Purification and characterization of rat epididymal-fluid α-D-mannosidase: similarities to sperm plasma-membrane α-D-mannosidase

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We have previously reported the occurrence and partial characterization of a novel α-D-mannosidase activity on rat sperm plasma membranes [Tulsiani, Skudlarek and Orgebin-Crist (1989) J. Cell Biol. **109**, 1257–1267]. Here, we report the presence of a similar α-D-mannosidase activity in a soluble form in rat epididymal fluid. The soluble enzyme was purified nearly 500-fold with 9–12% recovery to a state approaching homogeneity using: (1) $(NH_4)_2SO_4$ precipitation; (2) affinity chromatography on immobilized mannan and D-mannosamine; (3) ion-exchange (DE-52) column chromatography; (4) molecular-sieve chromatography. The enzyme was eluted from the final column (Sephacryl S-400) at an apparent molecular mass of 460 kDa. When resolved by SDS/PAGE (under denaturing conditions), the enzyme showed a major protein band (115 kDa) and few very minor bands. The polyclonal antibody raised against the major protein band was found to cross-react with the α-D-mannosidase activity present in epididymal fluid (soluble) and detergent-solubilized spermatozoa from the rat and mouse. This result suggested that the soluble and membrane-bound enzyme activities shared a common antigenic site(s). The antibody was used to characterize further the α-D-mannosidase activity(ies) present in the rat epididymal fluid and rat sperm plasma membranes. Data from these studies show that the two forms are similar in (a) subunit molecular mass, (b) substrate specificity and (c) inhibitory effect of several sugars. These similarities suggest that the soluble and membrane-bound α-D-mannosidase activities are isoforms. Immunoprecipitation studies after solubilization of the testis and epididymal particulate fraction from sexually immature rats show that the testis (but not the epididymis) contains the immunoreactive α-D-mannosidase activity. This result and the fact that spermatozoa from the rat rete testis show α-D-mannosidase activity indicate that the sperm enzyme is synthesized in the testis during spermatogenesis.

INTRODUCTION

Several α-D-mannosidase activities, which are involved in the processing of asparagine-linked oligosaccharides present on glycoproteins, have been identified, mostly in rat liver. For instance, endoplasmic reticulum α-D-mannosidase [1,2], Golgi mannosidases IA [3–5] and IB [4] show preference for the α1,2-linked mannosyl residues. Golgi mannosidase II shows little or no activity towards the oligosaccharide substrates containing α1,2-linked terminal mannosyl residues but cleaves α1,3- and α1,6-linked mannosyl residues from $GlcNAcMan_5GlcNAc$ [5–7]. Rat liver Golgi membranes also contain an α-D-mannosidase activity with a broad substrate specificity [8,9]. The physiological role of this enzyme is not yet known. In addition to these processing enzymes, lysosomal α-D-mannosidase, active at pH 4.5, which shows much greater activity towards linear than branched oligosaccharides [10,11], is believed to be involved in the degradation of glycoproteins in lysosomes. Recently, we have described a novel α-D-mannosidase activity in the rat sperm plasma membrane, active at pH 6.2–6.5, which is thought to have a receptor-like role in sperm–egg interaction during the fertilization process [12]. Attempts to solubilize the sperm enzyme for purification purposes were not successful. However, in our initial work, we noted that 20% of an apparently similar enzyme activity was present in soluble form in the epididymal fluid [12]. Since the characteristics of the α-D-mannosidase activities present in sperm plasma membrane and epididymal fluid were quite different from other known mannosidases, we postulated that the epididymal-fluid enzyme could be used as a source for the purification of the novel α-D-mannosidase activity.

In this report, we describe the purification of the soluble form of the enzyme, the preparation of an antiserum specific for the soluble form of the enzyme and the ability of the antibody to cross-react with the membrane form of α-D-mannosidase activity. The antibody was used to demonstrate that the soluble and plasma-membrane α-D-mannosidase activities have similar characteristics.

MATERIALS AND METHODS

Chemicals and antibodies

Sprague–Dawley rats (sexually immature 28–32-day-old and sexually mature 10–12-week-old), obtained from Sasco, Omaha, NE, U.S.A., were kept in our animal facilities for at least 3 days before experiments were begun. Animals were killed by CO_2 asphyxiation. Each epididymis and testis was dissected free of most visible blood vessels and connective tissue under a dissecting microscope while the tissue was kept on ice (0–4 °C). The tissues were processed as described in each experiment. Concanavalin A–Sepharose 4B (10 mg of lectin/ml of gel), D-mannosamine and mannan were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Oligosaccharides, uniformly labelled with [^{3}H]-mannose, namely [^{3}H]$Man_9GlcNAc$, [^{3}H]$Man_8GlcNAc$, [^{3}H]-$Man_7GlcNAc$, [^{3}H]$Man_6GlcNAc$, [^{3}H]$Man_5GlcNAc$ and $GlcNAc$[^{3}H]$Man_5GlcNAc$, were prepared as described [13]. [^{3}H]Mannose-labelled glycoproteins were prepared by a previously published procedure [13]. Briefly, the residue left after repeated extraction of the labelled epididymal epithelial cells with chloroform/methanol/water [13] was dried under N_2, dissolved in 25 mM Tris/HCl buffer, pH 7.5, containing 1 mM

Abbreviation used: PNP, *p*-nitrophenyl.
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$CaCl_2$, 1 mM $MgCl_2$, and 0.5 M NaCl and fractionated on a column of concanavalin A–Sepharose 4B [10]. The radioactive peak eluted with 1 M methyl α-mannoside (nearly 35% of the total radioactivity applied) was extensively dialysed against distilled water, lyophilized, dissolved in 0.25 M NaCl and stored frozen at −20 °C. [^{3}H]Mannose-labelled glycopeptides were prepared by Pronase digestion of the cellular glycoproteins, followed by resolution on a column of Bio-Gel P-4 [13]. The [^{3}H]mannose-labelled glycopeptides present in Pool 2 of swainsonine-treated cells (see Figure 3 of ref. [13]) were fractionated on a column of concanavalin A–Sepharose 4B as described [10]. The labelled glycopeptide ([^{3}H]$Man_{9-5}GlcNAc_2$ Asn), eluted with methyl α-mannoside, was further purified by gel filtration [13]. The fractions containing radioactivity were pooled, lyophilized, dissolved in a small volume of distilled water and stored frozen at −20 °C. Vectastain reagents were from Vector Laboratories (Burlingame, CA, U.S.A.). All electrophoresis and electroblotting chemicals including molecular-mass marker proteins were from Bio-Rad (Richmond, CA, U.S.A.). Polyclonal antibody (rabbit IgG fractions) against rat liver Golgi mannosidase IA was prepared as described previously [5]. Anti-[rat epididymal (lysosomal) α-D-mannosidase] IgG was prepared as reported previously [11] or by affinity chromatography on a column of Protein G–Sepharose 4FF (Pharmacia LKB Biotechnology) using the manufacturer's protocol. *Staphylococcus aureus* cells (IgGSorb) were obtained from The Enzyme Center (Malden, MA, U.S.A.). Microgranular DE52 (Whatman Lab Sales, Hillsboro, OR, U.S.A.), Sephacryl S-400, AH-Sepharose 4B and epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.) were used as recommended by the manufacturers. Centricon 10 microconcentrator (10000 kDa molecular-mass cutoff) was from Amicon Corp. (Beverly, MA, U.S.A.). The salt-washed plasma-membrane-rich fraction was prepared from cauda spermatozoa by the procedure described [12]. All other chemicals used were obtained commercially and were of the highest purity available.

