Immunocytochemical localization and biochemical characterization of a novel plasma membrane-associated, neutral pH optimum α-L-fucosidase from rat testis and epididymal spermatozoa

Manuel AVILÉS*, Irene ABASCAL*, José Angel MARTÍNEZ-MENÁRGUEZ*, María Teresa CASTELLS*, Sheri R. SKALABAN†, José BALLESTA* and Jack A. ALHADEFF*†‡

*Section of Histology and General Embryology, Department of Cell Biology, Medical School, University of Murcia, Murcia, Spain E-30071, and †Division of Biochemical Sciences, Department of Chemistry, Lehigh University, Bethlehem, PA 18015, U.S.A.

1. Immunocytochemical and biochemical techniques have been used to localize and characterize a novel plasma membrane-associated, neutral-pH-optimum α-L-fucosidase from rat spermatozoa. Light and electron microscopy specifically localized the fucosidase on the plasma membrane of the convex region of the principal segment of testicular and cauda epididymal sperm heads. Immunoreactivity for α-L-fucosidase was also detected in the Golgi apparatus of spermatocytes and spermatids but no immunoreactivity was observed in the acrosome. 2. Fractionation of epididymal sperm homogenates indicated that over 90% of the α-L-fucosidase activity was associated with the 48000 ***g*** pellet. This pellet-associated activity could be solubilized with 0.5 M NaCl but not with 0.5% Triton X-100, suggesting that fucosidase is peripherally associated with membranes. Sucrose-density-gradient centrifugation of sperm homogenates indicated that fucosidase was enriched in the plasma membrane-enriched fraction. Analysis of α-L-fucosidase on intact epididymal sperm indicated that the enzyme was active, displayed linear kinetics and had a pH–activity curve (with an optimum near 7) which was comparable to that of fucosidase from epididymal sperm extracts. These results further suggest that fucosidase is associated with plasma membranes, and that its active site is accessible to fucoconjugates. Evidence that most of the fucosidase is associated with the exterior of the plasma membrane came from studies in which intact sperm had fucosidase activity comparable to that of sperm sonicates, and from studies in which approx. 90% of the fucosidase activity on intact sperm could be released from the sperm by gentle shaking with 0.5 M NaCl. Isoelectric focusing indicated that the NaCl-solubilized epididymal sperm fucosidase appears to have one major and one minor isoform with pIs near 7.2 and 5.2, respectively. SDS/PAGE and Western blotting indicated that the NaCl-solubilized extract of epididymal sperm contains two protein bands of 54 and 50 kDa which were highly immunoreactive with the IgG fraction of anti-fucosidase antibodies. Although the function of the novel sperm fucosidase is not known, its specific localization to the plasma membrane of the region of the rat sperm head involved in sperm–egg binding and its high enzymic activity at neutral pH on intact sperm suggest that this enzyme may have a role in sperm–egg interactions.

INTRODUCTION

α-L-Fucosidase (EC 3.2.1.51) is a lysosomal glycosidase which is ubiquitous in eukaryotic cells. This enzyme is usually found as a soluble component of the lysosome and functions as an acid hydrolase in the degradation of numerous and diverse fucoglycoconjugates (reviewed in ref. [1]). The importance of this enzyme in mammalian catabolism is indicated by the neurovisceral storage disease fucosidosis which results from absence or gross deficiency of α-L-fucosidase activity and the concomitant accumulation of fucoglycoconjugates [2,3]. Numerous studies have indicated that mammalian α-L-fucosidases are relatively large, multisubunit glycoproteins which exist in multiple molecular isoforms. In addition, almost all studies have indicated that α-L-fucosidases are soluble, found in the lysosome, and exhibit relatively acidic enzymic activity optima between pH values of 4 and 6 [1].

In the present investigation a novel α-L-fucosidase from testicular and epididymal sperm has been identified and characterized. Immunocytochemical techniques at the light and electron microscope level have been used to localize this fucosidase to the plasma membrane region of the rat sperm head thought to be involved in sperm–egg binding [4]. Biochemical studies have indicated that rat sperm fucosidase is unlike most mammalian fucosidases in that it is not soluble, not lysosomal and does not exhibit an acidic pH optimum. It appears to be associated with the exterior of the plasma membrane and to exhibit high activity on intact sperm at a neutral pH optimum near 7. Although the function of rat sperm α-L-fucosidase is not known, its unusual location and kinetic properties suggest that this enzyme may have a role in sperm–egg interactions in the reproductive tract of the female rat.

MATERIALS AND METHODS

Animals and tissues

Adult sexually mature male Sprague–Dawley rats ranging from 120 to 210 days of age and weighing 400–600 g were used. Rats were killed when needed by ether or CO_2 asphyxiation. Testes and total epididymes (caput, corpus and cauda) were removed, trimmed of adipose tissue and immediately used for homogenization and preparation of sperm, respectively. For some

‡ To whom correspondence and proofs should be addressed at: 111 Research Drive, Lehigh University, Bethlehem, PA 18015, U.S.A.

experiments, epididymes were subdivided into caput, corpus and cauda sections, and sperm from a specific section were prepared.

Preparation of epididymal sperm

Sperm were isolated by cutting and mincing the epididymes (or a specific section of epididymes) of one to two rats into small pieces with a scalpel in 7.5–15 ml of 10 mM NaH_2PO_4 buffer, pH 5.5 (phosphate buffer), which was kept on ice for 10–15 min (until the buffer turned cloudy). The cloudy solution was transferred to a test tube, allowed to stand on ice for 10 min (to allow settling of any tissue pieces), and the supernatant fluid containing the sperm was centrifuged at 400 ***g*** for 10 min. The pelleted sperm were washed twice by resuspension in phosphate buffer and recentrifugation at 400 ***g*** for 10 min. The washed sperm were used intact for α-L-fucosidase activity analysis, or lysed by sonication [5] using various conditions (sonicating 35 % or 70 % of the time for 3–5 min, at low or high setting, at 25 % or 50 % output on a Braun Labsonic U sonicator) in 100 vol. of distilled water, or the sperm were homogenized in 5 vol. of phosphate buffer using a Kinematica Polytron homogenizer (at a setting of 5) for three 30-s periods with 15-s intervals between periods on ice.

Release of α-L-fucosidase from intact epididymal sperm

Intact epididymal sperm were shaken gently (70 rev./min on a Tek-Pro Variable Rotator) for various lengths of time (0–120 min) at 20 °C in either 10 mM phosphate buffer, pH 5.5, or in phosphate buffer containing 0.5 M NaCl. After shaking, the suspensions were centrifuged (10000 ***g*** for 20 min), the resultant supernatant fluids and pellets were assayed for α-L-fucosidase activity, and the distributions of recovered fucosidase activity between supernatant fluids and pellets were calculated.

