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

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Glucosidase II is an endoplasmic-reticulum-localized enzyme that cleaves the two internally α -1,3-linked glucosyl residues of the oligosaccharide $\text{Glc}\alpha 1 \rightarrow 2\text{Glc}\alpha 1 \rightarrow 3\text{Glc}\alpha 1 \rightarrow 3\text{Man}_5\text{-}_9\text{GlcNAc}_2$ during the biosynthesis of asparagine-linked glycoproteins. We have purified this enzyme to homogeneity from the lactating bovine mammary gland. The enzyme is a high-mannose-type asparagine-linked glycoprotein with a molecular mass of approx. 290 kDa. Upon SDS/polyacrylamide-gel electrophoresis under reducing conditions, the purified enzyme shows two subunits of 62 and 64 kDa, both of which are glycosylated. The pH optimum is between 6.6 and 7.0. Specific polyclonal antibodies raised against the bovine mammary enzyme also recognize a similar antigen in heart, liver and the mammary gland of bovine, guinea pig, rat and mouse. These antibodies were used to develop a sensitive enzyme-linked immunosorbent assay for glucosidase II.

INTRODUCTION

The common feature for the post-translational modifications of the different types of asparagine-linked glycoproteins involves the action of two processing-specific glucosidases that clip the glucosyl residues from the terminally linked moiety $\text{Glc}\alpha 1 \rightarrow 2\text{Glc}\alpha 1 \rightarrow 3\text{Glc}$ on the tetramannosyl branch of the precursor oligosaccharide $\text{Glc}_3\text{Man}_5\text{-}_9\text{GlcNAc}_2$ (Grinna & Robbins, 1979; Snider, 1984). Glucosidase I triggers the processing reactions by releasing the outer α -1,2-linked residue. Subsequently, glucosidase II sequentially trims the two α -1,3-linked glucose residues. Both glucosidase I and glucosidase II are located in the rough endoplasmic reticulum and have been studied in a variety of tissues (Michael & Kornfeld, 1980; Burns & Touster, 1982; Brada & Dubach, 1984; Snider, 1984; Hino & Rothman, 1985; Lucocq *et al.*, 1986).

The growth and differentiation of the mammary gland are profoundly modulated by mammogenic and lactogenic hormones (Cowie *et al.*, 1980). An earlier study from our laboratory showed that three key glycosyltransferases, namely *N*-acetylglucosaminyl-1-phospho-, mannosyl- and glucosyl-transferase of the dolichol cycle, undergo differentiation-related activation during the ontogeny of the mouse mammary gland (Vijay & Oka, 1986). Since glucosidases I and II are the first two and the common processing enzymes for all asparagine-linked glycoproteins, these early enzymes in the post-translational modification phase are potential targets for hormonal regulation of the overall biosynthesis of these conjugated proteins in the mammary gland. The present paper describes the preparation and characterization of a homogeneous glucosidase II from the lactating bovine mammary gland. Evidence is presented to show that a monospecific polyclonal antibody raised against the bovine mammary enzyme shows tissue and species cross-reactivity with a similar antigen in several systems.

MATERIALS AND METHODS

Materials

UDP-[^{14}C]glucose (sp. radioactivity 284 Ci/mol) was purchased from Amersham International. Unlabelled nucleotide sugars, dolichol phosphate, Triton X-100, leupeptin, 1,10-phenanthroline, 4-methylumbelliferyl α -D-glucopyranoside, phenylmethanesulphonyl fluoride, Tris and other commonly used chemicals were obtained from Sigma Chemical Co. Concanavalin A-Sepharose 4B, DEAE-Sepharose, Sepharose S-300 and protein molecular-mass markers were the products of Pharmacia Fine Chemicals. Hydroxyapatite, CM-Affi-Gel Blue, horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody, 4-chloro-1-naphthol, nitrocellulose membranes, electrophoresis reagents and silver staining kit were from Bio-Rad Laboratories. Endo- β -*N*-acetylglucosaminidases H and F were purchased from Genzyme. Labelled oligosaccharides [$\text{Glc-}^{14}\text{C}$]Glc $_3$ Man $_5$ -GlcNAc $_2$, [$\text{Glc-}^{14}\text{C}$]Glc $_2$ Man $_5$ GlcNAc $_2$ and [$\text{Glc-}^{14}\text{C}$]Glc $_3$ Man $_5$ GlcNAc $_2$ were prepared in this laboratory (Vijay & Perdew, 1982). The lactating bovine mammary tissue was freshly obtained from a local slaughterhouse and transported to the laboratory on ice.

