Purification and characterization of two forms of β -D-galactosidase from rat epididymal luminal fluid: evidence for their role in the modification of sperm plasma membrane glycoprotein(s)

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Previous studies from this laboratory have identified rat epididymal luminal fluid acid β -D-galactosidase activity which also optimally hydrolyses a glycoprotein substrate at neutral pH [Skudlarek, Tulsiani and Orgebin-Crist (1992) *Biochem. J.* **286**, 907–914]. We have now separated the luminal fluid β -D-galactosidase into two molecular forms by ion-exchange chromatography on a column of DE-52. The separated enzyme activities were purified to an apparent homogeneity by molecular-sieve chromatography followed by affinity chromatography on a column of immobilized *p*-nitrophenyl β -D-thiogalactopyranoside. The purified forms, when resolved by SDS/PAGE under reducing conditions, showed apparent molecular masses of 84 and 97 kDa. Kinetic studies, including a pH-dependent substrate preference and pH-dependent association/dissociation, disclosed no differences between these two forms. The two forms had identical N-terminal amino acid sequences. However, the 97 kDa form contained much more total carbohydrate and sialic acid than the 84 kDa form. The carbohydrate moieties in the two forms were assessed by comparing their size on SDS/PAGE before and after treatment with endo-enzymes. The removal of N-linked glycans by treatment with N-glycanase or endoglyco-

sidase F generated de-N-glycosylated polypeptides of an apparent molecular mass of 70 kDa, and indicated that the two forms contained varying amounts of asparagine (N)-linked high mannose/hybrid-type and biantennary complex-type oligosaccharides. This result and the fact that the two molecular forms had identical N-terminal amino acid sequences indicated that the two forms probably have identical or very similar polypeptides. The potential role of the enzyme in modification of sperm plasma membrane (PM) glycoproteins was examined by resolving caput sperm PM proteins (before and after treatment *in vitro* of the membranes with the purified β -D-galactosidase) on SDS/PAGE, followed by staining with peanut agglutinin (PNA), a lectin which preferentially binds to Gal β 1,3GalNAc-linkages found in O-linked glycoproteins. The evidence presented in this report has indicated that a PNA-positive glycoprotein of an apparent molecular mass of 135–150 kDa present on the caput (but not cauda) sperm PM is degalactosylated by the digestion *in vitro* of the membranes with purified luminal fluid β -D-galactosidase. This result suggests a possible role for the epididymal luminal fluid β -D-galactosidases.

INTRODUCTION

Spermatozoa released from the seminiferous tubules and proximal regions of the epididymis are unable to fertilize an egg. They acquire progressive motility and fertilizing ability during epididymal transit [1,2]. The sperm plasma membrane (PM), a vital component in the early events of fertilization, undergoes extensive modifications as spermatozoa travel from the caput to the cauda region of the epididymis. Although details of these modifications are not fully understood, lectin binding studies provide evidence suggesting that sugar moieties of sperm PM glycoproteins are extensively modified as spermatozoa pass through the epididymis [3–6]. For instance, peanut agglutinin (PNA), a lectin known to preferentially bind to the terminal galactosyl residue of Gal β 1,3GalNAc-linkage found in O-linked glycoproteins, binds glycoproteins from the rat proximal caput (but not cauda) sperm PM [7]. The apparent loss of PNA affinity could either be due to masking of galactosyl residues, possibly by the addition of other sugar residue(s) by the luminal fluid glycosyltransferases [8], or the cleavage of terminal galactosyl residue(s) by the luminal fluid β -D-galactosidase [9]. This enzyme has recently been shown by us to be an acid β -D-galactosidase which optimally hydrolyses synthetic *p*-nitrophenyl β -D-galactoside at an acidic pH (3.5) and a glycoprotein substrate at neutral pH [9]. Competition studies

have provided evidence indicating that the epididymal luminal fluid β -D-galactosidase activity is capable of cleaving a variety of linkages found in O- and N-linked oligosaccharides. Subsequent studies, using the Western blotting approach, have revealed that the rat epididymal luminal fluid β -D-galactosidase exists in two molecular forms, a 97 kDa and an 84 kDa form [10]. The purpose of the studies presented in this report is to: (a) purify and chemically characterize each of the two forms of β -D-galactosidase activity from rat epididymal luminal fluid; and (b) use homogeneous preparations of the enzyme to examine mechanism(s) for the apparent loss of galactosyl residues from sperm surface glycoprotein(s) during the sperm maturation. The evidence presented in this report suggests a role for the luminal fluid β -D-galactosidase in modification of the sperm PM glycoproteins.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley retired breeder rats (Sasco, Omaha, NE, U.S.A.) were kept in our animal facility for at least 3 days before an experiment. UDP[4,5-³H]Gal (43.3 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.); Budget-Solve scintillation cocktail was from Research Product International

Abbreviations used: PM, plasma membrane; PNA, peanut agglutinin; endo, endoglycosidase; PNP, *p*-nitrophenyl; CBB, Coomassie Brilliant Blue.

