 50 μL. After incubation at 37 °C for appropriate times, 1 mL of 0.2 M Na_2CO_3 was added and the absorbance at 405 nm was determined.

Assay with Na^3H_4-Reduced Oligosaccharides. The assay mixtures contained 100 mM NaOAc, pH 6.0, 10 mM $CaCl_2$, 10 000 dpm of reduced oligosaccharide substrate, and enzyme in 20 μL total volume. The reactions were terminated at appropriate times by heating for 3 min in a boiling water bath, and the products were then separated by HPLC. A 250 × 4.6 mm amino-silica column (5 μ Econosil, Altech, Deerfield, IL) was equilibrated with 68% acetonitrile/32% water (v:v) and eluted for 10 min with this solvent after application of the sample. A linear gradient up to 100 min to 40% acetonitrile/60% water (v:v) was then applied. The flow rate was 1.0 mL/min. Fractions of 0.5 or 1.0 mL were

collected, and radioactivity in the fractions was determined by liquid scintillation counting.

Assay with Ovalbumin. The ability of the mannosidase to catalyze hydrolysis of mannosidic linkages, presumably α-1,2 linkages, in intact glycoproteins was determined by a coupled enzymatic assay with ovalbumin as the substrate. The released mannose was monitored by coupling the ADP formed in the hexokinase reaction to the pyruvate kinase reaction, and the pyruvate formed in the latter was monitored by NADH oxidation with lactate dehydrogenase. The initial assay mixture contained 15 mg of ovalbumin, 0.1 M sodium acetate, 5 mM $MgCl_2$, pH 6.0, and 5 μg of purified mannosidase in 0.3 mL (total volume). After a 3-h incubation at 37 °C, this mixture was added to 0.7 mL of a solution to provide the following final concentrations: 50 mM triethanolamine-HCl buffer, pH 7.6; 5 mM $MgCl_2$; 4 mM ATP; 0.5 mM PEP; 0.16 mM NADH; and 20 μg/mL each of yeast hexokinase, muscle pyruvate kinase, and muscle lactate dehydrogenase. The decrease in absorbance at 340 nm was measured as a monitor of the reaction.

Mannosidase Purification. All procedures were carried out at 4 °C unless otherwise specified. The Golgi-rich fraction from 180 g of cells was first extracted with stirring for 4 h into 10−15 mL of a solution containing 0.5% (v:v) Triton X-100, 5 mM $MgCl_2$, 0.25 mM phenylmethanesulfonyl fluoride, and 50 mM potassium phosphate buffer, pH 7.2.

The mixture was then centrifuged at 55 000 rpm for 30 min in a Beckman 30 rotor. The pellets were collected and extracted again with the detergent solution. This extraction procedure was repeated a total of four times. The pooled supernatant fluids were dialyzed against 10 mM potassium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 0.1% (v:v) Triton X-100 (buffer A).

Next, a DEAE-cellulose chromatography step was employed. A 10-mL column of DEAE-cellulose was equilibrated with buffer A. The dialyzed enzyme solution was applied, and the column was then washed with 50 mL of the same buffer solution. The column was next eluted with a 200-mL linear concentration gradient of 0−0.5 M NaCl in buffer A. Fractions of 4.5 mL were collected at a flow rate of 10 mL/h. Fractions 10−15 of the salt gradient contained enzyme activity. These samples were pooled and dialyzed against 10 mM potassium phosphate buffer, pH 5.8, containing 5 mM $MgCl_2$ and 0.1% (v:v) Triton X-100 (buffer B).

For the next step, a 10-mL column of phosphocellulose was equilibrated with buffer B. After application of the dialyzed enzyme solution, the column was washed with 50 mL of buffer B and then eluted with a 200-mL linear concentration gradient of 0−0.5 M NaCl in buffer B. Fractions of 4.5 mL were collected at a flow rate of 10 mL/h. Enzyme activity was found in fractions 8−12 of the salt gradient. These fractions were pooled and dialyzed against 10 mM potassium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 0.1% (v:v) Triton X-100 (buffer C).