Purification of glucosidase II

All purification steps were performed at 0–4 °C. The buffers used throughout tissue homogenization and enzyme purification contained a cocktail for inhibition of proteinases (final concentrations 2 μg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-1,10-phenanthroline and 1 mM-NaHSO $_3$).

Preparation of microsomal fraction. The preparation of a 48 000 *g* $\times$ 60 min membrane fraction from the lactating bovine mammary tissue was essentially as described previously (Vijay *et al.*, 1980).

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; e.l.i.s.a., enzyme-linked immunosorbent assay.

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Solubilization of glucosidase II by Triton X-100. The microsomal fraction was suspended in 10 mM-sodium phosphate buffer, pH 6.8, to a protein concentration of approx. 50 mg/ml. An equal volume of 1% Triton X-100, prepared in the same buffer, was added to the microsomal suspension. The mixture was gently stirred for 1 h. It was later centrifuged at 105 000 *g* for 60 min in a Beckman ultracentrifuge. The supernatant contained more than 80% of glucosidase II activity.

(NH₄)₂SO₄ fractionation. The supernatant from the above step was subjected to a 20–60%-satn.-(NH₄)₂SO₄ fractionation followed by centrifugation at 10 000 *g* for 30 min. The precipitate was dissolved in 10 mM-sodium phosphate buffer, pH 6.8, and dialysed for 48 h against the same buffer with three changes. The dialysis residue was centrifuged at 15 000 *g* for 10 min.

Concanavalin A-Sepharose 4B chromatography. The supernatant from the previous step was applied to a Concanavalin A-Sepharose 4B column (2.5 cm × 15 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 6.8, containing 100 mM-NaCl. The column was washed with 4 bed volumes of equilibrating buffer. The elution of glucosidase II activity was effected with 0.2 M-methyl α -mannopyranoside in equilibrating buffer. The active fractions were concentrated by ultrafiltration on a PM-30 membrane. The concentrated enzyme was dialysed against 10 mM-sodium phosphate buffer, pH 6.8, containing 10% (v/v) glycerol.

Hydroxyapatite column chromatography. The dialysed enzyme from the concanavalin A-Sepharose 4B eluate was applied to a hydroxyapatite column (2.5 cm × 20 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 6.8. After being washed with 3 bed volumes of 20 mM-sodium phosphate buffer, pH 6.8, the column was eluted with a linear gradient of 100–400 mM-sodium phosphate buffer, pH 6.8. Fractions showing glucosidase II activity were pooled and concentrated by ultrafiltration on a PM-30 membrane.

DEAE-Sephacel column chromatography. The concentrated enzyme from the previous step was applied to a DEAE-Sephacel column (1.5 cm × 12 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 6.8. The column was washed with 3 bed volumes of equilibrating buffer and then eluted with a linear gradient of 0–500 mM-NaCl in 50 mM-sodium phosphate buffer, pH 6.8.

Glucosidase II assay

The activity of glucosidase II was measured by the hydrolysis of 4-methylumbelliferyl α -D-glucopyranoside (Brada & Dubach, 1984). One unit of 4-methylumbelliferyl α -D-glucopyranosidase activity was defined as the amount of enzyme that would liberate 1 μ mol of 4-methylumbelliferone/min at 37 °C.

