Purification and characterization of glucosidase I involved in *N*-linked glycoprotein processing in bovine mammary gland

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Glucosidase I, the first enzyme involved in the post-translational processing of *N*-linked glycoproteins, was purified to homogeneity from the lactating bovine mammary tissue. The enzyme was extracted by differential treatment of the microsomal fraction with Triton X-100 and Lubrol PX. The solubilized enzyme was subjected to affinity chromatography on Affi-Gel 102 with *N*-5-carboxypentyldeoxynojirimycin as ligand and DEAE-Sephacryl CL-6B chromatography. Purified glucosidase I shows a molecular mass of 320–330 kDa by gel filtration on Sephacryl S-300. SDS/polyacrylamide-gel electrophoresis under reducing conditions indicates a single band of approx. 85 kDa, indicating that the native enzyme is probably a tetrameric protein. Several criteria, including pH optimum of 6.6–7.0, specific hydrolytic action towards $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, to release the terminally α -1,2-linked glucosyl residue, and total lack of activity towards $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ and $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$ saccharides, which are the biological substrates for processing glucosidase II, and 4-methylumbelliferyl α -D-glucopyranoside show the non-lysosomal origin and the processing-specific role of the purified enzyme. The enzyme does not require any metal ions for its activity. Hg^{2+} , Ag^+ and Cu^{2+} are potent inhibitors of the enzyme; this inhibition can be reversed by adding an excess of dithiothreitol. Among the saccharides tested, kojibiose ($\text{Glc}\alpha 1\rightarrow 2\text{Glc}$) was inhibitory to the enzyme. Polyclonal antibodies raised against the enzyme in rabbit were found to be specific for glucosidase I, as revealed by Western-blot analysis and by immunoadsorption with Protein A–Sephacryl. Anti-(glucosidase I) antibodies were cross-reactive towards a similar antigen in solubilized microsomal preparations from liver, mammary gland and heart from the bovine, guinea pig, rat and mouse.

INTRODUCTION

Glucosidase I is an endoplasmic-reticulum protein that carries out the first step in the processing of all three types, namely high-mannose, complex and hybrid *N*-linked, glycoproteins. It catalyses the removal of terminal α -1,2-linked glucose from the branched precursor oligosaccharide $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ (Snider, 1984). Subsequent processing reactions involve the stepwise removal of the two α -1,3-linked glucose residues by glucosidase II to give $\text{Man}_5\text{GlcNAc}_2$ -protein or the immediate precursor of high-mannose glycoproteins (Burns & Touster, 1982; Brada & Dubach, 1984). Additional processing by endoplasmic-reticulum and Golgi mannosidases and/or the action of Golgi glycosyltransferases is determined by the structural requirements of each individual glycoprotein (Kornfeld & Kornfeld, 1985).

As the first processing enzyme, glucosidase I is critically juxtaposed between the dolichol-linked assembly of the branched tetradecasaccharide $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$ and its protein-linked post-translational modifications. Thus it could play a crucial role in the overall regulation of glycoprotein biosynthesis. At present, the regulation of the biosynthesis of asparagine-linked glycoproteins is poorly defined. Even more significantly, little is known about the regulation of biosynthesis of this important class of glycoconjugates of diverse biological significance in hormonally responsive tissue. For investigating the biosynthesis and regulation of *N*-linked glycoproteins, we have focused our attention on the mammary gland, a

tissue that is the target of a variety of hormones for its growth and differentiation. The lactating gland elaborates a structurally and functionally well-characterized asparagine-linked glycoprotein, α -lactalbumin, in its secretion, i.e. milk.

