Novel α-D-Mannosidase of Rat Sperm Plasma Membranes: Characterization and Potential Role in Sperm–Egg Interactions

Daulat Ram P. Tulsiani, Marjorie D. Skudlarek, and Marie-Claire Orgebin-Crist

Center for Reproductive Biology Research, Department of Obstetrics and Gynecology, Vanderbilt School of Medicine, Nashville, Tennessee 37232-2633

Abstract. During the course of a study of glycoprotein processing mannosidases in the rat epididymis, we have made an intriguing discovery regarding the presence of a novel α-D-mannosidase on the rat sperm plasma membranes. Unlike the sperm acrosomal "acid" mannosidase which has a pH optimum of 4.4, the newly discovered α-D-mannosidase has a pH optimum of 6.2, and 6.5 when assayed in sperm plasma membranes and intact spermatozoa, respectively. In addition, the two enzymes show different substrate specificity. The acrosomal α-D-mannosidase is active mainly towards synthetic substrate, *p*-nitrophenyl α-D-mannopyranoside, whereas the sperm plasma membrane α-D-mannosidase shows activity mainly towards mannose-containing oligosaccharides. Evidence is presented which suggest that the sperm plasma membrane α-D-mannosidase is different from several processing mannosidases previously characterized from the rat liver.

The newly discovered α-D-mannosidase appears to be an intrinsic plasma membrane component, since washing of the purified membranes with buffered 0.4 M NaCl did not release the enzyme in soluble form. The enzyme requires nonionic detergent (Triton X-100) for complete solubilization. The enzyme is activated by Co^{2+} and Mn^{2+}. However, Cu^{2+} and Zn^{2+} are potent inhibitors of the sperm plasma membrane α-D-mannosidase. At a concentration of 0.1 mM, these divalent cations caused nearly complete inactivation of the sperm enzyme. In addition methyl-α-D-mannoside, methyl-α-D-glucoside, mannose, 2-deoxy-D-glucose, and D-mannosamine are inhibitors of the sperm surface α-D-mannosidase. The physiological role of the newly discovered enzyme is not yet known. Several published reports in three species, including the rat, suggest that the sperm surface α-D-mannosidase may have a role in binding to mannose-containing saccharides presumably present on the zona pellucida.

It is generally accepted that one step in the fertilization process requires interaction between complementary molecules present on the surface of the spermatozoon and the zona pellucida. The chemical nature of these complementary recognition sites is poorly understood, although there is growing evidence that carbohydrate moieties on surface membrane glycoconjugates are involved in these interactions (1, 34).

In mammals, several sperm proteins have been suggested to bind to zona pellucida (28). Some of these macromolecules have enzymatic activity and are thought to form a stable enzyme–substrate complex by binding to the oligosaccharide units present on the zona pellucida glycoproteins. In mice, galactosyltransferase present on the head region of the spermatozoa mediates sperm–egg binding by interacting with its substrate on the zona pellucida (23). There is also evidence that trypsin-like protease present on spermatozoa of mouse (4, 31) initiates sperm–egg binding. Mouse spermatozoa also contain sialyltransferase (13) and fucosyltransferase (30). The latter enzyme has been suggested to be involved in some aspect of sperm–egg recognition (Apter, F. M., J. M. Baltz, and C. F. Millette, unpublished data). Boar spermatozoa contain two fucosylated proteins of apparent relative molecular masses of 17 and 53 kD (10). One of these proteins (proacrosin, 53,000 M_r) interacts with the carbohydrate moiety of zona pellucida glycoprotein and is suggested to have a role in sperm–egg recognition (20).

In this report we present evidence for the occurrence of a previously uncharacterized α-D-mannosidase activity on the rat sperm plasma membranes and discuss the potential role of this enzyme in sperm–egg binding (Tulsiani, D. R. P., M. D. Skudlarek, and M.-C. Orgebin-Crist, unpublished data).

Materials and Methods

Materials

Sprague-Dawley rats (250–300 g) were from Sasco, Omaha, NE. Animals were killed by CO_2 asphyxiation. Each epididymis was removed, dissected from the testis and the fat pad, and used as described in each experiment. Uniformly labeled oligosaccharides, namely [^{3}H]Man$_9$GlcNAc, [^{3}H]Man$_8$-GlcNAc, and [^{3}H]Man$_5$GlcNAc were prepared as described (38). Sialylated hybrid oligosaccharide was prepared after endo-*N*-acetyl-β-D-glucosaminidase treatment of [^{3}H]mannose-labeled glycopeptides prepared from the rat epididymal epithelial cells cultured in the presence of swainsonine (10 μg/ml of the culture medium) as described previously (38). This oligosaccharide (NANAGalGlcNAcMan$_5$GlcNAc) was treated with sialidase

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and β-D-galactosidase as described (38), and the resulting oligosaccharide ($GlcNAcMan_5GlcNAc$) was purified by high resolution gel filtration on a column of Bio-Gel P-4 (42). Swainsonine isolated from *Rhizoctonia leguminicola* (32) was provided by Dr. H. P. Broquist of this university. 1-Deoxymannojirimycin was from Boehringer Mannheim Diagnostics, Inc., Houston, TX. Highly specific polyclonal antibody (IgG fraction) against rat liver Golgi mannosidase IA was prepared as described (41). This antibody has been shown to cross-react with Golgi mannosidases IA and IB (41). Anti-mannosidase II IgG was prepared as previously reported (24). Highly specific polyclonal antibody (IgG) against rat epididymal α-D-mannosidase was prepared as reported (44). This antibody has been shown to cross-react with the rat liver lysosomal α-D-mannosidase (44) and rat sperm acrosomal α-D-mannosidase (36). Anti-rat liver cytosolic α-D-mannosidase prepared as described (35) was a gift from Dr. Oscar Touster of this university. In addition to cytosolic α-D-mannosidase, this antibody cross-reacts with the novel α-D-mannosidase present in rat brain microsomes (39). The anti-rat liver-soluble α-D-mannosidase antibody was kindly provided by Dr. Rosalind Kornfeld of Washington University, St. Louis, MO. This antibody has previously been shown to cross-react with the liver-soluble and endoplasmic reticulum α-D-mannosidase (6). *Staphylococcus aureus* cells (IgGSorb) obtained from Enzyme Center, Inc. (Malden, MA) were washed (38) before suspending in 20 mM Tris-HCl buffer, pH 7.5 containing 1% Triton X-100, and 0.5 M NaCl (10% suspension). Methyl-α-D-mannoside, methyl-α-D-glucoside, D-mannose, and 2-deoxy-D-glucose were from Sigma Chemical Co., St. Louis, MO; D-mannosamine was from Aldrich Chemical Co., Milwaukee, WI. All other chemicals used were obtained commercially and were of the highest purity available.

Surgical Procedures

Mature, 16-wk-old male rats were anesthetized with an intraperitoneal injection of 30 mg nembutal and the animals were randomly unilaterally castrated under sterile conditions through a scrotal incision as previously described (16). Briefly, blood vessels and ductuli efferentes were tied close to the epididymis and cut close to the testis and the testis was removed. The epididymis was replaced in the scrotal sac, and the tunica, vaginalis, and scrotal skin were sutured, respectively. The procedure for efferent duct ligation was similar except that the testis and the testicular blood vessels were left intact. The animals were kept warm under a heat lamp (~25°C) at the time of surgery. After surgery, the animals were injected intramuscularly with 1 ml penicillin-streptomycin (10,000 U/ml). After 1 wk, each animal was placed with a sexually mature female rat to accelerate removal of caudal spermatozoa from the epididymis. The female was removed after 1 wk and replaced with a new female. Epididymal tissue was harvested 5 wk after surgery. In contrast to the contralateral epididymis, no spermatozoa were seen by phase contrast microscopy in minced tissue from the operated epididymis.