A 5-mL column of hydroxyapatite was equilibrated with buffer C. The dialyzed enzyme solution was applied, and the column was then washed with 50 mL of buffer C. A 100-mL linear concentration gradient from 10 to 500 mM potassium phosphate in buffer C was then applied. Fractions of 1.0 mL were collected at a flow rate of 4 mL/h. Enzyme activity was found in fractions 23−27. These were pooled and concentrated to 1 mL in an Amicon ultracentrifugation cell.

Table 1: Purification of (α1,2)-Mannosidase from Golgi of IPLB-SF-21AE Cells

step	total protein (mg)	total act. (DPM/min)	sp. act. (DPM/min/mg)	yield (%)	purification (x-fold)
Golgi	91	39 902	438	100	1
Triton extract	74	36 531	494	91	1
DEAE-52	19	23 347	1228	58	3
phosphocellulose	2	9856	4928	25	11
hydroxyapatite	0.12	6372	53 100	16	121

General Methods. $DodSO_4$/PAGE was carried out in 12.5% (v:v) polyacrylamide gels as described (Laemmli, 1970) and the gels were stained with Coomassie Blue. Protein concentrations were determined with the Bio-Rad protein assay reagent and bovine serum albumin as the standard. Chromatography on ConA-Sepharose was carried out on a 2.5-mL column that had been equilibrated with 10 mM potassium phosphate, pH 7.2, containing 5 mM $CaCl_2$ and 0.1% (v:v) Triton X-100. After the enzyme solution was applied, the column was eluted with 25 mL of the equilibration buffer and then with 10 mL of 0.75 M methyl α-D-mannoside.

RESULTS

A Golgi-rich fraction of infected SF-21AE cells was prepared and employed as the starting material for purification of an (α1,2)-mannosidase which converts $Man_6GlcNAc_2$ to $Man_5GlcNAc_2$, an activity that we have previously identified in these cells (Davidson et al., 1991). For this purification, we selected chromatographic fractions for activity against the substrate $Man_6GlcNAc_2$, assuring that the material that we ultimately purified would possess the desired activity. To optimize the sensitivity of our assays, the reducing end of the substrate was treated with NaB^3H_4 of very high specific radioactivity, and the conversion of [^{3}H]$Man_6GlcNAc_2$-ol to [^{3}H]$Man_5GlcNAc_2$-ol was readily followed by HPAEC-PAD with two columns in tandem. In such a situation, baseline separation of the substrate and product was obtained.

After the Golgi-rich fraction of the baculovirus-infected cells was obtained, four routine chromatographic steps were needed to obtain a homogeneous protein fraction. These are listed in Table 1, with a typical chromatogram of the final hydroxyapatite step provided in Figure 1. As a result of these procedures, we found that, beginning with approximately 180 g of cells, approximately 120 μg of enzyme with the desired activity was obtained in a 16% yield. From examination of the final purification achieved, we approximate that this product represents 0.8% (w:w) of the total Golgi-rich protein.

The high degree of homogeneity of the purified protein is illustrated by the reduced and nonreduced $DodSO_4$/PAGE analysis of Figure 2, where we also show that the molecular mass of the (α1,2)-mannosidase was approximately 63 000 Da. The material was not retarded on a column of immobilized ConA, suggesting that it is not glycosylated, or does not contain appropriate high-mannose type oligosaccharides.

Some enzymatic characteristics of the insect Golgi (α1,2)-mannosidase have been determined. As seen in Figure 3, the pH optimum of the enzyme is approximately 6.0, and according to Figure 4, the enzyme is stimulated by the