Preparation of the plasma membrane-enriched fraction

A plasma membrane-enriched fraction was prepared from epididymal sperm using the procedure of Tulsiani et al. [6]. In brief, epididymal sperm from one rat were homogenized as described above in 5 vol. of 10 mM phosphate buffer, pH 5.5, for three 30-s periods using a Kinematica Polytron homogenizer. The homogenate was centrifuged at 400 ***g*** for 90 s and the resulting supernatant fluid was carefully removed and centrifuged at 48000 ***g*** for 30 min. The resulting membrane-containing pellet was resuspended in 2.0 ml of phosphate buffer and rehomogenized for one 20-s period. A portion of this homogenate (1.0 ml) was placed on a sucrose density gradient containing 4.5 ml each of 15 % and 50 % sucrose in phosphate buffer. The gradient was centrifuged at 105000 ***g*** for 90 min using a SW41 rotor, the tube was punctured, and 0.32 ml fractions were collected and assayed for α-L-fucosidase activity. The fractions at the 15 %/50 % sucrose interface were combined, diluted in 3 vol. of phosphate buffer, and centrifuged at 105000 ***g*** for 30 min. A portion of the resulting pellet was diluted 1:1 in 1 % phosphotungstic acid in 0.1 M HCl, dried over a formvar carbon-coated nickel grid, and analysed by transmission electron microscopy for the presence of membrane vesicles using a negative-staining method according to the method of Mercer and Birbeck [7]. In addition, the pellet was assayed for α-L-fucosidase activity, protein concentration and for 5′-nucleotidase, a plasma membrane marker [8]. 5′-Nucleotidase was assayed at pH 7.5 using 5 mM 5′-AMP as substrate and inorganic phosphate was determined using the method of Lanzetta et al. [9]. Aliquots of the homogenate were also assayed for fucosidase, 5′-nucleotidase and protein concentration so that specific activities and enzyme-enrichment factors could be determined.

Tissue extraction and fractionation

Epididymal sperm homogenates prepared as described above were centrifuged using a Sorvall RC-5B centrifuge at either 24000 ***g*** or 48000 ***g*** for 30 min. Testicular tissue was homogenized for three 60-s periods (at a setting of 5) using 5 vol. of phosphate buffer per g of tissue, and the homogenate was centrifuged as described above for epididymal sperm. The pellets resulting from centrifugation were washed by resuspension in the phosphate buffer and recentrifuged. The washed pellets were extracted by rehomogenization (as described above) in phosphate buffer alone or phosphate buffer containing various concentrations (0.25 M, 0.50 M, 0.75 M) of NaCl and/or Triton X-100 (0.5 %, w/v). The resulting homogenates were recentrifuged at 48000 ***g*** for 30 min and the distribution of α-L-fucosidase activity between the supernatant fluid and resuspended pellet fractions was determined.

Immunocytochemistry

Light microscopy

Spermatozoa were prepared from cauda epididymis as described above and washed three times in pH 7.4 PBS. Spermatozoa were smeared on clean glass slides coated with poly-L-lysine. Smears were also obtained from minced portions of testis. The smears were allowed to dry at room temperature for 30 min and fixed in 100 % methanol for 10 min. The testes of rats were also obtained and fixed in Bouin's solution (12 h) and embedded in paraffin wax.

Endogenous peroxidase was blocked with 0.3 % hydrogen peroxide in PBS, pH 7.2. After washing in PBS, paraffin sections and smears were incubated with the enriched IgG fraction of anti-(human liver α-L-fucosidase) polyclonal antibodies from goat [10] for 1 h. These polyclonal antibodies are cross-reactive with, and able to immunoprecipitate, rat liver α-L-fucosidase to the same extent (93 %) as human liver α-L-fucosidase [11]. Negative controls were done using (1) serum from an unimmunized goat, and (2) PBS instead of the anti-(human liver α-L-fucosidase) antibodies. After washing in PBS, slides were incubated for 2 h with rabbit biotin-conjugated anti-(goat IgG) antibodies (Sigma Chemical Co.) diluted 1:200 in PBS. Slides were washed in PBS and incubated with peroxidase-conjugated avidin (6 μg/ml) for 1 h (Vector Labs, Burlingame, CA, U.S.A.). Peroxidase was developed with 0.05 % 3,3′-diaminobenzidine and 0.015 % hydrogen peroxide [12].

Electron microscopy

Preparation of colloidal gold and Protein A–gold complex. Monodisperse colloidal gold solutions with a mean particle diameter of 15 nm were prepared according to the method of Frens [13]. Protein A–gold complexes were prepared by mixing 10 ml of colloidal gold with 1 ml of double-distilled water containing 1 mg of protein at pH 6.0. The minimal amount of protein needed for stabilization of colloidal gold and the optimal pH were estimated by the method of Geoghegan and Ackerman [14]. After conjugation, 0.1 % poly(ethylene glycol) was added and the solution was centrifuged at 60000 ***g*** for 1 h at 4 °C. The supernatant was discarded and the pellets were resuspended in 1.5 ml of PBS containing 0.02 % poly(ethylene glycol) and 0.02 % (w/v) sodium azide and stored at 4 °C.

Immunogold labelling of testis. Samples of testis were fixed in

0.5% or 2% glutaraldehyde in PBS for 2 h at 4 °C. After washing in PBS, tissue samples were immersed in 0.5 M NH_4Cl in PBS for 1 h to block free aldehyde groups. Then, the specimens were washed again in PBS and embedded in Lowicryl K4M at low temperature according to the method of Carlemalm et al. [15].

Ultrathin sections were incubated for 10 min in 0.5% BSA (Sigma Chemical Co.) and then transferred to a drop of the IgG fraction of unlabelled anti-α-L-fucosidase antibodies diluted (1:10) in PBS for 1 h. Negative controls were done using (1) serum from an unimmunized goat, and (2) PBS instead of the anti-(human liver α-L-fucosidase) antibodies. After washing, the grids were floated on a drop of rabbit anti-(goat IgG) antibodies (Sigma Chemical Co.; 1:10) for 1 h. Finally, gold-labelled Protein A (1:50) was applied. After washing in PBS and double-distilled water, the grids were counterstained with uranyl acetate and lead citrate.

'*In toto*' *preparation*. Sperm from cauda epididymis were fixed in 2% paraformaldehyde in PBS for 15 min. After washing in PBS, they were adsorbed to carbon-coated formvar film mounted on nickel grids. Prior to spermatozoa adsorption these grids were coated with a poly-L-lysine film (0.1% solution for 1 h). Grids were incubated for 10 min in 0.5% BSA and then transferred to a drop of the IgG fraction of unlabelled anti-α-L-fucosidase antibodies diluted (1:500) in PBS for 1 h. Negative controls were done using (1) serum from an unimmunized goat and (2) PBS instead of the anti-(human liver α-L-fucosidase) antibodies. After washing, the grids were floated on a drop of rabbit anti-(goat IgG) antibodies (1:200) for 1 h. Finally, gold-labelled Protein A (1:50) was applied. After washing in PBS and double-distilled water, the grids were examined at 100 kV in a Zeiss EM 10c transmission electron microscope.