Preparation of Epididymal Particulate Fraction

The tissue homogenate was prepared in 0.25 M sucrose containing 5 mM Tris-HCl buffer, pH 8.0 (4 vol/g tissue) using a glass homogenizer fitted with a mechanically driven teflon pestle. Moderate pressure was applied to push the pestle rotating at 1,200 rpm for five up and down passes. The cytoplasmic extract was prepared by the low speed centrifugation (800 *g* for 10 min) of the homogenate as described (11). The supernatant was removed by aspiration, and the residue was homogenized and centrifuged as above. This process was repeated one more time. The particulate fraction was obtained by centrifuging the pooled cytoplasmic extract at 105,000 *g* for 30 min.

Solubilization of the Particulate Fraction

The epididymal particulate fraction was extracted by suspending the fraction in potassium phosphate buffer (10 mM, pH 7.2) containing 0.5% Triton X-100 and 0.25% sodium deoxycholate (1.5 vol/g tissue) followed by homogenizing in a glass homogenizer with a teflon pestle rotating at 1,200 rpm (five up and five down strokes). The suspension was centrifuged at 165,000 *g* for 30 min and the supernatant was removed by aspiration. The pellet was resuspended in a small volume of the above buffer (1 ml/g tissue), again homogenized, and centrifuged as above. The combined extract was designated as solubilized particulate fraction.

Preparation of Spermatozoa

The epididymis was removed and the cauda region (proximal and distal) was dissected and placed into a petri dish containing PBS and maintained either at room temperature or 0-4°C as indicated in each experiment. The epididymal cauda was minced several times with a razor blade to release spermatozoa in the PBS solution. The supernatant containing sperm cells was collected by aspiration and the spermatozoa were pelleted by centrifugation at 500 *g* for 10 min. The resultant sperm pellet was suspended in the desired buffer as described in each experiment.

Preparation of Subcellular Fractions from the Cauda Spermatozoa

The sperm pellet was suspended in 0.25 M sucrose containing 10 mM Pipes buffer (piperazine-*N*-N^1-bis 2-ethane sulfonic acid, pH 7.0, 4°C) and the suspension was sonicated for three 5-s bursts in a sonicator (model W-375; Heat Systems-Ultrasonic Inc., Farmingdale, NY) with the setting at energy level 5. The mild sonication conditions used here were designed to detach sperm membranes without causing extensive damage to the acrosomes as judged by their sedimentation with the residual fraction (see Results). The sonicated spermatozoa were centrifuged at 600 *g* for 10 min. The supernatant (cytoplasmic extract) was removed by aspiration and the pellet (residual fraction) was washed once by suspending in a small volume (1.0 ml) of the sonication buffer and centrifugation as above. The combined postresidual supernatant and wash was centrifuged at 105,000 *g* for 30 min. The supernatant (cytosolic fraction) was removed from the pellet (crude membranes) by aspiration. The nuclear pellet and crude membranes were suspended in a small volume of the sonication buffer without the sucrose. Aliquots from the membrane fractions and cytosolic fractions were analyzed for α-D-mannosidase activities and protein. Aliquots from the crude membrane fraction were pelleted in an airfuge (100,000 *g* for 30 min) and used for ultrastructural studies.

Isolation of the Sperm Plasma Membranes

The spermatozoa obtained from the cauda epididymidis of three rats were suspended in 30 ml of ice-cold PBS. Plasma membranes were prepared by the procedure of Olson et al. (26). Briefly, the spermatozoa were disrupted by nitrogen cavitation using a pressure of 400 psi and an equilibration period of 10 min (26). The cavitated sperm suspensions were centrifuged for 15 min at 500 *g* and the supernatant was removed by aspiration. Aliquots (8 ml) of the supernatant were layered on top of 2-ml steps of a 15% (wt/vol) and 50% (wt/vol) sucrose solution in 20 mM Pipes buffer, pH 7.2. The gradient was centrifuged in an SW 41 rotor (Beckman Instruments Inc., Palo Alto, CA) at 30,000 rpm for 90 min. The plasma membranes accumulated as a dense white band at the interphase between the 15 and 50% sucrose solutions. This band was collected by aspiration, diluted with 3 vol PBS and centrifuged at 105,000 *g* for 30 min in a 70.1 rotor (Beckman Instruments, Inc.). The resulting pellet was used for biochemical and ultrastructural studies.

Ultrastructural Studies

The isolated sperm membranes were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C as described (26). Thin sections, stained with uranyl acetate and lead citrate, were examined in a Hitachi H-600 electron microscope.

Immunoprecipitation Studies

The washed IgGSorb (100 μl) was incubated at room temperature for 60 min with or without antibody to rat liver Golgi mannosidase IA, antibody to mannosidase II, anti-rat epididymal α-D-mannosidase or anti-rat liver cytosolic/soluble α-D-mannosidase. The mixture was centrifuged in an Eppendorf microcentrifuge (Brinkman Instruments, Inc., Westbury, NY) for 1 min. The pellet was mixed with the small volume (50-75 μl) of the soluble epididymal particulate fraction and the mixture was kept at room temperature for 60 min with occasional mixing. The supernatant obtained after centrifugation was assayed for PNP-mannosidase/[^{3}H]Man_9-mannosidase activities. Similar protocol has been described in our publications (36, 39, 41, 44).

Percoll Density Gradient Centrifugation

Rat spermatozoa free of cytoplasmic droplets and other contaminant cells (epididymal epithelial cells, blood cells, etc.) were prepared by Percoll density gradient centrifugation (22) with some modification. Briefly, cauda segments from three rats were dissected and placed in a petri dish containing 8 ml of PBS (at room temperature). The tissue was minced several times

to allow release of spermatozoa in PBS. The sperm cells present in the supernatant were collected by aspiration. 4 ml of the sperm suspension were layered on top of a discontinuous Percoll gradient. The gradient was prepared in an ultra-clear 16 × 102-mm centrifuge tube (Beckman Instruments, Inc.) immediately before use and consisted of 2 ml of 80% Percoll, 2.5 ml of 60% Percoll, 3 ml of 40% Percoll, and 3 ml of 10% Percoll. Two such tubes were centrifuged at 1,500 g for 45 min in a centrifuge (model HN-S II; International Equipment Co., Needham Heights, MA) at room temperature. After centrifugation, the gradient tube was placed on a fraction recovery system (Beckman Instruments, Inc.). Fractions (0.6 ml) were collected from the bottom of the tube. Aliquots from each fraction were used for biochemical and morphological studies. The latter was done by phase contrast microscopy.

Enzyme Assays

PNP-α-D-mannosidase activity was routinely assayed by measuring the release of p-nitrophenol in a standard incubation mixture (0.5 ml) containing 4 mM p-nitrophenyl α-D-mannoside and the desired buffer as described (43). After incubation for 15–60 min at 37°C, the reaction was stopped by the addition of 1.0 ml of an alkaline buffer adjusted to pH 10.7 (43). The p-nitrophenol released was quantitated by measuring the absorbance at 400 nm. Enzyme and substrate blanks were run with all assays. 1 U of the PNP-α-D-mannosidase activity is the enzyme that catalyzes release of 1 μmol of p-nitrophenol per hour.

Acidic glycosidases, namely α-D-mannosidase, β-D-glucuronidase, and β-D-galactosidase were assayed by fluorometric method essentially as described (36). 1 U of enzyme activity is the amount of enzyme that catalyzes the release of 1 μmol of 4-methylumbelliferone per hour at 37°C.

