Purification and properties of α-mannosidase II from Golgi-like membranes of baculovirus-infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells

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An α-mannosidase II-like activity was identified in baculovirus-infected *Spodoptera frugiperda* (IPLB-SF21-AE) cells. The enzyme responsible was purified from Golgi-type membranes to apparent homogeneity by using a combination of steps including DEAE-cellulose, hydroxyapatite, concanavalin A–Sepharose and gel filtration chromatography. The molecular mass of this purified protein was approx. 120 kDa by SDS/PAGE under reducing conditions and approx. 240 kDa under non-reducing conditions, indicating that the enzyme is a disulphide-linked dimer. Substrates demonstrated to undergo hydrolysis with this enzyme were GlcNAc-Man_5-GlcNAc-GlcNAc (non-reduced and reduced) and *p*-nitrophenyl α-D-mannopyranoside. The oligosaccharide substrate was converted into GlcNAc-Man_3-GlcNAc-GlcNAc through an intermediate GlcNAc-Man_4-GlcNAc-GlcNAc. Treatment of the isolated intermediate oligosaccharide with endoglycosidase H resulted in its conversion into GlcNAc-Man_4-GlcNAc. This indicated that it contained the α-1,3-linked mannose residue on the α-1,6-linked mannose arm and showed that the α-1,6-linked mannose residue on the α-1,6-linked mannose arm had been preferentially hydrolysed by the mannosidase. The oligosaccharide lacking the β-1,2-linked GlcNAc residue on the α-1,3-linked mannose arm (Man_5-GlcNAc-GlcNAc) was not hydrolysed in the presence of the enzyme. Metal ions were not required for enzymic activity on any of the substrates, but Cu^{2+} was strongly inhibitory. The activity of the enzyme was inhibited at low concentrations of swainsonine, but much higher concentrations of 1-deoxymannojirimycin were required to achieve inhibition. All of these properties are characteristic of mannosidase II enzymes from other eukaryotic tissues. The presence of mannosidase II in lepidopteran insect cells would allow entry of N-linked glycoproteins into the complex processing reaction pathway or into the terminal Man_3-GlcNAc-GlcNAc pathway.

INTRODUCTION

The biosynthesis of the asparagine-linked oligosaccharide moieties of glycoproteins of different eukaryotic cells occurs through many common, but not identical, steps [1]. In general, a common precursor oligosaccharide containing GlcNAc, mannose and glucose residues (Glc_3-Man_9-GlcNAc-GlcNAc) is preassembled from sugar nucleotide precursors in the endoplasmic reticulum on dolichol phosphate [2]. This long-chain polyisoprenoid serves to anchor the growing oligosaccharide in the luminal space of the endoplasmic reticulum and allows the subsequent transfer of the oligosaccharide to an asparagine residue of the polypeptide as a co-translational event. Processing reactions that can ensue in the endoplasmic reticulum include the removal of the glucose residues by two specific glucosidases and the removal of one α-1,2-linked mannose residue by endoplasmic reticulum mannosidase I. This partly processed glycoprotein (Man_8-GlcNAc-GlcNAc-protein) can then be transported to the Golgi complex where the remaining three α-1,2-linked mannose residues of the oligosaccharide are susceptible to removal by Golgi mannosidase I. The resulting protein-linked oligosaccharide (Man_5-GlcNAc-GlcNAc) is then available for further processing reactions that lead to the complex type of protein-bound oligosaccharides [3,4]. For initiation of these reactions, the α-1,3-mannose arm is first elongated with a β-1,2-linked GlcNAc residue via the action of GlcNAc transferase I. The oligosaccharide product then becomes a substrate for mannosidase II, an enzyme that catalyses the removal of the remaining two non-reducing-end mannose residues linked α-1,6 and α-1,3 to the α-1,6-mannose arm. The product of mannosidase II, GlcNAc-Man_3-GlcNAc-GlcNAc-protein, can subsequently be converted into the simple biantennary complex-type structure, $GlcNAc_2$-Man_3-GlcNAc-GlcNAc, by the action of GlcNAc transferase II, a Golgi enzyme that catalyses the addition of β-1,2-linked GlcNAc to the α-1,6-mannose arm. In the Golgi apparatus, further branching on the core mannose arms with additional GlcNAc residues can follow, as can subsequent elongation with other sugars, such as galactose and sialic acid, to give a variety of complex oligosaccharide structures [5].