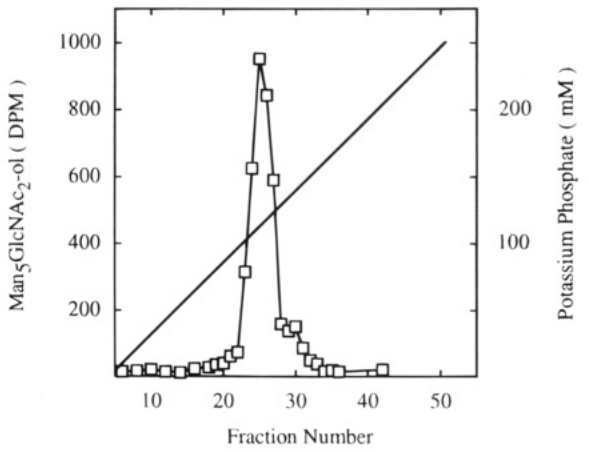

FIGURE 1: Hydroxyapatite chromatography of the insect Golgi (α1,2)-mannosidase at 4 °C. The active pool from the phosphocellulose chromatography step was dialyzed against a buffer containing 10 mM potassium phosphate, pH 7.2, and 5 mM $MgCl_2$/0.1% (v:v) Triton X-100 and applied to a 5-mL column of hydroxyapatite equilibrated with this same buffer. The column was washed with 50 mL of this buffer, followed by application of 100 mL of a linear gradient consisting of 50 mL of 10 mM potassium phosphate, pH 7.2, and 5 mM $MgCl_2$/0.1% (v:v) Triton X-100 as the start buffer and 50 mL of 500 mM potassium phosphate, pH 7.2, and 5 mM $MgCl_2$/0.1% (v:v) Triton X-100 as the limit buffer. Fractions (1 mL) were collected at a flow rate of 4 mL/h. The activity of each indicated fraction tested against the substrate, [^{3}H]$Man_6GlcNAc_2$-ol, liberating the product, [^{3}H]-$Man_5GlcNAc_2$-ol, is indicated on the graph.

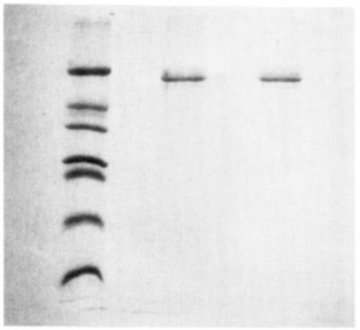

FIGURE 2: $DodSO_4$/PAGE of the purified insect Golgi (α1,2)-mannosidase. Lane 1, standard protein mixture; lane 2, reduced (α1,2)-mannosidase; lane 3, nonreduced (α1,2)-mannosidase. The standard proteins (along with their molecular weights) in lane 1, from top to bottom, are as follows: bovine serum albumin (66 000), egg albumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), bovine carbonic anhydrase (29 000), bovine pancreatic trypsinogen (24 000), soybean trypsin inhibitor (20 000), and bovine α-lactalbumin (14 200).

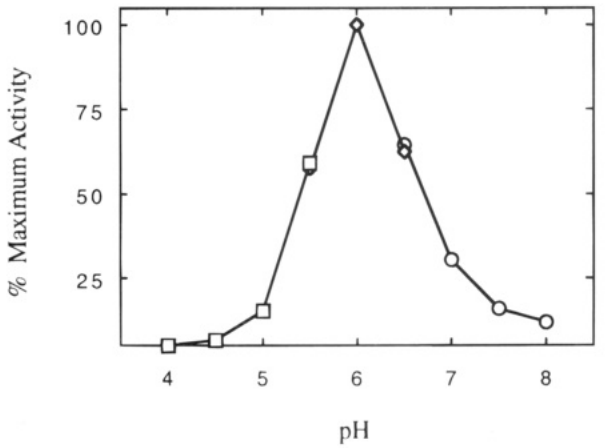

FIGURE 3: Dependence on pH of the activity of the purified insect Golgi (α1,2)-mannosidase. Approximately 1 μg of purified (α1,2)-mannosidase was added to the assay mixture (total volume 20 μL) containing 100 mM of the appropriate buffer, 5 mM $CaCl_2$/5 mM $MgCl_2$, and 8000−10 000 dpm [^{3}H]$Man_6GlcNAc_2$-ol. After incubation at 37 °C for 2 h, the assay tube was placed in a boiling water bath for 3 min, cooled, and centrifuged to remove any particulate material, and the supernate was used for the assays. The [^{3}H]$Man_6GlcNAc_2$-ol and [^{3}H]$Man_5GlcNAc_2$-ol in the supernatant fluid were then separated by HPAEC-PAD. The total dpm of the [^{3}H]$Man_5GlcNAc_2$-ol fraction as a function of the pH is illustrated on the graph. The buffers employed for the study were (□) 100 mM NaOAc, from pH 4.0 to 5.5; (◇) 100 mM Na^+-Mes, from pH 5.5 to 6.5; and (○) 100 mM Na^+-Hepes, from pH 6.5 to 8.0.