Glucosidase I has been partially purified and characterized in several systems (Snider, 1984; Szumilo *et al.*, 1986; Bause *et al.*, 1986). 1-Deoxynojirimycin is a basic analogue of glucose and a potent inhibitor of processing glucosidases I and II. Hettkamp *et al.* (1984) attached *N*-carboxypentyl-1-deoxynojirimycin to aminohexyl-(AH-)Sephacryl 4B and used the affinity matrix to purify calf liver glucosidase I to a high degree of purity. In the present study we have used a modification of the procedure of Hettkamp *et al.* (1984) to purify the glucosidase I from the microsomal preparation of lactating bovine mammary gland to electrophoretic homogeneity. A number of properties of the enzyme have been studied. We further report on a specific antibody raised against the purified bovine mammary enzyme that cross-reacts with the enzyme in several tissues of the bovine and other species.

MATERIALS AND METHODS

Materials

UDP-[^{14}C]glucose (sp. radioactivity 284 Ci/mol) was a product of ICN Laboratories. All other materials were obtained as follows: unlabelled nucleotide sugars, doli-

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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chol phosphate, Triton X-100, Lubrol PX, phospholipids, leupeptin, 6-aminohexanoic acid and other commonly used chemicals from Sigma Chemical Co.; Sephadex G-25, Sephacryl S-300, DEAE-Sepharose CL-6B and AH-Sepharose 4B from Pharmacia; Affi-Gel 102, CM-Affi-Gel Blue, dicyclohexylcarbodi-imide, nitro-cellulose membranes, horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody and colour-development reagents, SDS/PAGE reagents and Dowex 1-X4 from Bio-Rad Laboratories and 6-bromohexanoic acid from Aldrich Chemical Co. Bovine mammary tissue was freshly obtained from the local slaughterhouse. 1-Deoxynojirimycin was generously given by Dr. Schmidt and Dr. Truscheit, Bayer AG, Wuppertal, Germany. All other chemicals were of the highest purity commercially available.

Preparation of affinity matrix

N-5-Carboxypentyl-1-deoxynojirimycin was synthesized essentially as described by Hettkamp *et al.* (1984). The purity of the *N*-5-carboxypentyldeoxynojirimycin was checked by t.l.c. in propan-2-ol/acetic acid/water (29:4:7, by vol.) on silica-gel plates. ¹H-n.m.r. and melting-point data confirmed the purity of the product. *N*-5-Carboxypentyldeoxynojirimycin was coupled to Affi-Gel 102 via the carbodi-imide procedure (Bio-Rad Manual). The amount of ligand bound to the resin averaged 3–4 μmol/g of swollen gel.

Purification of glucosidase I

Unless stated otherwise, all operations were conducted at 4 °C. Microsomal fraction from the lactating bovine mammary tissue was prepared as described previously (Vijay & Fram, 1977) except that all the buffers used for tissue homogenization and enzyme purification contained the proteinase inhibitors leupeptin (2 μg/ml), phenylmethanesulphonyl fluoride (0.5 mM), *o*-phenanthroline (1 mM) and 6-aminohexanoic acid (1 mM). For solubilization, washed microsomal fraction was resuspended in 10 mM-sodium phosphate buffer, pH 6.8, containing 0.5 % Triton X-100 (buffer A) to a protein concentration of 10 mg/ml. The suspension was gently stirred on ice for 30 min and then centrifuged at 48 000 *g* for 1 h. The supernatant (Triton X-100 extract) was removed and the pellet was resuspended in 200 mM-sodium phosphate buffer, pH 6.8, containing 0.8 % Lubrol PX (buffer B) to a protein concentration of 6–7 mg/ml. After being kept on ice for 1 h, the suspension was centrifuged at 200 000 *g* for 1 h. The supernatant (Lubrol PX extract) was taken as the source of solubilized enzyme.

The solubilized glucosidase I was adsorbed by mixing the affinity gel with Lubrol PX extract. The suspension was stirred end-over-end on a rotary shaker for 14–16 h. The gel was separated by filtering on a sintered-glass funnel. It was washed sequentially with several volumes of buffer A and buffer B. Glucosidase I was eluted with 10 mM-deoxynojirimycin in buffer B. Deoxynojirimycin was removed by chromatography of the eluate on a column of Sephadex G-25 equilibrated with 10 mM-sodium phosphate buffer, pH 6.8, containing 0.5 % Lubrol PX (buffer C).