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(Mount Prospect, IL, U.S.A.); Endoglycosidase H (endo H) endoglycosidase F (endo F) and N-glycanase (peptide: N-glycosidase F) were from Genzyme (Boston, MA, U.S.A.); Endo- β -galactosidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.); DE-52 was from Whatman BioSystems (Hillsboro, OR, U.S.A.); Bio-Gel A 0.5m (200–400 mesh), Protein Assay Kit, electrophoretic chemicals and molecular mass marker proteins were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); bovine milk galactosyltransferase, *p*-aminophenyl β -D-thiogalactopyranoside (immobilized on 4% beaded agarose), *p*-nitrophenyl (PNP) β -D-galactopyranoside, and BSA (A 3803) were from Sigma (St. Louis, MO, U.S.A.); biotinylated-PNA was from EY Laboratories (San Mateo, CA, U.S.A.); Vectastain reagents (Vectastain ABC Kits PK 4000 and PK 4005) were from Vector Laboratories (Burlingame, CA, U.S.A.). Asialoagalactofetuin and asialoagalacto-ovomucoid were prepared by our published procedure [11]. All other reagents were obtained commercially and were of the highest purity available. Centricon 10 microconcentrator (10000 kDa molecular mass cut-off) was from Amicon (Beverly, MA, U.S.A.). Antibody to mouse liver β -D-galactosidase, prepared in goat [12], has been previously shown to cross-react with the acid β -D-galactosidase of rat epididymal luminal fluid [9,10]. Dulbecco's phosphate-buffered saline (10-fold concentrated, Gibco-BRL, Grand Island, NY, U.S.A.) was diluted 10-fold in glass-distilled water while the pH was adjusted to 7.4 to make a working PBS solution.

Preparation of epididymal luminal fluid

For enzyme purification, the luminal fluid was prepared as previously described [13]. In brief, epididymides from 11–12 rats (free of fat-pad, blood vessels and connective tissue) were cut with a sharp razor blade into approx. 1 mm³ pieces in PBS. The tissue pieces in PBS were gently shaken at room temperature to release spermatozoa and luminal fluid. The minced tissue was filtered through eight layers of cheesecloth (to remove tissue pieces) and the cheesecloth was washed with the above PBS solution. The pooled supernatant was centrifuged for 10 min at 600 *g*. The cloudy supernatant was removed by aspiration (approx. 50 ml), and stored, frozen at -20°C until ready to be used for enzyme purification.

Preparation of caput and cauda sperm PMs

The salt-washed PM-rich fraction from caput and cauda spermatozoa was prepared according to our published procedures [14]. In brief, the spermatozoa were disrupted by nitrogen cavitation in a Parr Disruption Bomb No. 4635 (Parr Instrument, Moline, IL, U.S.A.), and the released PMs purified by discontinuous sucrose density gradient centrifugation [14].

Preparation of [³H]Gal-labelled fetuin and ovomucoid substrates

[³H]Galactose was incorporated into 20 mg of asialoagalactofetuin or -ovomucoid essentially as described [9]. Briefly, the asialoagalactofetuin or asialoagalacto-ovomucoid was mixed with 10 μCi of UDP[³H]Gal and 0.5 unit of bovine milk galactosyltransferase in a total volume of 0.3 ml containing 100 mM sodium cacodylate buffer, pH 7.0, 40 mM 2-mercaptoethanol, 10 mM MnCl₂, and 2 mM ATP. The reaction mixture was incubated at 37°C for 24 h under two drops of toluene. Following the reaction, the mixture was kept at 60°C for 30 min to inactivate the galactosyltransferase. The sample was washed by precipitating with 10% trichloroacetic acid and then in ethanol/ether (1:1, v/v) as previously described [9]. The [³H]Gal-

labelled glycoproteins were dried under nitrogen, suspended in 2 M NH₄OH and neutralized with acetic acid. The neutralized sample was exhaustively dialysed at 4°C for 48 h, divided into small aliquots and stored frozen at -20°C .

Analytical SDS/PAGE

The purified β -D-galactosidases or sperm PM-enriched fractions were analysed by the SDS/PAGE system of Laemmli carried out under reducing conditions [15]. Polypeptides separated as above were identified either by staining with Coomassie Brilliant Blue (CBB) or silver nitrate according to the method of Merrill et al. [16].

Western blot analysis

The polypeptides separated by SDS/PAGE were electrophoretically transferred to a nitrocellulose membrane according to the method of Towbin et al. [17] as described before [10]. After the transfer, resolved polypeptides were identified by their ability to bind either the lectin (PNA) or the primary antibody (immunodetection). For the detection of PNA-binding sites, the nitrocellulose membrane was processed sequentially as follows. The membrane was equilibrated for 15 min in 50 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl, 10 mM CaCl₂ and 0.1% Tween-20. The membrane was incubated for 2 h in the above buffer containing 1% BSA, to block non-specific binding sites. After washing once in the above buffer, the nitrocellulose membrane was incubated with biotinylated-PNA (10 $\mu\text{l/ml}$) for 2 h followed by washing extensively in the above buffer containing 0.1% BSA, and then two times in the buffer alone. The washed membrane was incubated for 1 h in Vectastain reagent (Vectastain ABC Kit PK 4000, Vector Laboratories) and washed five times in Tris/HCl buffer alone. The lectin-binding sites were visualized by staining with diaminobenzidine solution (2.4 mM) in the above Tris/HCl buffer. The reaction was stopped by rinsing the membrane in distilled water. Goat anti-mouse liver β -D-galactosidase antibody was used as the primary antibody for the immunodetection of the 84 and the 97 kDa molecular forms by the procedure described previously [10] using biotinylated secondary antibody (Vectastain ABC kit, 4005).

Densitometry

The lectin blot was photographed, and each lane from the print was scanned using a Microtek Image Scanner (ScanMaker^B IIXE). The resulting image was digitized into an Apple Macintosh IIfx computer, and the relative density was traced using the NIH image program (version 1.33f) as previously described [18].

N-terminal amino acid sequence analysis

The purified β -D-galactosidases were separated by SDS/PAGE under reducing conditions and the resolved polypeptides electrotransferred to a polyvinylidene difluoride membrane [Problot, Applied BioSystems (ABI), Foster City, CA, U.S.A.] as described [17]. The polypeptide transblotted to the membrane was stained using CBB, then destained in 50% methanol. The visible band was excised and subjected to automated Edman degradation on ABI 475A protein sequencer equipped with an ABI 120A on-line analyser. Sequence data were collected and analysed using an ABI 900A data system.