[^{3}H]Man-mannosidase activity (oligosaccharide-cleaving activity) was assayed by measuring the hydrolysis of [^{3}H]mannose-labeled oligosaccharide in a standard incubation mixture (50 μl) containing radiolabeled substrate (∼3,000 cpm) and 100 mM of the desired buffer. After incubation for 1–4 h at 37°C, the reaction was stopped by placing the tube in boiling water (5–7 min). The released [^{3}H]mannose was separated from the labeled oligosaccharide by gel filtration on a column of Bio-Gel P-2 and quantitated as described (46). 1 U is the amount of enzyme that catalyzed the release of 1,000 cpm of [^{3}H]mannose per hour at 37°C. Adenylate cyclase was assayed essentially by the procedure described (18). 1 U is the amount of enzyme which catalyzes the production of 1 nmol of cyclic AMP per minute.

Protein was assayed by the fluorometric method (2) using BSA as standard.

Results

Mannosidases of the Mature Rat Epididymis

In preliminary studies, the particulate fraction was prepared from the epididymis of mature rats and solubilized as described in Materials and Methods. The solubilized extract contained >90% of the activities towards p-nitrophenyl α-D-mannoside substrate (PNP-mannosidase) and [^{3}H]mannose-labeled oligosaccharide substrate ([^{3}H]Man_8-mannosidase) originally present in the particulate fraction. That the PNP-mannosidase activity present in the detergent-salt extract reflected the presence of lysosomal-α-D-mannosidase was indicated by the following: (*a*) it possessed the acidic pH optimum (4.4) reported for lysosomal α-D-mannosidase (43); (*b*) it was likewise inhibited by swainsonine (44); and (*c*) it was selectively and nearly quantitatively immunoprecipitated with a highly specific antibody to the epididymal (lysosomal) α-D-mannosidase (Tables I and II). Attempts to characterize the enzyme activity towards [^{3}H]Man-labeled substrate with a pH optimum of 6.2 were partially successful. Only ∼30% of the oligosaccharide-cleaving activity present in the epididymal extract cross-reacted with the antibody to rat liver Golgi mannosidase IA (41). The remaining 70% of the oligosaccharide-cleaving activity showed no cross-reactivity with the antibodies to the mannosidases previously characterized in rat liver tissues (i.e., lysosomal α-D-mannosidase, liver Golgi mannosidase II, and cytosolic α-D-mannosidase). These preliminary studies provided evidence suggesting that the rat epididymis contained an α-D-mannosidase activity different from any of the several mannosidase activities previously characterized in rat tissues.

Table I. Assay for α-D-Mannosidase Activities in the Epididymis of Sexually Immature Rats

Assay	Solubilized particulate fraction*
	U/g epididymis
Lysosomal α-D-mannosidase‡	4.70 ± 0.73
Golgi mannosidase IA‖	8.29 ± 2.49
Newly discovered mannosidase activity§	1.44 ± 0.41
Tissue weight (g/epididymis)	0.082 ± 0.014

* Particulate fraction prepared from the epididymis of 30-d-old rats was solubilized by repeated extraction from a salt-detergent solution as described in Materials and Methods. Aliquots were assayed for enzyme activities before or after immunoprecipitation with the polyclonal antibodies to the specific mannosidase. The values reported are the average of three animals with ± indicating the range in values.

‡ The enzyme activity was assayed (before immunoprecipitation) at pH 4.4 using 4 mM p-nitrophenyl α-D-mannoside as substrate. This activity was nearly quantitatively immunoprecipitated with anti-rat epididymal (lysosomal) α-D-mannosidase antibody.

§ This activity ([^{3}H]Man_8-mannosidase) did not cross-react with the antibodies to the lysosomal α-D-mannosidase, Golgi mannosidase IA, Golgi mannosidase II, and cytosolic/soluble α-D-mannosidase. The enzyme activity reported here represents <4% of the activity normally found in the epididymis of the adult rat (also see Table II).

‖ The enzyme was assayed after immunoprecipitation with the antibody to liver Golgi mannosidase IA (41). The immunoprecipitated activity was quantitated by incubating antibody–antigen complex at pH 6.2 using ∼3,000 cpm of [^{3}H]Man_8GlcNAc as substrate.

Table II. Assay for α-D-Mannosidase Activities in the Epididymis of the Unilaterally Castrated or Unilaterally Ligated Mature Rat

Assay	Surgical procedures*		
	Normal	Castrated	Ligated
	U/g epididymis	*U/g epididymis*	*U/g epididymis*
Lysosomal α-D-mannosidase‡	3.85	3.71	–
	3.48	–	3.56
Golgi mannosidase IA‡	7.82	7.14	–
	9.05	–	12.30
Newly discovered mannosidase activity‡	41.25	0.93	–
	38.46	–	1.62
Tissue weight (g/epididymis)	0.35	0.20	–
	0.29	–	0.17

* 16-wk-old male rats were either unilaterally castrated or unilaterally ligated as described in Materials and Methods. Epididymal tissue from the normal and castrated/ligated side was excised 5 wk after the surgery and used to prepare the particulate fraction as described in Materials and Methods. The particulate fraction was solubilized and the aliquots were assayed for enzyme activities before or after immunoprecipitation with the polyclonal antibodies to the specific mannosidase. This table represents a typical experiment where results are reported for the unilaterally castrated or ligated epididymis and the contralateral control epididymis. Similar results were obtained in two additional experiments.

‡ See Table I for details of enzyme assays.

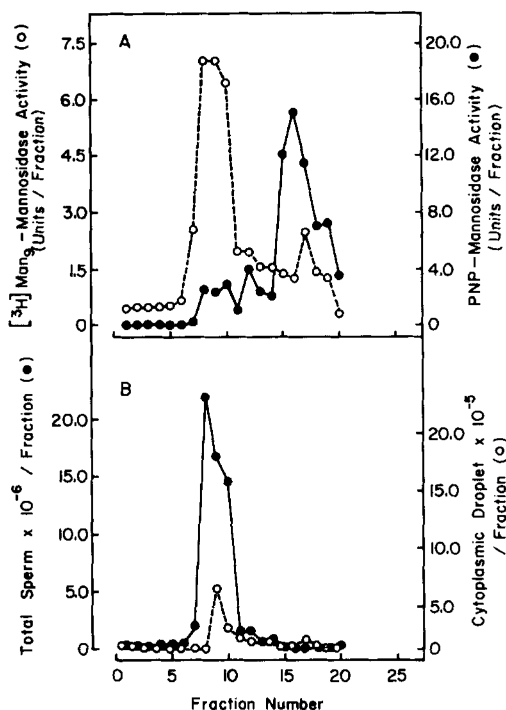

Figure 1. Distribution of PNP-mannosidase and Man_9-mannosidase activities after centrifugation of spermatozoa on a Percoll gradient. Rat spermatozoa were released from the cauda and separated on a Percoll density gradient as described in Materials and Methods. After centrifugation, the gradient tube was placed on a fraction recovery system. Fractions (0.6 ml) were collected from the bottom of the tube. Aliquots from each fraction were assayed for PNP-mannosidase activity (pH 4.4) and [^{3}H]Man_9-mannosidase activity (pH 6.5) as described in Materials and Methods (*A*). Aliquots from each fraction were fixed and the number of spermatozoa with or without the cytoplasmic droplets were examined by phase contrast microscopy (*B*).