This report is concerned with particular aspects of glycoprotein processing reactions that occur in lepidopteran insect cells. Traditionally, insects have been thought not to synthesize complex N-linked glycoproteins, but rather to process the N-linked oligosaccharides of proteins predominantly to a simple Man_3-GlcNAc-GlcNAc structure (with or without fucose) [6]. However, other earlier reports have documented the ability of cultured lepidopteran cells that have been infected with recombinant baculovirus to synthesize and secrete recombinant glycoproteins that have N-linked complex oligosaccharides [7,8]. The structures that were determined for several of these oligosaccharides were consistent with the presence in the insect cells of the genetic information needed to support the complex processing pathway. We therefore undertook a study of the processing enzymes that can be found in non-infected and baculovirus-infected insect cells. An α-1,2-mannosidase that catalysed the removal of the four α-1,2-linked mannose residues in Man_9-GlcNAc-GlcNAc

Abbreviations used: endo H, endoglycosidase H; HPAEC, high-performance anion-exchange chromatography; Nph-α-Man, *p*-nitrophenyl-α-D-mannopyranoside.

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was detected [9]. This enzyme was purified to homogeneity and shown to have some preference for hydrolysis of the one remaining α-1,2-linked mannose residue on the α-1,3-mannose arm in Man_6-GlcNAc-GlcNAc [10]. Additionally, activity of GlcNAc transferase I was demonstrated in detergent-solubilized cellular membranes [11], and evidence for the presence of GlcNAc transferase II has been reported by others [12]. If the early processing events in insect cells, at least up to the formation of the biantennary $GlcNAc_2$-Man_3-GlcNAc-GlcNAc protein-linked oligosaccharide, are the same as has been elucidated in higher animals, then a mannosidase II activity should also be present (to generate the substrate for GlcNAc transferase II from the product of GlcNAc transferase I); indeed, this has been recently demonstrated in unfractionated homogenates of three insect cell lines [13]. The central importance of this enzyme in processing insect cell-derived glycoproteins required the elucidation of its properties and substrate specificity. Thus we embarked on a characterization of SF-21-AE cell α-mannosidase II. Here we report a summary of our findings.

EXPERIMENTAL

Materials

[^{3}H]Sodium borohydride and UDP-[6-^{3}H(N)]GlcNAc were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.); Man_5-GlcNAc-GlcNAc, (β-1,2)-*N*-acetylglucosaminidase (*Diplococcus pneumoniae*) and endo-*N*-acetylglucosaminidase H (recombinant) from Oxford GlycoSystems (Rosedale, NY, U.S.A.); hydroxyapatite and Bio-Gels from Bio-Rad (Hercules, CA, U.S.A.); DEAE-cellulose (DE52) from Whatman (Maidstone, Kent, U.K.); concanavalin A–Sepharose, UDP-GlcNAc, swainsonine, 1-deoxymannojirimycin and *p*-nitrophenyl-α-D-mannopyranoside (Nph-α-Man) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and Centricon-10 concentrators from Amicon (Beverly, MA, U.S.A.).

HPLC methods

The Dionex liquid-chromatography system, high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection was used with either a single CarboPac PA-1 column or two CarboPac PA-1 columns linked in tandem. For both arrangements the columns were equilibrated with 47% A/50% B/3% C (by vol.), where solution A was 0.2 M NaOH, solution B was 1 mM NaOH and solution C was 1 M sodium acetate/0.2 M NaOH. The columns were eluted with the equilibration solvent mixture for 20 min, during which time the saccharides of interest were recovered. To regenerate the column, a linear gradient with a limit solution of 25% A/50% B/25% C was then applied over 20 min (up to 40 min total elapsed time). This limit solvent mixture was continued for another 20 min (up to 60 min total elapsed time) and the column was then equilibrated with the starting solvent. A flow rate of 1 ml/min for the single column and 0.8 ml/min for the tandem-column arrangement was maintained. Fractions were collected during the first 12 min of elution, every 0.5 min (0.5 ml) for the single-column system and every 0.5 min (0.4 ml) for the tandem-column system.

An additional HPLC system with an amino-silica column (250 mm × 4.6 mm, 5 μm particle size) was used for separation of the corresponding Man_4 and Man_3 products derived from both oligosaccharide substrates 1 and 2 (see below) by action of the mannosidase. The column was equilibrated with 68% acetonitrile/32% water (v/v) and eluted with this solvent for 10 min after application of the sample. A linear gradient up to 100 min (total elapsed time) to 40% acetonitrile/60% water was then applied. A flow rate of 1 ml/min was maintained and fractions of 0.5 ml were collected.