Purification of human liver α-L-fucosidase

α-L-Fucosidase was purified to apparent homogeneity from normal human liver by affinity chromatography using agarose-ε-aminocaproylfucosamine resin (Miles Scientific, Naperville, IL, U.S.A.) as described [16]. Human adult liver was obtained at autopsy from an individual whose tissue appeared normal upon gross pathological examination and was stored at −20 °C until used. The procedures that involved human tissue were approved by an institutional Committee on Investigation/Activities Involving Human Subjects. This purified α-L-fucosidase, which had a specific activity of 24000 units/mg of protein, was used as an authentic standard for SDS/PAGE and Western blotting analyses (see below). α-L-Fucosidase activity was assayed as described [16] under linear conditions (for amount of enzyme and incubation time at 37 °C) using 1 mM 4-methylumbelliferyl α-L-fucopyranoside (Sigma Chemical Co.) as substrate. Assays were done in 0.1 M citric acid/sodium citrate buffer, pH 5.0, (for human liver α-L-fucosidase) or in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0 (for the testicular and sperm extracts). Fluorescences were read on a Hitachi F-4500 fluorimeter (using wavelengths of 360 and 460 nm for excitation and emission, respectively) and fluorescences were corrected by subtracting tissue and substrate blanks. One unit of α-L-fucosidase activity is defined as the amount of enzyme necessary to hydrolyse 1 nmol of substrate per min at 37 °C (under the above-defined conditions). Protein concentration was determined by the bicinchoninic acid method using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, U.S.A.) and the protocol of the manufacturer. BSA (Sigma Chemical Co.) was used as the protein standard.

Stability, pH optimum and isoelectric focusing studies

The stability of α-L-fucosidase activity in the soluble and NaCl-solubilized extracts of epididymal sperm was determined after storage of aliquots at either 2–4 °C or −20 °C for various lengths of time up to 31 days. The pH–activity curves for these epididymal sperm extracts and intact epididymal sperm, as well as for soluble and NaCl-solubilized extracts of rat testis, were determined using three buffers with overlapping pH values: 0.1 M oxalic acid/sodium oxalate (pH values 2.0–3.7), 0.1 M citric acid/sodium citrate (pH values 3.1–6.1), and 0.1 M NaH_2PO_4/Na_2HPO_4 (pH values 6.1–8.1). Assays were done in duplicate for 5–10 min at 37 °C, and actual pH values of a third set of mock tubes were recorded. Fluorescences were corrected for tissue and substrate blanks. Isoelectric focusing was performed on aliquots of soluble and NaCl-solubilized extracts of epididymal sperm containing 15–40 units of α-L-fucosidase activity. Focusing was done essentially as described in [11] at 2–4 °C in a 40 ml column with 2% (v/v) Ampholine ampholytes (pH range 5–8; Pharmacia LKB Biotechnology, Bromma, Sweden) and a 0–67% (w/v) sucrose gradient. Electrofocusing was conducted at 600 V (3–4 mA starting amperage) for 18–19 h after which time 0.3–0.4 ml fractions were collected. The pH of each fraction was determined and 20 μl of each fraction was assayed for α-L-fucosidase activity for 45–60 min at 37 °C. The data were plotted as α-L-fucosidase activity versus isoelectric point (pI).

SDS/PAGE analysis

Slab SDS/PAGE was done as described in [17] on homogenates of epididymal sperm made using 1 vol. of SDS sample buffer [2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol, 62.5 mM Tris/HCl buffer, pH 6.8, 10% (v/v) glycerol] and three 30-s periods of homogenization as described above. In addition, slab SDS/PAGE was done on supernatant fluids of centrifuged (48000 ***g***, 30 min) epididymal sperm phosphate buffer homogenates (soluble extract) and on 0.5 M NaCl extracts of washed pellets (NaCl-solubilized extract) concentrated 11- to 27-fold by Centricon concentrators (Amicon Inc., Beverly, MA, U.S.A.). In brief, 4% stacking and 12% separating gels were used and run using 25 mM Tris/0.2 M glycine buffer, pH 8.6, containing 0.1% SDS for 1 h at 175 V at room temperature. Molecular-mass standards were from Sigma Chemical Co. (Prestained SDS/PAGE proteins) and included *Escherichia coli* β-galactosidase (123 kDa), rabbit muscle fructose-6-phosphate kinase (89 kDa), chicken muscle pyruvate kinase (67 kDa), chicken egg ovalbumin (50 kDa), and rabbit muscle lactic dehydrogenase (37.5 kDa). Gels were stained with 0.1% Coomassie Blue R-250 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) prepared in methanol/acetic acid/water (2:3:35, by vol.) and destaining was accomplished in the same solvent system without Coomassie Blue.

Western blot analysis

Western blot analysis of α-L-fucosidase in the SDS sample buffer homogenate of epididymal sperm, and in the concentrated soluble and NaCl-solubilized extracts of epididymal sperm, was done essentially as described in [17]. In addition, Western blotting was performed on a portion of the concentrated NaCl-solubilized sperm extract after immunoprecipitation of α-L-fucosidase using anti-(human liver α-L-fucosidase) polyclonal antibodies as previously described in detail [11]. In brief, the SDS/PAGE gels (run as described above) were equilibrated for 30 min in 25 mM Tris/192 mM glycine in 20% (v/v) methanol, and proteins were electrotransferred from the gel to 0.45 μm-pore-size Immobilon-P membrane (Sigma Chemical Co.) at 100 V for 1 h. After

appropriate washings and blocking with 0.25 g of BSA in 50 ml of 10 mM Tris-buffered saline, pH 7.4, the membrane was incubated with a 10^3-fold dilution of the IgG fraction of anti-(human liver α-L-fucosidase) polyclonal antibodies from a goat [10]. The secondary antibody used in the present study was horseradish peroxidase-conjugated rabbit anti-(goat IgG) antibody (Sigma Chemical Co.). Visualization was accomplished by putting the membrane in 0.05% 3,3′-diaminobenzidine and 0.015% hydrogen peroxide in PBS for 2–5 min [12]. The molecular-mass standards described above for SDS/PAGE and authentic human liver α-L-fucosidase were used as negative and positive controls, respectively, for blotting analysis.

RESULTS

Immunolocalization of α-L-fucosidase

Smears of rat cauda epididymal sperm showed an intense α-L-fucosidase immunoreactivity on the plasma membrane over the convex region of the sperm head (Figure 1a). A similar immunoreactive pattern was detected in smears of testis sperm (Figure 1b). At the ultrastructural level, an '*in toto*' preparation of epididymal sperm showed a strong gold labelling over the convex region of the principal segment of the sperm head (arrows; Figure 1c). No labelling was observed in the apical segment (arrowheads) and postacrosomal region (Figure 1c). Paraffin sections of rat testis demonstrated the existence of α-L-fucosidase immunoreactivity in some cells of the spermatogenic lineage. The round Golgi apparatus of pachytene spermatocytes (arrows) showed a strong reactivity to anti-α-L-fucosidase (Figure 2a). A weak staining was also detected in the cytoplasm. The Golgi apparatus of both early and late spermatids (arrowheads) was also stained (Figure 2a). A non-reactive area (arrowheads) was observed between the Golgi apparatus and the nucleus in early spermatids (Figure 2a, inset). Electron microscopic studies demonstrated that this unreactive region corresponded to the acrosome (Figure 2b). The Golgi complex showed a moderate labelling (Figure 2b). The Golgi apparatus was identified in our studies using antibodies against *cis*-Golgi (p58, p53, Rab 2) and *trans*-Golgi (TGN 38, clathrin) markers (results not shown) as previously described for rat spermatids [18]. No immunoreactivity was seen in the two negative controls (using either serum from an unimmunized goat or PBS instead of anti-fucosidase antibodies) for both the light and electron microscope studies.