Dulbecco's phosphate-buffered saline (10-fold concentration; Gibco, Grand Island, NY, U.S.A.) was diluted 10-fold in glass-distilled water while the pH was adjusted to 7.4 to make working PBS solution.

Preparation of epididymal luminal fluid

For enzyme purification, the luminal fluid was prepared as previously described [14]. In brief, the tissue (free of fat-pad, blood vessels and connective tissue) was cut with a sharp razor blade into approximately 1 mm^3 pieces in PBS containing 25 mM benzamidine. The tissue pieces in the solution were shaken for 15 min at room temperature to release spermatozoa and luminal fluid. The tissue minces were filtered through four layers of cheesecloth (to remove epididymal tissue pieces) and the cheesecloth was gently washed with the above PBS solution. The pooled supernatant was centrifuged for 10 min at 600 *g*. The cloudy supernatant (enriched luminal fluid) was used for enzyme purification. In some experiments, luminal fluid from the cauda epididymidis of sexually mature rats was prepared by retrograde flushing of the vas deferens, and centrifugation at 600 *g* for 10 min.

Preparation of epididymal and testicular particulate fraction

The tissue homogenate was prepared at 4 °C in 0.25 M sucrose containing 10 mM Tris/HCl buffer, pH 7.5, and 25 mM benzamidine (3 vol./g of tissue) by homogenizing for 50 s with a Polytron homogenizer (type PT 10 20 3500; Brinkman Instruments) set at position 5. The homogenate was centrifuged at 800 *g* for 10 min. The supernatant was removed by aspiration and the residue was homogenized and centrifuged as above. The process was repeated a third time. The particulate fraction was obtained by centrifuging the pooled cytoplasmic extract at 105000 *g* for 60 min. The supernatant was discarded, and the residue was washed by suspending in 5 vol. of the homogenizing solution, and centrifuging as above. The washed particulate fractions obtained from the epididymis or testis were used for biochemical studies.

Solubilization of the membrane fractions

The epididymal and testicular particulate fractions were solubilized by repeated extraction in potassium phosphate buffer (10 mM, pH 7.5) containing 1% Triton X-100, 0.25% sodium deoxycholate and 0.25 M NaCl as previously described [12]. The plasma-membrane-rich fraction (from cauda spermatozoa) was solubilized by suspension in a small volume of the above phosphate buffer containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.25 M NaCl, followed by homogenization in a glass homogenizer with a Teflon pestle rotating at 1200 rev./min (five up and five down strokes). The homogenate was centrifuged at 165000 *g* for 30 min and the supernatant was removed by aspiration. The pellet was suspended in a small volume of the above buffer, homogenized and centrifuged as above. The combined supernatants were designated the solubilized membrane fraction.

Immunoprecipitation studies

In these studies, the appropriate antibody (IgG fraction) was preadsorbed to washed IgGSorb as described [15]. Excess antibody was removed by washing in detergent/salt solution. The washed pellet was mixed with a small volume of the luminal fluid or solubilized membrane fractions, and the mixture was kept at room temperature for 60 min with occasional mixing. The supernatant obtained after centrifugation was used for quantifying enzyme activities. However, if the antibody–antigen complex was used for enzyme assays, the pellet was washed twice to remove adsorbed proteins [15]. The washed pellet (immobilized antibody–antigen complex) suspended in the desired buffer was mixed with appropriate [^{3}H]mannose-labelled substrate and incubated at 37 °C for the indicated time.

SDS/PAGE

The system of Laemmli [16] was used to resolve the proteins under reducing conditions. Briefly, the sample was solubilized by mixing in SDS buffer containing 2.5% 2-mercaptoethanol, and placing the tube in boiling water for 5 min. The heat-treated sample was centrifuged in a microcentrifuge for 2 min. The solubilized material removed by aspiration was resolved on a 1.5 mm-thick polyacrylamide gel along with the high-molecular-mass marker proteins as described [15]. Gels were stained for protein with 0.5% Coomassie Blue in 20% methanol/10% acetic acid and destained in 10% methanol/7% acetic acid.

Electrotransfer and immunostaining

The resolved polypeptides were electrophoretically transferred to 0.45 μm-pore nitrocellulose sheets by the method of Towbin et al. [17]. After transfer, the immunoreactive polypeptides were identified by our published procedure [15] using antibody (IgG fraction) to the 115 kDa subunit (see below) as primary antibody.

Production of antiserum

The purified enzyme prepared from the epididymal fluid of 20 rats (approx. 40 μg of enzyme protein) was first resolved by SDS/PAGE under reducing conditions along with one lane of prestained protein markers. The gel was cut along the line of the position of the major protein band, and used as a source of antigen. Before immunization, a female (virgin) white rabbit (approx. 2 kg) was bled via an ear vein, and the blood (15–20 ml) was used for preparation of preimmune serum. The gel pieces containing the antigen were homogenized in 1.5 ml of 10 mM potassium phosphate buffer, pH 7.2, containing 0.25 M NaCl and the homogenate was emulsified in an equal volume of Freund's complete adjuvant. The rabbit was injected (multiple sites) subcutaneously on the back. The animal received booster injections of the same amount of antigen (see above), emulsified in Freund's incomplete adjuvant (subcutaneously on the back, and intramuscularly in the thighs) at day 10, 25 and 35. At 10 days after the last booster, the animal was bled by cardiac puncture, and serum was prepared as described [18]. The γ-globulin (IgG fraction) was prepared from the preimmune and immune serum by affinity chromatography as described above.

Immobilization of D-mannosamine and mannan

Coupling of D-mannosamine to Sepharose 4B

AH-Sepharose 4B gel was extensively washed with glass-distilled water on a sintered glass filter. The washed gel (40 ml of settled gel) was suspended in 200 ml of 0.1 M succinic anhydride solution and the pH adjusted to 6.0 using 1 M NaOH. The mixture was kept overnight at room temperature with gentle rocking. The gel was washed with distilled water and allowed to settle. Mannosamine (1.5 g) dissolved in 20 ml of glass-distilled water was added to 20 ml of the settled gel, and the pH of the mixture adjusted to 4.7. Carbodi-imide solution (0.8 g in 5 ml of distilled water) was added to the gel/mannosamine mixture over a period of 30 min. The pH was maintained at 4.7 for an additional 30 min and the reaction was allowed to proceed at room temperature with gentle rocking. After 24 h, the gel was washed with 1 litre each of distilled water and 1 M NaCl. The gel was stored in 1 M NaCl containing 0.02% sodium azide at 0–4 °C.