Glucosidase II activity with labelled biological substrate [*Glc*-¹⁴C]Glc₂Man₉GlcNAc₂ was assayed by incubating the enzyme with 7000 c.p.m. of [*Glc*-¹⁴C]Glc₂Man₉GlcNAc₂ for 15 min at 37 °C in a total volume of 100 μ l containing 25 mM-sodium phosphate buffer, pH 6.8. After termination of the reaction in a boiling-water bath, the released [¹⁴C]glucose was determined (Saunier *et al.*, 1982). Glucosidase I activity at different

stages of enzyme purification was assayed similarly except that the substrate used was [*Glc*-¹⁴C]Glc₃Man₉GlcNAc₂.

Electrophoresis

Analytical non-denaturing PAGE was carried out according to the method of Davis (1964). SDS/PAGE was performed under reducing conditions in accordance with Laemmli (1970). Gels were stained with either Coomassie Brilliant Blue R-250 or AgNO₃ for proteins. Periodate/Schiff-base staining was employed for staining glycoproteins (Pharmacia Technical Bulletin: Polyacrylamide Gel Electrophoresis—Laboratory Techniques, p. 53).

Molecular-mass determination by gel filtration

Gel filtration on a Sephacryl S-300 column (2.2 cm × 44 cm) was performed to determine the molecular mass of glucosidase II with standards ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and chymotrypsin (25 kDa). The column was equilibrated and eluted with 10 mM-sodium phosphate buffer, pH 6.8, containing 100 mM-NaCl.

Endo- β -N-acetylglucosaminidase digestions

The digestion of 15 μ g of pure glucosidase II with endo- β -N-acetylglucosaminidase H (Vijay *et al.*, 1980) and endo- β -N-acetylglucosaminidase F (Elder & Alexander, 1982) was conducted at 37 °C for 16 h under toluene and terminated in a boiling-water bath. The products were analysed by SDS/PAGE by the procedure of Laemmli (1970).

Preparation of antiserum

For raising antibodies, 100 μ g of pure glucosidase II dissolved in 0.5 ml of 10 mM-sodium phosphate buffer, pH 6.8, was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously in multiple sites on the back of a New Zealand White female rabbit. This was followed by three subcutaneous and intradermal booster injections, given 1 week apart, starting 1 month after the first injection. For boosters, 200 μ g of glucosidase II was mixed with an equal volume of Freund's incomplete adjuvant. Blood was collected 1 week after the last booster and checked for immunological activity by Ouchterlony (1949) double immunodiffusion. The IgG fraction in the antiserum was partially purified by column chromatography on CM-Affi-Gel Blue (Bio-Rad Tech Bulletin no. 1197) followed by precipitation by 40% saturation with (NH₄)₂SO₄.

To examine the specific inhibition of glucosidase II activity by the partially purified IgG, 250 μ g of Triton X-100-solubilized microsomal protein was incubated with various amounts of the antibody at 4 °C for 6 h in a total volume of 150 μ l. The incubation tubes were centrifuged and the supernatants were checked for glucosidase II activity with [*Glc*-¹⁴C]Glc₂Man₉GlcNAc₂ as substrate. Control incubations were set up with pre-immune IgG fraction and examined for glucosidase II activity under identical conditions.

Immunoblotting

Transfer of proteins from SDS/polyacrylamide gel to nitrocellulose membrane was carried out as described by Burnette (1981). After transfer, the nitrocellulose membrane was immersed in 3% gelatin in Tris-buffered saline

(500 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.5) for 1 h at 37 °C. This was followed by overnight incubation at 37 °C with 1:500 dilution of the anti-(glucosidase II) serum. After being washed with Tris-buffered saline containing 0.05 % Tween 20, the membrane was incubated with the second antibody solution consisting of horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody at a 1:3000 dilution for 1 h at 37 °C. The immunoreactive proteins were detected by using 4-chloro-1-naphthol reagent.

Cross-reactivity of various tissue preparations with antibodies against bovine mammary glucosidase II was checked by dot-immunoblot assay in accordance with the Bio-Rad Manual. Solubilized microsomal preparations were made from freshly obtained tissues of bovine, guinea pig, rat and mouse as given above. A 5 µg portion of protein was applied to the nitrocellulose membrane. α -Lactalbumin, bovine serum albumin and purified glucosidase I from the bovine mammary gland served as controls.