Enzymic digestion of β -D-galactosidases

Endo H, endo F, and N-glycanase digestions were carried out according to the manufacturer's instructions in the presence of

Figure 1 Separation of acid β -D-galactosidase activities by ion-exchange column chromatography

The clear supernatant obtained after dialysis and high speed centrifugation of rat epididymal luminal fluid (see the Materials and methods section) was applied to a DE-52 column (1 cm $\times$ 22 cm) equilibrated with buffer A. After the column was washed with buffer A, it was eluted with a linear NaCl gradient. The enzyme activities separated into effluent (peak I) and NaCl-eluted (peak II) fractions. Other details are described in the Materials and methods section.

10 μ l toluene as described before [18]. Endo- β -galactosidase digestion was carried out by incubating each form at 37 $^{\circ}$ C with 10 m-units of the endo-enzyme in a total volume of 30 μ l containing 50 mM sodium citrate buffer, pH 5.5, 1.3 % Nonidet P-40 and 10 μ l toluene. Additional enzyme (10 m-units) was added after 24 h, and the mixture incubated for an additional 24 h at 37 $^{\circ}$ C. Following enzymic digestions, the samples were mixed with SDS buffer and processed for SDS/PAGE as above.

Treatment of caput sperm PMs with the purified β -D-galactosidase

Rat caput sperm PMs suspended in 100 mM sodium cacodylate buffer were heat treated (80 $^{\circ}$ C for 1 h) to inactivate endogenous β -D-galactosidase and proteases. Aliquots containing 75 μ g of membrane proteins were mixed with the purified enzyme (4 units of 97 kDa form) and 10 μ l toluene, and the mixture was incubated at 37 $^{\circ}$ C. Additional enzyme (4 units) was added after incubation for 4 h and 8 h, and the incubation continued for 12 h. The reaction was stopped by heat treatment at 80 $^{\circ}$ C for 10 min and the samples processed for SDS/PAGE as described above.

Enzyme assays

Unless otherwise indicated, PNP β -D-galactosidase (PNP galactosidase) activity was assayed by measuring the release of *p*-nitrophenol in a standard incubation mixture (0.5 ml) containing 5 mM substrate, enzyme, and 100 mM sodium citrate buffer, pH 3.5, as described before [19]. After incubation at 37 $^{\circ}$ C for the desired time, the reaction was stopped by the addition of 1.0 ml of alkaline buffer containing 0.133 M glycine, 0.083 M Na_2CO_3 and 0.067 M NaCl, pH 10.7. The released *p*-nitrophenol was quantified by measuring the absorbance at 400 nm. Enzyme and substrate blanks were run in all assays. One unit was the amount of enzyme which catalysed the release of 1 μ mol of *p*-nitrophenol/h.

[^3H]Gal- β -D-galactosidase activity was assayed by measuring the hydrolysis of [^3H]Gal-labelled fetuin or ovomucoid (approx. 5000 c.p.m.) in a standard incubation mixture (0.1 ml) by our published procedure [9]. The cleaved [^3H]galactose was separated from the protein as previously described [9] and the radioactivity

measured by liquid scintillation spectroscopy, the counting efficiency of which was 48 % for tritium. Substrate blanks incubated under similar assay conditions showed little (< 0.2 %) release of total radioactivity as free [^3H]Gal. One unit was the amount of enzyme that cleaved 5 % of the added substrate/h.

Carbohydrate analysis

Total carbohydrate in unhydrolysed enzyme samples was assayed by the phenol/sulphuric acid method [20], scaled down 4-fold with mannose as standard. Sialic acid was assayed by the procedure of Warren [21] as described previously [22]. Briefly, the purified forms of β -D-galactosidase (50–70 μ g of enzyme protein) were hydrolysed in 0.05 M H_2SO_4 for 1 h at 80 $^{\circ}$ C. Standard *N*-acetylneuraminic acid was subjected to the same acid treatment. The released sialic acid was quantified by the thiobarbituric acid method [21].

Protein was measured by the colorimetric method of Bio-Rad according to the manufacturer's instructions with BSA as standard.

Purification of epididymal fluid β -D-galactosidases

Unless otherwise indicated, all purification steps were carried out at 0–4 $^{\circ}$ C. The following buffers were used throughout the purification scheme: buffer A, pH 7.0 (10 mM Tris/HCl buffer); buffer B, pH 4.0 (25 mM sodium acetate buffer containing 100 mM NaCl); buffer C, pH 4.3 (20 mM potassium phosphate/citrate buffer containing 100 mM NaCl); buffer D, pH 4.3 (same as buffer C but containing 7 M urea).

Step 1: separation of two forms of β -D-galactosidases by DE-52 column chromatography

The frozen epididymal fluid (approx. 50 ml) prepared from 11–12 rats (see above) was thawed at room temperature, and the slightly turbid fluid was dialysed against 50 vol. of buffer A for 6 h with four changes of the buffer. After dialysis, the sample was subjected to high-speed centrifugation (105 000 *g* for 30 min). The clear supernatant was removed by aspiration and applied to a DE-52 column (1 cm $\times$ 22 cm) equilibrated with the above

Figure 2 Purification of the separated β -D-galactosidases by chromatography on a column of Bio-Rad A 0.5 m

