Rat epididymal luminal fluid acid β -D-galactosidase optimally hydrolyses glycoprotein substrate at neutral pH

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Several glycosidases, purified and characterized from mammalian tissues, have been shown to be optimally active under acidic conditions when *p*-nitrophenyl (PNP) or 4-methylumbelliferyl glycosides are used as substrates. Although high levels of the glycosidases are present in the epididymal lumen, their physiological role remains uncertain. To be functional, the glycosidases are expected to be enzymically active at or near the physiological pH of luminal fluid. In this report, we demonstrate that the rat epididymal luminal fluid β -D-galactosidase, optimally active toward PNP β -D-galactoside at pH 3.5, shows maximum activity towards a glycoprotein substrate ([Gal- 3 H]fetuin) at neutral pH. Several lines of evidence, including immunoprecipitation studies using antibody to the acid β -D-galactosidase, and substrate competition studies, indicate that PNP galactosidase and [3 H]Gal galactosidase activities are caused by a single enzyme, and that the two substrates are probably cleaved by the same catalytic site(s). Competition studies with various disaccharides indicate that this enzyme is capable of cleaving a variety of galactose linkages found in both *O*- and *N*-linked oligosaccharides. Molecular-sieve column chromatography of the β -D-galactosidase of luminal fluid under several conditions of buffer and pH show that, whereas the enzyme eluted as a tetramer (apparent M_r 320000) under acidic conditions (pH 3.5–4.3), only dimers and monomers (apparent M_r 180000 and 92000 respectively) were observed in neutral conditions (pH 6.8). This aggregation/dissociation phenomenon is reversible. These studies indicate that β -D-galactosidase is present in the luminal fluid in dissociated forms, and is therefore optimally active towards glycoprotein substrates at physiological pH. The potential role of the enzyme in modification of sperm surface glycoproteins is discussed.

INTRODUCTION

Mammalian spermatozoa, as they leave the testis, are unable to fertilize eggs. They acquire progressive motility and fertilizing ability during passage through the epididymis [1,2]. The maturation process in the epididymis involves several biochemical changes including modification of the sperm surface glycoproteins (for reviews see [3–6]). Two sets of enzymes, namely glycosyltransferases, which add sugar residues, and glycosidases, which cleave sugar residues from the existing glycoconjugates, are present in high concentrations in the epididymal luminal fluid that surrounds spermatozoa [7,7a]. Thus, it has been suggested that glycosyltransferases, in conjunction with glycosidases, could have a role in the modification of sperm surface glycoproteins during epididymal maturation [7–11].

Although glycosyltransferases are functional at the physiological pH of the epididymal lumen, glycosidases have only been shown to be optimally active at acidic pH when assayed using the commercially available substrates, *p*-nitrophenyl (PNP) glycosides or 4-methylumbelliferyl glycosides. It is not known whether they would be active with a natural substrate at neutral pH. To investigate whether one of these acid glycosidases could be active toward a glycoprotein substrate, we prepared and used a galactose-labelled glycoprotein substrate ([Gal- 3 H]fetuin). Interestingly, our results indicate that the β -D-galactosidase of epididymal luminal fluid, which optimally cleaves PNP β -D-galactoside at pH 3.5, shows maximum activity at pH 6.8 when [Gal- 3 H]fetuin was used as the substrate. In addition, we present evidence indicating a pH-dependent association/dissociation of the enzyme. Our studies indicate that although the β -D-galactosidase form which is active toward PNP β -D-galactoside shows optimal activity when in an aggregated form, the β -D-galactosidase form which is active towards glycoprotein substrates is optimally active when in a dissociated form. These

results are consistent with the possibility that the β -D-galactosidase present in the epididymal lumen is enzymically active *in vivo*.

EXPERIMENTAL

Materials and chemicals

Male Sprague–Dawley retired breeder rats (Sasco, Omaha, NE, U.S.A.) were kept in our animal facility for several days before an experiment. [4,5- 3 H]UDPGal was from New England Nuclear (Boston, MA, U.S.A.); NCS solubilizer (Amersham, Arlington Heights, IL, U.S.A.); Budget-Solve scintillation cocktail was from Research Products International Corp. (Mount Prospect, IL, U.S.A.); Protein G Sepharose 4FF and concanavalin A-Sepharose 4B (approx. 10 mg of gel sediment/ml) were from Pharmacia (Piscataway, NJ, U.S.A.); DE-52 was from Whatman BioSystems Ltd. (Hillsboro, OR, U.S.A.); Bio-Gel A-0.5m (200–400 mesh) and Protein Assay Kits were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); bovine milk galactosyltransferase and disaccharides were from Sigma (St. Louis, MO, U.S.A.); natural *N*-Glycanase enzyme was from Genzyme (Boston, MA, U.S.A.); silica gel G t.l.c. plates were from Analteck (Newark, DE, U.S.A.). Other reagents were of the highest purity commercially available. Antibody to mouse liver β -D-galactosidase, prepared in goat [12,13], has been shown previously to cross-react with the acid β -D-galactosidase of rat epididymal epithelial cultured cells [8].

Preparation of epididymal luminal fluid

Rats were killed by CO₂ asphyxiation and each epididymis was removed, dissected free of the testis and fat, and used as described in each experiment. For routine assays, the luminal fluid was prepared as previously described [14]. Briefly, the tissue was cut with a sharp razor into pieces approx. 1 mm³ in size whilst in

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Fig. 1. Effect of pH on β -galactosidase activity

The reaction mixture contained rat epididymal luminal fluid (30 μ g of protein) and either 5000 c.p.m. of [3 H]-fetuin substrate or 5 mM-PNP galactoside in the appropriate buffer as indicated: 0.1 M-sodium citrate pH 3.0–3.8, 0.1 M-sodium acetate pH 4.0–6.0, 0.1 M-sodium cacodylate pH 6.2–8.0. The mixture was incubated at 37 °C for 1 h and the two activities assayed as described in the Experimental section. Maximum activity was approx. 1.6 units for PNP galactosidase (○) and 4.8 units for [3 H]Gal galactosidase (●).