The enzyme eluted from Sephadex G-25 was applied to a column (1.5 cm × 10 cm) of DEAE-Sepharose CL-6B, equilibrated with buffer C. The column was washed with 2 bed volumes of buffer C and then eluted with a linear gradient of 0–500 mM-NaCl in buffer C. The

fractions containing glucosidase I activity were pooled and concentrated by ultrafiltration on PM-30 membrane in an Amicon filtration apparatus.

Glucosidase assays

The activities of both glucosidase I and glucosidase II were determined essentially as described by Saunier *et al.* (1982). The crude microsomal fraction or fractions at different stages of enzyme purification were incubated with 7000 c.p.m. of purified [*Glc*-¹⁴C]Glc₃Man₉GlcNAc₂ for glucosidase I or [*Glc*-¹⁴C]Glc₃Man₉GlcNAc₂ for glucosidase II activity determination. One unit of enzyme activity is defined as the amount of enzyme that releases 100 c.p.m. of [¹⁴C]glucose/h under the conditions of the assay.

General methods

SDS/PAGE was done under reducing conditions in accordance with Laemmli (1970). Gels were stained for protein with Coomassie Brilliant Blue G-250 or AgNO₃ (Bio-Rad manual). Native PAGE was conducted as given by Newby *et al.* (1978), with some modifications. The gel polymerization mixture contained 7.3 % acrylamide, 0.2 % *NN'*-methylenebisacrylamide, 125 mM-imidazole/HCl buffer, pH 6.5–7.5, and 25 % (w/v) sucrose. After degassing, *NNN'*-tetramethylethylenediamine (1 μl/ml), Lubrol PX (final concn. 0.1 %) and ammonium persulphate (final concn. 0.07 %) were added to polymerize the gels in tube gel apparatus. After electrophoresis, one set of gels was stained with Coomassie Brilliant Blue and another set was sectioned into 2 mm slices. The slices were incubated at 4 °C for 2 h in glucosidase I assay mixture (minus substrate). The enzyme reaction was started with [*Glc*-¹⁴C]Glc₃Man₉GlcNAc₂ and carried out for 1 h at 37 °C. The product was analysed as given above. The molecular mass of purified glucosidase I was determined by gel filtration on a column of Sephacryl S-300 in buffer B. Ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and RNAase (13.5 kDa) were used as reference standards.

Kinetics of the glucosidase I reaction

A 5 μg portion of purified glucosidase I was incubated with 12 000 c.p.m. of [*Glc*-³H]Glc₃Man₉GlcNAc₂ in standard assay mixtures for various times at 37 °C. The reactions were terminated by adding 20 μl of acetic acid. The quenched reaction mixtures were deproteinized and desalted over 1 ml columns of AG501 resin and freeze-dried. After being redissolved, the products were analysed by paper chromatography in butan-1-ol/pyridine/water (4:3:4, by vol.) as described previously (Prakash *et al.*, 1984).

Preparation of anti-(glucosidase I) antibodies

Antibodies against bovine mammary glucosidase I were raised as follows. A 100 μg portion of purified enzyme in 1 ml of a 1:1 mixture of Freund's complete adjuvant and phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.2) was injected into multiple sites intradermally on the back of a female New Zealand rabbit. Booster injections of 70–80 μg of enzyme in Freund's incomplete adjuvant were given at 2–3-week intervals for a total of three times. Blood was collected 1 and 3 weeks after the final booster injection. The IgG fraction was partially purified

from the immune serum by column chromatography on CM-Affi-Gel Blue followed by precipitation by 40 % saturation with $(\text{NH}_4)_2\text{SO}_4$.