Coupling of mannan to Sepharose 6B

Epoxy-activated Sepharose 6B (4 g) was extensively washed with distilled water, suspended in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.0) and the gel allowed to settle at room temperature. Then 10 ml of the settled gel was mixed with 30 ml of the above buffer containing 1 g of mannan. The mixture was gently shaken at 30 °C for 20–22 h. After the reaction, the gel was washed with 600 ml of distilled water and 200 ml of the above Na_2CO_3/$NaHCO_3$ buffer. The washed gel was suspended in 50 ml of the above buffer containing 1 M ethanolamine, and the mixture kept at room temperature for 12–15 h. The coupled gel was washed thoroughly with distilled water (200 ml), 0.1 M sodium acetate buffer containing 0.1 M NaCl (approx. 300 ml) and 0.1 M Na_2CO_3/$NaHCO_3$ buffer containing 0.5 M NaCl (approx. 500 ml). The gel was stored at 0–4 °C in 0.1 M NaCl solution containing 0.02% sodium azide.

Estimation of the immobilized ligands

For the estimation of D-mannosamine, the conjugated resin was thoroughly washed in distilled water and hydrolysed for 6 h at 100 °C in 4 M HCl [19]. The released amino sugar was separated from the interfering substances by ion-exchange chromatography and assayed after deamination with nitrous acid [20], with D-mannosamine as standard. The resin was found to contain 12.8 mg of covalently bound D-mannosamine per ml of packed gel. The amount of mannan covalently bound to Sepharose was determined by the phenol/sulphuric acid method [21], scaled down 4-fold, with mannan as standard. Briefly, samples from a 5% suspension of the washed gel (before and after immobilization of the mannan) were analysed for carbohydrate content. A value of 15 mg of mannan per ml of packed gel was calculated from the difference in absorbance (490 nm) before and after conjugation of the ligand.

Enzyme assays

Unless otherwise indicated, *p*-nitrophenyl α-D-mannosidase (PNP-mannosidase) activity was quantified by measuring the release of *p*-nitrophenol [22]. The reaction mixture (0.5 ml) contained the desired buffer (100 mM), 4 mM PNP-mannosidase and 0.2% Triton X-100. After the incubations were carried out for 15–60 min at 37 °C, the reaction was stopped by adding 1 ml of an alkaline buffer adjusted to pH 10.7 [22]. The amount of *p*-nitrophenol released was quantified by measuring the absorbance at 400 nm. One unit is the amount of enzyme that catalyses the release of 1 μmol of *p*-nitrophenol/h.

[^{3}H]Man-mannosidase activity ([^{3}H]mannose-labelled oligosaccharide, glycopeptide and glycoprotein-cleaving activity) was assayed by measuring the amount of [^{3}H]mannose cleaved in a standard incubation mixture (50 μl) containing labelled substrate, desired buffer (100 mM) and enzyme. After incubation for the indicated time, the reaction was stopped by placing the tube in boiling water (3–5 min). The released [^{3}H]mannose was separated from the labelled substrates by gel filtration on a column of Bio-Gel P-2, and quantified as described [4]. One unit is the amount of enzyme that catalyses the release of 1000 c.p.m. of [^{3}H]mannose/h at 37 °C.

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

Purification of epididymal-fluid α-D-mannosidase

Unless otherwise indicated, all purification steps were carried out at 0–4 °C. The following buffers were used: buffer A, pH 7.4 (10 mM Hepes buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.5 mM $CoCl_2$); buffer B, pH 7.4 (same as buffer A containing 10 mM-NaCl); buffer C, pH 7.4 (same as buffer A containing 0.05 M NaCl); buffer D, pH 7.4 (same as buffer A containing 0.1 M NaCl); buffer E, pH 7.4 (same as buffer A containing 0.5 M NaCl); buffer F, pH 7.5 (10 mM Tris/HCl containing 0.1 M NaCl).

Step 1: $(NH_4)_2SO_4$ precipitation

The epididymal fluid (approx. 50 ml) was prepared from 10 or 11 rats as described above. The slightly turbid fluid was mixed with solid $(NH_4)_2SO_4$ with constant stirring to achieve 45% saturation. The mixture was kept at 0–4 °C for 30 min, and the precipitate was collected by high-speed centrifugation (165000 *g* for 30 min). The pelleted enzyme was suspended in 20–25 ml of buffer A, and 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 as above, and the clear supernatant removed by aspiration.

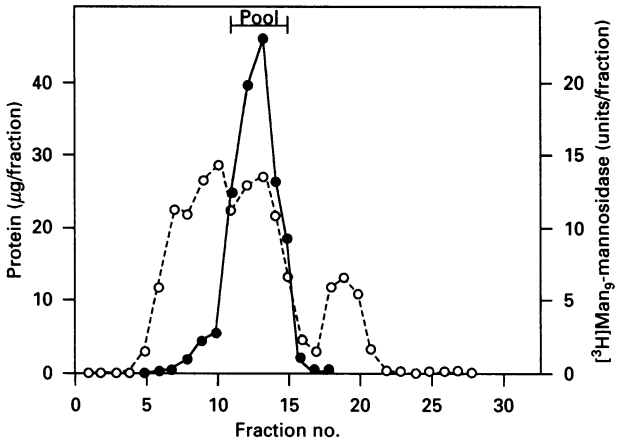

Figure 1 Chromatography of epididymal fluid α-D-mannosidase on a column of DE-52

Approximately 210 units of the enzyme was applied to the column (1 cm × 4 cm) and eluted with a linear gradient of NaCl. Other details are described in the text. ○, Protein; ●, [^{3}H]Man$_9$-mannosidase activity.

Table 1 Purification of α-D-mannosidase from rat epididymal fluid

The fluid was prepared from the epididymides of ten rats. The enzyme from various purification steps was assayed using 3000 c.p.m. of [^{3}H]Man$_9$GlcNAc at pH 6.5 as described in the Materials and methods section.

Purification step	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg)	Enzyme recovery (%)	Purification (fold)
Epididymal fluid	141.0	354	2.5	100	1
$(NH_4)_2SO_4$ (0–45%)	17.2	417	24.2	118	10
Mannan column	2.5	230	92.0	65	37
Mannosamine column	0.35	210	600.0	59	240
DE-52 column	0.13	92	697.0	26	279
Sephacryl S-400	0.026	32	1231.0	9	492

Step 2: immobilized mannan column chromatography

To the clear supernatant, stock NaCl solution (500 mM) was added to achieve a salt concentration of 10 mM. The sample was applied to the immobilized affinity column (0.5 cm × 8 cm) equilibrated with buffer B. After the column had been washed with 25–35 ml of buffer B, the enzyme was eluted with 20–35 ml of buffer C. Samples were saved for determination of enzyme activity and protein contents.