Homogenization, fractionation and solubilization of α-L-fucosidase from rat epididymal sperm and testis

Preliminary experiments were done to determine the best conditions for homogenizing and extracting α-L-fucosidase activity from rat epididymal sperm and testis. The results indicated that the largest amount of recoverable activity occurred for three 30-s periods of homogenization for sperm, and three 60-s periods for testis. Centrifugation of homogenates at 24000 ***g*** or 48000 ***g*** for 30 min gave comparable recoveries of α-L-fucosidase activity, but the higher centrifugal field resulted in firmer pellets which were more readily recovered. Using the optimized conditions from these preliminary experiments, the activity and specific activity of α-L-fucosidase in the sperm and testis homogenates were determined, and the fractionation of fucosidase activity between supernatant fluids (soluble) and resuspended 48000 ***g*** pellets (pellet-associated) was investigated. The average fucosidase activity in two epididymal sperm homogenates (180 ± 35 units/ml) was approximately 10-fold higher than the average activity (17 ± 1.2 units/ml) in two testis homogenates. The differences were even greater when fucosidase activities were normalized to the amount of protein. The epididymal sperm homogenate specific activity (53 units/mg of protein) was approximately 40-fold higher than the testis homogenate specific activity (1.4 units/mg of protein). In five fractionation experiments on epididymal sperm, approximately 90% of the homogenate fucosidase activity was recovered and 92 ± 2% (mean ± mean deviation) of this recovered activity was pellet-associated. For four fractionation experiments on testis, essentially all of the homogenate fucosidase activity was recovered, but a much smaller percentage (68 ± 2%) of this recovered α-L-fucosidase activity was pellet-associated when compared with the epididymal sperm fucosidase.

The 48000 ***g*** pellets from epididymal sperm were washed in phosphate buffer and rehomogenized in phosphate buffer with or without various concentrations of NaCl and/or Triton X-100 (as described in the Materials and methods section) to determine the best conditions for solubilizing the pellet-associated α-L-fucosidase activity. Preliminary experiments indicated that NaCl and Triton X-100, at the concentrations employed, had no stimulatory or inhibitory effect on α-L-fucosidase activity (results not shown). Recovery of fucosidase activity from rehomogenized pellets was essentially quantitative, and phosphate buffer containing 0.5 M NaCl solubilized approximately 90% of the fucosidase activity compared with approximately 10% for phosphate buffer containing 0.5% (v/v) Triton X-100. Experiments using phosphate buffer containing various concentrations of NaCl (0.25–0.75 M) indicated that 0.5 M was optimal for solubilizing α-L-fucosidase with maximal recovery of enzymic activity. Homogenization of pellets with phosphate buffer containing both 0.5 M NaCl and 0.5% Triton X-100 did not result in increased amounts of solubilized fucosidase activity. Rehomogenization of washed 48000 ***g*** pellets from rat testis with phosphate buffer containing 0.5 M NaCl was effective in solubilizing approximately 70% of the pellet-associated α-L-fucosidase activity.

Release of α-L-fucosidase from intact epididymal sperm

Gentle shaking of intact sperm for 30–120 min in phosphate buffer containing 0.5 M NaCl led to the release of almost all the fucosidase activity to the supernatant fluid (after centrifugation of shaken sperm suspensions). In three separate experiments with shaking for 60 min, 87 ± 4% (mean ± mean deviation) of the recovered fucosidase activity was found in the supernatant fluid (compared with 24 ± 5% for a control in which sperm were shaken for 60 min in phosphate buffer alone). Comparable results were found for shaking times of 30, 90 and 120 min. Even for the zero time-point control in which intact sperm were placed in phosphate buffer containing 0.5 M NaCl and centrifuged immediately, approximately 80% of the recovered fucosidase activity was found in the supernatant fluid (compared with 3% for phosphate buffer alone).

Sucrose-density-gradient centrifugation of epididymal sperm homogenates

A plasma membrane-enriched fraction was prepared from epididymal sperm homogenates by an established sucrose-density-gradient-centrifugation procedure [6]. In three separate experiments, α-L-fucosidase activity was found in the gradient as a single sharp peak at the 15%/50% sucrose interface where plasma membranes are found [6]. Recentrifugation of this interface at 105000 ***g*** for 30 min yielded a final plasma membrane-

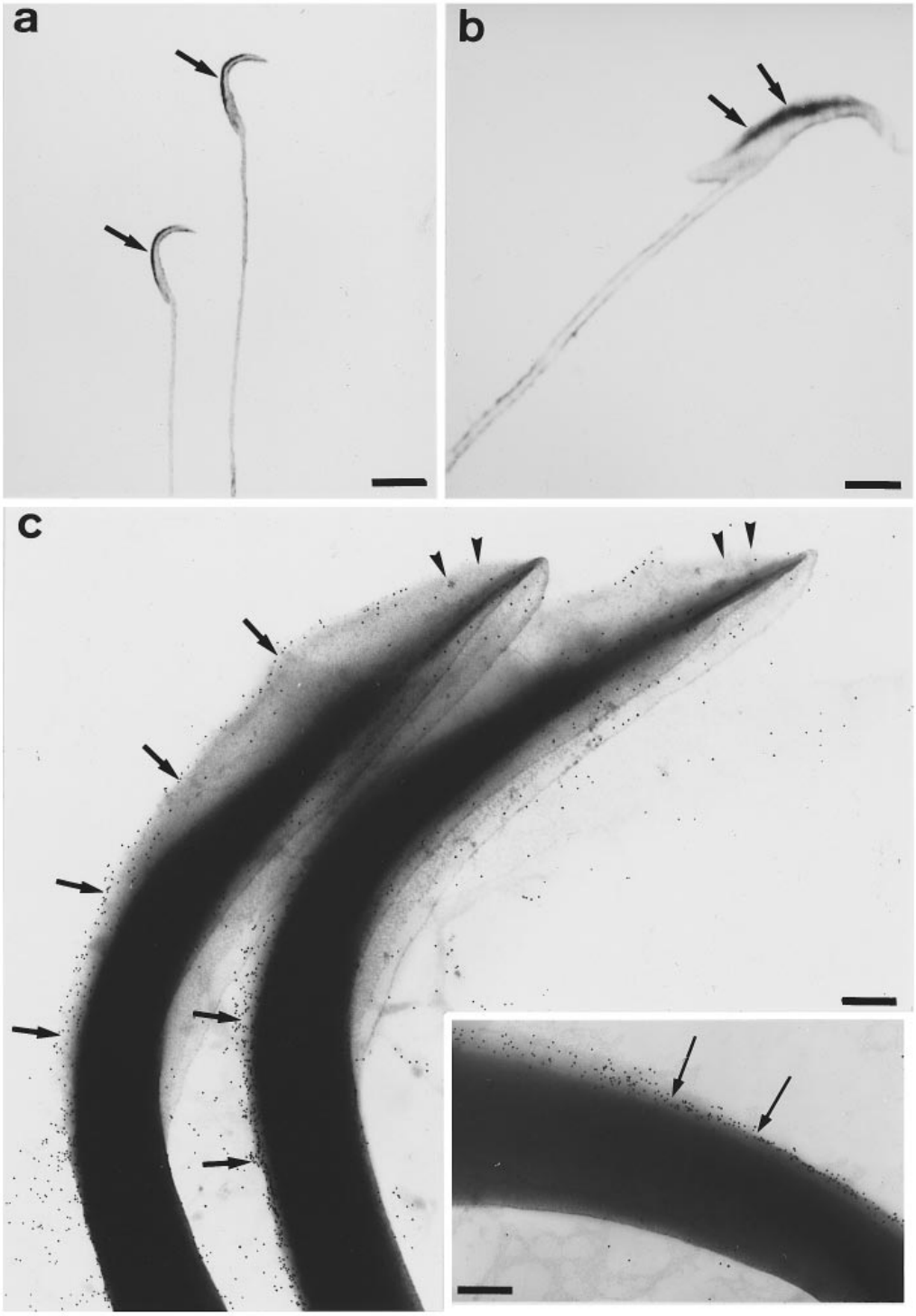

Figure 1 Immunocytochemical localization of α-L-fucosidase in the sperm plasma membrane