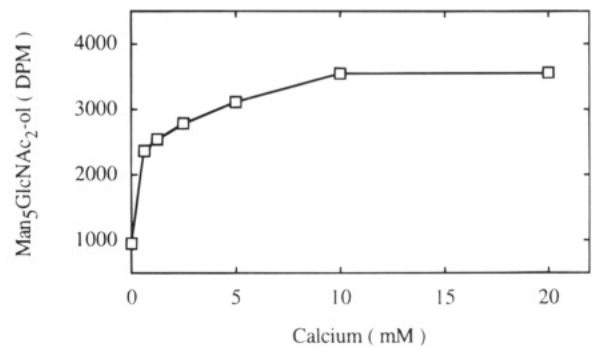

FIGURE 4: Calcium dependency of the purified insect Golgi (α1,2)-mannosidase. Approximately 1 μg of purified (α1,2)-mannosidase was added to the assay mixture (total volume 20 μL) containing 100 mM NaOAc, pH 6.0, 5 mM $MgCl_2$, and 8000−10 000 dpm [^{3}H]$Man_6GlcNAc_2$-ol. After incubation at 37 °C for 2 h, the assay tube was placed in a boiling water bath for 3 min, cooled, and centrifuged to remove any particulate material. The [^{3}H]Man_6-$GlcNAc_2$-ol and [^{3}H]$Man_5GlcNAc_2$-ol in the supernatant fluid were then separated by HPAEC-PAD. The total dpm of the [^{3}H]Man_5-$GlcNAc_2$-ol fraction as a function of the concentration of calcium is illustrated on the graph.

presence of Ca^{2+}. The residual activity in the absence of divalent cations seen in Figure 4 is likely due to the presence of endogenous divalent cation in the assay mixture, since addition of 1 mM EDTA completely inhibits this activity. Enzyme activity is nearly completely restored as a result of addition of 10 mM Ca^{2+} to this assay mixture, and partially reestablished upon inclusion in the assay of 10 mM Mg^{2+} or 10 mM Sr^{2+} (Table 2). Addition of Zn^{2+}, Mn^{2+}, Co^{2+}, or Cu^{2+} did not significantly restore the activity of the mannosidase. The enzyme activity was sensitive to the presence of dMJ, with 50% inhibition resulting at an inhibitor concentration of 20 μM. No inhibition resulted from addition of swainsonine, up to a concentration of 3 mM (Table 2).

The substrate specificity of the isolated (α1,2)-mannosidase was examined against a series of reduced oligosaccharides, ranging from $Man_9GlcNAc_2$-ol to $Man_5GlcNAc_2$-ol, that would be expected to be present on glycoproteins during normal trimming and processing. Since we were not able to achieve the desired degree of separation of these reduced glycans on HPAEC-PAD, HPLC was employed for this purpose. Excellent separation of all glycans was then accomplished as shown by the example in Figure 5. Using this technique, we examined the temporal relationships between substrate loss and product formation of each of these substrates when incubated with the purified enzyme. An example of the data obtained upon incubation for 120 min of [^{3}H]$Man_7GlcNAc_2$-ol with the purified (α1,2)-mannosidase is illustrated in Figure 6. Clearly present are the major products, [^{3}H]$Man_6GlcNAc_2$-ol and [^{3}H]$Man_5GlcNAc_2$-ol, as well as the internal standard [^{3}H]$Man_3GlcNAc_2$-ol. Data of this type was employed to generate the graphs of Figure 7,

Table 2: Properties of Purified (α1,2)-Mannosidase

property	characteristic
metal ion effect	
1 mM EDTA	0% activity
1 mM EDTA/10 mM Ca^{2+}	80% activity
1 mM EDTA/10 mM Mg^{2+}	30% activity
1 mM EDTA/10 mM Sr^{2+}	30% activity
1 mM EDTA/10 mM Zn^{2+}	<5% activity
1 mM EDTA/10 mM Mn^{2+}	<5% activity
1 mM EDTA/10 mM Co^{2+}	<5% activity
1 mM EDTA/10 mM Cu^{2+}	<5% activity
10 mM EDTA/10 mM Ca^{2+}	0% activity
10 mM EDTA/10 mM Sr^{2+}	0% activity
inhibitors	
20 μM dMJ[a]	50% activity
3 mM swainsonine	no inhibition
molecular weight	63 000 Da (reduced or nonreduced)
ConA−Sepharose	no binding
Nph-α-D-Man[b]	no hydrolysis

[a] 1-Deoxymannojirimycin. [b] Nph-α-D-Man, p-nitrophenyl α-D-mannoside.