Insect cell culture

IPLB-SF-21AE cells were maintained and infected with wild-type AcMNPV virus as previously described [10].

Preparation of substrate oligosaccharides

[^{3}H]GlcNAc-Man_5-GlcNAc-GlcNAc (substrate 1, non-reduced and containing tritiated GlcNAc on the Man-α1,3-arm) was prepared from Man_5-GlcNAc-GlcNAc and UDP-[^{3}H]GlcNAc with the activity of GlcNAc transferase I present in rat liver Golgi membranes. These membranes were isolated from a rat liver by the procedure previously used for insect cells [11]. The following components in 50 μl were incubated at 37 °C for 10 h: 10 μg of Man_5-GlcNAc-GlcNAc, 0.2 nmol of UDP-[^{3}H]GlcNAc (60 Ci/mmol), 50 mM Tris/HCl (pH 6.0), 0.2% (v/v) Triton X-100, 5 mM $MnCl_2$, 5 mM 5′-AMP, 100 mM GlcNAc, 25 μg/ml swainsonine and 0.1 mg of Golgi membrane protein. After incubation the sample was placed in a boiling-water bath for 3 min, cooled and centrifuged to remove any denatured protein. The supernatant was passed through a 0.3 cm × 2 cm column of Dowex-1 (formate) to remove residual sugar nucleotide, and the eluted neutral fraction was then applied to a 1.4 cm × 32 cm column of Bio-Gel P-4 (400 mesh) in water and eluted with water at room temperature. A single radioactive peak that was included in the column volume was collected.

GlcNAc-Man_5-GlcNAc-[^{3}H]GlcNAc-OH [substrate 2, reduced and containing ^{3}H on the (reduced) terminal *N*-acetylglucosaminitol] was prepared in a similar manner to substrate 1 except that Man_5-GlcNAc-GlcNAc was first reduced with NaB^3H_4. The resulting Man_5-GlcNAc-[^{3}H]GlcNAc-OH was then used as an acceptor substrate for GlcNAc transferase I with non-radioactive UDP-GlcNAc as the donor substrate. The resulting product (substrate 2) was purified on a Bio-Gel P-4 column as for substrate 1.

Substrates 1 and 2 were each further characterized by chromatography on a single-column Dionex HPLC system. Each substrate was eluted as a single radioactive component. Treatment of substrate 1 with *Diplococcus pneumoniae* (β-1,2)-*N*-acetylglucosaminidase resulted in a single peak of radioactivity being eluted earlier than the parent substrate and at the position of standard GlcNAc. Treatment of substrate 2 with the same glycosidase resulted in a single peak of radioactivity being eluted earlier than the parent substrate and at the position of $NaBH_4$-reduced standard Man_5-GlcNAc-GlcNAc.

Assay of mannosidase II activity

Enzyme assays with either substrate 1 or substrate 2 were performed in a total volume of 20 μl containing 0.1 M sodium acetate buffer, pH 6.0, appropriate amounts of enzyme and 8000 d.p.m. of substrate. After incubation at 37 °C for different durations, the sample was placed in a boiling-water bath for 3 min, cooled and then analysed by HPAEC. A single-column system was employed for substrate 1; 0.5 ml fractions (0.5 min) were collected. For substrate 2, a tandem-column system was employed and fractions of 0.4 ml (0.5 min) were collected. Aliquots of each fraction were analysed for radioactivity by liquid-scintillation counting.

Assays with Nph-α-Man as substrate were performed in a total volume of 0.1 ml containing 0.1 M sodium acetate buffer, pH 6.0, enzyme and 4 mM substrate. After incubation at 37 °C

for various durations, 0.4 ml of 0.2 M sodium carbonate was added and the absorbance at 405 nm was determined.

Gel electrophoresis and activity stain

PAGE was performed by the Laemmli procedure [14] with 10 % slab gels. Proteins were stained with Coomassie Blue. Mannosidase II activity was detected on gels after electrophoresis by the following procedure. The Laemmli system was used with 10 % polyacrylamide (w/v) gels but SDS was replaced with sodium dodecyl sarcosinate, and the enzyme sample was not reduced. After electrophoresis the gel was rinsed with a solution of 10 % (v/v) isopropanol in 10 mM Tris/HCl buffer, pH 7.0. The gel lane containing the enzyme sample was then cut into 0.5 cm slices and individual slices were placed in small tubes. To each tube was added 0.2 ml of substrate solution (4 mM Nph-α-Man in 0.1 M sodium acetate buffer, pH 6.0). After appropriate durations of incubation at 37 °C, 0.2 M sodium carbonate was added and the absorbance at 405 nm of the liquid component was determined. This procedure resulted in the recovery, after electrophoresis, of approx. 60 % of the enzyme units that were applied to the gel.