0.25 M-sucrose, 0.01 M-Tris/HCl, pH 7.2 (1:2, w/v), to release spermatozoa and luminal fluid. Tissue pieces in buffer were shaken for 15 min at room temperature and were then centrifuged for 2 min at 2700 g . The supernatant was then centrifuged for 20 min at 165000 g (4 °C). The clear supernatant (enriched luminal fluid) was dialysed against the appropriate buffer as described in each experiment and then used in the following studies. In some experiments, luminal fluid from the cauda epididymidis was prepared by retrograde flushing of the vas deferens followed by centrifugation. When the purified luminal fluid was assayed for PNP galactosidase and [3 H]Gal galactosidase, the two activities were similar to those observed in the enriched luminal fluid used in subsequent studies.

Preparation of [3 H]Gal-labelled fetuin substrate

Asialoagalactofetuin was prepared by our previously published method [15]. [3 H]Gal was incorporated into 20 mg of asialoagalactofetuin by incubation of the latter with 10 μ Ci of UDP[3 H]Gal (specific radioactivity 43.3 Ci/mmol) and 0.5 unit of bovine milk galactosyltransferase (1,4-specific) in a total volume of 0.3 ml containing 100 mM-sodium cacodylate buffer, pH 7, 40 mM-2-mercaptoethanol, 10 mM-MnCl₂ and 2 mM-ATP. The reaction was carried out by incubation at 37 °C for 24 h under 2 or 3 drops of toluene. Following incubation, the sample was kept at 60 °C for 30 min to abolish the transferase activity. The sample was then washed extensively by suspending in 2 ml of ice-cold 10% (v/v) trichloroacetic acid and centrifuged for 10 min at 3000 g (six washes). Excess trichloroacetic acid was removed by three washings with ethanol/ether (1:1, v/v) and centrifugation carried out as above. The washed pellet was dried at room temperature under nitrogen, suspended in 2 M-NH₄OH (3 ml) and neutralized with acetic acid. The neutralized sample was dialysed at 4 °C for 48 h against five changes, 100 volumes each, of distilled water. The dialysed samples were counted for 3 H radioactivity and aliquots were frozen at -20 °C.

The chemical nature of the galactose-labelled oligosaccharides in [3 H]Gal-fetuin was established by *N*-Glycanase (Peptide-*N*-glycosidase F) treatment. We incubated 20 μ g of the substrate with 1.0 unit of *N*-Glycanase (following the manufacturer's instructions) for various times up to 12 h, with additions of 0.5

units of *N*-Glycanase every 24 h. Protein was precipitated with three volumes of ethanol at 4 °C, centrifuged for 5 min at 3500 g , the pellet washed with 0.1 ml of 80% (v/v) ethanol and dried. The supernatant was treated with 2 vol. of diethyl ether at 4 °C, centrifuged for 5 min at 3500 g , and the pellet dried at 60 °C. Both pellets were dissolved in water and aliquots were counted for radioactivity.

Assays

PNP β -D-galactosidase activity was determined by measuring the release of *p*-nitrophenol in a standard incubation mixture (0.5 ml) containing 5 mM-PNP β -D-galactoside, 0.2% Triton X-100, and the desired buffer as described in the legend to Fig. 1. After incubation for 1 h at 37 °C, the reaction was stopped by the addition of 1.0 ml of alkaline buffer containing 0.133 M-glycine, 0.083 M-Na₂CO₃ 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 is the amount of enzyme which catalyses the release of 1 μ mol of *p*-nitrophenol/h.

[3 H]Gal β -D-galactosidase activity was assayed by measuring the hydrolysis of [3 H]Gal-labelled fetuin in a standard incubation mixture (100 μ l) containing radiolabelled substrate (approx. 5000 c.p.m.), 0.2% Triton X-100, and 0.1 M of the desired buffer. Following incubation for 1 h at 37 °C, the reaction mixture was placed on ice and the reaction was stopped by adding 10 μ l of 1% (w/v) BSA solution (carrier protein), and 100 μ l of 1% (w/v) phosphotungstic acid prepared in 0.5 M-HCl. After being kept on ice for 10 min, the mixture was centrifuged for 10 min at 3000 g . The supernatant was removed by aspiration, neutralized to pH 7.0 with 2 M-NaOH and used for quantifying released [3 H]Gal. The pellet was washed twice with 0.5 ml of cold 10% (v/v) trichloroacetic acid and centrifugation as above. Trichloroacetic acid was removed from the pellet by suspending the residue in ethanol/light petroleum (b.p. range 37.4–55.4 °C) (1:1, v/v) followed by centrifugation. The washed pellet was air-dried and dissolved in 0.55 ml of 2 M-NH₄OH, neutralized to pH 7.0 with acetic acid, mixed with 1 ml of NCS solubilizer and 15 ml of Budget-Solve and the radioactivity was measured by liquid scintillation spectroscopy, the efficiency of which was 48% for 3 H. Substrate blanks incubated under similar assay conditions, showed less than 0.2% of total radioactivity as free [3 H]Gal. One unit is the amount of enzyme that cleaves 5% of the added substrate/h.