Step 3: immobilized mannosamine column chromatography

The eluted enzyme in step 2 was mixed with 1 M NaCl to achieve a final salt concentration of 0.1 M, and then applied to the affinity column (1 cm × 7 cm) equilibrated with buffer D. After application of the enzyme, the column was washed with 30–35 ml of buffer D, and the α-D-mannosidase activity eluted with 20–25 ml of buffer E.

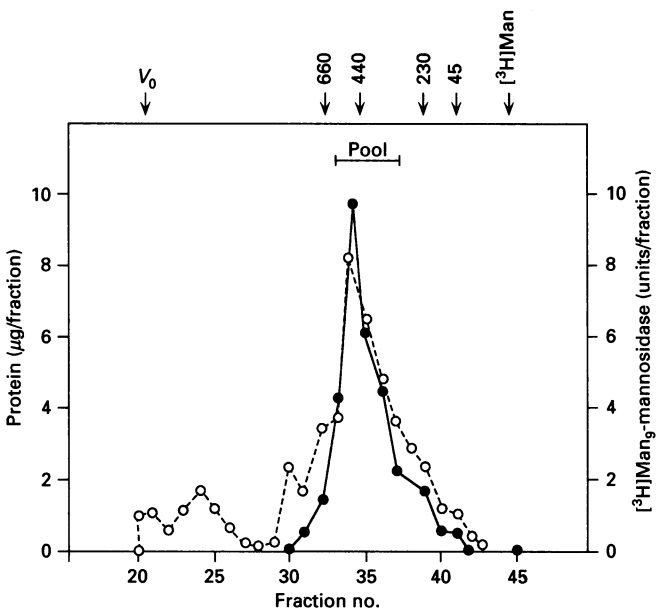

Figure 2 Purification of α-D-mannosidase by chromatography on a column of Sephacryl S-400

The enzyme from the pooled fractions in Figure 1 was precipitated with $(NH_4)_2SO_4$ (75% saturation) and collected by high-speed centrifugation. The precipitated enzyme was dissolved in 1.5 ml of buffer F and applied to the column (1.5 cm × 97 cm) equilibrated with the above buffer. The column void volume (V_0) was determined using Blue Dextran 2000. The standard marker proteins (see arrows) are: thyroglobulin, 660 kDa; ferritin, 440 kDa; catalase, 230 kDa; ovalbumin, 45 kDa. The column inclusion volume was determined with [^{3}H]mannose. Other details are described in the Materials and methods section. ○, Protein; ●, [^{3}H]Man$_9$-mannosidase activity.

Step 4: DE-52 column chromatography

The eluted enzyme was dialysed against 100 vol. of buffer C for 4–6 h with four changes of the buffer. The dialysed sample was applied to a DE-52 column (1 cm × 4 cm) equilibrated with buffer C. After the column had been washed with 30–35 ml of buffer C, it was eluted with 100 ml of a linear NaCl gradient (0.05–0.3 M) in buffer A. Fractions (3.6 ml) were collected at a flow rate of 15–20 ml/h. Portions of each fraction were checked for protein and [^{3}H]Man$_9$-mannosidase activity. The enzyme began to appear in fraction number 9 and the peak activity was present in fraction number 12 (Figure 1). Pooled fractions showed a specific activity of nearly 700 units/mg of protein, which corresponds to nearly 300-fold purification over the epididymal fluid (Table 1).

Step 5: molecular-sieve column chromatography

The [^{3}H]Man$_9$-mannosidase activity was precipitated from the pooled fractions (Figure 1) by adding solid $(NH_4)_2SO_4$ to achieve 75% saturation, and the precipitated enzyme collected by high-speed centrifugation (165000 *g* for 30 min). The residue was dissolved in 1.5–2.0 ml of buffer F, and resolved by gel filtration on a column (1.5 cm × 97 cm) of Sephacryl S-400 equilibrated with buffer F. Fractions (4.2 ml) were collected at a flow rate of 20 ml/h. Between 9 and 12% of the original enzyme activity was eluted from the column at an apparent molecular mass of 460 kDa (Figure 2). The enzyme-rich fractions were pooled, and concentrated to 0.1–0.2 ml using a microconcentrator.

Data obtained from a typical purification experiment are presented in Table 1. The enzyme activity towards the oligosaccharide ([^{3}H]Man$_9$GlcNAc) was purified nearly 500-fold

with a recovery of 9%. The purified enzyme showed no detectable activity when incubated with *p*-nitrophenyl α-D-mannoside at pH 4.5 or pH 6.5.

RESULTS

Evidence for the presence of [^{3}H]Man$_9$-cleaving mannosidase activity in the epididymal luminal fluid

In previous studies we observed that when rat cauda spermatozoa were fractionated using discontinuous Percoll density-gradient centrifugation, most of the oligosaccharide-cleaving activity was found as a peak in fractions containing spermatozoa (fractions 8–10 of Fig. 1A in [12]). However, most of the PNP-mannosidase activity and a little over 20% of the [^{3}H]Man$_9$-mannosidase activity was present (in soluble form) in distinct peaks on top of the gradient (fractions 15–20) [12]. Our preliminary attempts in the present studies were directed to characterizing the two activities (PNP-mannosidase and [^{3}H]Man$_9$-mannosidase) present in soluble form in the epididymal lumen. The luminal fluid was prepared from the rat cauda epididymidis by retrograde flushing of the vas deferens, and the clear fluid was obtained by high-speed centrifugation. The supernatant was assayed for PNP-mannosidase and Man$_9$-mannosidase activities over a wide range of pH, before and after immunoprecipitation of the acidic (lysosomal) α-D-mannosidase activity. The data from a typical experiment presented in Figure 3(a) confirmed the presence of PNP- and [^{3}H]Man$_9$-mannosidase activities in the cauda fluid. That the PNP-mannosidase activity present in the fluid reflected the presence of acidic (lysosomal) α-D-mannosidase was indicated by (a) its acidic (4.5) pH optimum (Figure 3a) and (b) its quantitative immunoprecipitation with a highly specific polyclonal antibody to the epididymal (lysosomal) α-D-mannosidase (Figure 3b). The oligosaccharide-cleaving activity left after immunoprecipitation of the PNP-mannosidase