The enzyme present in the pooled fractions of peak I and peak II (Figure 1) was precipitated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitated enzyme was collected by high-speed centrifugation. The residue from peak I and peak II was suspended in 3 ml of buffer B and the suspension was centrifuged at 105 000 g for 30 min. The clear supernatant was resolved by gel filtration on a column of Bio-Rad A 0.5 m (1.5 cm $\times$ 87 cm) equilibrated with 25 mM sodium acetate buffer, pH 4.0 containing 100 mM NaCl. Fractions (2.4 ml) were collected at a flow rate of 12 ml/h. Aliquots from each fraction were analysed for PNP β -D-galactosidase activity and protein. The elution profile of the enzyme activity and protein present in peak I and peak II are shown in (a) and (b) respectively. Standards (1–7) are: 1, Blue Dextran-2000; 2, ferritin (M_r 440 000); 3, catalase (M_r 232 000); 4, aldolase (M_r 158 000); 5, monomer of BSA (M_r 68 000); 6, ovalbumin (M_r 43 000); and 7, ribonuclease A (M_r 13 700).

buffer A. The column was eluted with buffer A at a flow rate of 15 ml/h until the absorbance at 280 nm was negligible. The column was then eluted with 100 ml of a linear NaCl gradient

(0–0.4 M) in buffer A. Fractions (2 ml) were collected at a flow rate of 15 ml/h. Aliquots from the alternate fractions of effluent and salt-gradient eluted fractions were checked for PNP galactosidase activity. The enzyme activity separated into two peaks as shown in Figure 1. The enzyme-rich fractions from peak I and peak II were pooled separately and used for further purification.

Step 2: molecular-sieve column chromatography

The β -D-galactosidase activity from the pooled fractions of Peak I and Peak II (Figure 1) was precipitated by adding solid $(\text{NH}_4)_2\text{SO}_4$ to achieve 70% saturation. The samples were kept on ice for 15–30 min, and the precipitated enzyme was collected by high-speed centrifugation (105 000 g for 30 min). The precipitated enzyme from the Peak I and Peak II fractions was suspended in 2.5–3.0 ml of buffer B and the suspension was centrifuged at 105 000 g for 30 min. The clear supernatant was removed by aspiration and resolved by gel filtration on a column of Bio-Rad A 0.5 m (1.5 cm $\times$ 87 cm) equilibrated with buffer B. Other details are in the legend to Figure 2. The enzyme-rich fractions from Figures 2(a) and 2(b) were pooled, and dialysed against 50 vol. of buffer C for 6 h with three changes.

Step 3: affinity column chromatography

The dialysed samples from Step 2 were separately applied to an immobilized *p*-aminophenyl β -D-thiogalactopyranoside column (1 cm $\times$ 14 cm) equilibrated with buffer C. The sample was applied at a flow rate of 4–5 ml/h followed by washing with buffer C. After the column had been washed with 60–70 ml of buffer C, it was eluted with buffer D. Fractions (3 ml) were collected at a flow rate of 6 ml/h. The enzyme activity routinely eluted in fractions 5–10.

The enzymatically active fractions were pooled and concentrated to 0.4 ml using a microconcentrator. The concentrated enzyme was dialysed against buffer A containing 0.1 M NaCl and 0.02% sodium azide, then stored at 0–4 °C.

Data obtained from a typical purification experiment are presented in Table 1. The two molecular forms of β -D-galactosidase were purified nearly 170-fold with a recovery of 9–10%.

RESULTS

Purification of β -D-galactosidases

Rat epididymal fluid was separated by ion-exchange chromato-

Table 1 Purification of rat epididymal luminal fluid β -D-galactosidases

The fluid was obtained from the epididymides of 12 rats. Various fractions obtained as described in the Materials and methods section were assayed at pH 3.5 with 5 mM PNP β -D-galactopyranoside.

Fraction	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg)	Enzyme recovery (%)	Purification (-fold)
Epididymal luminal fluid	190	1363	7.2	100	1
Separation of 84 kDa and 97 kDa β -D-galactosidase forms by DE-52 chromatography					
Effluent (peak I, 84 kDa form)	52	520	10.0	38	1.4
Salt-eluate (peak II, 97 kDa form)	26	720	26.7	52	3.8
Bio-Rad A 0.5 m chromatography					
84 kDa form (Figure 2a)	10.2	410	41.0	30	5.7
97 kDa form (Figure 2b)	7.4	650	87.8	48	12.2
Affinity chromatography					
84 kDa form	0.10	121	1210.0	9	168
97 kDa form	0.14	170	1214.0	10	169

Figure 3 Electrophoretic behaviour of the purified β -D-galactosidase

The purified, concentrated enzyme after affinity column chromatography was resolved by SDS/PAGE. The electrophoresis was performed using 5–10% linear gradients of acrylamide with the SDS system (reducing conditions) of Laemmli [15] as previously described [8]. Following electrophoresis, gels were stained for protein using silver nitrate as described in the Materials and methods section. Lane 1, 0.96 μ g of enzyme protein purified from peak I; lane 2, 1.39 μ g of enzyme protein purified from peak II. The position of prestained standard marker proteins is shown on the left.

graphy on a column of DE-52 (Figure 1). The β -D-galactosidase activities present in the effluent fractions (peak I) and the salt-eluate fractions (peak II) were further purified by gel filtration on a column of Bio-Rad A 0.5 m (Figure 2), followed by affinity chromatography on a column of immobilized *p*-aminophenyl β -D-thiogalactopyranoside. The two forms of β -D-galactosidase eluted from the affinity column with 7 M urea routinely showed similar specific activity ranging from 1150 to 1270 units/mg of protein (Table 1).