Enzyme-linked immunoassays

Different dilutions of purified glucosidase II and solubilized bovine mammary microsomal preparations were coated in 96-well polystyrene e.l.i.s.a. plates at pH 9.0 (100 mM-Na₂CO₃/NaHCO₃ buffer). After blocking with 1 % bovine serum albumin in phosphate-buffered saline (500 mM-NaCl/100 mM-sodium phosphate buffer, pH 7.2), 100 µl of anti-(glucosidase II) antibodies, appropriately diluted with 1 % bovine serum albumin in phosphate-buffered saline, was added and the plates were incubated for 16 h at 4 °C. The plates were washed five times with phosphate-buffered saline containing 0.05 % Tween 20, and 100 µl of horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody (dilution 1:3000) was added. After 2 h at 25 °C, the plates were washed five times with phosphate-buffered saline and the colour was developed with 4-chloro-1-naphthol for 10 min at 25 °C; colorimetric determinations were made at 414 nm in a Dynatech e.l.i.s.a. reader. All samples were run in duplicate and the results are presented as means $\pm$ S.D. for at least four determinations.

Protein determination

Protein was determined by the dye-binding assay (Bradford, 1976).

RESULTS

Purification of glucosidase II

The glucosidase II was purified to homogeneity from the bovine mammary gland by the six-step procedure outlined in Table 1. The final preparation had a specific activity of 96 units/g of protein, with 4-methylumbelliferyl α -D-glucopyranoside as substrate, and was purified 126-fold with a yield of 15 % from the original microsomal preparation. Each step of purification was analysed by electrophoresis in native polyacrylamide gels and staining for neutral α -glucosidase activity as well as glucosidase II activity with [Glc-¹⁴C]Glc₂Man₆GlcNAc₂ as substrate. The enzyme could be solubilized from microsomal fraction by 0.5 % Triton X-100 extraction, precipitated by 20–60 %-satn.-(NH₄)₂SO₄ fractionation and bound to a concanavalin A-Sepharose 4B column and eluted with 0.2 M-methyl α -mannopyranoside. Hydroxyapatite chromatography of concanavalin A-Sepharose 4B eluate enzyme showed two peaks (A and B) of activity (Fig. 1a) as fractions were analysed for activity with 4-methylumbelliferyl α -D-glucopyranoside as substrate. The major glucosidase II activity, as judged by the release of glucose from [Glc-¹⁴C]Glc₂Man₆GlcNAc₂, was found in fractions comprising peak B. This peak was selected for further purification on a DEAE-Sepharose column to a single symmetrical peak of glucosidase II activity eluted at approx. 310–360 mM-NaCl concentration (Fig. 1b).

The purified enzyme showed a single protein band upon native electrophoresis in 12.5 % polyacrylamide gel (Fig. 2) when stained with either Coomassie Brilliant Blue or AgNO₃. It also showed a single band of enzyme activity with identical mobility on the native polyacrylamide gel when tested with the fluorogenic substrate 4-methylumbelliferyl α -D-glucopyranoside.

Enzymic properties

The cleavage of 4-methylumbelliferyl α -D-glucopyranoside by the purified glucosidase II showed Michaelis-Menton kinetics and gave a K_m of 13.1 µM for the artificial substrate. It did not hydrolyse 4-methylumbelliferyl β -D-glucopyranoside and 4-methylumbelliferyl α -D-mannopyranoside. The cleavage of 4-methylumbelliferyl α -D-glucopyranoside by the enzyme was inhibited by maltose (12 % of control at 100 mM-maltose) and turanose (37 % of control at 100 mM-turanose), but was unaffected by trehalose at concentrations up to 100 mM. The enzyme showed no requirement for a

Table 1. Purification of glucosidase II from the bovine mammary gland

For experimental details see the text.