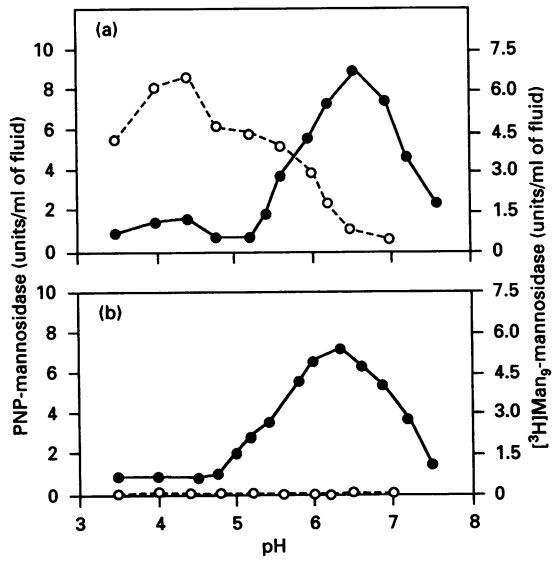

Figure 3 Effect of pH on α-D-mannosidase activities present in rat cauda fluid

Luminal fluid from the cauda epididymidis was prepared by retrograde flushing of vas deferens and high-speed centrifugation. Samples were incubated with 4 mM *p*-nitrophenyl α-D-mannoside (○) or 3000 c.p.m. of [^{3}H]Man$_9$GlcNAc (●) before (**a**) or after (**b**) removal of lysosomal (acid) α-D-mannosidase by immunoprecipitation. The reaction buffers were 0.1 M sodium citrate (pH 3.5), 0.1 sodium acetate (pH 4.0–5.8) and 0.1 M sodium cacodylate buffer (pH 6.0–8.0).

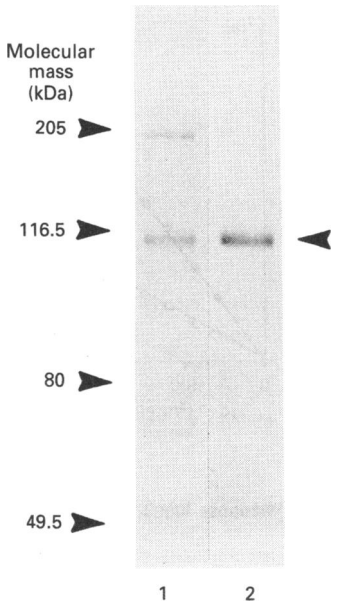

Figure 4 Electrophoretic behaviour of the purified α-D-mannosidase

SDS/PAGE (7.5% gel under reducing conditions [16]) was carried out on enzyme pool after DE-52 column chromatography (lane 1, 3.1 μg of enzyme protein containing 2.5 units of activity) or after Sephacryl S-400 column chromatography (lane 2, 3.2 μg of enzyme protein containing 3.9 units of activity). The positions of the prestained standard marker proteins are shown on the left.

showed optimal activity at pH 6.2–6.5, a value similar to the pH optimum of the novel α-D-mannosidase activity we have characterized on sperm plasma membranes of several species [12,23]. Furthermore, the soluble activity, like the sperm plasma-membrane [^{3}H]Man$_9$-mannosidase [12], showed little or no hydrolysis of the *p*-nitrophenyl α-D-mannoside substrate (Figure 3b) and was inhibited similarly by several sugars (see below). These similarities and the fact that our initial attempts to solubilize quantitatively the sperm plasma-membrane [^{3}H]Man$_9$-mannosidase activity were unsuccessful, prompted us to use the luminal fluid as a source of the enzyme in soluble (detergent-free) form for the purpose of purification, characterization and antibody production. Details of enzyme purification are described in the Materials and methods section.

Purity of the concentrated enzyme

Preliminary attempts to resolve the enzyme under non-denaturing conditions at pH 8.9 (Davis system [24]) proved unsuccessful. However, the purified enzyme, when resolved on SDS/PAGE under denaturing conditions and stained for proteins, showed a major protein band (apparent molecular mass 115 kDa) and two to three very faint bands (Figure 4, lane 2).

Kinetic properties of the purified enzyme

The concentrated enzyme is very unstable when stored at 0–4 °C (loss of 60–70% of the activity within 24 h). Addition of Co^{2+} ions (which stimulate the enzyme activity in crude preparations and plasma-membrane preparations [12]) did not improve the stability of the enzyme. The enzyme showed maximum activity at pH 6.2–6.5 with oligosaccharide ([^{3}H]Man$_9$GlcNAc) substrate as shown in Figure 3. This value is similar to the pH optimum of the rat sperm plasma-membrane α-D-mannosidase activity [12]. Under standard assay conditions, the rates of hydrolysis of

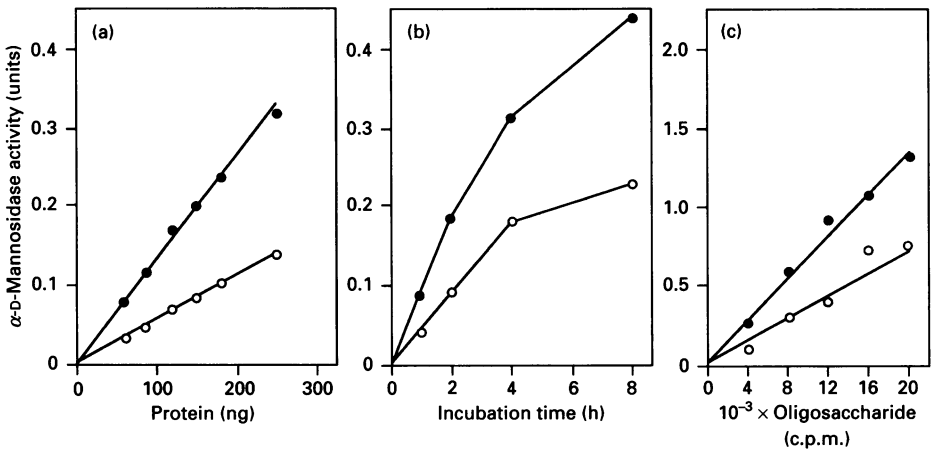

Figure 5 Hydrolysis of [^{3}H]mannose-labelled oligosaccharides by the epididymal fluid α-D-mannosidase

(**a**) Effect of protein concentration on rate of hydrolysis of oligosaccharides; (**b**) influence of incubation time on rate of hydrolysis; (**c**) influence of oligosaccharide concentration on rate of hydrolysis. ●, [^{3}H]Man_9GlcNAc; ○, [^{3}H]Man_5GlcNAc.

[^{3}H]Man_9GlcNAc and [^{3}H]Man_5GlcNAc were directly proportional to the concentration of enzyme protein (Figure 5a). With 150 ng of enzyme protein, the reaction was linear for up to 4 h (Figure 5b). The reaction remained linear when increasing concentrations of the two substrates were incubated for 4 h at 37 °C (Figure 5c). However, [^{3}H]Man_9GlcNAc was more readily hydrolysed by the enzyme than the [^{3}H]Man_5GlcNAc, a result consistent with the assumption that the former substrate has more susceptible mannosyl residues than are present in [^{3}H]Man_5GlcNAc.