Two sets of experiments indicated that nearly all of the newly discovered enzymatic activity observed in the epididymis of the mature rat was associated with the spermatozoa. First, the epididymis from sexually immature rats (30 d old) contained no significant level of the uncharacterized mannosidase activity(ies), although the tissue showed presence of the acidic (lysosomal) α-D-mannosidase and Golgi mannosidase I (Table I). Second, the newly discovered α-D-mannosidase activity in the epididymis from unilaterally ligated or unilaterally castrated rats showed a gradual decrease with time (data not shown). 5 wk after these surgical procedures, when the ligated or castrated side of the epididymis was completely devoid of spermatozoa, <3% of the enzyme activity could be detected in the tissue extract. These surgical procedures, however, had little or no effect on the lysosomal α-D-mannosidase and Golgi mannosidase IA activities (Table II). These studies provided evidence suggesting the presence of α-D-mannosidase activity(ies) in rat spermatozoa.

Sperm-associated α-D-Mannosidase

Our next set of experiments was designed to determine whether the sperm-associated cytoplasmic droplets are contributing to the oligosaccharide-cleaving α-D-mannosidase activity. To remove cytoplasmic droplets, spermatozoa were fractionated using Percoll density gradient centrifugation as described in Materials and Methods. Aliquots from each fraction were assayed for PNP-mannosidase and [^{3}H]Man_9-mannosidase activities (Fig. 1). Each fraction was also examined by phase contrast microscopy to quantitate spermatozoa and cytoplasmic droplets. As shown in Fig. 1 *A*, most of the oligosaccharide-cleaving activity ([^{3}H]Man_9-mannosidase) was found in three fractions (fractions 8–10). These three fractions showed little or no other contaminating cells (i.e., epididymal cells, blood cells, etc.). Furthermore, >98% of the spermatozoa present in these three fractions contained no cytoplasmic droplets as examined by phase contrast microscopy (Fig. 1 *B* and Fig. 2), a result indicating that the newly discovered α-D-mannosidase of rat spermatozoa was not associated with the cytoplasmic droplets. It should be noted that most of the PNP-mannosidase activity was found in fractions 15–20 (Fig. 1 *A*), which showed little or no oligosaccharide-cleaving activity. These PNP-mannosidase-rich fractions were present at the top of the gradient and likely contain the acid α-D-mannosidase present in the luminal fluid (36) and also associated with the cytoplasmic droplets. A small amount of PNP-mannosidase activity observed in fractions 8–10 (Fig. 1 *A*) is likely due to the enzyme associated with the acrosomes.

Enzyme Activities in the Subcellular Fractions of Rat Spermatozoa

Next, we attempted to determine the localization of the oligosaccharide-cleaving mannosidase activity in rat spermatozoa. Subcellular fractions were prepared from the caudal spermatozoa by the procedure described in Materials and Methods. Assay of the three fractions, namely, residual, crude membrane, and cytosolic fraction for the PNP-mannosidase and [^{3}H]Man_8-mannosidase activities, showed that the "acid" α-D-mannosidase with a pH optimum of 4.4 (PNP-mannosidase) was present mainly in the residual fraction (Fig. 3). The other two fractions contained very little of the PNP-mannosidase activity. However, when these fractions were assayed for the oligosaccharide-cleaving activity ([^{3}H]Man-mannosidase at its pH optimum of 6.2), the crude membrane fraction showed highest relative specific activity, followed by the cytosolic fraction (Fig. 3). The reason for the presence of substantial amount of the oligosaccharide-cleaving activity in the cytosolic fraction is not yet known. Examination of the crude membrane fraction by electron microscopy showed the presence of membrane vesicles of several morphologic classes and scattered sperm fragments (data not shown). These preliminary studies provided evidence for the differential distribution of the PNP-mannosidase and [^{3}H]Man_8-mannosidase activities in the subcellular fractions of rat spermatozoa. Furthermore, these studies indicated that the oligosaccharide-cleaving α-D-mannosidase activity may be associated with the sperm plasma membranes.

We then prepared highly enriched plasma membrane fraction from the caudal spermatozoa by nitrogen cavitation as described in Materials and Methods. Examination of the purified membrane fraction by electron microscopy showed the presence of membrane vesicles free of contaminating acrosomal, nuclear, and mitochondrial membranes (Fig. 4). Two distinct classes of membrane vesicles are recognizable. The major class of vesicles appears to be round shaped and are believed to be derived from sperm plasma membrane

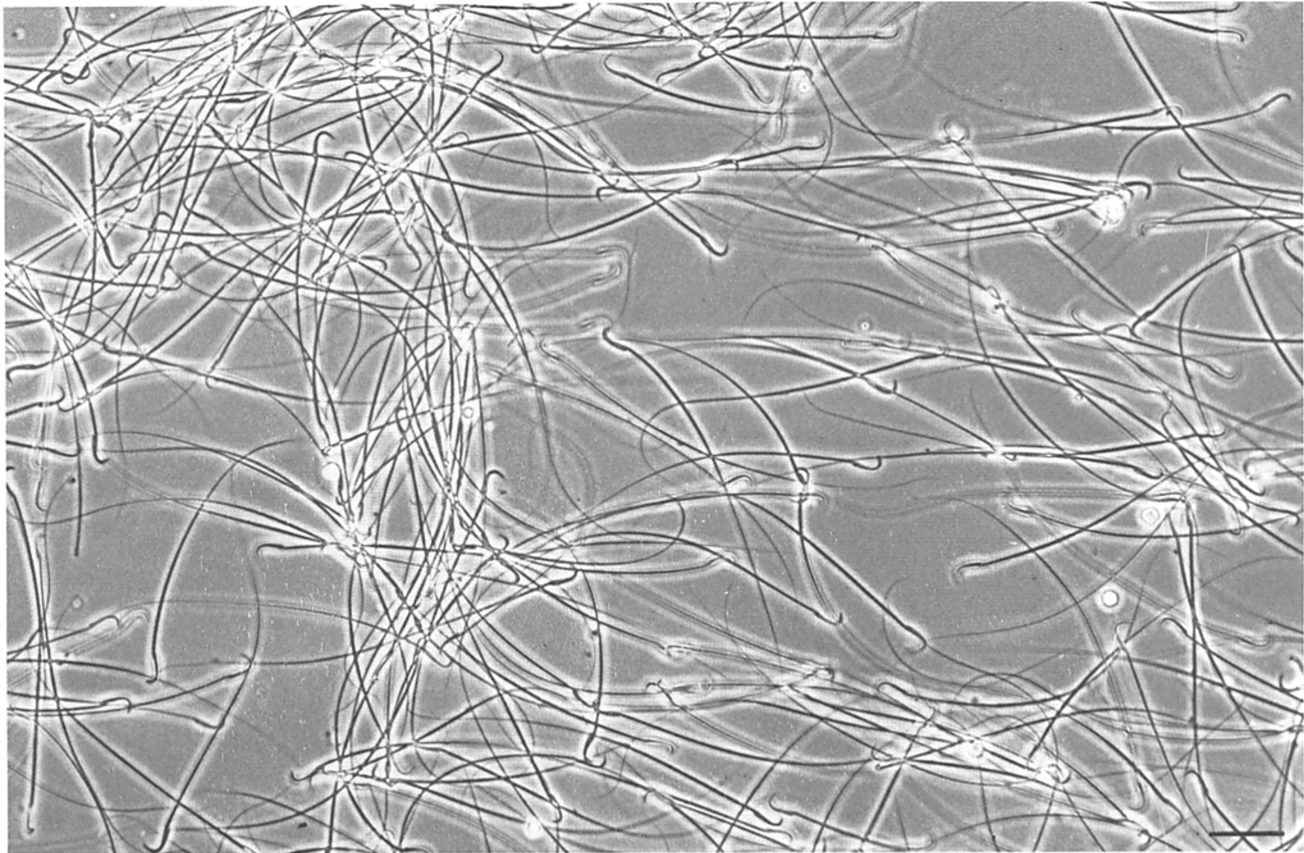

Figure 2. Microscopic appearance of rat caudal spermatozoa recovered after Percoll gradient centrifugation. Spermatozoa were obtained from the cauda and fractionated by Percoll density gradient centrifugation as described in Fig. 1. Aliquots of the suspended spermatozoa present in fraction 8 (Fig. 1 *B*) were photographed using phase contrast microscopy. Bar, 15.6 μm.