Fraction	Protein (mg)	Enzyme activity (munits)	Specific activity (munits/mg)	Yield (%)	Purification (fold)
1. Microsomal suspension	5583	4285	0.76	100	1
2. Triton X-100-solubilized enzyme	1665	3710	2.22	86	2.9
3. 20–60 %-satn.-(NH ₄) ₂ SO ₄ -fractionated enzyme	1284	2887	2.25	67	3.0
4. Concanavalin A-Sepharose 4B eluate enzyme	143	2520	17.6	58	23
5. Hydroxyapatite eluate enzyme	39	1820	46.6	42	61
6. DEAE-Sepharose-purified glucosidase II	7	673	96	15	126

Fig. 1. Chromatographic steps during the purification of glucosidase II

(a) Hydroxyapatite column chromatography. The dialysed enzyme from the concanavalin A-Sepharose 4B eluate was applied to a hydroxyapatite column (2.5 cm $\times$ 20 cm). Glucosidase II was eluted by increasing the concentration of phosphate buffer (----). (b) DEAE-Sephacel column chromatography. The enzyme fractions eluted from the hydroxyapatite column were pooled, concentrated by ultrafiltration, and applied to a DEAE-Sephacel column (1.5 cm $\times$ 12 cm). After being washed with equilibrating buffer, the column was eluted with a linear gradient of NaCl (----). Elution profiles of glucosidase II activity, measured with 4-methylumbelliferyl α -D-glucopyranoside as substrate (●), and protein (A_{280}) (○) were determined.

bivalent ion; heavy-metal ions, e.g. Ag^+ and Hg^{2+} , were potent inhibitors. It was totally inactive against $[\text{Glc-}^{14}\text{C}]\text{-Glc}_3\text{Man}_9\text{GlcNAc}_2$, the specific biological substrate of glucosidase I, but hydrolysed both $[\text{Glc-}^{14}\text{C}]\text{-Glc}_1\text{-Man}_9\text{-GlcNAc}_2$ and $[\text{Glc-}^{14}\text{C}]\text{-Glc}_2\text{Man}_9\text{GlcNAc}_2$ at a linear rate for 15 min. Under standard conditions of assay, nearly 75% of the radioactivity from $[\text{Glc-}^{14}\text{C}]\text{-Glc}_2\text{Man}_9\text{GlcNAc}_2$ could be released as ^{14}C glucose in 45 min. All of these data are consistent with the specificity of the enzyme for α -1,3-linked glucosyl residues in the dodeca- and trideca-saccharide during glycoprotein processing. Because of the limitations of quantity and the lack of uniformity of labelling of glucosyl residues in the $\text{Glc}_1\text{-}$ and $\text{Glc}_2\text{-Man}_9\text{GlcNAc}_2$ substrates (Vijay & Per-dew, 1982), it was not possible to conduct a detailed study of the kinetics with these substrates. The purified

Fig. 2. PAGE of purified glucosidase II under non-denaturing conditions in 12.5% gel

The gel was stained for protein with Coomassie Brilliant Blue.

enzyme exhibited a pH optimum at 6.5–7.0 when tested with 4-methylumbelliferyl α -glucopyranoside and $[\text{Glc-}^{14}\text{C}]\text{-Glc}_2\text{Man}_9\text{GlcNAc}_2$. These results clearly indicate that the purified glucosidase II is not of lysosomal origin.

Molecular mass and subunit characteristics

The apparent molecular mass of the native enzyme is 290 kDa, as determined by gel filtration (Fig. 3). Reductive SDS/12.5%-PAGE of the purified enzyme showed a doublet migrating in a position corresponding to 64 kDa and 62 kDa. Under similar conditions of reductive SDS/PAGE, the enzyme gave a partially resolved doublet on 10% gel and a single band corresponding to 63 kDa on 7.5% gel (Fig. 4).

Glycoprotein nature of purified glucosidase II

The binding of glucosidase II to concanavalin A-Sepharose 4B and its elution with 0.2 M-methyl α -mannopyranoside during purification indicated that the enzyme is an asparagine-linked glycoprotein. After reductive SDS/PAGE on 12.5% gels, the doublet of the purified enzyme gave a positive reaction for periodate/Schiff-base staining. Digestion of the enzyme with endo- β -N-acetylglucosaminidases H and F followed by analysis on reductive SDS/12.5%-PAGE showed a distinct shift in the migration position of the doublet (Fig. 5). These results show that glucosidase II is an asparagine-linked glycoprotein of the high-mannose type; further, glycosylation is on both of its component polypeptides.