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

T.l.c. analysis

Cleaved product from [3 H]Gal-fetuin substrate in the [3 H]Gal β -D-galactosidase assay was identified by t.l.c. on a glass plate coated with silica gel (20 cm $\times$ 20 cm) as described in [16]. Briefly, plates were pre-run in 0.1 M-sodium bisulphite and allowed to air dry at room temperature. Rat epididymal luminal fluid (1.4 mg of protein), was incubated with [3 H]Gal-fetuin (100000 c.p.m.) for 24 h at 37 °C. The samples were deproteinized with ethanol, centrifuged and lyophilized. Radioactive sample and standard sugars (5 μ g of galactose, glucose or mannose), dissolved in 70% (v/v) ethanol, were pipetted to a reference line on the plate. The plate was allowed to run at room temperature in *n*-butanol/pyridine/water (6:4:3, by vol.) for 5 h. The plates were allowed to air dry, were sprayed with phenol (0.3 M) and sulphuric acid (5%, v/v) in ethanol, were air dried again and were then heated at 100 °C for 10 min to develop the colour. Sections (1 cm²) were scraped, extracted in 0.5 ml of H₂O and centrifuged for 2 min at 6500 g . The pellets were extracted a second time and

centrifuged as above. The combined supernatants were dissolved in 20 ml of Budget-Solve and radioactivity counted. T.l.c. of single samples or co-chromatography of cleavage products and standard sugars together gave similar mobility results.

Concanavalin A-Sepharose 4B, DEAE cellulose (DE-52) and Bio-Gel A-0.5m column chromatography

These columns were prepared according to the manufacturer's instructions. Other details are in the figure legends.

Immunoprecipitation studies

Immunoprecipitation was carried out using our previously published procedure [17] except that Protein G-Sepharose 4FF was the antibody absorbant and 10 mM-Tris/HCl, pH 7.4, with 0.25 M-NaCl was used as buffer in the incubation mixture (150 μ l). The supernatant obtained after centrifugation was assayed for enzyme activity.

RESULTS

β -D-Galactosidase activities in rat epididymal luminal fluid

The previously reported high levels of epididymal β -D-galactosidase activity were determined using the synthetic substrates, PNP galactoside and 4-methylumbelliferyl galactoside [7,7a]. It was of interest to compare the β -D-galactosidase activities in rat epididymal luminal fluid using the PNP galactoside (PNP galactosidase) and the galactose-labelled fetuin ($[^3\text{H}]\text{Gal}$ galactosidase) substrates. The effect of pH on these activities is shown in Fig. 1. It was obvious that when PNP galactoside was used as a substrate, the enzyme showed optimal activity at pH 3.5, and less than 10% of the maximum activity was detected at pH 6.6 and over. In contrast, when $[\text{Gal-}^3\text{H}]\text{fetuin}$ was used as the substrate, optimal cleavage was observed between pH 6.4 and 6.8, a value similar to the physiological pH of rat epididymal luminal fluid [18]. A smaller peak of $[^3\text{H}]\text{Gal}$ galactosidase activity was observed at pH 3.5, the pH optimum of the PNP galactosidase (Fig. 1). These results suggested to us that either the β -D-galactosidase present in the luminal fluid has multiple substrate specificities or that the two substrates are hydrolysed by two different enzymes.

Optimizing conditions for PNP- and $[^3\text{H}]\text{Gal}$ galactosidase assays

Since many glycosidases (including β -D-galactosidase) are known to carry out glycosyl transfer reactions [19], it was important to optimize assay conditions for the two substrates. The amount of radioactivity released as free $[^3\text{H}]\text{Gal}$ was directly

Fig. 2. Effect of substrate concentration on $[^3\text{H}]\text{Gal}$ galactosidase activities

$[^3\text{H}]\text{Gal}$ galactosidase activity was assayed as described in the Experimental section, except that increasing amounts of $[\text{Gal-}^3\text{H}]\text{fetuin}$ were added to the reaction mixture. The assay was linear for up to 3 μg of substrate protein/0.1 ml, giving a K_m of 1.5 μg of substrate protein/0.1 ml.

proportional to the amount of luminal fluid added over a range of 1–10 μg of protein, and was linear with respect to the incubation time for at least 2 h (results not shown). Longer incubation times (24 h) resulted in cleavage of more than 97% of the radiolabelled substrate ($[\text{Gal-}^3\text{H}]\text{fetuin}$). The cleaved sugar was identified as $[^3\text{H}]\text{Gal}$ by comparing its mobility with the mobility of standard sugars using t.l.c. (results not shown). Since nearly all of the radioactivity remained as free $[^3\text{H}]\text{Gal}$, it is reasonable to assume that the radioactive sugar enzymically cleaved from the $[\text{Gal-}^3\text{H}]\text{fetuin}$ was not transferred to other components of the epididymal luminal fluid. For the $[^3\text{H}]\text{Gal}$ galactosidase assay, we routinely used 1.6 μg of $[\text{Gal-}^3\text{H}]\text{fetuin}$ (approx. 5000 c.p.m.) as substrate/0.1 ml assay volume, a value similar to the K_m value of 1.5 μg of substrate protein/0.1 ml of sample obtained by the double-reciprocal plot (Fig. 2).

The substrate-concentration studies using PNP galactoside generated a linear double-reciprocal plot with an apparent K_m of 0.52 mM (results not shown), a value similar to the one reported for mouse liver β -D-galactosidase [20]. This value is well below the saturating concentration of 5 mM used in routine assays.

Evidence suggesting that the PNP- and $[^3\text{H}]\text{Gal}$ galactosidase activities are caused by a single enzyme

Our initial studies, reported above, raised the question of whether the two substrates are hydrolysed by a single enzyme or whether there are two different β -D-galactosidases in the epididymal luminal fluid. We therefore attempted to characterize further the PNP galactoside and $[^3\text{H}]\text{Gal}$ galactoside hydrolysing activities.