Purity of the concentrated enzymes

The purity of the two molecular forms of β -D-galactosidase was examined by SDS/PAGE under denaturing conditions followed by staining for proteins. Each form resolved into a broad protein

band as revealed by silver staining (Figure 3). Apparent molecular masses of 84 kDa (lane 1) and 97 kDa (lane 2) were estimated via extrapolation from the relative mobility (R_f) versus log molecular mass curve of the standards shown in Figure 3. The 84 kDa form was virtually free of the 97 kDa form. However, the latter isoform appeared to contain a small amount of the 84 kDa form. The 97 kDa value reported here was calculated using the mid-point of the high molecular mass band rather than the broad band shown in lane 2.

Kinetic properties of the purified β -D-galactosidase activities

Both molecular forms of β -D-galactosidase were very stable when stored at 0–4 °C in concentrated enzyme solutions (0.2–0.3 mg of protein/ml of 10 mM Tris/HCl buffer containing 0.1 M NaCl and 0.02 % sodium azide). The two forms retained all of the enzymic activities even after 3 months. However, nearly complete inactivation of both forms occurred if the purified enzymes were stored frozen at –20 °C even for a few days.

The β -D-galactosidase activity present in the epididymal luminal fluid was found to optimally cleave PNP β -D-galactoside at an acidic pH of 3.5, and [3 H]Gal-fetuin at a nearly neutral pH of 6.4–6.8 [9]. The separation of β -D-galactosidase activity into two molecular forms raised the possibility that these isoforms may have different substrate preferences. It was therefore important to determine the pH-dependent substrate preference for the two isoforms. The effect of pH on PNP galactosidase and [3 H]Gal galactosidase activities has been presented in Figure 4. It was obvious that the two forms had similar pH-dependent substrate preferences. Both forms showed maximum hydrolysis of the PNP β -D-galactoside substrate at an acidic pH of 3.5, and less than 10 % of the substrate was cleaved at pH 6.5 and higher. In contrast, the [3 H]Gal-fetuin was maximally cleaved between pH 6.4 and 6.8. The [3 H]Gal-ovomucoid substrate was optimally cleaved by the two forms at a pH slightly lower than that for [3 H]Gal-fetuin (Figure 4). Nonetheless, the two forms of β -D-galactosidase had a similar pH-dependent substrate preference.

The substrate concentration studies using PNP galactoside generated a linear double-reciprocal plot for the two forms with an apparent K_m of 0.34 for the purified 84 kDa and 97 kDa forms. This value was quite similar to the value of 0.52 mM obtained with the crude luminal fluid β -D-galactosidase [9].

Figure 4 Effect of pH on purified β -D-galactosidase activities

The purified, concentrated enzyme [(a) 84 kDa form; (b) 97 kDa form] was mixed with 5 mM PNP β -D-galactopyranoside substrate or approx. 5000 c.p.m. of glycoprotein ([3 H]Gal-fetuin or [3 H]Gal-ovomucoid) substrates in the appropriate buffer as follows: 0.1 M sodium citrate, pH 3.0–3.8, 0.1 M sodium acetate buffer, pH 4.0–6.0 or 0.1 M sodium cacodylate buffer, pH 6.2–8.0. The reaction mixture was incubated at 37 °C for 15–60 min and the enzymic activities assayed as described in the Materials and methods section. Maximum activity was 2.5 units with PNP β -D-galactoside and 6.2 and 5.4 units with [3 H]Gal-fetuin and [3 H]Gal-ovomucoid substrates, respectively. Substrates are: PNP β -D-galactoside (●); [3 H]Gal-fetuin (▲); [3 H]Gal-ovomucoid (△). Other details are described in the Materials and methods section.

Figure 5 pH-dependent association/dissociation of the purified (97 kDa form) β -D-galactosidase

Purified β -D-galactosidase (50 units of PNP galactosidase) in 1 ml of the appropriate buffer (20 mM containing 50 mM NaCl and 0.02% sodium azide) was fractionated on a column of Bio-Gel A 0.5 m (1.5 cm $\times$ 89 cm) equilibrated with the sample buffer. The column was eluted each time with the sample buffer. Fractions (2.4 ml) were collected at a flow rate of 12 ml/h. Aliquots from each fraction were analysed for the PNP galactosidase and [3 H]Gal galactosidase activities under optimal conditions. The buffers are: pH 4.0–5.6, sodium acetate; pH 6.2–7.4, sodium cacodylate. The letters T, D and M indicate expected elution positions of tetrameric, dimeric and monomeric forms of β -D-galactosidase, respectively. The numbers in each panel represent standard M_r marker proteins which are the same as in Figure 2. The molecular mass of β -D-galactosidase (β -Gal; shown by arrow) as a function of pH was determined from the slope of elution behaviour versus molecular properties of the marker proteins: K_{av} versus molecular mass on a logarithmic scale. The K_{av} value was calculated by using the equation: $K_{av} = V_0 - V_i \div V_i - V_0$, where V_0 is the elution volume for β -D-galactosidase and the marker proteins. V_0 (void volume) and V_i (total volume of the gel bed) were determined using Blue Dextran-2000, and Phenol Red dye, respectively.

Table 2 Partial amino acid sequence of β -D-galactosidases from epididymal luminal fluid*

Phe	Tyr	Xaa†	Val	Ser	Gln	Arg	Thr	Phe	Leu	Glu	84 kDa
Phe	Tyr	Xaa	Val	Ser	Gln	Arg	Thr	Phe	Leu	Glu	97 kDa
Ile	Tyr	Asn	Val	Thr	Gln	Arg	Thr	Phe	Leu	Lys	Mouse β -Galactosidase precursor

* Alignment of partial amino acid sequence data obtained from analysis of the 84 and 97 kDa molecular forms, and mouse β -D-galactosidase precursor as reported previously [23]. Identical amino acid residues are boxed.

† Xaa, unidentified. It could be Asn but is unidentified due to glycosylation.