Immunological characterization of glucosidase II

Antibodies raised against purified enzyme gave a single precipitin line with the pure enzyme and Triton X-100-solubilized microsomal fraction, having a line of identity on Ouchterlony double-diffusion analysis (Fig. 6 inset).

Fig. 3. Determination of oligomeric molecular mass of purified glucosidase II by using a calibrated Sephacryl S-300 column (2.2 cm $\times$ 44 cm)

The eluted fractions were assayed for enzyme activity (●) and protein concentration (A_{280}) (○). The inset shows the standard curve constructed from the elution volumes of marker proteins: 1, chymotrypsinogen (25 kDa); 2, bovine serum albumin (67 kDa); 3, aldolase (158 kDa); 4, catalase (232 kDa); 5, ferritin (440 kDa). The arrow indicates the elution of glucosidase II (×).

Fig. 4. SDS/PAGE of purified glucosidase II under reducing conditions at 12.5% (lanes 1 and 2), 10% (lanes 3 and 4) and 7.5% (lanes 5 and 6) gel concentrations

Lanes 1, 3 and 5 show the migration of the standards: A, thyroglobulin (330 kDa); B, ferritin half-unit (220 kDa); C, bovine serum albumin (67 kDa); D, catalase (60 kDa); E, lactate dehydrogenase (36 kDa); F, ferritin (18.5 kDa). Lanes 2, 4 and 6 show the migration of glucosidase II. The gel was stained for protein with Coomassie Brilliant Blue.

To examine the specific inhibition of glucosidase II by the antibodies, increasing concentrations of antibodies were added to Triton X-100-solubilized microsomal fraction. After the formation of antigen-antibody immune complex, the mixture was centrifuged and the

Fig. 5. Reductive SDS/12.5%-PAGE showing the effect of endo- β -N-acetylglucosaminidases H and F on purified glucosidase II

Lane 1, endo- β -N-acetylglucosaminidase H-treated enzyme; lane 2, endo- β -N-acetylglucosaminidase F-treated enzyme; lane 3, untreated pure glucosidase II; lane 4, molecular-mass markers (the same standards as were used in Fig. 4); lane 5, endo- β -N-acetylglucosaminidase H-treated ovalbumin; lane 6, untreated ovalbumin. The gel was stained for protein with Coomassie Brilliant Blue.

glucosidase II activity was assayed in the supernatant. The results given in Fig. 6 show that the antibodies precipitated glucosidase II until, at an antibody/solu-

Fig. 6. Inhibition of glucosidase II activity in detergent-solubilized microsomal proteins by immunoprecipitation with antibodies against the enzyme (●) and the IgG fraction in the pre-immune serum (○)

Details of the experimental procedure are given in the Materials and methods section. The inset shows Ouchterlony agarose-gel double-diffusion analysis of purified glucosidase II. The wells contained the following: A, glucosidase II; B, Triton X-100-solubilized microsomal proteins; C, antibodies against glucosidase II.

Fig. 7. Western blot of glucosidase II subunit

Detergent-extracted microsomal proteins (lane 1), concanavalin A-Sepharose 4B eluate fractions containing enzyme activity (lane 2) and the purified glucosidase II (lane 3) were subjected to SDS/7.5%-PAGE under reducing conditions. The resolved proteins were transferred to a nitrocellulose membrane. The membrane was treated with antiserum against glucosidase II, followed by decoration with a horseradish-peroxidase-conjugated goat antiserum against rabbit immunoglobulins. The replica was washed and treated with 4-chloro-1-naphthol for colour development.

bilized microsomal protein ratio of 0.9, nearly 70% of the enzyme activity had been inhibited. Controls with IgG fraction from pre-immune serum showed no inhibition.