Concanavalin A-Sepharose 4B column chromatography. Since acid β -D-galactosidase in other tissues has been shown to be a glycoprotein containing high-mannose/hybrid-oligosaccharide units [12,21–25], we first attempted to separate the PNP galactosidase from $[^3\text{H}]\text{Gal}$ galactosidase activities by affinity chromatography on a column of immobilized concanavalin A. Rat luminal fluid was applied to the column which was then extensively washed with the column buffer as described in the legend to Fig. 3. Essentially all of the PNP- and $[^3\text{H}]\text{Gal}$ galactosidase activities bound to the column (< 1% of the two activities was present in the void volume of the column). When the column was eluted with a linear gradient (0–1 M) of α -methyl mannoside (Fig. 3), more than 80% of the two activities eluted from the column. Moreover the elution profiles of the two activities were similar. This result is consistent with the possibility that the catalytic domain for the two activities resides on a molecule(s) containing similar N-linked high-mannose/hybrid-type units.

DEAE-cellulose column chromatography. Next we attempted to resolve the two activities by ion-exchange chromatography on a column of DE-52. Rat epididymal luminal fluid was applied to the anion-exchange resin and, after the column was washed with the column buffer, it was eluted with a linear NaCl gradient in the column buffer. Results from these studies (Fig. 4) show that both activities eluted in a single peak. The ratio of the two activities remained fairly constant, a result indicating no net negative-charge difference between the two activities.

Immunoprecipitation studies. We then tried the immunoprecipitation approach using anti-mouse liver acid galactosidase antibody in an attempt to separate the two activities. Results from these studies, presented in Fig. 5, show that the antibody cross-reacted with the PNP galactosidase activity (assayed at pH 3.5) and $[^3\text{H}]\text{Gal}$ galactosidase activity (assayed at pH 3.5 or 6.6), indicating that the two activities possess the same antigenic determinant(s).

Thermostability of PNP- and $[^3\text{H}]\text{Gal}$ galactosidase activities. We then attempted to differentiate the two activities on the basis of their thermostability. However, both activities were stable

Fig. 3. Fractionation of PNP galactosidase and $[^3\text{H}]\text{Gal}$ galactosidase activities from rat epididymal luminal fluid on a column of concanavalin A-Sepharose 4B

An aliquot (2 ml) of the rat epididymal luminal fluid (4.8 mg of protein) in 0.01 M-Tris/HCl buffer pH 7.4, 0.1 M-NaCl, 1 mM- CaCl_2 and 1 mM- MgCl_2 was placed on a column (1 cm $\times$ 14 cm) of concanavalin A-Sepharose 4B previously washed and equilibrated in the same buffer. After washing with 20 ml of the buffer, the column was eluted with 100 ml of a linear α -methyl mannoside gradient (0–1.0 M) in the above buffer. Fractions (1 ml) were collected and alternate fractions assayed for both PNP- (○) and $[^3\text{H}]\text{Gal}$ - (●) galactosidase activities at pH 3.5 and 6.8 respectively.

Fig. 4. Fractionation of PNP galactosidase and $[^3\text{H}]\text{Gal}$ galactosidase activities on a column of DE-52

A sample (2 ml) of the rat luminal fluid (4.6 mg of protein) in 0.25 M-sucrose and 10 mM-Tris/HCl pH 7.2 was applied to a column of Whatman DE-52 (1 cm $\times$ 9 cm) previously equilibrated in 0.01 M-Tris/HCl pH 7.2 buffer. The column was washed with 30 ml of the above buffer and then eluted with 100 ml of a linear NaCl gradient 0–0.4 M) in the same buffer. Fractions (1 ml) were collected at a flow rate of 30 ml/h. Aliquots were analysed for PNP galactosidase (○) and $[^3\text{H}]\text{Gal}$ galactosidase (●) at pH 3.5 and 6.8 respectively.

when the luminal fluid was subjected, before assay, to heat treatment at 45 °C for at least 60 min at either pH 3.5 or 7.2 (Fig. 6). Both activities were less stable at higher temperatures. When the heat treatment was carried out at 60 °C in sodium citrate buffer, pH 3.5 (Fig. 6a), 20–40% of the two activities was lost after 60 min. Both activities were even more sensitive to the heat treatment (60 °C) in sodium cacodylate buffer at pH 7.2 (Fig. 6b). Nearly 90% of the two activities was lost after 20 min, and almost 100% after a 60 min heat treatment. These studies suggest that the β -D-galactosidase activities towards PNP galactoside and $[\text{Gal-}^3\text{H}]\text{fetuin}$ substrates show very similar thermostabilities.

Taken together, the above results suggest the presence of a

Fig. 5. Immunoprecipitation of β -galactosidase activities from rat epididymal luminal fluid

Aliquots containing a constant amount of the luminal fluid protein (0.1 mg) and Protein G-Sepharose 4FF preabsorbed with increasing concentrations of antibody (prepared against mouse liver acid β -D-galactosidase). The mixture was kept at room temperature for 60 min and was then centrifuged. The residual activities present in the supernatant were assayed at the indicated pH: PNP galactosidase activity at pH 3.5 (○), and $[^3\text{H}]\text{Gal}$ galactosidase activity at pH 3.5 (▲), and pH 6.8 (●).

Fig. 6. Thermostability of PNP galactosidase and $[^3\text{H}]\text{Gal}$ galactosidase activities

Rat epididymal luminal fluid in 10 mM-sodium citrate buffer, pH 3.5 (a), or 10 mM-sodium cacodylate buffer, pH 7.2 (b), was incubated at either 45 °C or 60 °C for various time periods. Aliquots from the preheated enzyme were assayed for PNP galactosidase at pH 3.5, and $[^3\text{H}]\text{Gal}$ galactosidase at pH 6.8.

common molecular form of β -D-galactosidase in rat epididymal luminal fluid which is capable of hydrolysing both PNP-galactoside and $[\text{Gal-}^3\text{H}]\text{fetuin}$ as substrates.