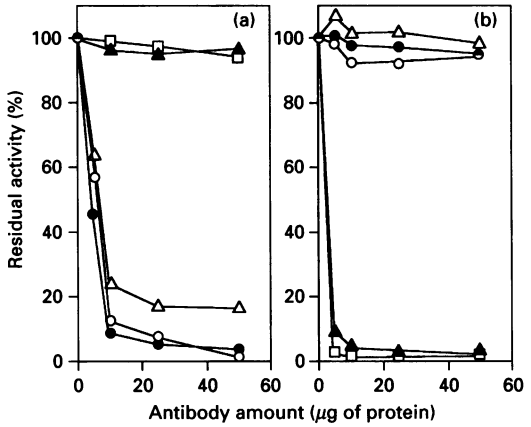

Figure 6 Immunoprecipitation of rat epididymal fluid and sperm mannosidases using affinity-purified anti-(115 kDa subunit) antibody (a) or affinity-purified anti-[epididymal (lysosomal) α-D-mannosidase] antibody (b)

The cauda fluid or detergent-solubilized spermatozoa was added to 100 μl of prewashed IgGsorb to which the indicated amount of antibody had been preabsorbed. After 60–90 min at room temperature, the mixture was centrifuged in a Microfuge for 2 min. The enzyme activity remaining in the supernatant was determined as described in the Materials and methods section, and plotted against the concentration of the antibody. Man_9-mannosidase activity (pH 6.5) in rat cauda fluid (○), detergent-solubilized rat (●) and mouse (△) spermatozoa; PNP-mannosidase activity (pH 4.5) in rat cauda fluid (▲) and detergent-solubilized rat spermatozoa (□). Nearly all of the [^{3}H]Man_9-mannosidase activity was recovered in the supernatant solution when IgG from preimmune serum was used.

Table 2 Effects of various sugars on purified epididymal fluid α-D-mannosidase

The stock sugar solution was added to the purified enzyme solution containing 100 mM sodium cacodylate buffer, pH 6.5. After the mixture had been kept at 0–4 °C for 10–15 min, substrate (4000 c.p.m. of [^{3}H]Man_9GlcNAc) was added, and the reaction mixture incubated for 4 h at 37 °C. The reaction was stopped by heat-treatment (2–3 min in boiling water), and released [^{3}H]mannose separated from the oligosaccharide by gel filtration was quantified as described in the Materials and methods section.

Addition	Concentration (mM)	[^{3}H]Mannose released (%)	Relative activity (%)
None	–	21.2	100
D-Mannose	10	15.5	73
	50	11.9	56
Methyl α-glucoside	10	16.4	77
	50	10.4	49
Methyl α-mannoside	10	12.4	59
	50	9.6	45
D-Mannosamine	1	9.3	44
	5	7.4	35
Mannan	50	14.3	67
	100	11.3	53

Comparison between epididymal fluid and sperm plasma-membrane [^{3}H]Man_9-mannosidase activities

Effect of inhibitors

Several sugars have previously been shown to have a dose-dependent inhibitory effect on rat sperm plasma-membrane mannosidase [12]. It was therefore important to study the effect of these sugars on the enzyme purified from the epididymal fluid. Data presented in Table 2 show that, like the sperm plasma-membrane α-D-mannosidase, the soluble enzyme is inhibited by D-mannose, methyl α-glucoside, methyl α-mannoside and D-mannosamine in a dose-dependent manner. In addition, mannan (a polymer of mannose) showed inhibition of the enzyme at higher concentrations (Table 2).

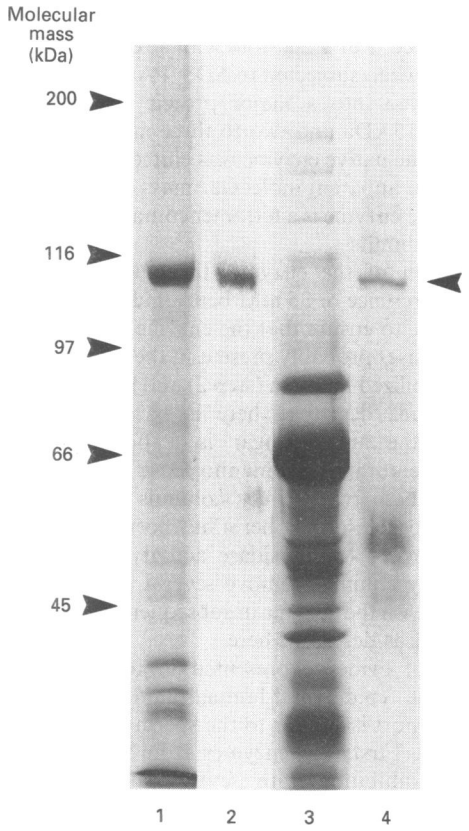

Figure 7 Immunodetection of α-D-mannosidase present on sperm plasma membrane and in cauda fluid

The sperm plasma membranes (approx. 42 μg of protein containing 3.0 units of activity in lanes 1 and 2) and epididymal fluid (approx. 200 μg of protein containing 1.2 unit of activity in lanes 3 and 4) were resolved on SDS/PAGE, as described in the legend to Figure 4, and the polypeptides visualized by protein staining (lanes 1 and 3) or immunostaining using anti-(115 kDa protein band) (2 μg of IgG protein/ml) as primary antibody (lanes 2 and 4). The positions of the high-molecular-mass marker proteins are shown on the left. The 115 kDa protein band seen in lanes 2 and 4 was not observed when preimmune IgG was used. However, the preimmune IgG stained the minor bands seen between 66 and 45 kDa standards in lanes 2 and 4. Other details are described in the Materials and methods section.

Immunological relationship

Polyclonal antibody (IgG fraction) prepared against the major protein band (see Figure 4 and the Materials and methods section) was used to determine immunological relationships among the PNP-mannosidase and [^{3}H]Man_9-mannosidase activities present in the epididymal fluid and spermatozoa of the rat and mouse. Data from these studies presented in Figure 6 indicate that the antibody cross-reacted with the [^{3}H]Man_9-mannosidase activities present in the rat epididymal fluid and spermatozoa from these two rodent species, a result suggesting that the enzymes present in the epididymal fluid and spermatozoa share a common antigenic site(s). No cross-reactivity was observed with the acidic (lysosomal/acrosomal) α-D-mannosidase (PNP-mannosidase) present in the fluid and spermatozoa.

Structural relationship

The antibody was also used to determine the structural relationship between the luminal fluid and sperm plasma-membrane α-D-mannosidase. In these studies, the cauda fluid and purified sperm plasma membranes were resolved by SDS/PAGE, transferred to nitrocellulose sheets, and the antigen was detected using antibody to the soluble enzyme. Data from these studies are presented in Figure 7.

Table 3 Substrate specificity of rat epididymal fluid and sperm plasma-membrane α-D-mannosidase activity

Cauda fluid (approx. 200μg of protein) or salt/detergent-solubilized sperm plasma membranes (approx. 25 μg of protein) were incubated with washed IgGSorb that had been preadsorbed with 10 μg of the anti-(epididymal fluid 115 kDa subunit) antibody. The immobilized antibody–antigen complex was washed to ensure the removal of acidic (lysosomal/acrosomal) α-D-mannosidase present in the fluid and sperm plasma membranes. The antibody–antigen complex was suspended in 25–35 μl of 100 mM sodium cacodylate buffer, pH 6.5, mixed with approx. 4000 c.p.m. of the appropriate substrate and incubated at 37 °C for 4 h or 24 h. Free [^{3}H]mannose was separated from the partially cleaved oligosaccharides, glycopeptide and glycoprotein, and quantified as described in the Materials and methods section.