The partially purified IgG fraction was serially diluted with phosphate-buffered saline and incubated for 3 h at 4 °C with the solubilized microsomal preparation in buffer B containing bovine serum albumin (1 mg/ml). At the end of incubation, tubes were centrifuged at 2000 *g* for 5 min and the supernatant fractions were checked for glucosidase I activity. Protein A-Sepharose, washed and equilibrated with buffer B, was added to the remaining mixture (200 μl of wet gel/mg of antibody) and incubated for 2 h at 4 °C. The mixture was centrifuged and glucosidase I activity was determined in the supernatant. Control experiments with non-immune sera were carried out under identical conditions.

Immunoblotting

The purified glucosidase I was analysed on an SDS/7.5 %-polyacrylamide gel. It was then electrophoretically transferred to nitrocellulose paper. After incubation of the membrane with 3 % gelatin in Tris-buffered saline (500 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.5) at 37 °C for 2 h, the nitrocellulose paper was incubated at 20 °C with anti-(glucosidase I) antibody. The filter was washed with Tris-buffered saline containing 0.05 % Tween 20 and then treated with horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody for 1 h. The immunoreactive bands were detected by using horseradish-peroxidase colour-development reagent.

For dot immunoblotting, portions (1–5 μg of protein) of various solubilized microsomal preparations were applied to the nitrocellulose paper in a Bio-Rad dot-blot apparatus. Subsequent treatment with gelatin solution, anti-(glucosidase I) antibody, horseradish-peroxidase-conjugated Protein A and colour development to detect the immunoreactive spots was carried out as per the manufacturer's instructions.

Analytical methods

Dol-PP-[^{14}C]Glc $_1$ - $_3$ Man $_9$ GlcNAc $_2$ (where Dol represents dolichol) was synthesized by using microsomal fractions from bovine mammary tissue (Vijay & Perdew, 1980). Free oligosaccharides were obtained by mild acid hydrolysis as described in Prakash *et al.* (1984), and purified by paper chromatography with butan-1-ol/pyridine/water (4:3:4, by vol.) as solvent. Protein was determined by BCA Protein Assay (Pierce Chemical Co. Manual), with bovine serum albumin as the standard.

RESULTS

Purification of glucosidase I

Lactating mammary gland was chosen as the source for glucosidase I since it contains high levels of microsomal enzyme activity and is readily available. Even though this enzyme has been partially purified from several sources, the highest purification accomplished thus far has been with the calf liver enzyme by Hettkamp *et al.* (1984). These workers purified the enzyme after differential solubilization of the microsomal fraction with detergents followed by affinity chromatography on AH-Sepharose 4B coupled with *N*-carboxypentyl-1-deoxynojirimycin. The purified enzyme preparation showed several faint bands in addition to the enzyme

band on SDS/PAGE. These bands were accounted for by non-specific binding of proteins to the affinity resin. We made a similar observation with the bovine mammary enzyme. Also, we obtained non-specific binding of the enzyme and several other proteins to AH-Sepharose 4B. On the other hand, if Affi-Gel 102 was used as the support medium in the construction of the affinity matrix, non-specific binding of the proteins was decreased considerably. Instead of an aminohexyl group, as in AH-Sepharose 4B, Affi-Gel 102 has an *N*-terminal six-atom hydrophilic arm. Thus the nature of the spacer arm contributes to non-specific binding of hydrophobic proteins solubilized from microsomal membrane preparation to some extent. The affinity-eluted glucosidase

Fig. 1. Chromatography of glucosidase I on DEAE-Sepharose CL-6B

Samples of each fraction were examined for glucosidase I activity (●) and for protein content by BCA reagent (○). A linear gradient of 0–500 mM-NaCl (—) was used to elute the enzyme. Glucosidase I activity was eluted from the column at 150–250 mM-NaCl.

Fig. 2. SDS/PAGE of samples at different stages of purification of glucosidase I

Lane 1, Lubrol PX extract; lane 2, Triton X-100 extract; lane 3, supernatant after affinity adsorption; lane 4, purified glucosidase I after chromatography on DEAE-Sepharose CL-6B; lane 5, molecular-mass standards [ovalbumin (45 kDa), bovine serum albumin (67 kDa), phosphorylase *b* (92.5 kDa), β -galactosidase (116 kDa) and myosin (200 kDa)].