(**a**) Smear of cauda epididymal sperm. A strong staining is specifically observed over the convex region of the sperm head plasma membrane (arrows). Scale bar: 11 μm. (**b**) Smear of testis sperm. Similar staining (arrows) to that observed in epididymal sperm. Scale bar: 4 μm. (**c**) Ultrastructural '*in toto*' preparation of epididymal sperm. Gold particles are deposited over the convex region of the principal segment of the sperm head plasma membrane (arrows). Note that the apical segment is not immunoreactive (arrowheads). Scale bar: 0.5 μm. Inset: Detail of the principal segment. Note specific labelling over the convex region (arrows). Scale bar: 0.5 μm.

enriched pellet which contained $85 \pm 1\%$ of the recovered fucosidase activity and in which fucosidase was enriched approximately 5-fold, as determined by the increase in its specific activity. 5′-Nucleotidase was found to be enriched approximately 10-fold in the final plasma membrane-enriched pellet. The overall recoveries of fucosidase and 5′-nucleotidase activities from the

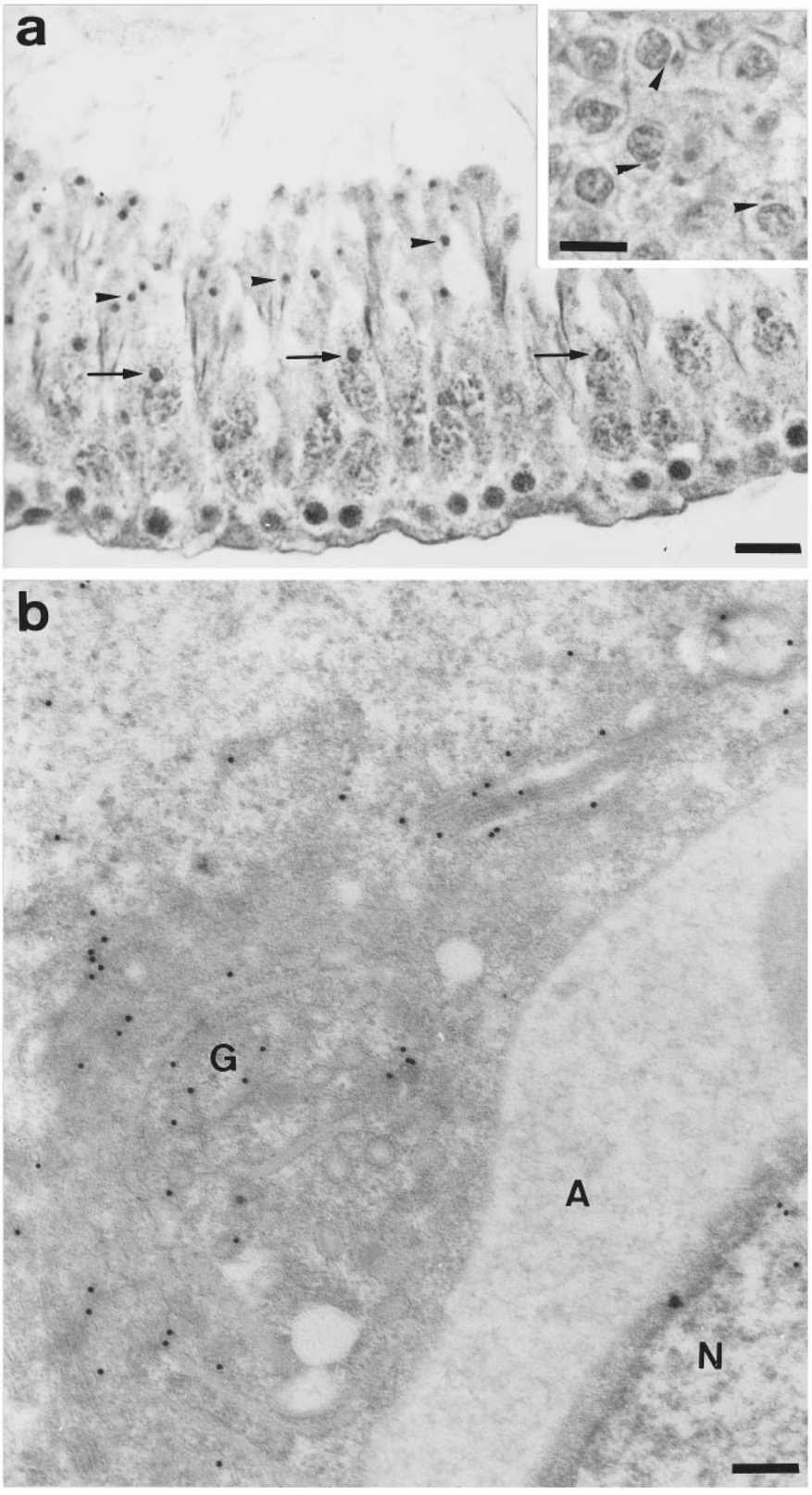

Figure 2 Immunocytochemical localization of α-L-fucosidase in the Golgi apparatus of spermatocytes and spermatids

(**a**) Paraffin section of a seminiferous tubule. The Golgi apparatus of both pachytene spermatocytes (arrows) and late spermatids (arrowheads) is strongly stained. Scale bar: 18 μm. Inset: Note the non-stained area between the nucleus and the Golgi apparatus (arrowheads) in early spermatids. Scale bar: 13.5 μm. (**b**) Ultrathin section of an early spermatid. The Golgi apparatus (G) is moderately labelled. No immunoreactivity is detected in the acrosome (A). Abbreviation: N, nucleus. Scale bar: 0.2 μm.