The specificity of the antibody was further examined by a Western-blot analysis. The solubilized microsomal proteins, the enzymically active fractions eluted from the concanavalin A-Sepharose 4B column and the purified glucosidase II were analysed by SDS/7.5%-PAGE under reducing conditions. After transfer to nitrocellulose membrane, the proteins were subjected to immunoblotting and colour development (Fig. 7). The antibody recognizes a polypeptide with a mobility of molecular mass 63 kDa in all the fractions. In addition, the solubilized microsomal fraction contains a polypeptide of approx. 75 kDa that also reacts with the antibody. This polypeptide could either represent a precursor form of a subunit of glucosidase II or represent the intact subunit of glucosidase II that was initially present in the microsomal fraction and was subsequently proteolysed to yield the 62–64 kDa subunits despite the presence of proteinase-inhibitor cocktail in the purification buffers. The significance of this observation is given in the Discussion section.

The immuno-cross-reactivity of the antibody with a similar antigen in preparations of different tissues of cow, guinea pig, rat and mouse was examined by dot-blot analysis. The results given in Fig. 8 show that the antibody is cross-reactive to the solubilized microsomal protein in several tissues of the different animal species. Control preparations showed that the antibody did not cross-react with purified bovine mammary glucosidase I, bovine serum albumin or bovine α -lactalbumin. Pre-

Fig. 8. Dot-blot analysis showing immuno-cross-reactivity of antibodies against bovine mammary glucosidase II with solubilized microsomal proteins from bovine mammary gland (1A), liver (1B), heart (1C) and the purified bovine mammary glucosidase II (1D), guinea-pig mammary gland (2A), liver (2B) and heart (2C), rat mammary gland (3A) and liver (3B), and mouse mammary gland (4A) and liver (4B)

Controls run simultaneously included bovine serum albumin (5A), α -lactalbumin (5B) and bovine glucosidase I (5C). Dot 5D shows the control in which the purified glucosidase II was treated with pre-immune rabbit serum in place of the antiserum against glucosidase II.

Fig. 9. Dose-response curves (a) pure glucosidase II and (b) detergent-solubilized microsomal protein on colour development in the e.l.i.s.a.

Details of the assay are given in the Materials and methods section. The bars represent the standard deviation for four different determinations.

immune serum did not show any reactivity with the different tissue preparations. All of these results strongly show the specificity of the antibody. Next, micro-titre plates were coated with increasing amounts of purified glucosidase and later developed through the double-antibody immunoadsorption followed by the colour-development reaction. As shown in Fig. 9(a) a nearly straight-line relationship is obtained in which the antibody can detect up to 500 ng of the enzyme. Subsequently, micro-titre plates were coated with increasing volumes of the Triton X-100-extracted microsomal proteins and subjected to e.l.i.s.a. as before. The results shown in Fig. 9(b) indicate that a linear relationship is obtained for the solubilized microsomal protein and absorbance at 414 nm.

DISCUSSION

This paper reports the purification and characterization of a neutral α -glucosidase from the lactating bovine mammary gland. Several lines of evidence, as described in the Results section, indicate that the enzyme is the processing-specific glucosidase II in this tissue. A proteinase-inhibitor cocktail used by Hino & Rothman (1985) for the purification of the rat liver glucosidase II was included in all buffers during tissue homogenization and enzyme purification. The purified enzyme showed a single band on non-denaturing PAGE. When examined by SDS/PAGE under reducing conditions, it migrated as a single band of about 63 kDa, which split into two bands of 62 kDa and 64 kDa as the gel concentration was raised to 12.5%. This is probably due to the sieving