Fig. 3. Native PAGE of purified glucosidase I under different pH conditions

(a) pH 6.5; (b) pH 7.0; (c) pH 7.5. After electrophoresis the gel was cut into 2 mm slices and the enzyme activity was determined as described in the Materials and methods section.

I still contained a few other contaminating proteins. However, further chromatography on DEAE-Sepharose CL-6B (Fig. 1) separated the glucosidase I activity from these contaminating proteins. The enzyme preparation was now homogeneous as judged by a reductive SDS/7.5%-PAGE (Fig. 2). For comparison, the gel also shows the pattern of proteins in crude microsomes, Lubrol PX extract and the run-through fraction from the affinity-chromatography column. The protein band corresponding to glucosidase I is retained on the affinity matrix, indicating the binding of glucosidase I to *N*-5-carboxypentyldeoxynojirimycin. The unbound fraction from the affinity column was missing several bands that were present in the original material. This could be attributed to the non-specific binding to the affinity matrix. The native PAGE of the purified enzyme, conducted under different pH conditions, showed a single protein band. This band coincided with the peak of enzyme activity (Fig. 3). These results confirm the purity of the enzyme preparation. The purification of the enzyme from 1.5 g of microsomal protein of the lactating bovine mammary gland is summarized in Table 1. Overall, a 211-fold purification of enzyme over its activity in the microsomal fraction was achieved. The increase in total activity of the enzyme after solubilization with Lubrol PX as compared with the total activity in the microsomal fraction probably results from activation of enzyme by the detergent.

Properties of glucosidase I

Stability. The purified enzyme could be stored at 4 °C for a week without significant loss of activity. It was stable at -20 °C for several months. At 25 °C, the enzyme lost more than 50 % of its activity after 24 h; however, when the enzyme was stored in buffer containing 1 % poly(ethylene glycol) 4000, it retained nearly 75 % of its activity after 72 h at 25 °C.

Molecular size. The molecular mass of the native glucosidase I is in the range 320–330 kDa as determined by comparison of the elution position of glucosidase I with the elution positions of standard proteins (Fig. 4).

Table 1. Purification of glucosidase I from bovine mammary tissue

For experimental details see the text.

Step	Volume (ml)	Total protein (mg)	10 ⁻³ × Total activity of glucosidase (units)		Specific activity of glucosidase (units/mg)		Purification of glucosidase I (fold)	Yield of glucosidase I (%)
			I	II	I	II		
Crude microsomal fraction	75	1500	54.97	433.00	37	289	1	100
Triton X-100 extract	127	953	7.20	279.00	8	293	0.2	13
Lubrol PX extract	75	413	75.58	87.14	183	211	5	137
Supernatant after affinity adsorption	70	364	8.93	67.70	25	186	0.7	16
Affinity-eluted fraction	11.6	1.39	13.61	0.08	9791	58	265	25
DEAE-Sepharose CL-6B fraction	6	0.42	3.28	0.0	7809	0	211	6

Fig. 4. Determination of apparent molecular mass of native glucosidase I by gel filtration

A 400 μ g portion of the purified glucosidase I was applied on a calibrated column (2.2 cm $\times$ 44 cm) of Sephacryl S-300. The eluted fractions were assayed for enzyme activity (●) and protein concentration (▲). The inset shows the standard curve constructed from the elution volumes of marker proteins: a, ferritin (440 kDa); b, catalase (232 kDa); c, ferritin subunit (220 kDa); d, aldolase (158 kDa); e, bovine serum albumin (67 kDa); f, RNAase (13.5 kDa).