Evidence suggesting that the PNP galactoside and $[\text{Gal-}^3\text{H}]\text{fetuin}$ substrates are cleaved by the same catalytic sites

We then investigated whether the two substrates are cleaved by the same catalytic site or by two catalytic sites: one which optimally hydrolyses PNP galactoside at pH 3.5, and the other which optimally hydrolyses $[\text{Gal-}^3\text{H}]\text{fetuin}$ at pH 6.8. In these studies, we assayed $[^3\text{H}]\text{Gal}$ galactosidase activity in the absence and presence of increasing concentrations of PNP galactoside. Epididymal luminal fluid was incubated at pH 3.5, 4.0 or 6.8 with or without PNP galactoside for 5 min at 0–4 °C. After these incubations, the radiolabelled substrate ($[\text{Gal-}^3\text{H}]\text{fetuin}$) was added, and the $[^3\text{H}]\text{Gal}$ galactosidase activity was assayed at pH 3.5, 4.0 or 6.8. Results from these substrate competition studies, presented in Table 1, show that (a) the added PNP galactoside substrate caused a concentration-dependent inhibition of the $[^3\text{H}]\text{Gal}$ galactosidase activity assayed at three

Table 1. Inhibition of rat luminal fluid [^3H]Gal β -D-galactosidase activity by PNP galactoside and various galactose-linked disaccharides

[^3H]Gal galactosidase activity was assayed as described in the Experimental section, except that epididymal luminal fluid was incubated in the standard incubation mixture at pH 3.5, 4.0 or 6.8 (omitting the radiolabelled substrate) with or without the potential inhibitor for 5 min at 4 °C. [^3H]Gal galactosidase activity was then determined by the addition of [^3H]fetuin and incubation for 1 h at 37 °C. The released [^3H]Gal was separated and quantified as described in the Experimental section.

Addition	Concn. (mM)	Linkage	Assay conditions...	Inhibition* (%)		
				pH 3.5	pH 4.0	pH 6.8
PNP- β -D-galactoside	1			34	32	40
	2			58	62	64
	5			68	66	69
<i>N</i> -Acetyl-lactosamine	1	Gal β 1,4GlcNA $\dagger$		33	32	26
	2			55	53	50
	5			62	57	55
Acetamido-2-deoxy-3- <i>O</i> - (β -D-galactopyranosyl)- D-glucopyranose	1	Gal β 1,3GlcNA $\dagger$		38	31	28
	2			60	54	52
	5			65	62	65
Acetamido-2-deoxy-3- <i>O</i> - (β -D-galactopyranosyl)- D-galactopyranose	1	Gal β 1,3GalNA $\dagger$		47	42	46
	2			72	70	69
	5			74	68	76
D-Galactose	1			3	6	4
	2			4	8	6
	5			12	10	15

* Under standard assay conditions (without additions), the enzyme released 15%, 17% and 30% of the added substrate ([^3H]fetuin) as free [^3H]Gal at pH 3.5, 4.0 and 6.8 respectively.

$\dagger$ Gal β 1,4GlcNAc linkage is found in both *O*- and *N*-linked oligosaccharides; Gal β 1,3GlcNAc is found only in *N*-linked oligosaccharides; and Gal β 1,3GalNAc is found only in *O*-linked oligosaccharides.

different pH conditions; (b) the observed inhibition of over 60% at a concentration of 5 mM cannot be explained as a result of any significant inhibition by the product (galactose), since the sugar at 5 mM concentration had little or no inhibitory effect on the [^3H]Gal galactosidase activity (Table 1). These results provide evidence indicating that at pH 3.5, 4.0 or 6.8 the two substrates are cleaved by the same catalytic site(s).

Characterization of the oligosaccharide moiety(ies) in [^3H]fetuin substrate

Next we examined the chemical nature of the Gal-labelled oligosaccharide moiety(ies) of the [^3H]fetuin substrate used in these experiments. When the substrate was treated exhaustively with *N*-Glycanase (Peptide-*N*-glycosidase F), an endo-enzyme known to cleave all *N*-linked oligosaccharides [26], more than 95% of the radioactivity was present in the ethanol-soluble oligosaccharide fraction indicating that most of the [^3H]Gal had been incorporated into *N*-linked oligosaccharides. This was not surprising since the bovine milk galactosyltransferase used to incorporate [^3H]Gal into the substrate is known to be a β -1,4-specific enzyme. No further attempt was made to characterize the remaining (less than 5%) radiolabelled galactose present in the ethanol-precipitable protein fraction.

Competition studies with disaccharides

Since less than 5% of the radioactive galactose in the [^3H]fetuin substrate was presumably *O*-linked, competition studies were done to characterize the specific linkages cleaved by epididymal fluid β -D-galactosidase. In these studies we used increasing concentrations of several galactose-containing disaccharides to study their inhibitory effect on [^3H]Gal galactosidase activity. The results in Table 1 indicate that regardless of the pH of the assay mixture, disaccharides containing a variety of galactose linkages found in both *O*- and *N*-linked oligosaccharides, show a concentration-dependent inhibition of [^3H]Gal galactosidase. These results provide strong evidence that

epididymal fluid β -galactosidase is capable of cleaving galactose residues present in both *O*- and *N*-linked oligosaccharides.