Purification of mannosidase II

Insect cells (300 g wet weight) were harvested 72 h after infection; Golgi membranes were isolated by the modified procedure of Velardo et al. [11] as previously described [10] for the purification of mannosidase I. These membrane preparations could be stored frozen at −20 °C for several weeks without apparent loss of activity. All of the subsequent procedures were performed at 4 °C. Enzyme activity was monitored with the non-reduced oligosaccharide substrate 1. Extraction of mannosidase II from the Golgi membranes with 0.5 % Triton X-100 and chromatography of the solubilized enzyme on a column of DEAE-cellulose in potassium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 0.1 % Triton X-100, were performed as previously described for purification of mannosidase I from SF-21AE cells [10]. Mannosidase II was not retained by the DEAE-cellulose at pH 7.2, therefore allowing its efficient separation from the retained mannosidase I.

The mannosidase II fraction was dialysed against a buffer containing 10 mM Tris/HCl, pH 8.0, 5 mM $MgCl_2$ and 0.1 % Triton X-100 (buffer A). This sample was applied to a DEAE-cellulose column (10 ml) that had been equilibrated with buffer A; the column was then eluted with a 200 ml linear gradient of 0–0.5 M NaCl in buffer A. Fractions containing enzyme activity were pooled, dialysed against 10 mM potassium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 0.1 % Triton X-100 (buffer B), and applied to a 5 ml column of hydroxyapatite that had been equilibrated with buffer B. The column was eluted with a 100 ml linear gradient of 0–0.5 M potassium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 0.1 % Triton X-100. Fractions containing enzyme activity were pooled. After dialysis against 10 mM potassium phosphate buffer, pH 7.2, containing 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.5 M NaCl and 0.1 % Triton X-100 (buffer C), the sample was applied to a 3 ml column of concanavalin A–Sepharose equilibrated with buffer C. Enzyme activity was retained on the column and was eluted with 3 column volumes of 0.5 M methyl-α-D-mannopyranoside in buffer C. This fraction was concentrated in a Centricon-10 concentrator, dialysed against 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 M NaCl, 5 mM $MgCl_2$ and 0.1 % Triton X-100 (buffer D), and applied to a column (1.6 cm × 120 cm) of Sephacryl 200 that had been equilibrated with buffer D. An elution flow rate of 6 ml/h was maintained and 15 min fractions were collected. Fractions containing enzyme activity were collected and stored frozen at −20 °C.

RESULTS

Baculovirus-infected SF-21 cells were used to prepare a subcellular membrane fraction that has operationally been referred to as a Golgi-rich membrane fraction. This membrane fraction was observed to contain an activity that catalysed the hydrolysis of Nph-α-Man and was thus suggestive of the presence of mannosidase II. Purification of this mannosidase, by using the non-reduced radioactive oligosaccharide substrate 1 to monitor the presence of the enzyme, was readily accomplished by use of conventional chromatographic procedures. A key step that allowed separation of the mannosidase II activity from the previously purified mannosidase I was chromatography on DEAE-cellulose at pH 7.2. Unlike mannosidase I, the mannosidase II was not retained on this anion-exchange column. Further purification of mannosidase II was then achieved by chromatography on DEAE-cellulose at pH 8.0 and on hydroxyapatite at pH 7.2. A second key step in the purification was the adsorption of the mannosidase II to concanavalin A–Sepharose and elution with methyl-α-D-mannoside. This allowed the removal of residual mannosidase I, as it is not retained by concanavalin A [10]. A final purification step on Sephacryl 200 removed the last contaminating proteins. A summary of the purification procedure is presented in Table 1. Starting from the Golgi membrane fraction, an overall purification of 353-fold was achieved with an 18 % yield of enzyme activity. Approx. 50 μg of enzyme protein was obtained from 90 mg of Golgi membrane proteins, which in turn were obtained from 300 g wet weight of virus-infected insect cells.

The activity of mannosidase II with the non-reduced oligosaccharide substrate 1 was also observed in a Golgi-rich membrane fraction isolated from non-infected cells. On a per mg of protein basis in detergent-solubilized Golgi membranes, the mannosidase II activity was 2.0–2.5-fold higher in infected cells than in non-infected cells. For this reason, purification of the mannosidase was undertaken with infected cells.