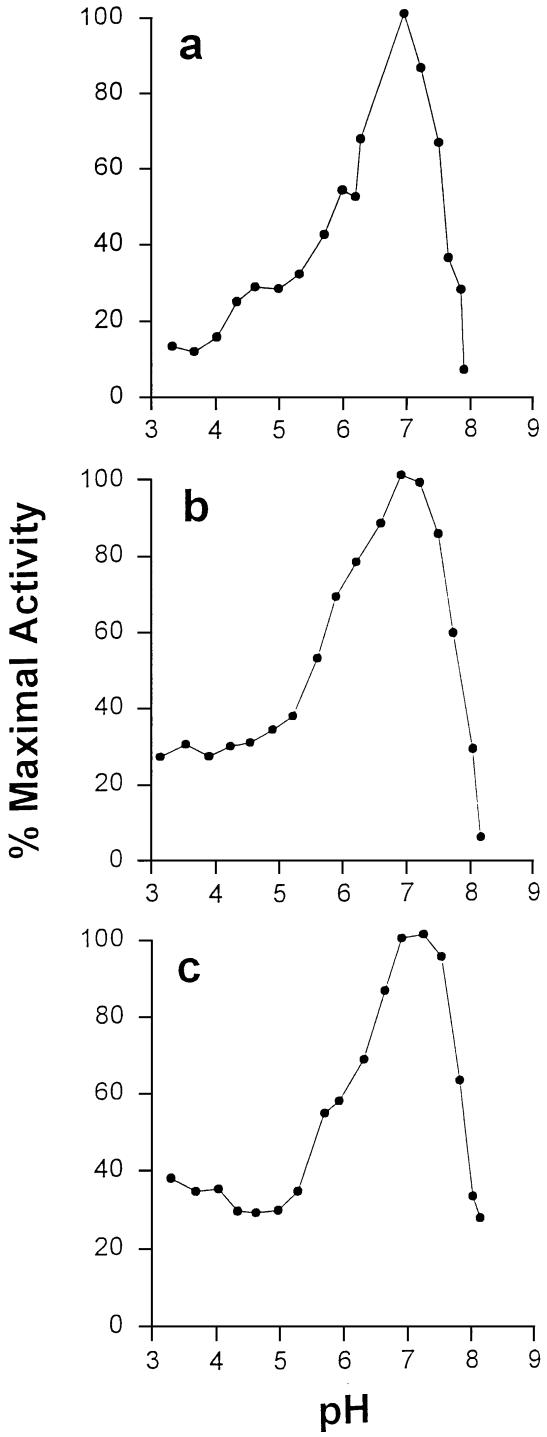

Figure 3 pH–activity curves of α-L-fucosidase in soluble (a) and NaCl-solubilized (b) extracts of rat epididymal sperm, and of α-L-fucosidase in intact rat epididymal sperm (c)

See the Materials and methods section for details.

original homogenate were $25 \pm 4\%$ and $55 \pm 5\%$ respectively. Electron microscope analysis of the final plasma membrane-enriched pellet by a negative-staining method using phosphotungstic acid [7] indicated the presence of only membrane vesicles (results not shown).

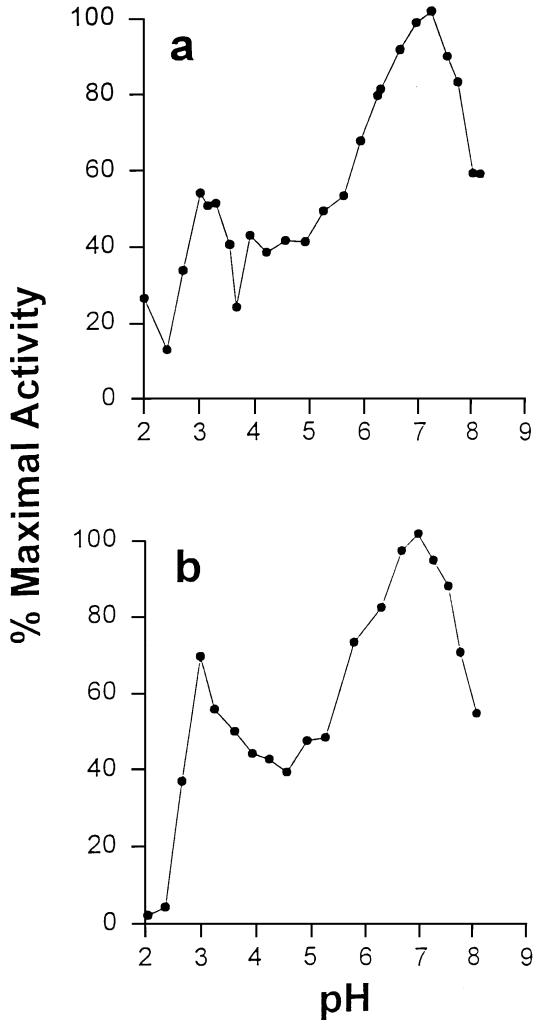

Figure 4 pH–activity curves of α-L-fucosidase in soluble (a) and NaCl-solubilized (b) extracts of rat testis

See the Materials and methods section for details.

Stability, kinetic and isoelectric focusing analysis of α-L-fucosidase from epididymal sperm and testis

α-L-Fucosidases from soluble and 0.5 M NaCl-solubilized extracts of epididymal sperm and/or testis were subjected to stability and kinetic analyses. Preliminary experiments indicated that the fucosidase extracts were quite stable when stored at 2–4 °C and retained 80–100% of their enzymic activity for at least 31 days (results not shown). However, storage of the extracts in a frozen condition at −20 °C led to loss of the majority of the fucosidase activity within 1 week. Therefore, all studies were performed on fresh extracts of sperm or testis, with the exception of the SDS/PAGE and Western blotting studies which were performed on extracts stored at 2–4 °C for up to 2 weeks.

pH–activity curves for α-L-fucosidase were run at least three times for each extract and representative curves for the epididymal sperm extracts and intact epididymal sperm are depicted in Figure 3. The soluble (Figure 3a) and NaCl-solubilized (Figure 3b) extracts have very similar fucosidase pH–activity curves with neutral optima between pH 6.9 and 7.1,

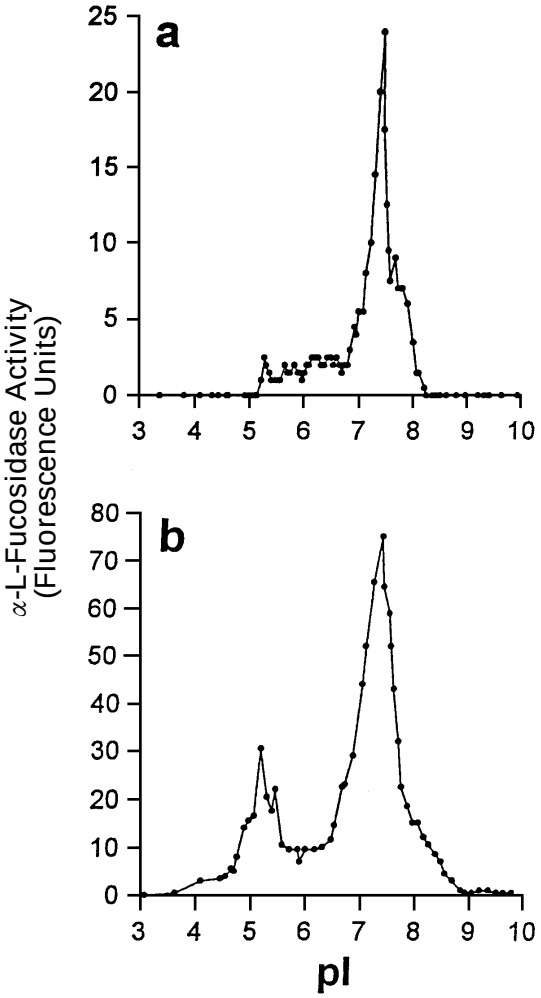

Figure 5 Isoelectric focusing profiles of α-L-fucosidase in soluble (a) and NaCl-solubilized (b) extracts of rat epididymal sperm

See the Materials and methods section for details.

a possible shoulder of activity near pH 6.0, and relatively low activity ($\leqslant 30\,\%$ of maximal) between pH values of 3 and 5. No significant buffer effects were seen at the overlapping pH values between the citrate and phosphate buffers. The pH–activity curve of α-L-fucosidase for intact epididymal sperm (Figure 3c) is very similar to the curves for the soluble and NaCl-solubilized sperm fucosidases, with an optimum between pH 6.9 and 7.2, a possible shoulder of activity near pH 6.0, and relatively little activity ($\leqslant 40\,\%$ of maximal) between pH values of 3 and 5. The intact epididymal sperm displayed linear kinetics for fucosidase activity with regard to amount of sperm and length of incubation time at 37 °C. In three separate experiments, aliquots of intact sperm diluted 10-fold in phosphate buffer had essentially the same fucosidase activity (95–105 %) as comparable aliquots of sperm sonicated in 10 vol. of distilled water as described in the Materials and methods section. Centrifugation of the putative intact sperm and sonicate at 500 ***g*** for 5 min led to recovery of approximately 80 % and 15 % of the fucosidase activity in the pellet for intact sperm and sonicate, respectively. These results indicate that the great majority of sperm in the intact preparations remained intact, and that the great majority of sperm in the sonicate had been disrupted.