Evidence for pH-dependent association/dissociation of epididymal luminal fluid β -D-galactosidase

It has been shown previously that mouse liver lysosomal acid β -D-galactosidase (assayed with PNP galactoside) is present as a tetramer (apparent M_r 240 000) in acidic conditions [27]. However, the enzyme (assayed with the same substrate) dissociates in neutral conditions at pH 7.2, and elutes as a dimer (apparent M_r 113 000) from a gel-filtration column [27]. To determine if the β -D-galactosidase of epididymal luminal fluid shows similar pH-dependent association/dissociation, we fractionated the rat luminal fluid on a column of Bio-Gel A-0.5 m-agarose under two sets of varying conditions with regard to buffer and pH. In brief, the fluid was resolved by gel filtration under either acidic (pH 3.5) or neutral (pH 6.8) conditions, and various fractions were assayed for the PNP galactosidase and [^3H]Gal galactosidase activities under their optimal pH conditions (pH 3.5–4.0 for PNP galactosidase and pH 6.8 for [^3H]Gal galactosidase). Results from these studies, presented in Fig. 7, clearly show that whereas the enzyme activities eluted in an associated form (an apparent tetramer of M_r 320 000) in acidic conditions, only dissociated dimers and monomers (apparent M_r 180 000, and 92 000 respectively) were observed when the gel filtration was carried out at neutral pH. These results indicate that β -D-galactosidase in luminal fluid, like the mouse liver enzyme, shows pH-dependent association/dissociation. To demonstrate reversibility of the association/dissociation phenomenon, we obtained the associated (tetrameric) form of the enzyme by gel filtration carried out under acidic conditions, precipitated the enzyme with ammonium sulphate, and resolved it by chromatography at pH 6.8. The eluted fractions, when assayed, showed activity eluting at the position of dissociated forms (results not shown). These results suggest that the association/dissociation of the enzyme is reversible. In addition, the results show that the epididymal enzyme

Fig. 7. Fractionation of PNP galactosidase and $[^3\text{H}]\text{Gal}$ galactosidase activities by Bio-Gel A-0.5m column chromatography

A sample (2 ml) of the epididymal luminal fluid (5.5 mg of protein) in acidic (10 mM-sodium citrate buffer, pH 3.5 or 4.0, containing 0.1 M-NaCl) or neutral (10 mM-sodium cacodylate buffer, pH 6.8, containing 0.1 M-NaCl) solutions was applied to a column of Bio-Gel A-0.5m (1.5 cm $\times$ 87 cm). The column was either equilibrated in the acidic (a) or the neutral buffers (b) and eluted with the same buffers. Fractions (2 ml) were collected at a flow rate of 10 ml/h. Aliquots from each fraction were analysed for the PNP galactosidase (○) and $[^3\text{H}]\text{Gal}$ galactosidase (●) activities under optimal conditions. The numbers on top of the Fig. are standard M_r markers: 1, Blue Dextran-2000 (column void volume); 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); and 6, ovalbumin (M_r 43 000). T, D and M, indicate the elution position of tetrameric, dimeric and monomeric forms of β -D-galactosidase, respectively. When the rat luminal fluid was fractionated by molecular sieve column chromatography at pH 5.2 or pH 7.2, as described for mouse liver [27], the enzyme eluted in an aggregated form in the acidic conditions, and in dissociated forms in the alkaline conditions (results not shown). Reversibility of the association/dissociation phenomenon was shown by isolation of the tetrameric form from a gel filtration column under acidic conditions, precipitation with 75% (w/v) ammonium sulphate, and rechromatography under neutral conditions (pH 6.8). The eluted fractions, when assayed showed activity eluting at the position of dissociated forms as in (b).

is optimally active toward a natural substrate in a dissociated form.

DISCUSSION

The purpose of the work presented here was to characterize the β -D-galactosidase found in abundant quantities in the epididymal luminal fluid. Since the enzyme has been shown to be optimally active in acidic conditions when assayed using PNP or 4-methylumbelliferyl β -D-galactoside as substrate [20,28], we wondered if the enzyme was active at neutral pH when a

glycoprotein is used as a substrate. Such a substrate ($[\text{Gal-}^3\text{H}]\text{fetuin}$) was synthesized and used in the studies described here. When we compared the effect initially of pH on the enzyme activities of the rat luminal β -D-galactosidase, the results showed that the pH of the reaction mixture had a great effect on the rate at which these two substrates were hydrolysed. These pH-dependent substrate preference studies suggested two likely possibilities: (a) The luminal fluid contained one enzyme active towards the substrates PNP galactoside and $[\text{Gal-}^3\text{H}]\text{fetuin}$ (b). The luminal fluid contained two β -D-galactosidases, one active towards the synthetic substrate and the other active towards the glycoprotein substrate.

Several lines of evidence including concanavalin A and ion-exchange chromatography, thermolability studies, and immunoprecipitation with antibody raised to mouse liver acid β -D-galactosidase, suggest that the two substrates are hydrolysed by the same enzyme. The substrate competition studies, showing that PNP galactoside inhibited the hydrolysis of $[\text{Gal-}^3\text{H}]\text{fetuin}$ over a wide range of pH, allow us to suggest that these two substrates are cleaved by the same catalytic site(s).

Comparative studies using synthetic (PNP glycoside) and natural (glycoprotein/oligosaccharide) substrate have provided evidence that natural substrates are hydrolysed at a slightly higher pH. For example, acid mannosidase from rat liver [29], kidney [30] and epididymis [31] optimally cleaves PNP-mannoside at pH 4.4. A slightly higher pH optimum (pH 5.0) has been reported with the natural substrate [29–31]. However, the epididymal luminal fluid β -D-galactosidase reported here is the first glycosidase which shows a strikingly different pH-dependent substrate preference (pH 3.5 with PNP galactoside versus pH 6.8 with $[\text{Gal-}^3\text{H}]\text{fetuin}$).