Analysis of the final purified enzyme fraction from Sephacryl 200 by SDS/PAGE under reducing conditions and staining with Coomassie Blue revealed a single protein band migrating as a 120 kDa component (Figure 1, lane 3). A small amount of a second protein migrating as a 65 kDa band was also occasionally observed. Detection of enzyme activity with Nph-α-Man on gels run under non-reducing conditions and with sodium dodecyl sarcosinate revealed activity only in gel slices that corresponded

Table 1 Purification of mannosidase II from Golgi membranes of SF-21 cells

Step	Total protein (mg)	10^{-9} × Total activity (μmol/min)	10^{-9} × Specific activity (μmol/min per mg)	Yield (%)	Purification (fold)
Golgi	96	131	1.36	100	1
Triton X-100 extract	85	122	1.43	93	1
DEAE-52, pH 7.2	41	116	2.83	88	2
DEAE-52, pH 8.0	11	81	7.4	62	6
Hydroxyapatite	1.5	41	27	31	20
Concanavalin A–Sepharose	0.4	38	95	29	70
Sephacryl S-200	0.05	24	480	18	353

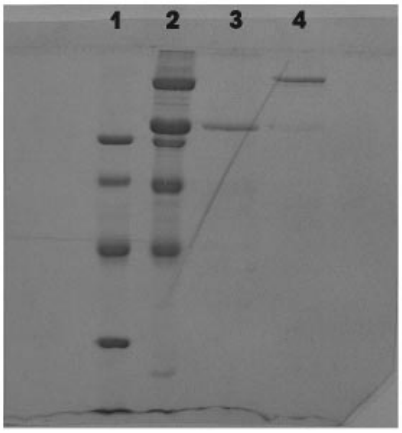

Figure 1 PAGE of purified mannosidase II

Lane 1, low-molecular-mass protein standards; lane 2, high-molecular-mass protein standards, lane 3, 2-mercaptoethanol-reduced purified mannosidase II; lane 4, non-reduced purified mannosidase II. The low-molecular-mass proteins in lane 1, from top to bottom, were phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), hen egg ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). The high-molecular-mass standard proteins in lane 2, from top to bottom, were myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa) and hen egg ovalbumin (45 kDa).

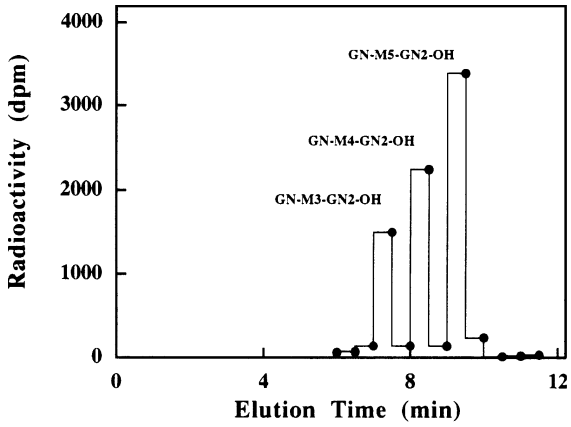

Figure 2 Elution profile from HPAEC analysis of substrate and products of the mannosidase II reaction

Substrate 2 was incubated for 120 min with mannosidase II in the presence of 0.1 M sodium acetate buffer, pH 6.0. The tandem CarboPac PA-1 column was used to separate the oligosaccharides. Fractions of 0.4 ml were collected every 0.5 min and an aliquot counted for radioactivity. Abbreviations: GN, *N*-acetylglucosamine; M, mannose.

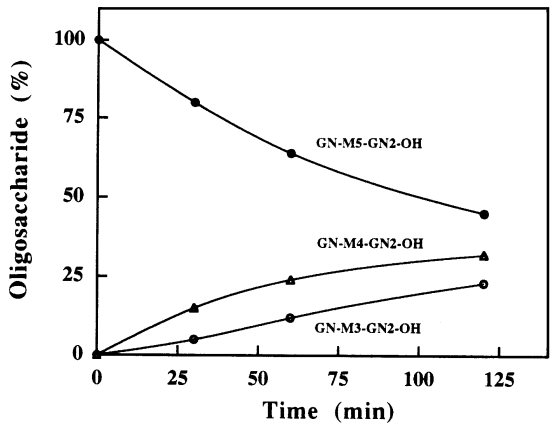

Figure 3 Time course of the reaction catalysed by mannosidase II

The reaction mixtures contained substrate 2 and 0.1 M sodium acetate buffer, pH 6.0. Aliquots of the reaction mixture were taken at 30, 60 and 120 min and each sample was analysed by the HPAEC pulsed amperometric detection tandem-column assay for the amount of remaining substrate and the amounts of products formed. Peak areas of radioactivity were determined from the chromatogram for each duration of incubation. Abbreviations: GN, *N*-acetylglucosamine; M, mannose.

to a 240 kDa component. This suggested that the active enzyme is a dimer of 120 kDa subunits.