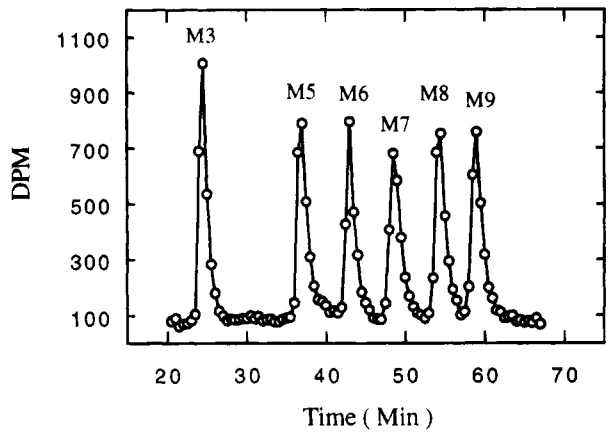

FIGURE 5: HPLC resolution of the standard [^{3}H]oligosaccharides. A 250 × 4.6 mm amino-silica column was equilibrated with 68% acetonitrile/32% water (v:v) and eluted for 10 min with this solvent after application of the sample. A linear gradient composed of 68% acetonitrile/32% water (v:v, start solution) and 40% acetonitrile/60% water (v:v, limit solution) was then applied up to 100 min. The flow rate was 1.0 mL/min. Fractions of 0.5 mL were collected, and their radioactivity contents were determined by liquid scintillation counting. Peaks: M3, [^{3}H]$Man_3GlcNAc_2$-ol; M5, [^{3}H]$Man_5GlcNAc_2$-ol; M6, [^{3}H]$Man_6GlcNAc_2$-ol; M7, [^{3}H]$Man_7GlcNAc_2$-ol; M8, [^{3}H]$Man_8GlcNAc_2$-ol; M9, [^{3}H]$Man_9GlcNAc_2$-ol.

which demonstrate that the preferred substrate is the $Man_6GlcNAc_2$-ol isomer that we employed in the assays, namely, Man(α1,2)Man(α1,3)[[Man(α1,3)[Man(α1,6)]Man(α1,6)]]-Man(β1,4)GlcNAc(β1,4)-GlcNAc-ol.

Finally, the incubation of the purified insect Golgi mannosidase with ovalbumin did result in release of mannose, as indicated by the coupled assay to NADH oxidation. For the longest time period of incubation carried out, it was calculated that 0.1 mol of mannose/mol of ovalbumin was released. No NADH was oxidized in controls for the assay system in which ovalbumin was omitted or in which mannosidase was omitted.

DISCUSSION

The question as to the full nature of the factors that control the types of N-linked oligosaccharide that are assembled on relevant consensus sequences in proteins is not fully resolved. On the basis of investigations with tissue-type plasminogen activator (Parekh et al., 1989a,b) and other glycoproteins (Yet & Wold, 1990), it has been proposed that the protein structure carries within it information as to the types of oligosaccharide(s) assembled at a given site. In this regard, certainly of great importance is the presence of the proper consensus sequence (Asn-X-Thr/Ser-X_1) needed for N-linked glycosylation (Marshall, 1972), but the presence of a Pro residue at the X or X_1 position prevents the assembly of glycan on Asn (Bause, 1983). Additionally, the conformation adopted by the consensus tripeptide plays a role in its ability to