	α-D-Mannosidase activity (% of [^{3}H]mannose released)			
	Epididymal fluid		Sperm plasma membrane	
Substrate	4 h	24 h	4 h	24 h
[^{3}H]Man_9GlcNAc	18	62	26	70
[^{3}H]Man_8GlcNAc	17	65	21	68
[^{3}H]Man_7GlcNAc	15	60	19	65
[^{3}H]Man_6GlcNAc	14	48	21	52
[^{3}H]Man_5GlcNAc	9	18	12	25
GlcNAc[^{3}H]Man_5GlcNAc	7	16	9	23
[^{3}H]Man-glycopeptide*	15	38	16	34
[^{3}H]Man-glycoprotein†	3	7	2	5

* Treatment with jack-bean α-D-mannosidase as described [4] released 48 and 70% of [^{3}H]mannose after incubation for 4 h and 24 h respectively.

† Nearly 32% of [^{3}H]mannose was cleaved when the glycoprotein was treated with jack-bean α-D-mannosidase for 24 h as described [4].

Substrate specificity

The cross-reactivity of the anti-(rat epididymal fluid α-D-mannosidase) antibody with the sperm plasma-membrane enzyme made it possible to examine the substrate specificity of the enzyme from the two sources using the immunoprecipitation approach as described [15]. Solubilized sperm plasma membranes (approx. 25 μg of protein) and cauda fluid obtained by flushing (approx. 200 μg of protein) were incubated with IgGsorb that had been preadsorbed with the antibody as described in the Materials and methods section. The mixture was kept at room temperature for 60–90 min with occasional mixing. The immobilized antibody–antigen was extensively washed by suspension in the salt/detergent solution and centrifugation. The immobilized antigen was incubated with uniformly [^{3}H]mannose-labelled oligosaccharides for two time periods (4 h and 24 h), and the released [^{3}H]mannose quantified as described in the Materials and methods section. The results presented in Table 3 show that the α-D-mannosidase from these two sources cleaves α1,2-linked mannosyl residues present in Man_{9-6}GlcNAc substrates in a time-dependent manner. In addition, the immobilized enzyme cleaves nearly 25% of [^{3}H]mannose from Man_5GlcNAc and GlcNAcMan_5GlcNAc in 24 h, the two substrates that contain α1,3- and α1,6-linked mannosyl residues. We also studied the release of free [^{3}H]mannose from labelled glycopeptide and glycoprotein prepared as described in the Materials and methods section. Data from these studies are also presented in Table 3.

Table 4 Assay for α-D-mannosidase activities in the testis and epididymis of sexually immature rats

Particulate fraction prepared from the testis and epididymis of 28–30-day-old rats was solubilized by extraction with salt/detergent solution. Samples were assayed for enzyme activities before and after precipitation with the polyclonal antibodies to the specific mannosidase. The values reported are averages of three different experiments with the range indicated.

Assay	Enzyme activity (units/g of tissue)	
	Testis	Epididymis
Lysosomal α-D-mannosidase*	0.16 ± 0.01	4.13 ± 0.95
Golgi mannosidase IA†	5.60 ± 2.50	24.61 ± 0.55
Residual activity	8.40 ± 1.84‡	2.63 ± 1.40§
Tissue weight (g/tissue)	0.51	0.062

* Assayed at pH 4.4 using 4 mM *p*-nitrophenyl α-D-mannoside as substrate. The activity was quantitatively immunoprecipitated with anti-[rat epididymal (lysosomal) α-D-mannosidase] antibody.

† This activity, assayed at pH 6.5 using approx. 5000 c.p.m. of [^{3}H]Man_9GlcNAc, was immunoprecipitated with anti-(rat Golgi mannosidase IA) antibody.

‡ Over 60% of this activity cross-reacted with anti-(115 kDa protein) antibody.

§ Less than 25% of this activity cross-reacted with the antibody to 115 kDa antigen.

Source of sperm α-D-mannosidase activity

The presence of similar α-D-mannosidase activities in a soluble form and in a sperm membrane form raised the possibility that the enzyme present in the luminal fluid may become associated with the spermatozoa during epididymal transit. This possibility was explored by carrying out two sets of experiments. First, the rete testis fluid collected by a published procedure [25] showed the presence of [^{3}H]Man_9-mannosidase activity in the fluid and spermatozoa (results not shown). Second, the particulate (membrane-bound) fraction prepared from the testis of 28–30-day-old rats contained a significant level of [^{3}H]Man_9-mannosidase activity, most of which cross-reacted with the antibody to 115 kDa protein (Table 4). However, the particulate fraction prepared from the epididymides of these rats contained very little α-D-mannosidase activity which cross-reacted with this antibody.

DISCUSSION

Previous studies from this laboratory produced evidence suggesting that the novel α-D-mannosidase activity present on spermatozoa of several species is an intrinsic plasma-membrane component [12,23]. Because of the potential role of the sperm surface enzyme in fertilization, attempts were first made to solubilize the enzyme for the purpose of purification and antibody production. In these studies, rat spermatozoa were repeatedly extracted in 1% Triton X-100, with or without 0.5 M NaCl, and high-speed centrifugation. The preliminary studies suggested that, regardless of the extraction solution used, only about 50–60% of the sperm-associated mannosidase could be solubilized. In subsequent studies, reported in the Results section, we were able to demonstrate the occurrence of an α-D-mannosidase activity in soluble form in the epididymal fluid which had properties similar to the sperm plasma-membrane mannosidase. Thus the epididymal fluid provided an excellent source of the enzyme in soluble (detergent-free) form for the purpose of purification and antibody production. The soluble enzyme was purified nearly 500-fold with a recovery of 9%. The purified enzyme, when subjected to SDS/PAGE under reducing conditions, resolved into a major protein band of apparent molecular mass 115 kDa and two to three minor bands (Figure 4). The fact that the native enzyme was eluted from a molecular-sieve column at an apparent molecular mass of 460 kDa (Figure 3) suggests that the enzyme is a tetramer containing four identical or very similar subunits.

For purification of the enzyme, the epididymal fluid was prepared in the presence of 25 mM benzamidine, an inhibitor of serine proteinases, to ensure that the enzyme was not altered by the serine proteinases probably present in the epididymal lumen. The use of immobilized mannan (step 2) and mannosamine (step 3) affinity columns described here is based on our report indicating that the amino sugar is a potent inhibitor of sperm plasma-membrane α-D-mannosidase activity [12]. The effectiveness of these two affinity columns (under conditions different from those described here) has been described for the purification of an α-D-mannosidase activity from plants [26]. These two affinity columns remove several proteins which are otherwise eluted with the enzyme in subsequent purification steps and must be used as described here.