(26). The flattened vesicles (Fig. 4) are fewer in number and are likely derived from the cytoplasmic droplet (26). Since the newly discovered α-D-mannosidase is not associated with the cytoplasmic droplet (see above), it is reasonable to assume that any enzyme activity in this fraction would be associated with the sperm plasma membrane vesicles.

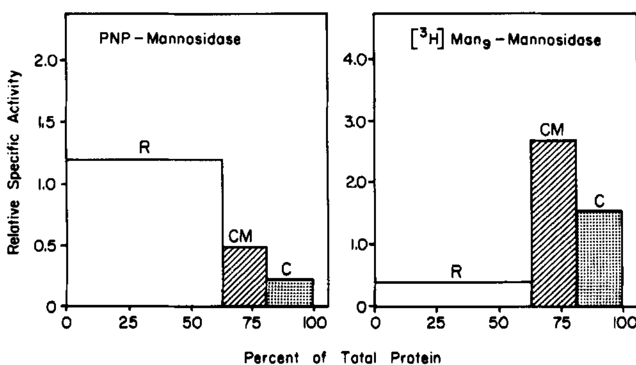

Figure 3. Intracellular distribution of PNP-mannosidase and [³H]Man₉-mannosidase activities in rat spermatozoa. The fractions, namely, residue (*R*), crude membranes (*CM*), and cytosol (*C*), were prepared from caudal spermatozoa as described in Materials and Methods. Ordinate: relative specific activity (percentage of total activity/percentage of total protein) of reactions; abscissa: relative protein content of the fractions. The enzyme activities and protein content were determined as described in Materials and Methods.

The isolated membrane fraction was also analyzed for the newly discovered mannosidase activity ([³H]Man₉-mannosidase) and adenylate cyclase, a plasma membrane marker enzyme (18), and three acidic glycosidases that are presumed to be acrosomal marker enzymes. The results from these studies presented in Table III show that the membrane fraction contained <1.5% of the acidic glycosidases originally present in the cavitated spermatozoa. However, >40% of the newly discovered mannosidase, and nearly 80% of the adenylate cyclase activity, originally presented in the spermatozoa was present in the plasma membrane–enriched fraction (Table III). These membranes were enriched 17- and 31-fold for the [³H]Man₉-mannosidase and adenylate cyclase, respectively. These data suggest that, like adenylate cyclase, the newly discovered enzyme is localized on the sperm plasma membrane.

The novel α-D-mannosidase appears to be an intrinsic plasma membrane component, since washing of the plasma membranes with buffered salt solution (0.4 M NaCl) did not release the enzyme in the salt wash. The enzyme requires nonionic detergent (0.5% Triton X-100) for complete solubilization. In related studies, we compared the [³H]Man₈-mannosidase activity in rat caudal spermatozoa before (intact spermatozoa) and after lysis with 0.2% Triton X-100 (total activity). The intact spermatozoa showed >70% of the enzyme activity observed in the lysed spermatozoa (data not

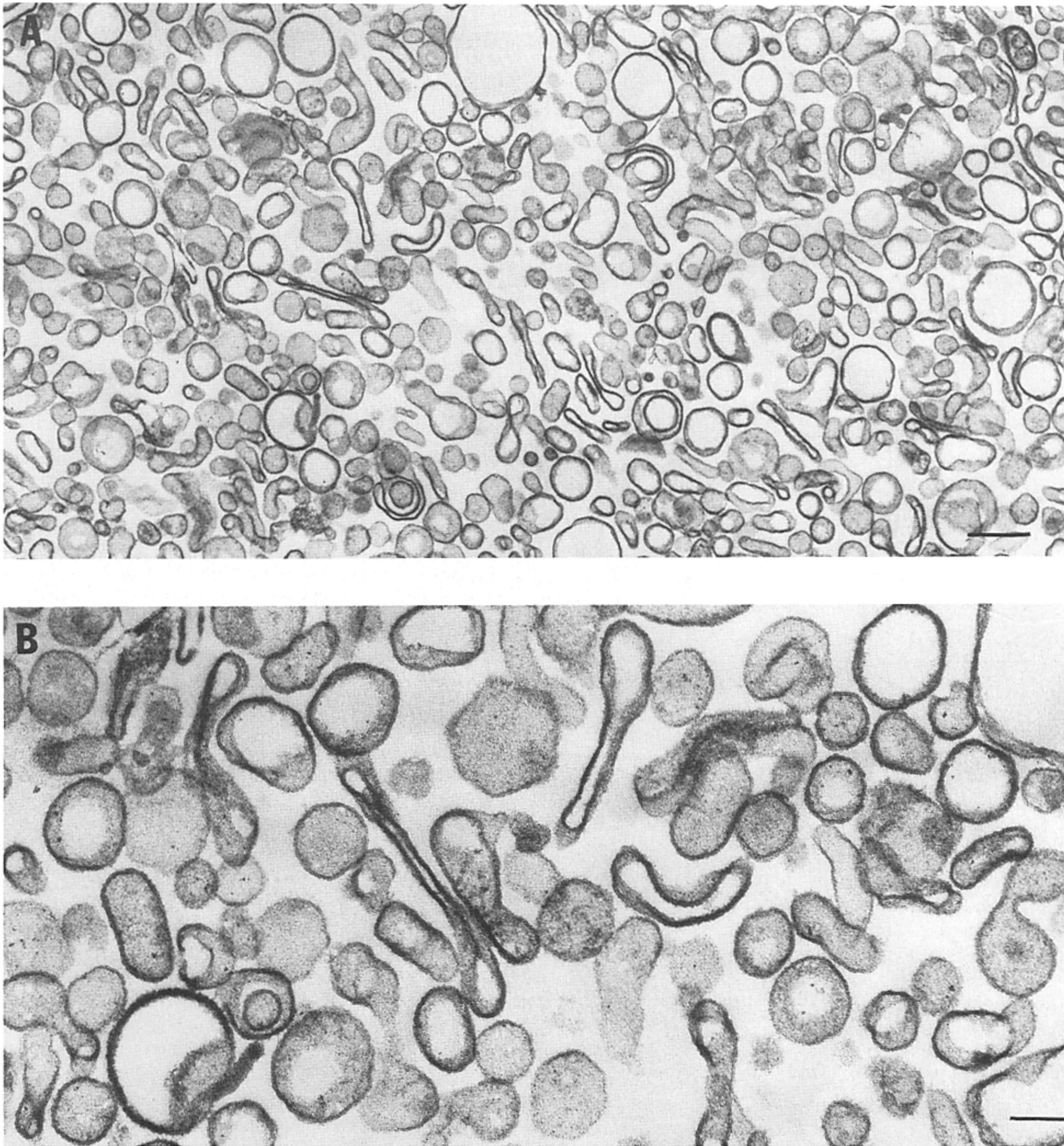

Figure 4. Electron micrograph of rat sperm plasma membrane–enriched fraction. The caudal spermatozoa were disrupted by nitrogen cavitation at 400 psi for 10 min. *A* represents a low power view of the membrane pellet and demonstrates the presence of only membrane vesicles. *B* shows higher power magnification of the same membrane pellet. Bars: (*A*) 0.36 μm; (*B*) 0.14 μm.

shown). This last study suggests that the catalytic domain of the enzyme is oriented towards sperm surface and that the enzyme will be functional in the physiological setting, both in the epididymal lumen as well as the female genital tract.