Additional evidence that the enzyme is a disulphide-linked dimer was obtained by SDS/PAGE. As shown in Figure 1, lane 3, the protein that had been reduced with 2-mercaptoethanol migrated as a single component with an apparent molecular mass of 120 kDa. Most of the non-reduced protein migrated as a component with an approximate molecular mass of 240 kDa (Figure 1, lane 4), although a small amount of the 120 kDa protein was also observed. A molecular mass of 240 kDa for the (dimeric) enzyme is consistent with its elution position from the Sephacryl 200 column close to the void volume of the column.

Other properties of the enzyme were determined. The pH optimum was 6 for activity with substrate 1 (the non-reduced oligosaccharide) in the presence of Mes buffer. With substrate 1, no inhibition of enzyme activity was observed in the presence of 1 or 10 mM EDTA and no activation of activity was observed on the addition of several different bivalent cations (10 mM final concentrations of Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+} or Co^{2+}). There is therefore no apparent requirement for a metal ion for this mannosidase activity. At 10 mM, Zn^{2+} resulted in a 40% inhibition of activity but at 2.5 mM no inhibition (and no activation) was observed. Enzyme activity with substrate 1 was inhibited by 90% by 0.1 mM Cu^{2+}. The activity with Nph-α-Man as substrate was more sensitive to Cu^{2+}, with 50% inhibition resulting at approx. 10 μM concentration. The activity of the purified insect mannosidase II was quite sensitive to swainsonine, with 50% inhibition of hydrolysis of substrate 1 occurring at approx. 20 nM inhibitor. Deoxymannojirimycin did not inhibit the enzyme activity at such low concentrations, but did inhibit activity by approx. 50% at 0.4 mM.

The rates of hydrolysis by the enzyme of both substrate 1 and substrate 2 were monitored by HPLC. Depending on the time of incubation of enzyme with substrate, two radioactive products were observed as being formed from either substrate. This suggested that either the α-1,6-linked mannose residue or the α-1,3-linked mannose residue on the α-1,6-mannose arm was being preferentially hydrolysed so that an intermediate GlcNAc-Man_4-GlcNAc-GlcNAc product was being formed and observed. The results of such an experiment with substrate 2 are shown in Figure 2. The product identified as GlcNAc-Man_4-GlcNAc-[^{3}H]GlcNAc-OH by HPAEC appeared at a short duration (30 min) of incubation with the enzyme at a greater concentration than did GlcNAc-Man_3-GlcNAc-[^{3}H]GlcNAc-OH. In this experiment both products then continued to be formed at approximately equal rates up to 120 min (Figure 3). In other experiments with higher enzyme concentrations and extended times of incubation, over 95% of substrate 2 was converted into the final GlcNAc-Man_3-GlcNAc-[^{3}H]GlcNAc-OH product.

To explore the structure of the intermediate species observed in the Dionex HPLC profile, this radioactive component was collected from chromatographic runs on the HPLC system with

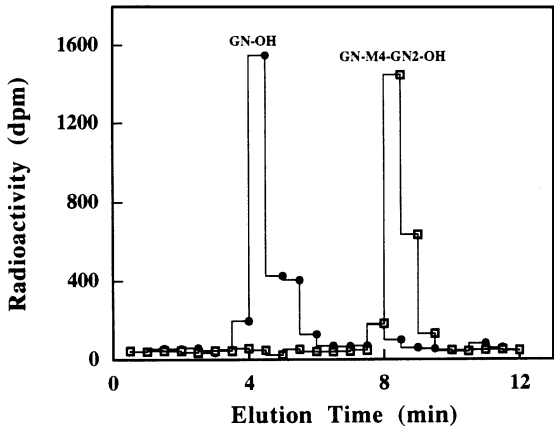

Figure 4 Elution profiles from HPAEC analyses of products from endo H treatment of mannosidase II intermediate product