Molecular-sieve column chromatography under acidic and neutral conditions showed a reversible, pH-dependent association/dissociation of the rat epididymal luminal fluid β -D-galactosidase. This result was not entirely surprising since similar pH-dependent aggregation has been reported for acid β -D-galactosidase purified from human and mouse liver. In mouse, the acid β -D-galactosidase occurs in an aggregate (tetramer, apparent M_r 240 000) form under acidic conditions, which dissociates into dimers (apparent M_r 113 000) under neutral conditions [27,28]. Human liver β -D-galactosidase, an oligomer of apparent M_r 660 000, dissociates into dimers (apparent M_r 150 000–180 000) and monomers (apparent M_r 65 000–80 000) [24,32,33]. These studies suggest that the pH-dependent association/dissociation is a common occurrence among mammalian β -D-galactosidases. It is noteworthy that the β -D-galactosidase from rat epididymal luminal fluid optimally cleaves PNP galactoside at an acidic pH of 3.5, when the enzyme is present only in an associated (tetrameric) state. However, $[\text{Gal-}^3\text{H}]\text{fetuin}$ is optimally hydrolysed at pH 6.8, a state when nearly all of the enzyme is present in dissociated forms. Thus it appears that the pH-dependent conformational changes in the enzyme molecule are necessary for the optimal cleavage of the two substrates. Since the physiological pH of the epididymal lumen has been shown to be 6.8 [18], it is likely that under conditions *in vivo*, the enzyme is present in dissociated forms, and is active mainly towards a glycoprotein substrate. In addition, since the enzyme was less thermostable at pH 6.8, it is reasonable to suggest that the β -D-galactosidase is less stable in the neutral conditions which favour dissociation.

The apparent M_r of the monomer (M_r 92 000) observed by gel filtration under neutral conditions is similar to the subunit M_r of the enzyme secreted by rat epididymal epithelial cells [8] and the subunit M_r of the rat epididymal luminal fluid β -D-galactosidase observed by SDS/PAGE (M. D. Skudlarek, D. R. P. Tulsiani & M.-C. Orgebin-Crist, unpublished work). We have not detected

a high M_r moiety or the 32000–40000- M_r protective protein observed by others in several tissues [34]. However, the subunit M_r of β -D-galactosidase from epididymal luminal fluid is significantly higher than that reported for mouse liver and macrophages [35,36], human fibroblast [35] precursor forms of β -D-galactosidase (M_r 82000– M_r 84000) and the enzyme secreted by mouse macrophages (M_r 63000) [37]. These differences are probably caused by differential glycosylation of the enzyme. Since the oligosaccharide moieties of the glycosidases are important in the intracellular transport and packaging of the enzymes [38,39], it will be of interest to study the oligosaccharide composition of the β -D-galactosidase purified from the tissue lysosomes, and from the epididymal luminal fluid.

In addition to the lysosomal acid β -D-galactosidase activity discussed above, human liver [32,33] has been shown to contain another β -D-galactosidase activity in the tissue cytosol. Unlike the lysosomal enzyme, the cytosolic β -D-galactosidase, optimally hydrolyses PNP galactoside at a neutral pH [40], does not show affinity for concanavalin A, and shows no cross-reactivity with the antibody to the acid β -D-galactosidase [41,42]. The luminal fluid enzyme described here is similar to the liver lysosomal acid β -D-galactosidase and not the cytosolic β -D-galactosidase.

The acid β -D-galactosidase and other glycosidases are usually present inside lysosomes where they are known to be degradative enzymes [43,44]. However, these enzymes have been reported to be present in extracellular fluids, such as blood [45,46] and epididymal luminal fluid [7,7a]. Their degradative role, when present in a neutral environment, has not been elucidated. Our results seem to suggest that at least one glycosidase, the acid β -D-galactosidase, could be functional in an acidic environment (inside the lysosomes) as well as in the neutral environment of the epididymal lumen. It will be important to examine whether other glycosidases present in the luminal fluid are optimally active at or near neutral pH when assayed using a glycoprotein substrate.

Spermatozoa acquire the ability to fertilize during transit through the epididymis [1]. It is generally believed that the maturational process includes modifications of the sperm plasma membrane to permit sperm-egg interaction. Although the details of these modifications are still unclear, there is growing evidence that glycoprotein-modifying enzymes (glycosidases and glycosyltransferases) along with proteases are involved in these modifications. Lectin binding studies have provided strong evidence suggesting that the sperm surface glycoproteins are extensively modified in the various regions of epididymis [4–6,47,48]. For instance, peanut agglutinin, a lectin which preferentially binds to the Gal β 1, 3GalNAc-linkage found in O-linked glycoproteins, stains two major glycoproteins from proximal caput sperm plasma membrane but not from cauda sperm plasma membrane [48]. The apparent loss of the galactosyl residue(s) during sperm maturation may be caused by the luminal fluid β -D-galactosidase, which is capable of cleaving galactosyl residues from O-linked (Gal β 1,3GalNAc) as well as N-linked glycoproteins.