Immunoprecipitation Studies

These studies, which gave negative results, are described briefly. The rat tissues have been shown to contain several α-D-mannosidases. Liver endoplasmic reticulum α-D-mannosidase (5), liver Golgi mannosidase IA and IB (37, 41, 46), and Golgi mannosidase II (24, 46) are all processing enzymes, whereas lysosomal (27) and cytosolic (35) α-D-mannosidases are believed to be degradative enzymes (40). A novel processing mannosidase was recently reported in rat brain microsomes (39). We used monospecific polyclonal antibodies to various mannosidases to examine the chemical nature of the sperm plasma membrane mannosidase. In these studies, the enzyme was solubilized from the sperm plasma membranes by detergent-salt extraction, and the cross-reactivity of the solubilized enzyme examined by immunoprecipitation studies as described in Materials and Methods. As expected from our preliminary study, highly specific

Table III. Newly Discovered α-D-Mannosidase and Marker Enzymes in Caudal Spermatozoa and Sperm Plasma Membranes

Assay	Spermatozoa*		Plasma membrane*		
	Enzyme activity (total units)	Specific activity (A)	Specific activity (B)	Enrichment (B/A)	Recovery
		U/mg protein		-fold	%
α-D-mannosidase‡	44.20	4.20	2.30	0.6	1.3
β-D-galactosidase‡	7.10	0.67	0.09	0.1	0.3
β-D-glucuronidase‡	0.41	0.04	0.02	0.5	1.2
Adenylate cyclase	32.48	3.09	96.95	31.4	80.0
Newly discovered mannosidase‖	63.50	5.72	97.26	17.0	41.4
Protein (mg)	10.50	–	–	–	2.6

* Spermatozoa recovered from the cauda region of the epididymis from three adult rats were subjected to nitrogen cavitation to detach plasma membranes that were isolated by sucrose density gradient centrifugation as described in Materials and Methods. Enzyme activities and protein content were measured in the disrupted spermatozoa and purified plasma membranes. This table records the results of a typical experiment. Similar results were seen in two additional experiments.
‡ The three enzymes were assayed by fluorometric method (36).
‖ Assayed by our published procedure using [^{3}H]Man$_8$GlcNAc as substrate (46).

polyclonal antibodies (IgG fraction) to the rat liver Golgi mannosidase IA (41), Golgi mannosidase II (24), epididymal (lysosomal) α-D-mannosidase (44), and liver cytosolic/soluble α-D-mannosidase (6, 35) showed no cross-reactivity with the sperm plasma membrane mannosidase (data not included). This study suggests that the enzyme described here does not share a common subunit with other known mannosidases, and further supports our conclusion that the sperm plasma membrane mannosidase is different from other mannosidases identified in the rat tissues.

Kinetic Studies

Sperm plasma membrane α-D-mannosidase showed optimal activity between pH 6.0 and 6.5, when [^{3}H]Man-labeled oligosaccharide was used as substrate (Fig. 5). The pH activity curve for the salt-washed plasma membrane fraction and for the intact sperm is shown in Fig. 5; very low activity was detected at pH 4.4, the pH optimum of the "acid" acrosomal α-D-mannosidase (36). However, nearly 70% of the enzyme remained active at pH 7.0–7.4 (Fig. 5). Under standard assay conditions, the rate of hydrolysis of [^{3}H]Man$_9$GlcNAc, [^{3}H]Man$_8$GlcNAc, and [^{3}H]Man$_5$GlcNAc was directly proportional to the plasma membrane protein concentration (5–20 μg of protein). The reaction was linear for at least 4 h.

Stability

The enzyme solubilized by the detergent-salt extraction of the sperm plasma membranes (see Materials and Methods for details) is unstable at 0–4°C or if stored frozen at −20°C. Addition of 1 mM Co^{2+} seems to stabilize the enzyme. The enzyme solutions containing the divalent metal ion could be stored at 0–4°C for several days without appreciable loss of activity.

Effectors of Mannosidase Activity

Mg^{2+} and Ca^{2+} had no effect on enzyme activity of sperm plasma membrane. Cu^{2+}, Zn^{2+} are potent inhibitors, whereas Co^{2+} and Mn^{2+} are activators (Table IV). *p*-Chloromercuriphenylsulfonic acid, iodoacetamide, and ethylenediaminetetraacetic acid are potent inhibitors of the enzyme. At 1 mM concentration, these three reagents caused 70–100% inhibition of the sperm plasma membrane mannosidase (Table IV).

Swainsonine, a potent inhibitor of lysosomal α-D-mannosidase, and Golgi mannosidase II (45), and a relatively less potent inhibitor of rat kidney cytosolic α-D-mannosidase (40), had little or no effect on the sperm surface mannosidase. 1-Deoxymannojirimycin, an inhibitor of the α-1,2-mannosidase activities in culture cells (17) and Golgi mannosidases IA and IB (41) had no significant effect on the sperm mannosidase (data not shown).

A number of sugars, including mannose, mannitol, mannonic y-lactone, mannosamine, galactono lactone, arabinose, 2-deoxy-D-glucose, D-galactose, fucose, α-methyl mannoside, and α-methyl glucoside, were tested at several concentrations for their effect on sperm surface mannosidase. The results presented in Fig. 6 show that D-mannosamine and, to some extent, α-methyl mannoside are the potent inhibitors of the enzyme, whereas mannose, α-methyl glucoside, and 2-deoxyl-D-glucose show significant inhibition only at concentrations of 10 mM and higher (Fig. 6). The other sugars tested had little or no effect on sperm surface mannosidase.

Substrate Specificity

Several α-D-mannosidases have been identified in mammalian tissues that show rather specific substrate specificity.

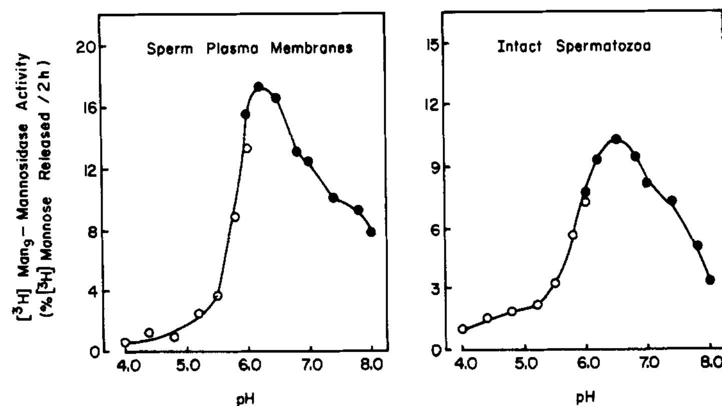

Figure 5. Influence of pH on [^{3}H]Man-mannosidase activity present in the sperm plasma membranes and intact spermatozoa. Salt-washed (0.4 M NaCl) plasma membranes (suspended in 0.4% Triton X-100) or caudal spermatozoa were suspended in PBS and aliquots containing ~15 μg of the plasma membrane protein or ~1 × 10^6 sperm were incubated in 0.1 M sodium acetate buffer (pH 4.0–6.0) or 0.1 M sodium cacodylate buffer (pH 6.0–8.0). After the addition of [^{3}H]Man$_9$GlcNAc (~3,000 cpm), the reaction mixtures were incubated at 37°C for 2 h. The [^{3}H]mannose released was quantitated as described in Materials and Methods.