Similar substrate-concentration studies using [3 H]Gal-glycoprotein as substrate also generated a linear reciprocal plot for the [3 H]Gal-fetuin with an apparent K_m of 3.5 μ g/0.1 ml of the assay mixture for both forms. The two forms showed some differences when [3 H]Gal-ovomucoid was used as substrate. K_m values of 4.5 μ g/0.1 ml, and 7.1 μ g/0.1 ml were obtained for the 84 kDa and 97 kDa forms, respectively.

In a previous report [9] we presented evidence indicating that rat epididymal β -D-galactosidase existed in dissociated forms under neutral conditions and in associated forms under acidic conditions. In the present study, we found that both molecular forms eluted from the gel filtration column as tetramers under acidic conditions. A detailed study carried out to examine pH-

dependent association/dissociation of the 97 kDa form of β -D-galactosidase is shown in Figure 5. The purified enzyme existed in associated forms under acidic conditions (pH 4.0–5.6) and dissociated forms in less acidic (pH 6.2) and neutral (pH 6.8 and 7.4) conditions.

Amino acid sequence analysis

Results of N-terminal amino acid sequence analysis presented in Table 2 have shown an identical amino acid sequence for the two molecular forms. In data base searches of the terminal sequences, a significant similarity (residue identity 63%) was found with mouse β -D-galactosidase precursor [23].

Table 3 Carbohydrate composition of β -D-galactosidase from rat epididymal fluid*

Composition	84 kDa β -D-galactosidase (mg/100 mg)	97 kDa β -D-galactosidase (mg/100 mg)
Total sugars	13.4	28.3
<i>N</i> -acetylneuraminic acid	1.9	6.3

* Data are means of two separate experiments.

Figure 6 Assessment of oligosaccharide moieties of purified galactosidases

The purified enzyme, (a) 1.2 μ g of protein of the 84 kDa form, (b) 1.74 μ g of protein of the 97 kDa form, was treated with (lanes 2–5) or without (lane 1) endo-enzymes as described in the Materials and methods section. Following the treatment, the sample was mixed with SDS buffer, resolved by electrophoresis on SDS/PAGE (7.5–12.5% acrylamide gradient) under denaturing conditions, and transferred to a nitrocellulose membrane. The bands were visualized by immunodetection using goat anti-mouse acid β -D-galactosidase as primary antibody. Other details are described in the Materials and methods section. Endo β -gal = endo- β -D-galactosidase.

Carbohydrate analysis

The appearance of the two enzymes as broad bands in Figure 3 suggests to us that the two molecular forms are glycosylated. Results of carbohydrate analysis presented in Table 3 show that the 97 kDa form contains twice as much total sugars, and nearly three times higher sialic acid as the 84 kDa isoform. The observed difference reflects differential glycosylation of the two molecular forms (also see below).

Asparagine N-linked glycans of β -D-galactosidases

In this study we report results of experiments carried out to quantitatively characterize the N-linked oligosaccharide moieties of the purified β -D-galactosidases. The two molecular forms of β -D-galactosidase were digested in the absence (lane 1, Figures 6a and 6b) and presence (lanes 2–5, Figures 6a and 6b) of a variety of endo-enzymes as described in the Materials and methods section, mixed with SDS buffer, and resolved on SDS/PAGE. The resolved peptides were electrophoretically transferred to a

Figure 7 Lectin (PNA) blot analysis of caput and cauda sperm plasma membranes

Plasma membranes were prepared from caput and cauda spermatozoa as described in the Materials and methods section. Aliquots from the membrane suspension containing 50 μ g of membrane protein were electrophoresed on SDS/PAGE (7% acrylamide) under reducing conditions. Following transfer to a nitrocellulose sheet, the blot was probed with biotinylated-PNA and the PNA-positive bands revealed with Vectastain reagents followed by staining with diaminobenzidine as described in the Materials and methods section. Lane 1, caput sperm PM; lane 2, cauda sperm PM. The position of standard marker proteins is shown on the left. The arrow indicates the position of PNA-positive glycoprotein of an apparent molecular mass of 135–150 kDa present in the caput (but not cauda) sperm PMs.

nitrocellulose sheet, and the immunoreactive polypeptides detected using goat anti-mouse β -D-galactosidase as primary antibody [10]. Results from a typical experiment are summarized as follows. (1) Treatment of the 84 kDa and 97 kDa forms with N-glycanase (an endo-enzyme known to cleave all types of N-linked glycans including high mannose/hybrid, bi-, tri-, and tetra-antennary complex-type oligosaccharides) (lane 2), or endo F (an endo-enzyme known to cleave only high mannose/hybrid-type and N-linked biantennary complex-type) (lane 3), caused the two forms to resolve at an apparent molecular mass of 70 kDa. This result was consistent with the carbohydrate composition of the two β -D-galactosidases and indicated that the two molecular forms are differentially N-glycosylated. Moreover, the fact that digestion of the two molecular forms with N-glycanase or endo F gave similar polypeptides (apparent molecular mass 70 kDa) suggests that the two forms mainly contain high mannose/hybrid-type, and biantennary complex-type oligosaccharides. (2) The two forms of β -D-galactosidase are sensitive to endo H (lane 4), a result indicating that the two forms contain high mannose/hybrid-type glycans. It should be noted that the two molecular forms show some difference in resistance to the endo H treatment. This result suggests that the 97 kDa form contains more biantennary glycans than the 84 kDa form. (3) The two isoforms appear to be resistant to endo β -D-galactosidase (lane 5), an endo-enzyme known to cleave repeat units of lactosamine [24], a result suggesting that the two forms of β -D-galactosidase do not contain N-linked polylactosaminylated glycans.