effect of the higher gel concentration. Burns & Touster (1982) also observed what appeared to be an unresolved doublet of approx. 65 kDa for the rat liver enzyme by SDS/PAGE. The native bovine mammary enzyme migrated with a molecular mass of 290 kDa on Sephacryl S-300, similar to the size of the rat liver enzyme (Burns & Touster, 1982). Thus the native enzyme might be a tetramer. On the other hand, Hino & Rothman (1985) reported a subunit size of 123 kDa for the rat liver enzyme. Limited proteolysis of this enzyme with trypsin yielded a 73 kDa fragment that retained its catalytic activity. Although the 123 kDa subunit was a glycoprotein, the 73 kDa fragment was non-glycosylated. Brada & Dubach (1984) reported a subunit size of 100 kDa for the pig kidney glucosidase II. The native kidney enzyme showed protein bands corresponding to 400, 200 and 100 kDa on PAGE with dissociation of the 400 kDa form to 200 kDa and 100 kDa forms on storage. Martiniuk *et al.* (1985) observed a molecular mass of 205 kDa for purified human placenta glucosidase II on Sepharose 6B. It is clear that glucosidase II from different sources is a high-molecular-mass anionic *N*-linked glycoprotein.

Apart from an inherent characteristic of the bovine mammary enzyme, several other possibilities might also explain the smaller size observed here. There may be an endogenous proteinase(s) in the preparation from the bovine mammary tissue that could be resistant to the proteinase inhibitors employed in this study. The results of Western blot shown in Fig. 7 would support this reasoning, since the polyclonal antibodies raised against the purified glucosidase II also recognize a larger 75 kDa polypeptide in the detergent extract of the microsomal fraction. This polypeptide is not present in the glucosidase II eluted from concanavalin A-Sepharose 4B. At the hydroxyapatite chromatography step, the enzyme activity, measured by the hydrolysis of the artificial substrate, was eluted in two peaks (Fig. 1a). The major glucosidase II activity, as measured by the release of labelled glucose from [14 C]Glc₂Man₉GlcNAc₂, was eluted in peak B. The fractions comprising this peak were therefore pooled for further purification. It is conceivable that peak B contained a proteolytically degraded but catalytically enhanced enzyme. This argument would be consistent with the results of the Western blot. Subsequent chromatography of the hydroxyapatite eluate enzyme on DEAE-Sephacel yielded one peak of activity. Importantly, we were able to obtain a catalytically active form of glucosidase II that could be used for the immunological studies reported in the present paper.

Polyclonal antibodies raised against the purified glucosidase II in rabbit were specific against the enzyme, as indicated by the single precipitin line of identity with the pure enzyme and the detergent-extracted microsomal proteins on Ouchterlony double-diffusion analysis. The inhibition of glucosidase II activity in the detergent-solubilized microsomal proteins by the partially purified IgG fraction from the antiserum, the results of Western-blot analysis and the inability of the antibodies to react with the related but distinctly different processing glucosidase I provide additional evidence for the specificity of the antibodies. The antibodies also failed to react with two other proteins, namely bovine serum albumin and bovine α -lactalbumin.

The antibodies raised against the enzyme showed immuno-cross-reactivity with a similar antigen in several

tissues of bovine, guinea pig, rat and mouse. It would appear that the genetic machinery coding for this enzyme has been well conserved during evolution. The data reported also show that glucosidase II antibodies allow the quantification of this enzyme by sensitive e.l.i.s.a. methods. These results have important implications for studying the developmental regulation of glycoprotein biosynthesis in the mammary gland. This tissue is under intense hormonal regulation for its development. It has been shown by us that several of the key glycosyltransferases of the dolichol cycle in the mouse mammary gland are developmentally regulated and reach their peak activities at late lactation; further, prolactin appears to play a role in this regulation (Vijay & Oka, 1986). Both glucosidase I and glucosidase II are key processing enzymes, since none of the three different types of asparagine-linked glycoproteins, i.e. high-mannose, complex or the hybrid type, contains glucose residues in the oligosaccharide chains. Thus a study of the developmental regulation of either of these glycosidases should yield important information on the overall regulation of glycoprotein biosynthesis and processing in the mammary gland.

We thank Miss Margaret Kempf for her excellent secretarial work and Miss Manju Masson for her graphic skill. This research was supported by a grant (AM19682) from the National Institutes of Health. This paper is Scientific Article no. A4578, Contribution no. 7574, of the Maryland Agricultural Experiment Station.

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Received 29 December 1986/10 April 1987; accepted 21 July 1987