The GlcNAc-Man_4-GlcNAc-[^{3}H]GlcNAc-OH product that was derived from the digestion of GlcNAc-Man_5-GlcNAc-[^{3}H]GlcNAc-OH with mannosidase II was treated with endo H. Chromatography was performed with the HPAEC tandem column system. Symbols: □, radioactivity at 8–9 min in the intermediate product (GlcNAc-Man_4-$GlcNAc_2$-OH) before treatment with endo H; ●, radioactivity at 4–5 min in *N*-acetyglucosaminitol formed after treatment with endo H. Abbreviations: GN, *N*-acetylglucosamine; M, mannose.

the amino-silica column and then subjected to digestion with endoglycosidase H (endo H). It was expected that if the mannosidase had preferentially catalysed the hydrolysis of the α-1,6-linked mannose residue from the substrate, the resulting oligosaccharide intermediate would retain the α-1,3-linked mannose residue on the α-1,6-mannose arm and would therefore be susceptible to endo H-catalysed hydrolysis. Conducting this experiment with substrate 2 would result in the release of [^{3}H]GlcNAc-OH (from the NaB^3H_4-reduced end of the oligosaccharide) by endo H from the intermediate product and thereby allow a convenient assessment of endo H activity. As shown in Figure 4, radioactivity in the presumed GlcNAc-Man_4-GlcNAc-[^{3}H]GlcNAc-OH intermediate eluted from the Dionex column at 8–9 min. After treatment of the intermediate with endo H, 93% of the radioactivity was eluted at the position (4–5 min) of standard GlcNAc-OH, indicating that essentially all of the intermediate GlcNAc-Man_4-GlcNAc-[^{3}H]GlcNAc-OH contained the α-1,3-mannose unit on the α-1,6-mannose arm. Control experiments with Man_9-GlcNAc-[^{3}H]GlcNAc-OH and Man_7-GlcNAc-[^{3}H]GlcNAc-OH produced the same radioactive product, [^{3}H]GlcNAc-OH. These results show that the insect mannosidase II catalyses preferential hydrolysis of the α-1,6-linked mannose unit in the GlcNAc-Man_5-GlcNAc-GlcNAc substrate.

DISCUSSION

The baculovirus/insect cell expression system has become an important method for generating relatively large quantities of recombinant proteins. As many of the recombinant proteins contain N-glycosylation sites, they will predictably be expressed and secreted from the insect cells as glycosylated proteins. This is of obvious significance when considering the possible effects of the particular *N*-glycan moiety on the structure–function relationships of the protein and in particular on the possible antigenicity of the *N*-glycan in heterologous tissues. A full understanding of the native glycosylation pathway in insect cells is therefore of importance. Our approach has been to identify, purify and characterize the properties of key glycosylation-processing enzymes from the insect cells that are used in expression systems, with the goal of understanding possible regulatory features that could be involved in determining whether the end product will be a high-mannose glycoprotein or a complex-type glycoprotein.

The presence and properties of one of the key processing enzymes, mannosidase II, in insects are described in this paper. This enzyme provides a means of generating the Man_3-GlcNAc-GlcNAc oligosaccharide that is commonly found as a terminally processed N-linked oligosaccharide of insect glycoproteins [15,16] and would also provide a critical step in the biosynthesis of complex N-linked oligosaccharides. The origin of the N-linked oligosaccharides has been shown, at least in mosquito cells, to be a Glc_3-Man_9-GlcNAc-GlcNAc oligosaccharide that is preassembled on dolichol (pyro)phosphate and is then transferred to the appropriate asparagine residue in the protein [17–19]. The subsequent processing reactions have been implicated through the use of inhibitors and the correlation of structures with those known for higher animal systems, and in some cases demonstrated by the measurement of enzyme activity. Evidence now exists for the presence of processing enzymes (hydrolases and glycosyltransferases) that are comparable to those known in higher animal tissues. Included are glucosidase I and II [20], α-1,2-mannosidase [9,10], GlcNAc transferase I [11,12], mannosidase II ([13], and this study) and GlcNAc transferase II [12]. The presence of these enzymes would allow a simple N-linked biantennary oligosaccharide, terminated on each arm with β-1,2-linked GlcNAc, to be generated and to be available for processing to higher complex oligosaccharide structures. The presence of the β-1,2-GlcNAc on the α-1,3-linked mannose arm would also allow a fucosyltransferase to add the fucose that is linked α-1,6 to the N-linked GlcNAc residue of many insect glycoproteins [21]. Recently, the activity of an *N*-acetylglucosaminidase has been demonstrated in insects that results in a departure from the common higher-animal processing pathway [22]. This enzyme has been demonstrated to catalyse hydrolysis specifically of the β-1,2-linked GlcNAc residue from the α-1,3-linked arm of the biantennary $GlcNAc_2$-Man_3-GlcNAc-GlcNAc oligosaccharide or the monoantennary GlcNAc-Man_3-GlcNAc-GlcNAc oligosaccharide. With the latter oligosaccharide substrate, the resulting product would be the truncated Man_3-GlcNAc-GlcNAc structure that is commonly found in insect glycoproteins. However, the *N*-acetylglucosaminidase did not catalyse the hydrolysis of the β-1,2-linked GlcNAc from the α-1,3-linked mannose arm if the α-1,6-linked mannose arm was disubstituted with α-1,3- and α-1,6-linked mannose residues (the substrate structure for mannosidase II). The reaction catalysed by mannosidase II is therefore a critical step in furnishing an oligosaccharide substrate that could be directed either towards higher complex structures by the activity of GlcNAc transferase II or to the simple Man_3-GlcNAc-GlcNAc core structure (with or without fucose) by the activity of the specific *N*-acetylglucosaminidase. An understanding of the structure–function relationships of the insect mannosidase II protein, and even conclusively demonstrating its presence, is necessary and prerequisite for further correlations with gene structure and possible regulatory features in baculovirus-infected compared with non-infected insect cells.