Functional studies on the potential role of the β -D-galactosidases

The availability of homogeneous preparations of luminal fluid β -D-galactosidases has allowed us to examine the potential role of the enzymes in modification of sperm surface glycoproteins. In the first set of experiments, the caput and cauda sperm plasma

Figure 8 Time course of the treatment of caput sperm PMs with the purified β -D-galactosidase

Rat caput sperm PMs suspended in 100 mM sodium cacodylate buffer were heat treated (80 °C for 1 h). Aliquots from the mixture containing 50 μ g of membrane protein were digested with (a) or without (b) the purified (97 kDa form) β -D-galactosidase as described in the Materials and methods section. At the indicated time intervals, the reaction was stopped by heat treatment at 80 °C for 10 min, and the samples were processed by SDS/PAGE (7% acrylamide gel). The resolved peptides were transferred to a nitrocellulose sheet and the blot probed with biotinylated-PNA as described in the Materials and methods section. The position of standard marker proteins is shown on the left.

membranes were resolved by SDS/PAGE (under reducing conditions), and the glycoproteins containing a terminal galactosyl residue were detected by PNA-binding as described in the Materials and methods section. Data presented in Figure 7 are in general agreement with an earlier report [7] and show that the PNA-lectin bound to several glycoproteins on the caput and cauda sperm PMs. Several of these glycoproteins showed higher affinity for the lectin in the sperm plasma membranes from caput than cauda. However, whereas a glycoprotein of an apparent molecular mass 135–150 kDa could be detected in the caput sperm plasma membranes, this glycoprotein was not present in the cauda sperm plasma membranes.

Figure 9 Densitometry scanning of Figure 8

Each lane of the Figure was scanned as described in the Materials and methods section. Other details are the same as in Figures 8(a) and (b). The arrowhead in (a) indicates the position of the 135–150 kDa PNA-binding glycoprotein. Note its preferential disappearance with increasing time of treatment.

Next, we attempted to modify the glycoprotein by treatment *in vitro* of the caput sperm plasma membranes with the purified (97 kDa) acid β -D-galactosidase. In these studies, caput sperm plasma membranes were treated with or without the enzyme for various time periods as described in the Materials and methods section. Following the treatment, the samples were heat-treated in SDS buffer, resolved by SDS/PAGE, electrotransferred to nitrocellulose sheets, and the glycoproteins detected by staining with biotinylated-PNA. Data from this study presented in Figures 8 and 9 show a decrease in caput specific PNA-positive glycoproteins. However, the 135–150 kDa band is the most sensitive to the β -D-galactosidase treatment, which virtually disappears after 12 h of incubation.

DISCUSSION

Acid β -D-galactosidase is an exo-glycosidase that cleaves β -linked terminal galactosyl residues from a variety of natural and artificial substrates [25]. The enzyme has been reported in all mammalian tissues examined [26], including male reproductive tissues [27]. Recently, we have reported that the β -D-galactosidase activity present in luminal fluid exists in dissociated form and is optimally active towards a glycoprotein ($[^3\text{H}]\text{Gal-fetuin}$) substrate at the pH of the epididymal lumen [9]. Subsequently, we showed that the luminal fluid β -D-galactosidase exists in two molecular forms, a 97 kDa form and an 84 kDa form [10]. Since spermatozoa in the epididymis are in contact with luminal fluid, we proposed that β -D-galactosidase may degalactosylate sperm PM glycoproteins [9]. In this report, we have separated and purified the two molecular forms, and, attempted to: (1) further characterize purified forms of rat epididymal fluid β -D-galactosidase activity, and (2) examine the potential role of the purified enzymes in modification of sperm surface glycoproteins.

The presence of two forms raised the possibility that, although immunologically similar [10], they may be catalytically different with different kinetic properties. Attempts were then made to separate the two activities by ion-exchange chromatography on a column of DE-52. After several attempts under various conditions of buffer and pH, the 10 mM Tris/HCl buffer at pH 7.0 allowed the separation of two activities. The differential binding of the two forms to the DE-52 column provided two pools of activity (Figure 1). The separated activities were then purified nearly 170-fold by gel filtration followed by affinity chromatography on a column of *p*-aminophenyl β -D-thiogalactopyranoside. The overall recovery of each form was only 9–10% (Table 1). However, it should be noted that most of the enzyme activity loss occurred during affinity column chromatography (under 30% recovery for the two forms). The low recovery is likely to be due to the use of a denaturing agent (7 M urea) in the elution buffer which may have caused partial inactivation of the enzyme activity.

The two forms of purified β -D-galactosidase activities, when subjected to SDS/PAGE under reducing conditions, resolved into broad bands of 84 kDa and 97 kDa. Despite the difference in their molecular masses, several lines of evidence presented in the Results section indicated that the two molecular forms were chemically and catalytically very similar. First, the two forms showed similar K_m values for the PNP β -D-galactoside and glycoprotein ($[^3\text{H}]\text{Gal-fetuin}$ and $[^3\text{H}]\text{Gal-ovomucoid}$) substrates. Second, both forms showed pH-dependent substrate preference, optimally cleaving the artificial substrate at acidic pH (3.5) and glycoprotein substrates at a significantly higher pH. Third, the N-terminal sequence analyses studies gave identical amino acid sequences for the two forms with a 63% residue identity with the

mouse β -D-galactosidase precursor. Fourth, de-N-glycosylation of the two forms by treatment with N-glycanase or endo F generated a similar polypeptide of an apparent molecular mass of 70 kDa. Finally, the two isoforms cross-reacted with the anti-mouse liver acid β -D-galactosidase antibody. These similarities allowed us to suggest that the two isoforms were immunologically and catalytically very similar and were probably derived from a common precursor.