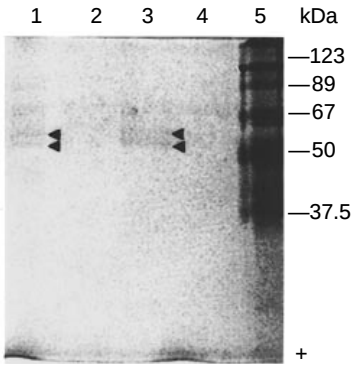

Figure 6 SDS/PAGE analysis of concentrated extracts of rat epididymal sperm

The NaCl-solubilized and soluble epididymal sperm extracts were concentrated 11- and 27-fold, respectively. Proteins were detected with Coomassie Blue R-250. Lane 1, NaCl-solubilized extract of epididymal sperm (approx. 3 μg of protein); lane 2, soluble extract of epididymal sperm (approx. 1.0 μg of protein); lane 3, purified human liver α-L-fucosidase (2 μg; 48 units); lane 4, purified human liver α-L-fucosidase (1 μg; 24 units); lane 5, Sigma Prestained Molecular Weight Proteins (80 μg of total protein). See the Materials and methods section for details.

Representative pH optimum curves for soluble and NaCl-solubilized fucosidase from rat testis are depicted in Figures 4(a) and 4(b), respectively. These curves are similar to those of the epididymal sperm fucosidases in that the major optima are at a neutral pH near 7.0 with a possible shoulder of activity near pH 6.0. However, both testis preparations contain a significant second optimum (with 55–70 % of maximal activity) near pH 3.0 which is not seen in the epididymal sperm curves. This second optimum was resolved by adding an oxalate buffer to extend the curve down to pH 2.0. No significant buffer effects were seen in the overlapping pH regions between the oxalate and citrate buffers, and between the citrate and phosphate buffers.

Isoelectric focusing profiles for α-L-fucosidase were run at least three times for soluble and NaCl-solubilized extracts of epididymal sperm from three different rats, and representative profiles are depicted in Figure 5. The soluble (Figure 5a) and NaCl-solubilized (Figure 5b) extract profiles were similar in that both contained one major isoform with a pI of 7.2 ± 0.1. The NaCl-solubilized fucosidase also had a minor, more acidic isoform with a pI of 5.2 ± 0.1 which was not detected in the soluble extract of epididymal sperm.

SDS/PAGE and Western blotting analysis of α-L-fucosidase from extracts of epididymal sperm

SDS/PAGE analysis of soluble and NaCl-solubilized extracts of rat epididymal sperm was done four times and representative results are depicted in Figure 6. The epididymal sperm extracts (lanes 1 and 2) contained very little protein but a few faint protein bands were present in the NaCl-solubilized sperm extract (lane 1) including two (arrowheads) with apparent molecular masses near 54 and 50 kDa (as determined by molecular-mass standards; lane 5). These two protein bands were very similar to the two protein bands (arrowheads; lane 3) corresponding to the subunits (56 and 51 kDa) of purified human liver α-L-fucosidase [17]. No protein bands were detected in the soluble epididymal sperm extract (Figure 6, lane 2) or for the smaller amount (1 μg) of human liver α-L-fucosidase (Figure 6, lane 4).

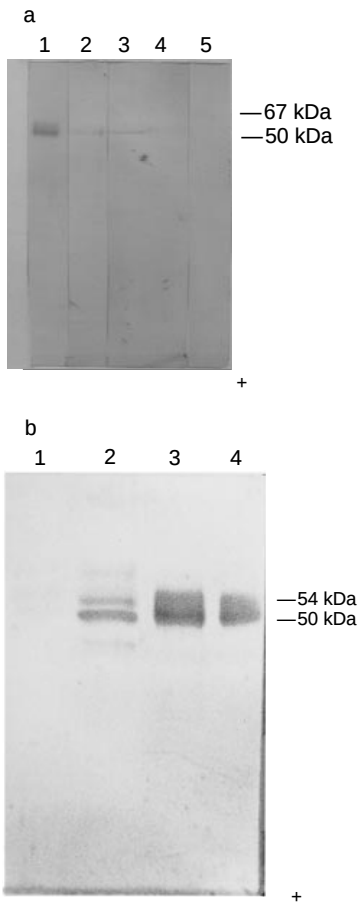

Figure 7 Western blot analysis of homogenates of epididymal sperm and concentrated extracts of rat epididymal sperm for α-L-fucosidase

The NaCl-solubilized and soluble epididymal sperm extracts were concentrated 11- and 27-fold, respectively. Immunoprecipitation of fucosidase from the NaCl-solubilized extract was done as described [11]. Detection of α-L-fucosidase was done using the enriched IgG fraction of goat anti-(human liver α-L-fucosidase) [10]. (**a**) Lane 1, purified human liver α-L-fucosidase (0.5 μg; 12 units); lane 2, SDS sample buffer homogenate of epididymal sperm (approx. 38 μg of protein); lane 3, NaCl-solubilized extract of epididymal sperm (30 μl; approx. 2 units of fucosidase activity); lane 4, NaCl-solubilized extract of epididymal sperm after immunoprecipitation of α-L-fucosidase activity (30 μl); lane 5, NaCl-solubilized extract of epididymal sperm using secondary antibody but no primary antibody (30 μl; approx. 2 units of fucosidase activity). (**b**) Lane 1, soluble extract of epididymal sperm (approx. 1 unit of fucosidase activity); lane 2, NaCl-solubilized extract of epididymal sperm (approx. 5 units of fucosidase activity); lane 3, purified human liver α-L-fucosidase (1 μg; 24 units); lane 4, purified human liver α-L-fucosidase (0.5 μg; 12 units). See the Materials and methods section for details.