The molecular mass of the enzyme as determined by SDS/7.5%-PAGE under reducing conditions yielded a value of 85 kDa. Therefore glucosidase I may be a tetrameric protein with a subunit size of 85 kDa. However, the possibility, which is less likely, that the higher molecular mass shown by gel filtration may be due to aggregation is not excluded.

pH-dependence. The optimum pH for glucosidase I was found to be in the range 6.6–7.0 (Fig. 5), consistent with its non-lysosomal origin. Similar values have been reported for processing glucosidase I in crude (Snider, 1984) or partially purified preparations (Hettkamp *et al.*, 1984).

Kinetics and specificity of glucosidase I. The purified glucosidase I was incubated with [Glc-¹⁴C]Glc₃Man₉GlcNAc₂ for various periods of time and the reaction products were analysed by paper chromatography (Fig. 6). There was a time-dependent cleavage of the substrate with a concomitant appearance of a second peak of radioactivity that co-chromatographed with the Glc₂Man₉GlcNAc₂ standard. There was no further hydrolysis of the product to give Glc₁Man₉GlcNAc₂. At the end of 120 min, nearly 50 % of the substrate had been converted into the product. These data clearly show that the purified enzyme was free of any contaminating glucosidase II. The purified enzyme did not show any activity towards [Glc-¹⁴C]Glc₁Man₉GlcNAc₂, [Glc-¹⁴C]Glc₂Man₉GlcNAc₂, *p*-nitrophenyl α -D-glucopyranoside or *p*-nitrophenyl β -D-glucopyranoside.

Effect of different saccharides on glucosidase I activity. A number of mono- and di-saccharides were tested as potential inhibitors of glucosidase I activity (Table 2).

Among the saccharides tested, only kojibiose, an α -1,2-linked glucose disaccharide, inhibited the enzyme significantly. Nigerose, Glc α 1 $\rightarrow$ 3Glc disaccharide and a potent inhibitor of glucosidase II, did not inhibit the purified glucosidase I.

Other properties. The enzyme did not show any requirement for a metal ion and was not affected by the chelators EDTA, $\alpha\alpha'$ -bipyridyl and *o*-phenanthroline. Heavy-metal ions, e.g. Co²⁺, Zn²⁺, Ag⁺ and Hg²⁺, were potent inhibitors of the enzyme activity; addition of an excess of dithiothreitol overcame the inhibition caused by heavy-metal ions. The enzyme was stimulated by

Fig. 5. Optimum pH for glucosidase I

For the pH range 4.5–6.0 25 mM-citrate/phosphate buffer was used, and for the pH range 6.0–7.5 25 mM-phosphate buffer was used.

Fig. 6. Kinetics of [^{14}C]Glc₃Man₅GlcNAc₂ hydrolysis by purified glucosidase I

At the given times samples were withdrawn and analysed by paper chromatography as described in the Materials and methods section.

Table 2. Effect of substrate analogues on the activity of glucosidase I

Analogue	Concn.	Inhibition (%)
Control	—	0
Maltose (α -1,4)	10 mM	29
Gentibiose (β -1,6)	10 mM	19
Cellobiose (β -1,4)	10 mM	29
Sucrose (α -1, β -2)	10 mM	33
Glucose	10 mM	13
Mannose	10 mM	0
Kojibiose	1 mM	76
Nigerose	1 mM	20
<i>p</i> -Nitrophenyl α -glucoside	0.5 μM	19
<i>p</i> -Nitrophenyl β -glucoside	0.5 μM	14
Deoxynojirimycin	5 μM	77
<i>N</i> -5-Carboxypentyldeoxynojirimycin	5 μM	47

phosphate ions up to a concentration of 25 mM and by detergents Triton X-100 and Lubrol PX. Several phospholipids were also examined for stimulation of

glucosidase I, but no significant activation was observed (results not shown).