Several lines of evidence presented in the Results section indicate that the epididymal-luminal-fluid α-D-mannosidase purified in this report is similar to the sperm plasma-membrane α-D-mannosidase. First, the enzymes from these two sources showed similar inhibition with several sugars including D-mannose, methyl α-glucoside, methyl α-mannoside and D-mannosamine. Secondly, the polyclonal antibody raised against the enzyme purified from the epididymal luminal fluid immunoprecipitated the α-D-mannosidase activities present in epididymal lumen and detergent-solubilized spermatozoa in a concentration-dependent manner (Figure 6a). The antibody, however, did not precipitate acid α-D-mannosidase activities present in the soluble and sperm extract, which were precipitated with anti-[epididymal (lysosomal) α-D-mannosidase] antibody (Figure 6b). Furthermore, the antibody recognized a 115 kDa polypeptide band when sperm plasma membranes or epididymal luminal fluid were resolved by SDS/PAGE and immunostained using anti-(epididymal fluid α-D-mannosidase) as primary antibody (Figure 7, lanes 2 and 4). Thirdly, the α-D-mannosidase activities from the two sources efficiently cleaved α-mannosyl residues from the oligosaccharides and glycopeptides but not from glycoproteins. Additionally, the two forms of enzyme cleaved at least one mannosyl residue from Man_5GlcNAc and GlcNAcMan_5GlcNAc (Table 3). These similarities allow us to suggest that the two forms of the enzyme are derived from a common precursor. Additional studies, such as tryptic mapping and/or partial amino acid sequencing, will be needed to establish the relationship between the α-D-mannosidase activities from the two sources.

The presence of similar enzyme activities in the epididymal fluid and sperm plasma membranes raises the possibility that the soluble enzyme may bind tightly to the sperm plasma membrane during the transit of spermatozoa through the epididymis. Two lines of evidence presented in the Results section allow us to conclude that the sperm plasma-membrane α-D-mannosidase is synthesized and expressed on the sperm surface during spermatogenesis. First, the spermatozoa collected from the rete testis exhibit α-D-mannosidase activity. Secondly, the particulate fraction prepared from the testis of 28–30-day-old rats, which contain no spermatozoa but do contain other germ cells, possesses membrane-bound α-D-mannosidase activity which cannot be immunoprecipitated with antibodies raised against epididymal

(lysosomal) α-D-mannosidase and Golgi mannosidase IA, but can be precipitated with antibody prepared against the 115 kDa protein band. However, most of the enzyme activity present in the epididymal particulate fraction was precipitated with the antibody to lysosomal and Golgi mannosidase IA. Combined, these results indicate that the membrane-bound novel α-D-mannosidase is expressed in the testis. Further studies are needed to examine the stage-specific synthesis and precise localization of the enzyme on testicular cells using an immunohistochemical approach. These studies, coupled with Western-blot analysis of the enriched populations of germ cells, will be needed to determine the site of synthesis and processing of the sperm surface α-D-mannosidase.

Nearly 25% of [^{3}H]Man_9-mannosidase activity of human semen is present in soluble form in the seminal plasma, and the remaining 75% is sedimented with the spermatozoa [23]. Although the origin of the soluble form of the enzyme is not yet known, it seems likely that this form, like the rat epididymal-fluid enzyme, comes from the testis. Since the polyclonal antibody prepared in this report cross-reacts with the human sperm α-D-mannosidase (D. R. P. Tulsiani, M. D. Skudlarek and M.-C. Orgebin-Crist, unpublished work), the antibody will be useful in establishing the relationship between the soluble and plasma-membrane α-D-mannosidase activities in human semen.

The presence of catalytically and immunologically similar α-D-mannosidase activities in soluble and plasma-membrane-bound forms deserves a comment. It has been known for quite some time that many proteins lead a dual existence, being present in both membrane-bound and soluble isoforms. The latter form is usually present in body fluids. A recent review article listed nearly 45 such proteins with a dual localization [27], including proteins of diverse functions such as neural-cell adhesion molecule [28–30], human epidermal growth factor receptor [31], nerve growth factor receptor [32,33] and Golgi sialyltransferase [34,35]. The membrane-bound and soluble isoforms are believed to be generated *in vivo* by either of two possible mechanisms. First, the two isoforms may be produced by separate biosynthetic pathways, by either alternate pre-mRNA splicing of a common transcript or transcription of closely related but distinct genes. Second, the release of soluble isoform may be a post-translational event resulting from hydrolytic cleavage (proteolysis) at a site adjacent to the membrane-spanning sequence [27]. Since the soluble and plasma-membrane-bound α-D-mannosidase activities are quite similar in their properties and subunit molecular mass, it seems likely that they are isoforms and that the soluble form is generated *in vivo* by either of the two mechanisms mentioned above. Additional studies on biosynthesis and processing of the two isoforms will probably disclose the mode of their generation.

That the sperm surface mannosidase has a receptor-like role in binding to the mannose-containing oligosaccharide units present on zona pellucida is suggested by several studies [36–40], including our recent study in the mouse [41]. The results of these studies include the following. (1) Treatment of hamster [36] and human [37] zona intact eggs with concanavalin A prevented sperm–egg binding. Since the lectin specifically binds to high-mannose/hybrid oligosaccharide unit(s), the results could be interpreted as saturation of the ligand site(s) for the sperm surface mannosidase. (2) Inclusion of methyl α-mannoside, methyl α-glucoside, D-mannitol and D-mannose (inhibitors of sperm plasma-membrane mannosidase) in the sperm–egg-binding assay caused a dose-dependent inhibition of sperm mannosidase and a similar dose-dependent inhibition in the number of spermatozoa bound per egg [38,41]. (3) Inclusion of polymannosylated oligosaccharide (a substrate for the sperm mannosidase) in the sperm–egg-binding assay caused a dose-dependent inhibition of the sperm mannosidase and a similar inhibition in sperm–egg binding [41]. (4) The presence of N-linked high-mannose/hybrid oligosaccharides, a potential substrate for the sperm enzyme on mouse zona pellucida [42], has been found. (5) Treatment of rat eggs (but not spermatozoa) with jack-bean α-D-mannosidase caused nearly complete inhibition of gamete binding [39]. Since the enzyme is known to cleave α-mannosyl residues, the observed effect is probably due to removal of the α-mannosyl residues from high-mannose/hybrid oligosaccharide chain(s) present on zona glycoconjugates. (6) There is evidence for the presence of mannose-binding antigen(s) on the spermatozoa from fertile (but not infertile) men [40]. Taken together, these results suggest that the sperm surface mannosidase has a receptor-like role in sperm–egg binding. The availability of polyvalent (polyclonal IgG) and univalent (Fab fragment) antibody will be useful in providing additional evidence for the functional significance of the sperm plasma-membrane mannosidase.

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