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REFERENCES

- Orgebin-Crist, M.-C. (1967) *Ann. Biol. Anim. Biochim. Biophys.* **7**, 373–389
- Bedford, J. M. (1966) *J. Exp. Zool.* **163**, 319–329
- Orgebin-Crist, M.-C. (1987) in *Morphological Basis of Human Reproductive Function* (deKretser, D. M. & Spera, G., eds.), pp. 155–174, Acta Medica, Plenum Press, New York
- Eddy, E. M. (1988) in *The Physiology of Reproduction* (Knobil, E. & Neill, J., eds.) vol. 1, pp. 27–68, Raven Press, New York
- Yanagimachi, R. (1988) in *The Physiology of Reproduction* (Knobil, E. & Neill, J., eds.) vol. 1, pp. 135–186, Raven Press, New York
- Jones, R. (1989) in *Oxford Reviews of Reproduction Biology* (Milligan, S. R., ed.) vol. 2, pp. 285–337
- Skudlarek, M. D. & Orgebin-Crist, M.-C. (1988) *J. Reprod. Fert.* **84**, 611–617
- Tulsiani, D. R. P., Skudlarek, M. K. D., Holland, M. K. & Orgebin-Crist, M.-C. (1991) *Biol. Reprod. Abstract* 505, p. 179, Society for the Study of Reproduction, Champaign, IL, U.S.A.
- Skudlarek, M. D. & Orgebin-Crist, M.-C. (1986) *Biol. Reprod.* **35**, 167–178
- Hamilton, D. W. (1980) *Biol. Reprod.* **23**, 377–385
- Hamilton, D. W. & Gould, R. P. (1982) *Int. J. Androl. (Suppl.)* **5**, 73–80
- Bernal, A., Torres, J., Reyes, A. & Rosado, A. (1980) *Biol. Reprod.* **23**, 290–293
- Skudlarek, M. D. & Swank, R. T. (1979) *J. Biol. Chem.* **254**, 9939–9942
- Meisler, M. H. (1978) *J. Biol. Chem.* **253**, 3129–3134
- Tulsiani, D. R. P., Skudlarek, M. D. & Orgebin-Crist, M.-C. (1989) *J. Cell. Biol.* **109**, 1257–1267
- Tulsiani, D. R. P., Skudlarek, M. D. & Orgebin-Crist, M.-C. (1990) *Biol. Reprod.* **42**, 843–858
- Tulsiani, D. R. P., Coleman, V. D. & Touster, O. (1990) *Arch. Biochem. Biophys.* **277**, 113–121
- Skudlarek, M. D., Orgebin-Crist, M.-C. & Tulsiani, D. R. P. (1991) *Biochem. J.* **277**, 213–221
- Levine, N. & Kelly, H. (1978) *J. Reprod. Fertil.* **52**, 132
- Distler, J. J. & Jourdain, G. W. (1973) *J. Biol. Chem.* **248**, 6772–6780
- Felton, J., Meisler, M. & Paigen, K. (1974) *J. Biol. Chem.* **249**, 3267–3272
- Distler, J. J., Guo, J., Sahagian, G. G. & Jourdain, G. W. (1989) *Anal. Biochem.* **182**, 432–437
- De Groen, P. C., Le Sage, G. D., Tietz, P. S. & La Russo, N. F. (1989) *Biochem. J.* **264**, 115–123
- Yamamoto, Y. & Nishimura, K. (1980) *J. Biochem. (Tokyo)* **88**, 705–713
- Heyworth, C. M., Neumann, E. F. & Wynn, C. H. (1981) *Biochem. J.* **193**, 773–779
- Berger, F. G., Paigen, K. & Meisler, M. H. (1978) *J. Biol. Chem.* **253**, 5280–5282
- Maley, F., Trimble, R. B., Tarentino, A. L. & Plummer, T. H., Jr. (1989) *Anal. Biochem.* **180**, 195–204
- Tomino, S. & Meisler, M. (1975) *J. Biol. Chem.* **250**, 7752–7758
- Lusis, A. J., Breen, G. A. M. & Paigen, K. (1977) *J. Biol. Chem.* **252**, 4613–4618
- Opheim, D. J. & Touster, O. (1978) *J. Biol. Chem.* **253**, 1017–1023
- Tulsiani, D. R. P. & Touster, O. (1987) *J. Biol. Chem.* **262**, 6506–6515
- Tulsiani, D. R. P., Coleman, V. D. & Touster, O. (1988) *Arch. Biochem. Biophys.* **267**, 60–68
- Cheetham, P. S. J. & Dance, N. E. (1976) *Biochem. J.* **157**, 189–195
- Frost, R. G., Holmes, E. W., Norden, A. G. W. & O'Brien, J. S. (1978) *Biochem. J.* **175**, 181–182
- Hoogveen, A. T., Verheijen, F. W. & Galjaard, H. (1983) *J. Biol. Chem.* **258**, 12143–12146
- Skudlarek, M. D., Novak, E. K. & Swank, R. T. (1984) in *Lysosomes in Biology and Pathology* (Dingle, J. T., Dean, R. T. & Sly, W., eds.), pp. 17–43, Elsevier Science Publishing, New York
- Skudlarek, M. D. & Swank, R. T. (1988) *J. Biol. Chem.* **263**, 11302–11305
- Skudlarek, M. D. & Swank, R. T. (1981) *J. Biol. Chem.* **256**, 10137–10144
- von Figura, K. & Hasilik, A. (1986) *Annu. Rev. Biochem.* **55**, 167–193
- Kornfeld, S. & Mellman, I. (1989) *Annu. Rev. Cell. Biol.* **5**, 483–525
- Ho, M. W., Seck, J., Schmidt, D., Veath, M. L., Johnson, W., Brady, R. O. & O'Brien, J. S. (1972) *Am. J. Hum. Genet.* **24**, 37–45
- Norden, A. G. W. & O'Brien, J. S. (1974) *Biochem. Biophys. Res. Commun.* **56**, 193–198

42. Norden, A. G. W., Tennant, L. L. & O'Brien, J. S. (1974) *J. Biol. Chem.* **249**, 7969–7976
43. Holtzman, E. (1976) *Lysosomes: A Survey*, pp. 4–25, Springer-Verlag, New York
44. de Duve, C. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), vol. 1, pp. 3–40, North-Holland Publishing Co., Amsterdam
45. Tulsiani, D. R. P. & Touster, O. (1983) *Arch. Biochem. Biophys.* **224**, 594–600
46. Belfiore, E., Napoli, E. & LoVecchio, L. (1972) *Diabetes* **21**, 1167–1172
47. Rankin, T. L., Holland, M. K. & Orgebin-Crist, M.-C. (1989) *Gamete Res.* **24**, 439–451
48. Srivastava, A. & Olson, G. E. (1991) *Mol. Reprod. Dev.* **29**, 357–364

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