Table IV. Effect of Activators/Inhibitors on Rat Sperm Surface Mannosidase Activity

Effector*	Concentration	Sperm plasma membrane mannosidase
	mM	*% activity*
None	–	100
$CoCl_2$	1	170
$CaCl_2$	1	90
$MgCl_2$	1	108
$MnCl_2$	1	141
$ZnCl_2$	0.1	4
$CuSO_4$	0.1	0
EDTA	5	24
2-Mercaptoethanol	5	113
p-Chloromercuriphenyl sulfonic acid	1	3
Iodoacetamide	5	31

* Aqueous solutions of the reagent under study were added to the reaction mixture (containing 100 mM sodium cacodylate buffer, pH 6.2, and sperm plasma membranes suspended in 0.2% Triton X-100) to the final concentration shown above. After preincubation for 5–10 min at 0–4°C, α-D-mannosidase was assayed by adding substrate (~3,000 cpm of [^{3}H]Man$_9$GlcNAc) and incubating at 37°C for 2 h. Free [^{3}H]mannose was separated from the oligosaccharide on a column of Bio-Gel P-2 and quantitated as described previously (46). All reagent solutions were prepared fresh and used within 4 h.

For instance, the liver endoplasmic reticulum α-D-mannosidase (5), Golgi mannosidase IA (37, 41, 46) and IB (46), are all processing mannosidases and show strong preference for the α1,2-linked mannosyl residues. Rat liver Golgi mannosidase II shows little or no activity towards the substrates containing α1,2-linked terminal mannosyl residues (46), but the enzyme cleaves α1,3-, and α1,6-linked mannosyl residues from Man$_5$GlcNAc only after the oligosaccharide has been glucosaminylated (38, 46). The rat brain microsomal α-D-mannosidase is quite different from the other processing mannosidases in that the brain enzyme shows rather broad substrate specificity, cleaving α1,2,- α1,3-, and α1,6-linked mannosyl residues at a similar rate (39). The limited substrate specificity studies carried out with the sperm plasma membrane α-D-mannosidase showed that the sperm plasma membrane–associated enzyme activity cleaves α1,2-linked mannosyl residues present in Man$_9$GlcNAc and Man$_8$GlcNAc very efficiently (Fig. 7). As a matter of fact, the sperm mannosidase cleaves nearly 70% of the [^{3}H]mannose from Man$_8$GlcNAc and Man$_9$GlcNAc. Since the mannosyl residues in these oligosaccharides are believed to be uniformly labeled (38), it appears that the sperm surface mannosidase is capable of cleaving nearly five and six mannosyl residues from Man$_8$GlcNAc and Man$_9$GlcNAc, respectively, leaving tetrasaccharide (i.e., Man$_3$GlcNAc). In addition, the sperm mannosidase cleaves a significant amount of mannose from Man$_5$GlcNAc without prior N-acetylglucosaminylation of the oligosaccharide, a reaction found necessary in the conversion of high mannose oligosaccharide to the complex type (38). The sperm mannosidase appears to cleave at least one mannosyl residue from Man$_5$GlcNAc and GlcNAcMan$_5$GlcNAc, as is evident by the release of nearly 20% of the free [^{3}H]mannose from these two oligosaccharides (Fig. 7). These two oligosaccharides (i.e., Man$_5$GlcNAc and GlcNAcMan$_5$-GlcNAc) contain terminal α1,3- and α1,6-linked mannosyl residues. The reason why only one mannosyl residue is cleaved from Man$_5$GlcNAc is not clear at the present time. One possible explanation could be that the sperm mannosidase hydrolyzes mannosyl residues in a particular sequence. Alternatively, the various oligosaccharides may have a differential stabilizing effect on the sperm mannosidase.

The novel α-D-mannosidase showed no activity towards *p*-nitrophenyl α-D-mannoside (PNP-mannosidase activity). Occasionally, when the purified plasma membranes were incubated with the synthetic substrate for 2–4 h at pH 6.2, the membranes showed very low activity towards this substrate. However, this activity had a pH optimum of 4.6, and was inhibited by swainsonine (data not included), a result suggesting that the activity towards the synthetic substrate is due to the presence of acid α-D-mannosidase as a contaminant (Table III).

Discussion

The data presented here demonstrates the presence of a novel

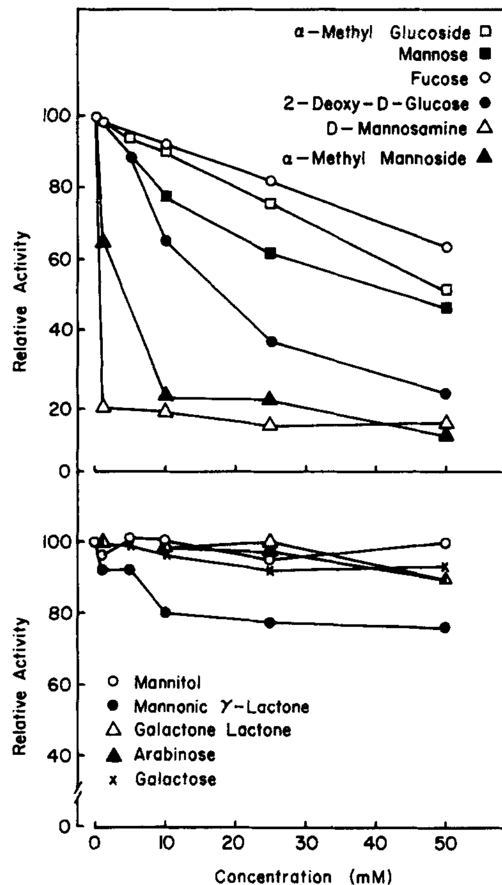

Figure 6. Effect of various sugars on rat sperm surface α-D-mannosidase. Caudal spermatozoa (~10^6) were first incubated (10 min at 0–4°C) with varying concentrations of the sugars near the physiological pH (100 mM sodium cacodylate buffer, pH 7.0). The reaction was started by addition of ~3,000 cpm of the substrate ([^{3}H]Man$_9$GlcNAc). After incubation for 2 h at 37°C, the reactions were stopped by heating at 100°C for 5–7 min and each mixture was applied to a Bio-Gel P-2 column to separate released [^{3}H]mannose from the oligosaccharide as described (46). Under these conditions, >12% of the added substrate was hydrolyzed and released as free [^{3}H]mannose (>360 cpm).

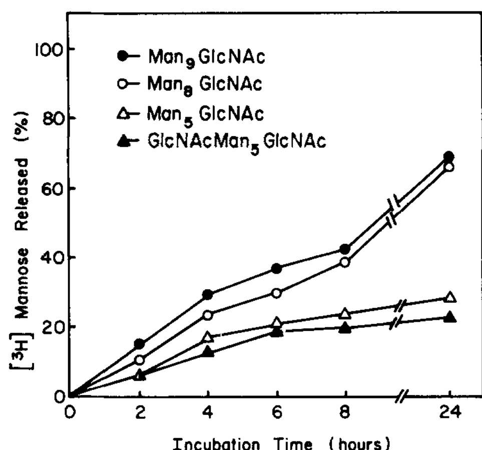

Figure 7. Time course of hydrolysis of [^{3}H]Man oligosaccharides by sperm plasma membrane α-D-mannosidase. Approximately 3,000 cpm of each oligosaccharide was incubated in sodium cacodylate buffer, pH 6.2, at 37°C with 20 μg of sperm plasma membrane protein. The incubation mixture also contained 1 mM $CoCl_2$ and 0.2% Triton X-100. At the indicated time interval, the reaction was stopped by heat inactivation (5–7 min in boiling water). Released [^{3}H]mannose was separated on a column of Bio-Gel P-2 and quantitated as described (46).

α-D-mannosidase activity on the sperm plasma membranes. This activity is clearly different from the sperm acidic α-D-mannosidase, which is active towards synthetic substrate, *p*-nitrophenyl α-D-mannoside, shows maximum activity at pH 4.5 (36), is inhibited by swainsonine (36, 44), and cross-reacts with the polyclonal antibody to the epididymal α-D-mannosidase (36).