Immunological studies

Immunological specificity. As judged by Ouchterlony double-diffusion, the rabbit antibody appeared to be highly specific for glucosidase I, and did not recognize glucosidase II from the bovine mammary tissue. The Western-blot analysis with solubilized enzyme, purified glucosidase I and the run-through fraction after affinity chromatography, devoid of glucosidase I activity, taken as control, revealed only one band in the solubilized microsomal preparation and one band in the glucosidase I lane (Fig. 7, lanes 1 and 3). The run-through fraction from affinity chromatography did not show the band corresponding to the migration of glucosidase I. These results show that the antibody prepared against the enzyme is highly specific for glucosidase I. In addition, incubation of the antiserum with solubilized microsomal fraction resulted in inhibition of the enzyme activity. Similarly, the addition of Protein A-Sepharose to the solubilized enzyme/antiserum mixture removed the glucosidase I activity by immunoadsorption (Figs. 8a and 8b). No inhibition and removal of enzyme activity was observed when non-immune serum was substituted for the antiserum. To eliminate the possibility that removal or inhibition of glucosidase I activity was caused by a non-specific binding of enzyme to the immune complexes, the Protein A-Sepharose containing antibody-antigen complex was washed extensively with buffer B. The glucosidase I activity bound to the Protein A-Sepharose immune complex was determined. Only glucosidase I, but not glucosidase II, activity was observed in the complex suspensions (results not shown). Thus the antibody is very specific towards glucosidase I and it does not recognize any other protein(s) in the solubilized enzyme preparation.

Fig. 7. Western blot of samples after SDS/PAGE at various stages of glucosidase I purification

Lane 1, Lubrol PX extract; lane 2, supernatant after affinity adsorption; lane 3, purified glucosidase I from bovine mammary tissue.

Fig. 8. Inhibition and precipitation of glucosidase I activity by partially purified anti-(glucosidase I) antibodies

A fixed amount of Lubrol PX extract was incubated with various amounts of antiserum or non-immune serum, as described in the Materials and methods section. Glucosidase I activity remaining in the supernatant before (a) and after (b) the addition of Protein A-Sepharose was determined. Each point in the graph is the average for three determinations. ●, With anti-(glucosidase I) antibody; ▲, with non-immune serum.

Immunological cross-reactivity. The immunological cross-reactivity of antibodies against bovine mammary enzyme towards glucosidase I in various tissues of several other species, namely, mouse, rat, guinea pig and cow, was examined by dot-immunoblotting assay. As shown in Fig. 9, the anti-(bovine mammary glucosidase I) antibodies show positive immunological signals towards solubilized microsomal extracts of different tissues of the four species. Serum albumin, α -lactalbumin and purified mammary glucosidase II, all from the bovine species, were tested as controls. None of the control antigens showed immunological reactivity with the antibodies. Non-immune serum did not show any reactivity with different tissue preparations. Thus the antiserum is quite specific against glucosidase I. The immunoreactive species in the extracts most probably represents glucosidase I in the tissues of the different species examined.

Fig. 9. Dot-blot analysis showing immunological cross-reactivity of antibodies against bovine mammary glucosidase I with solubilized microsomal proteins from bovine mammary gland, liver, heart and the purified bovine mammary glucosidase I (Glu I), guinea-pig mammary gland, liver and heart, rat mammary gland and liver, and mouse mammary gland and liver

Controls run simultaneously included bovine serum albumin (BSA), α -lactalbumin (α -LA) and bovine glucosidase II (Glu II). Controls with non-immune serum did not show any reactivity with the different tissue preparations given above.