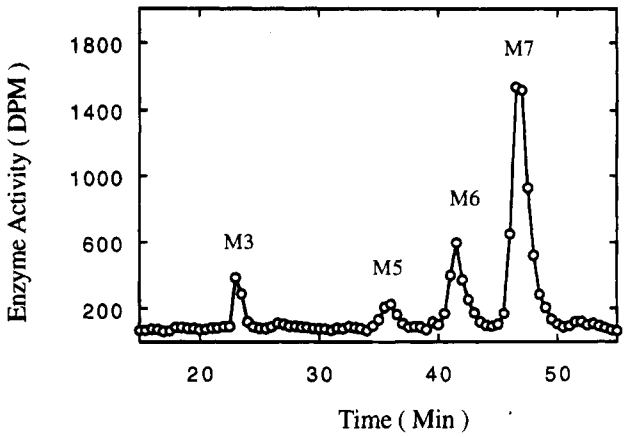

FIGURE 6: Example of an assay of the glycosidase activity of the purified insect Golgi (α1,2)-mannosidase. The substrate was [^{3}H]$Man_7GlcNAc_2$-ol (M7) assayed after 120 min of incubation with the enzyme. The assay mixtures contained 100 mM NaOAc, pH 6.0, 10 mM $CaCl_2$, 10 000 dpm of reduced oligosaccharide substrate, and 1 μg of enzyme. The reaction was terminated by heating for 3 min in a boiling water bath, and the products were then separated by HPLC as described in Figure 5. The product peaks correspond to M5 ([^{3}H]$Man_5GlcNAc_2$-ol) and M6 ([^{3}H]$Man_6GlcNAc_2$-ol). The M3 ([^{3}H]$Man_3GlcNAc_2$-ol) was added as an internal standard.

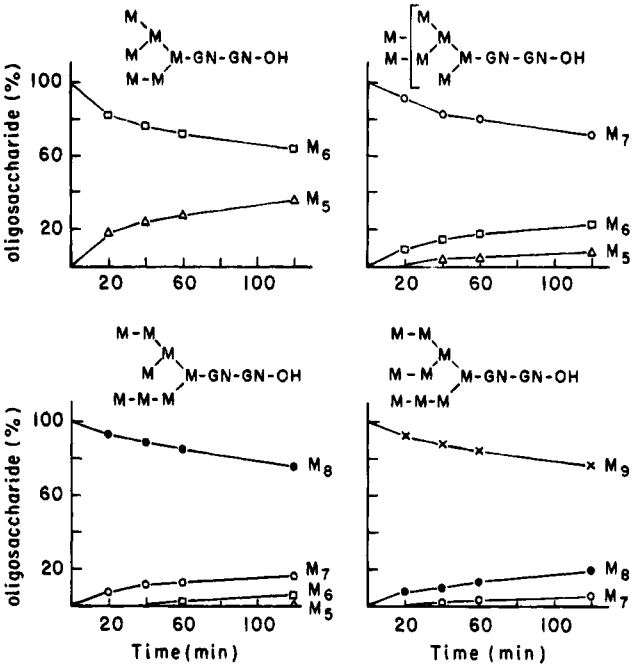

FIGURE 7: Activity and product analysis of the purified insect Golgi (α1,2)-mannosidase against reduced oligosaccharides. The assay conditions were as in Figure 6, and the conditions for separation of products by HPLC are as in Figure 5. The same initial concentrations of substrates (10 000 dpm) were present in each case. The structure of the initial substrate is indicated above each of the graphs. M5, [^{3}H]$Man_5GlcNAc_2$-ol; M6, [^{3}H]$Man_5GlcNAc_2$-ol; M7, [^{3}H]$Man_7GlcNAc_2$-ol; M8, [^{3}H]$Man_8GlcNAc_2$-ol; M9, [^{3}H]$Man_9GlcNAc_2$-ol.

become glycosylated (Imperiali & Shannon, 1991). Other, more subtle effects of the resident protein on glycan assembly have been proposed that involve steric accessibility of mannosidases and glycosyltransferases to the oligosaccharide present at a given location in the protein (Hubbard, 1988); the ability of the protein to undergo secondary interactions with an assembled or partially assembled glycan, thus affecting accessibility to glycosyltransferases (Savvidou et al., 1984); the positions in the amino acid sequence wherein the consensus sequence resides, especially the proximity of the consensus sequence to the protein amino-terminus (Pollack & Atkinson, 1983) and/or the carboxy-terminus (Glabe et al., 1980); the localized conformation around the glycan anchoring site (Leonard et al., 1990); and the quaternary structure of the protein (Dahms & Hart, 1986). As yet unknown is whether a recognition signal exists in the cDNA or protein structure for activation of oligosaccharide trimming and processing enzymes. In this regard, some evidence for the importance of protein recognition signals for phosphorylation of Man residues in lysosomal enzymes has been presented (Lang et al., 1984). Other factors that affect cell function, such as glucose availability [reviewed in Goochee and Monica (1990)], have also been found to be of importance to protein glycosylation.