The purification of a mannosidase II from baculovirus-infected SF-21 cells has been accomplished as reported here. Interestingly, many of the catalytic properties observed for the purified insect enzyme are the same as, or very similar to, those previously reported by others for the purified rat liver Golgi enzyme [23,24], for the purified mung bean seedling enzyme [25] and for enzyme activity in homogenates of insect cells [13]. These common

properties include: a requirement for the β-1,2-linked GlcNAc residue on the α-1,3-linked mannose arm of the Man_5-GlcNAc-GlcNAc substrate; preferential hydrolytic activity on the α-1,6-linked mannose residue on the α-1,6-linked mannose arm; utilization of aryl-α-D-mannosides as substrate; no activation by bivalent metal ions or inhibition by EDTA of activity; inhibition of activity by Cu^{2+}; and inhibition of activity by swainsonine. The mannosidase I inhibitor deoxymannojirimycin is shown here to inhibit the insect enzyme, but only at relatively high concentrations (approx. 50% inhibition of activity, with Nph-α-man as substrate, by 0.5 mM deoxymannojirimycin). These observations are in agreement with the reported inhibition of rat liver mannosidase II, either purified or in Golgi membranes, by deoxymannojirimycin [26]. In those studies, the inhibitor was shown to be non-competitive with Nph-α-Man and exhibited approx. 50% inhibition at 0.2 mM concentration.

The structural properties of the insect mannosidase II protein that have been determined are also similar to those of other mannosidase II proteins. As reported here, the insect mannosidase II protein has a subunit molecular mass of approx. 120 kDa as observed by SDS/PAGE of the reduced protein. This compares closely to the molecular mass of approx. 125 kDa that has been observed for the rat liver enzyme [24], the enzyme from 3T3 and from HeLa cells [27], and the mung bean seedling enzyme [25]. All of the mannosidase II proteins so far studied, including the insect enzyme of this report, exhibit some characteristics of being glycosylated proteins, and thus the exact molecular masses may vary depending on the extent of glycosylation. Additionally, the insect enzyme exhibited a molecular mass of approx. 250 kDa on non-reduced gel electrophoresis, indicative of the enzyme's existence as a disulphide-linked dimer. Evidence that the rat liver enzyme exists as a disulphide-linked dimer has previously been presented [24], suggesting that this might be a common structural feature. The similarities of the lepidopteran enzyme to a variety of other α-mannosidase II enzymes suggests that no unusual events occur in this regard in these insect cells and that further oligosaccharide processing can occur. One difference is the apparent increase in levels of this enzyme in baculovirus-infected cells, a factor that would be consistent with the higher order of processing in infected cells that was previously observed [7]. These similarities in properties also suggest that this enzyme can be cloned through homology regions, and, in fact, a preliminary report on the cloning of the insect (Sf9) mannosidase II has been published in abstract form [28]. Having available a gene and protein source of this enzyme will allow future studies of its transcriptional and translational regulation with the expectation of understanding its functions in infected and non-infected cells.

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