DISCUSSION

The glucosidase purified to homogeneity from the lactating bovine mammary microsomal fraction represents the glucosidase I involved in the processing of asparagine-linked glycoproteins of this tissue for several reasons. First, the pH optimum of 6.6 is indicative of its non-lysosomal origin. This is also similar to the pH optimum observed for processing glucosidases that have been investigated in the microsomal fraction of different tissues (Kornfeld & Kornfeld, 1985). Secondly, among the different sugars tested, kojibiose is the best inhibitor of the enzyme activity. This is consistent with the nature of the glucosidic linkage cleaved by the enzyme in the tetradecasaccharide $\text{Glc}_3\text{Man}_6\text{GlcNAc}_2$ substrate. Thirdly, the enzyme does not show any reactivity with *p*-nitrophenyl α - or β -D-glucoside. Fourthly, the enzyme cleaves only the first glucose residue of $\text{Glc}_3\text{Man}_6\text{GlcNAc}_2$ and is totally ineffective against $\text{Glc}_2\text{Man}_6\text{GlcNAc}_2$ and $\text{Glc}_1\text{Man}_6\text{GlcNAc}_2$ saccharides. The latter two saccharides serve as substrates for the cleavage of α -1,3-linked glucosyl residues by processing glucosidase II.

The 211-fold purification of the glucosidase I activity from the bovine mammary microsomal fraction is most probably an underestimate. The tetradecasaccharide used as substrate for assaying glucosidase was prepared by incubating UDP-[^{14}C]glucose with bovine mammary microsomal fraction and isolating the products (Vijay & Perdew, 1982). Structural analysis of this oligosaccharide had shown that all the three glucosyl residues were labelled; further, the density of the label in the glucosyl residues of the $\text{Glc}\alpha 1 \rightarrow 2\text{Glc}\alpha 1 \rightarrow 3\text{Glc}\alpha 1 \rightarrow 3(\text{Man}_6)\text{GlcNAc}_2$ structure was in the proportions 1:0.9:0.8. The microsomal fraction from the bovine mammary gland contains both glucosidase I and glucosidase II activities; an assay of glucosidase I with the labelled $\text{Glc}_3\text{Man}_6\text{GlcNAc}_2$ substrate is subject to interference by the hydrolytic action of glucosidase II on

the product $\text{Glc}_2\text{Man}_9\text{GlcNAc}_2$. Since release of [^{14}C]-glucose from the labelled tetradecasaccharide was used as a measure of glucosidase I activity in the lactating bovine mammary membranes, the value given for the activity of the enzyme in the initial microsomal fraction is an overestimate of the activity of the glucosidase I.

The purified glucosidase I shows only one band of 85 kDa as determined by SDS/PAGE under reducing conditions; a single protein band coinciding with enzyme activity was observed when the preparation was examined by native PAGE at three different pH values. However, it is eluted with a molecular mass of 320–330 kDa during gel filtration on Sephacryl S-300. It is possible that the native enzyme is a tetramer; on the other hand, the larger size observed during gel filtration could result from an aggregation of the native enzyme. This is a less likely possibility, since a smaller, i.e. 85 kDa, form of the native enzyme was not observed during gel filtration.

The antibody raised against bovine mammary glucosidase I was quite specific and recognized only a single polypeptide of 85 kDa during immunoblotting of detergent-solubilized proteins of the bovine mammary microsomal fraction. It should be noted that the antibodies did not react with a related but distinctly different protein, glucosidase II, which is also present in the detergent-solubilized bovine mammary microsomal fraction. Importantly, the antibodies cross-reacted with an antigen in the detergent-solubilized extracts of microsomal fractions prepared from various tissues of mouse, rat, guinea pig and cow. It would, then, appear that the genetic machinery coding for this enzyme has been well conserved during evolution. Further, the availability of these antibodies might be useful in studying a potential regulation of this enzyme during growth and differentiation of the mammary gland under the influence of mammogenic and lactogenic hormones. We have observed a significant activation of several glycosyltransferase activities of the dolichol cycle during the development of the mouse mammary gland *in vivo*. Further, prolactin appears to play a role in this regulation (Vijay & Oka, 1986).

The availability of the antibodies described in the present paper should allow subcellular localization of glucosidase I. This study also illustrates the usefulness of immunological techniques in detecting and potentially isolating glucosidase I from various animal species. Since

the antibodies are cross-reactive towards glucosidase I from several other animal species, the antibodies should be useful in developing a sensitive enzyme-linked immunosorbent assay/radioimmunoassay for this enzyme.

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