While some or all of the above parameters may be important to protein glycosylation in cells, clearly the availability of processing mannosidases and glycosyltransferases would be among the determining factors in directing the types of glycans assembled on proteins. Glucosidases and mannosidases are necessary to trim and process the $Glc_3Man_9GlcNAc_2$ that is initially transferred to the relevant asparagine residue of the protein consensus site, to the $Man_5GlcNAc_2$ intermediate—the preferred substrate for GlcNAc transferase I and the committed step toward complex-type glycan formation. For this reason, we are interested in characterizing the types of mannosidases that are present in lepidopteran insect cells. We are especially concerned with revealing the nature of a $Man_6GlcNAc_2$-specific mannosidase that we found to exist in baculovirus-infected SF-21AE cells (Davidson & Castellino, 1991), since if processing to complex-type glycans is to occur, such a high-mannose intermediate must be further processed.

We were successful in purification of a mannosidase with activity toward $Man_6GlcNAc_2$-ol from the Golgi-rich fraction of baculovirus-infected SF-21AE cells employing essential elements of processes that have been generally successful in mannosidase I purifications. Our purification factor from Golgi, of approximately 121-fold, is slightly lower than that obtained (186-fold) for purification from Golgi of a rat liver α-mannosidase (Tulsiani & Touster, 1988). Since markers of lepidopteran insect Golgi have not been identified, our operational definition of Golgi is based on isolation of this cell fraction by procedures effective for mammalian cell Golgi and the fact that our Golgi preparations contain GlcNAc-TI, an enzyme generally identified in the Golgi apparatus. The enzyme that we purified has features of some (α1,2)-mannosidases. Its pH optimum of approximately 6.0 is typical of the nonlysosomal (α)-mannosidases, which possess pH optima between 5.5 and 6.5, and the fact that dMJ serves as an effective inhibitor indicates that the enzyme is an (α1,2)-mannosidase. The insect mannosidase did not interact with a column of ConA-Sepharose, suggesting that it is either not a high-mannose glycoprotein, similar to the calf liver (α1,2)-mannosidase (Schweden et al., 1986), or that it does not contain optimal glycan structures for interaction with this lectin to occur (Baenziger & Fiete, 1979). The molecular mass of the purified enzyme, of approximately 63 000 Da under reducing and nonreducing conditions, is similar to the full-length (α1,2)-mannosidase from pig liver (Schweden & Bause, 1989a), the Man_9-mannosidase from calf liver (Schweden et al., 1986), and the processing (α1,2)-mannosidase from yeast (Camirand et al., 1991). The size of this insect Golgi protein is slightly smaller than those (*ca.* 73 000) predicted from the cDNA sequences of murine and rabbit (α1,2)-mannosidases (Herscovics et al., 1994; Lal et al., 1994) and from the cDNA sequence of a human kidney Man_9-mannosidase (Bause et al., 1993). Thus, some commonalty in size is present in this family of processing mannosidases, despite their somewhat different substrate specificities, the wide range of species in which they are found, and their disparate subcellular localizations.

The Ca^{2+} dependency of the insect (α1,2)-mannosidase also places this protein in a group of similar enzymes isolated from rabbit (Forsee & Schutzbach, 1981), pig (Schweden & Bause, 1989), and calf liver microsomes (Schweden et al., 1986). These mannosidases are distinguished from (α1,2/1,3/1,6)-mannosidases which possess activity in the absence of added Ca^{2+} and which have higher molecular weights of approximately 110 000−117 000 Da (Bischoff & Kornfeld, 1986; Monis et al., 1987; Bischoff et al., 1990; Bonay & Hughes, 1991).