Despite similarities, the two isoforms were significantly different in total carbohydrate content. The observed differences could be accounted for by the presence of varying amounts of high mannose/hybrid-type and complex-type glycans. This difference in the N-linked oligosaccharide chains, as expected, had no detectable effect on the catalytic properties of the two isoforms.

We have previously reported [9] that the unpurified epididymal luminal fluid β -D-galactosidase, like β -D-galactosidases from other tissues [28–30], undergoes pH-dependent association/dissociation. Data have suggested that in the epididymal lumen, where the pH is reported to be 6.6–6.8 [31], the enzyme is present in dissociated (monomeric/dimeric) forms and is therefore maximally active towards glycoprotein substrate. In this report we have confirmed the pH-dependent substrate preference and pH-dependent association/dissociation using the purified β -D-galactosidase and shown that dissociation occurs at pH 6.2 and higher. It should be noted that although [3 H]Gal-fetuin and [3 H]Gal-ovomucoid show somewhat different pH optimum curves, the two glycoprotein substrates are optimally cleaved at a pH which favours dissociation.

The epididymal fluid obtained from different regions of the epididymis has been shown by us to contain different molecular forms of β -D-galactosidase. For instance, the proximal and distal caput fluid contain one form (97 kDa), corpus and proximal cauda fluid contain two forms (97 kDa and 84 kDa) and the distal cauda fluid contains mainly the 84 kDa form. Since the two forms are different in oligosaccharide moieties, it is possible that the 97 kDa form present in the proximal regions of the epididymis is modified to the 84 kDa form by the cleavage of oligosaccharide chains. Alternatively, the two molecular forms may be selectively synthesized and secreted in various regions of the epididymis. Additional studies are needed to resolve this issue.

In human fibroblasts [32] and rat epididymal epithelial cells [33], the acid β -D-galactosidase is synthesized in an 84–85 kDa precursor form which is processed via intermediates into a mature form of an apparent molecular mass of 63–64 kDa. Similarly, precursor forms (82–84 kDa forms) are processed to a mature enzyme (63 kDa) secreted by the mouse macrophages [34]. In lysosomes, it has been reported that a 'protective protein' interacts with β -D-galactosidase monomers affecting their multimerization into a high-molecular-mass aggregate of 600–800 kDa [35]. We have obtained no evidence for the presence of high molecular mass aggregates or the presence of a protective protein of 32–40 kDa observed by others in several tissues [35]. Furthermore, the apparent molecular mass of luminal fluid acid β -D-galactosidase monomers (84 kDa and 97 kDa) and de-N-glycosylated monomers (70 kDa) is significantly higher than the low levels of the monomer (63 kDa) form of the enzyme present in the rat epididymal luminal fluid [9], and secreted from the human fibroblasts [32], rat epididymal epithelial cells [33], and mouse macrophages [34]. Whether the 63 kDa form is a normal physiological product is not yet known. Nonetheless, these differences indicate that the 97 kDa and 84 kDa forms secreted in the luminal fluid are chemically different from the 63 kDa form of the acid β -D-galactosidase.

Spermatozoa from proximal regions of the epididymis are

unable to bind to zona pellucida, an extracellular glycocalyx which surrounds the egg PM, and fertilize the egg [2]. The fertilizing ability is acquired as the sperm plasma membrane undergoes biochemical modifications in the distal corpus and cauda epididymidis. Although all details of these modifications are not yet known, there is growing evidence that sperm surface glycoproteins are modified during sperm maturation [3–8,36]. Two sets of glycoprotein-modifying enzymes, namely glycosyltransferases and glycohydrolases, present in high concentration in the luminal fluid, are believed to be involved in the glycosylation/deglycosylation of sperm surface glycoproteins [8,25]. The former enzymes are considered synthetic enzymes as they modify existing glycoproteins by adding sugar residue(s) from the nucleotide sugar (sugar donor) to the sugar acceptor glycoproteins [37,38] and the latter enzymes are considered degradative as they modify existing glycoproteins by cleaving terminal sugar residue(s). Acid β -D-galactosidase activities described here will be expected to modify the existing glycoproteins by cleaving terminal β -D-galactosidase residues (degactosylation).

A recent report from another laboratory has presented evidence suggesting that the caput sperm PM glycoprotein(s) may be degactosylated as spermatozoa transit from caput to cauda epididymidis [7]. Since the reported modification of the caput sperm surface glycoproteins may be important for developing functionally mature spermatozoa, it was important to determine whether the degactosylation is caused by the luminal fluid β -D-galactosidases. Treatment of the caput sperm PM *in vitro* with the purified β -D-galactosidase resulted in a gradual and preferential disappearance (degactosylation) of a 135–150 kDa PNA-reactive band. Other PNA-positive glycoproteins decreased also with increased time of treatment but not to the same extent as the 135–150 kDa band which virtually disappeared after 12 h of treatment. It should be noted that only the 97 kDa isoform was used for digestion of caput sperm PMs *in vitro*. This molecular form was chosen primarily because it is present in the fluid from different regions of epididymis [10]. Moreover, since the two forms were catalytically very similar, it was reasonable to assume that the digestion of caput sperm plasma PMs *in vitro* with the 84 kDa form would give results similar to those reported with the 97 kDa form.

We do not know at the present time whether the removal of galactosyl residue(s) from the 135–150 kDa glycoprotein of caput sperm plasma membrane is physiologically significant. It will be interesting to chemically characterize the glycoprotein and examine its localization and function. Nonetheless, this study demonstrates that purified luminal fluid β -D-galactosidase can modify caput sperm plasma membrane glycoprotein(s). These data are consistent with the proposed role for the β -D-galactosidase in modification of sperm surface glycoprotein(s) during maturation.

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