The sperm plasma membrane mannosidase is also different from the rat liver endoplasmic reticulum α-D-mannosidase (5), the cytosolic α-D-mannosidase (35), and the Golgi mannosidase II (24), which, unlike the sperm enzyme, hydrolyzes *p*-nitrophenyl α-D-mannoside very efficiently. In addition, the polyclonal antibodies to the three rat liver mannosidases showed no cross-reactivity with the solubilized sperm plasma membrane mannosidase.

The sperm membrane mannosidase appears to be different from the rat liver Golgi mannosidases IA and IB (37, 41). Although both the sperm mannosidase and the liver-processing enzymes cleave α1,2-linked mannosyl residues from [^{3}H]Man$_9$GlcNAc and [^{3}H]Man$_8$GlcNAc (Fig. 8), the sperm mannosidase cleaves α1,3-(α1,6-) linked mannosyl residue from [^{3}H]Man$_5$GlcNAc and GlcNAc[^{3}H]Man$_5$GlcNAc, whereas the processing mannosidases IA and IB do not. It is not known at the present time whether the sperm mannosidase shows preference for the α1,3- or α1,6-linked mannosyl residues. Additional studies with the purified enzyme are needed and are expected to provide information on the precise substrate specificity of the novel mannosidase. In addition, 1-deoxymannojirimycin, a potent inhibitor of processing mannosidases IA and IB (41), had little or no effect on the sperm mannosidase. Finally, the highly specific polyclonal antibody to the processing mannosidase IA (41) showed no cross-reactivity with the sperm membrane mannosidase. This last study indicates that the sperm membrane mannosidase and the liver-processing enzymes do not share a common subunit. The sperm membrane mannosidase appears to be different from the α1,2-specific mannosidases purified from the calf liver microsomes (33) and rabbit liver microsomes (15).

A neutral mannosidase has recently been characterized from bull semen and reproductive organs (19). The enzyme present in semen is sperm associated and like the rat plasma membrane mannosidase, is activated by Co^{2+} ions. However, whereas the bull neutral mannosidase is localized in the sperm cytosol, hydrolyzes the synthetic substrate, *p*-nitrophenyl α-D-mannoside, and showed double optima at pH 5.5 and 6.0–7.0 (19), the enzyme described in this report is membrane bound, has no activity towards the synthetic substrate, and showed a single pH optimum (6.2) when assayed using oligosaccharide substrate (Fig. 5).

Although the novel mannosidase, like the adenylate cyclase, was enriched in the sperm plasma membranes (Table III), it is not known why the recovery and the enrichment of the two enzymes is so different (17-fold enrichment with 42% recovery for the mannosidase compared with 31-fold enrichment and nearly 80% recovery for the adenylate cyclase). One explanation is the possibility that the two enzymes are present on the plasma membranes of morphologically distinct regions of the spermatozoon. An alternate interpretation of these results could be that the spermatozoa contains yet another mannosidase capable of hydrolyzing the oligosaccharide substrate. Additional studies are needed and are expected to resolve this issue.

The involvement of this novel α-D-mannosidase on the sperm plasma membranes in the fertilization process can be surmised from earlier observations. First, treatment of the zona intact eggs with concanavalin A (a lectin known to recognize and bind to exposed high mannose and hybrid oligosaccharides (29) prevented sperm–egg binding in vitro (25). These studies could be interpreted as due to blocking of the sperm receptor sites (presumably high mannose/hybrid oligosaccharide side chains) present on the zona pellucida. These oligosaccharide chains on zona pellucida are presumably the recognition sites for the sperm surface mannosidase. Second, inclusion of α-methyl mannoside and D-mannose in the sperm–egg binding assay prevented the binding of the gametes both in the mouse (21) and rat (34). Since these two sugars have inhibitory effect on the sperm surface mannosidase (Fig. 6), the above results could be due to inhibition of the sperm mannosidase. Third, treatment of the zona intact rat eggs (but not spermatozoa) with the jack bean α-D-mannosidase, an enzyme known to cleave α-linked mannosyl residues from mannose-rich oligosaccharides (38), caused nearly complete inhibition of sperm–egg binding (34). Collectively, these studies suggest that the sperm surface mannosidase may have a role in the interaction of gametes.

Our preliminary studies with the mouse and hamster spermatozoa support the proposed role for the newly discovered mannosidase. The spermatozoa from these two species, like the rat spermatozoa, contain the novel mannosidase. Incubation of the mouse and hamster spermatozoa with the sugars, namely α-methyl mannoside, α-methyl glucoside, and D-mannose, resulted in a dose-dependent inhibition of the sperm surface mannosidase and a dose-dependent decrease in the number of sperm bound per egg in an in vitro sperm–egg binding assay (Cornwall, G. A., D. R. P. Tulsiani, and

M.-C. Orgebin-Crist, unpublished data). Galactose, which did not inhibit sperm surface mannosidase had no effect on the sperm–egg interactions. These data are consistent with the possibility that the novel mannosidase may be important for the interaction of the spermatozoon and the zona pellucida.

To the best of our knowledge, no information is available on the composition and chemical nature of the glycoconjugates present on rat zona pellucida. However, the mouse zona pellucida consists of three glycoproteins (ZP1, ZP2, and ZP3), but only ZP2 and ZP3 have sperm receptor activities (7, 8, 48). The receptor activity in the mouse ZP3 has been shown to be associated with only O-linked oligosaccharide chains with an apparent relative molecular mass of 3.9 kD (14). Treatment of the oligosaccharide unit with α-galactosidase or α-fucosidase destroys its ability to inhibit sperm–egg binding (9), a result suggesting that galactosyl and fucosyl residues present on O-linked oligosaccharide chains are important in sperm–egg recognition and binding. In contrast, treatment of mouse ova with almond glycopeptidase, an endo-enzyme that cleaves the *N*-acetylglucosaminyl asparagine linkage in N-linked glycoproteins, greatly reduces sperm–egg binding (49). This last study implies that N-linked oligosaccharide/glycoprotein also has a role in the interaction of gametes.

Most investigators agree that sperm surface proteins mediate the sperm–egg interactions by binding with high affinity and specificity to the glycoconjugate receptor(s) present on egg zona pellucida. The evidence for the presence of several binding proteins on the surface of mouse sperm (4, 23, 28, 31) supports the argument that more than one receptor is involved and that the recognition and binding of gametes is perhaps regulated by the sum of all molecular interactions. Understanding the mechanisms underlying gamete recognition will require knowledge of all the interacting molecules on the opposite gametes and the structural basis for these interactions.

In view of the biological significance of our finding, studies on the sperm surface mannosidase are continuing in our laboratory. Only when the enzyme has been obtained in a homogeneous form and monospecific antibody prepared will it be possible to gain insight into the precise role of this unique mannosidase.

The excellent secretarial assistance of Mrs. Loreita Little and Mrs. Reesha Leone is gratefully acknowledged. The authors are indebted to Dr. Gary Olson for a critical reading of the manuscript and to Dr. Loren Hoffman for the electron micrographs done in this manuscript. The electron microscopic studies were performed by the Electron Microscopy Core Laboratory of the Vanderbilt Center for Reproductive Biology Research, and adenylate cyclase assays were done by the Protein Phosphorylation/Cyclic Nucleotide Core Laboratory of the same center.

This work was supported in part by a research grant from The Andrew W. Mellon Foundation, and grant HD03820 and a Biomedical Research Support grant RR-05424 from the National Institutes of Health, United States Public Health Service.

Received for publication 30 January 1989 and in revised form 6 April 1989.

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