The substrate specificity of the purified insect mannosidase has some unique features. Similar to the Ca^{2+}-dependent mannosidases previously discussed, *e.g.*, pig liver Man_9-mannosidase (Schweden & Bause, 1989b), Nph-α-D-Man does not serve as a substrate for the insect enzyme. This represents another difference from the group of mannosidases with (α1,2/1,3/1,6)-Man hydrolytic activity, *e.g.*, rat brain mannosidase (Tulsiani & Touster, 1985), members of which do react with this synthetic substrate. Of the series of glycans examined, the preferred substrate for the insect Golgi mannosidase is the $Man_6GlcNAc_2$-ol isomer that is found on glycoproteins, namely, Man(α1,2)Man(α1,3)[[Man(α1,3)-[Man(α1,6)]Man(α1,6)]]Man(β1,4)GlcNAc(β1,4)-GlcNAc-ol. In this case, only the nonreducing terminal Man(α1,2) residue is liberated, forming a stable product, $Man_5GlcNAc_2$-ol, that is, Man(α1,3)[[Man(α1,3)[Man(α1,6)]Man(α1,6)]]-Man(β1,4)GlcNAc(β1,4)GlcNAc-ol. Other high-mannose substrates that exist in glycoproteins, specifically $Man_9GlcNAc_2$-ol, $Man_8GlcNAc_2$-ol, and the mixed isomers of $Man_7GlcNAc_2$-ol (Figure 7), were cleaved less readily by the purified enzyme. Under the conditions used, of limiting but approximately equal substrate concentrations, 18% of the $Man_6GlcNAc_2$-ol substrate was hydrolyzed in the first 20 min, whereas only 7−8% of the other glycan substrates were hydrolyzed to the next smaller oligosaccharide during this same time interval. A definitive comparison of rates of hydrolysis of the different (α1,2)-Man-containing processing intermediates requires the availability of considerably larger quantities of these oligosaccharides. Nevertheless, the current studies indicate that the Golgi apparati of these cells possess a probable late-acting (α1,2)-Man-specific mannosidase. Such an occurrence has been suggested earlier on the basis of processing of IgA in MOPC 315 cells (Hickman et al., 1984) and on the glycosylation pattern found on hydroxymethylglutaryl-CoA reductase (Bischoff et al., 1990). Another indication that this last (α1,2)-mannose residue

might require an additional Golgi mannosidase results from a past investigation, which showed that a calf liver processing (α1,2)-mannosidase only removes three of the four (α1,2)-Man residues from $Man_9GlcNAc_2$, yielding $Man_6GlcNAc_2$ (Schweden et al., 1986), and from our previous observation that such an enzyme might be a regulated feature of infected SF-21AE cells (Davidson et al., 1991).

While the current focus on identification of the substrate specificity of this new insect (α1,2)-mannosidase was with isolated oligosaccharides, we have also demonstrated herein that incubation of this enzyme with ovalbumin results in release of mannose. The single *N*-glycosylation site on ovalbumin contains a mixture of oligosaccharides that can be resolved, in glycopeptide form, into five major fractions. Glycopeptide fractions III and IV have been shown to contain nonreducing-end mannose residues in α1,2 linkages (Tai et al., 1975, 1977), and it is presumably these mannose residues that were released by treatment with the mannosidase. While only 0.1 mol of mannose/mol of ovalbumin was released by the insect (α1,2)-mannosidase, this value would be consistent with the small percentage of Man(α-1,2) linkages in ovalbumin. While we cannot be certain at this time whether this does indeed reflect the actual content of (α1,2)-linked Man residues in ovalbumin, the results do demonstrate that the mannosidase has the potential to function *in vivo* as a late processing enzyme.

In summary, we have purified a unique mannosidase from lepidopteran insect (SF-21AE) cells that possesses optimal activity with the substrate $Man_6GlcNAc_2$-ol. This enzyme has been further characterized to be a Ca^{2+}-dependent (α1,2)-Man-specific mannosidase that most likely exists in the Golgi apparatus. This is the first example of the characterization of such an enzyme in these types of cells. Further elucidation of the existence and properties of trimming and processing mannosidases of Lepidoptera are critical to developing a cohesive understanding of the nature of the control mechanisms that may be applied in glycoprotein assembly in these cell types.

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