Western blotting analysis of the rat epididymal sperm homogenates and extracts was done three times and representative results are shown in Figure 7. Figure 7(a) summarizes the evidence that the anti-(human α-L-fucosidase) polyclonal antibodies cross-react with and are specific for rat sperm α-L-fucosidase. Lane 2, which is a blot of the sperm homogenate, indicates the presence of one-to-two closely spaced immunoreactive bands which co-migrate with authentic human liver α-L-fucosidase (Figure 7a, lane 1). Blotting of the NaCl-solubilized sperm extract before (Figure 7a, lane 3) and after (lane 4) immunoprecipitation of α-L-fucosidase activity with the polyclonal antibodies indicates the presence of the immunoreactive band(s) which co-migrate(s) with authentic fucosidase prior to, but not after, immunoprecipitation (which removed 90% of the fucosidase activity). Blotting of the NaCl-extract with the secondary antibody but without the primary antibody (Figure 7a, lane 5) resulted in no immunoreactive bands. Figure 7(b) depicts a blot of the NaCl-solubilized sperm extract with better resolution of the immunoreactive bands. Lane 2 contains two major immunoreactive bands with apparent molecular masses comparable to the 54 and 50 kDa protein bands seen in the SDS/PAGE analysis (Figure 6, lane 1), and very similar to the two immunoreactive subunits of human liver fucosidase (lanes 3 and 4) which were not well-resolved due to excess antigen. Two minor immunoreactive bands may also be present in the NaCl-solubilized epididymal sperm extract (Figure 7b, lane 2), one with a molecular mass above 54 kDa and the other with a molecular mass below 50 kDa. However, it is difficult to determine whether these faint bands are real or artefactual. No distinct immunoreactive bands were detected in the soluble sperm extract (lane 1), probably due to the very low concentration of fucosidase in this extract. In addition, the SDS/PAGE molecular mass standards from Figure 6 which were used as a negative control for Western blotting were not immunoreactive with the anti-(human liver fucosidase) antibodies (results not shown).

DISCUSSION

In the present investigation, a novel α-L-fucosidase has been identified and characterized in rat testicular and epididymal spermatozoa. Immunocytochemical techniques have been employed to demonstrate that α-L-fucosidase is localized in the Golgi apparatus and plasma membrane of rat testicular spermatocytes and spermatids, and in the plasma membrane of cauda epididymal spermatozoa. In addition, α-L-fucosidase was found to be specifically enriched on the convex region of the plasma membrane of the rat sperm heads, a region thought to be involved in sperm–egg binding in the rat [4]. However, fucosidase was not detected over the acrosome, a lysosome-like organelle in sperm which contains degradative enzymes [19]. This latter finding is different from the 'unpublished observation' of Hancock et al. [20] that fucosidase was present in the acrosome of both testicular germ cells and epididymal sperm. The results of our immunocytochemical studies suggest that α-L-fucosidase is present in pachytene spermatocytes, is targeted to plasma membranes in early spermatids, and is found enriched on the convex region of testicular and cauda epididymal sperm heads. Biochemical studies provided further complementary evidence for an association of α-L-fucosidase with the plasma membrane of rat spermatozoa. Fractionation studies of epididymal sperm homogenates indicated that almost all of the α-L-fucosidase activity was associated with the 48000 ***g*** pellet. This is a very novel finding for this enzyme since the great majority (90–100%) of α-L-fucosidase activity in almost all mammalian tissues investigated is in the soluble fraction [1]. Human brain is an exception in that it contains a pellet-associated α-L-fucosidase but it only represents approximately 30% of the recovered activity from brain homogenates [21]. Approximately 90% of the epididymal sperm pellet-associated α-L-fucosidase activity could be extracted with phosphate buffer containing 0.5 M NaCl compared with only 10% of the activity with phosphate buffer containing 0.5% Triton X-100. These results suggest that α-L-fucosidase is not an integral membrane protein and that the enzyme is probably peripherally associated with membranes, primarily by ionic-type interactions. Our findings for rat sperm fucosidase are different from those for another rat sperm glycosidase, α-D-mannosidase, which was found by extraction and phase separation studies to be an integral membrane protein

[6,22]. Additional evidence that α-L-fucosidase is associated with rat sperm plasma membranes was provided by demonstrating an enrichment of this enzyme in a plasma membrane-enriched fraction prepared by sucrose-density-gradient centrifugation. As previously demonstrated by Tulsiani et al. [6], whose centrifugation methodology we employed, we were able to confirm by electron microscopy the presence of membrane vesicles in the putative plasma membrane-enriched fraction. We were also able to demonstrate an enrichment of the plasma membrane marker 5′-nucleotidase. It is unclear why 5′-nucleotidase was enriched approximately 2-fold more than fucosidase in the plasma membrane-enriched fraction but the differential enrichment may be due to the lower overall recovery of α-L-fucosidase activity (25 %) compared with that for 5′-nucleotidase activity (55 %).

α-L-Fucosidases from soluble and NaCl-solubilized extracts of rat epididymal sperm and testis were characterized comparatively in an attempt to provide a better understanding of the novel fucosidases found in these extracts. pH–activity curves indicated that both the soluble and NaCl-solubilized epididymal sperm fucosidases possessed neutral pH optima near 7 with little activity at acidic pH values. Our findings are very unusual since almost all previous studies have found that mammalian fucosidases are associated with lysosomes and have relatively acidic pH optima between 4 and 6 [1]. The pellet-associated human brain fucosidase has a pH optimum of 5.5–6.0 [21] and purified rat liver [23] and cerebral cortex [24] α-L-fucosidases have pH optima of 5.7–5.9 and 4.3, respectively. In addition and more specifically, previous studies on rat epididymal fucosidase [25–27] have found pH optima (5.5–6.5) lower than the pH 7.0 value which we have observed. We are currently unable to explain these differences but they may be due in part to the analysis of different isoforms of rat epididymal sperm fucosidase (as described in the next paragraph) in the various studies.

Isoelectric focusing indicated one major isoform of α-L-fucosidase with an approximate pI of 7.2 for both the soluble and NaCl-solubilized extracts of epididymal sperm. The latter extract also contained a minor isoform with a pI near 5.2 which was not detected in the former extract, possibly due to the significantly lower fucosidase activity present in the soluble extract. Our results are different from previous results on rat epididymal fucosidase in which investigators have found either one isoform at pI 6.3 [25] or two major and four minor isoforms with pIs between 6.0 and 7.0 [27]. In addition, our rat sperm isoform results are different from previous isoform results for other rat tissues including liver, in which multiple fucosidase isoforms are present with pIs between approximately 3 and 7 [11,23], and spleen and brain, in which the major isoforms are present at pIs of 4.5 and 4.3, respectively [28].

An analysis of intact epididymal sperm indicated that they possessed substantial α-L-fucosidase activity, and that this activity displayed linear kinetics even for short reaction times. The fucosidase pH–activity curve for the intact sperm was essentially the same as the curves for the two epididymal sperm extracts with an optimum near pH 7.0 and with little activity at the acidic pH values. These kinetic results suggest that at least some of the sperm fucosidase is associated with the exterior of the plasma membrane since it is unlikely that such results would occur if substrate had to enter into, and product exit from, intact sperm cells. Evidence that the great majority of α-L-fucosidase is associated with the plasma membrane came from studies in which 0.5 M NaCl released almost all the fucosidase activity from intact sperm, and from studies in which intact sperm had fucosidase activity comparable with that of sperm sonicate. Analysis of fucosidase activity in pellets and supernatant fluids derived from intact sperm and sonicated preparations (after a low-speed spin to pellet whole sperm) indicated the integrity of sperm in the former preparation, and the disruption of sperm in the latter. The α-L-fucosidase pH–activity curves of the soluble and NaCl-solubilized testis extracts indicated that the major pH optimum was at pH 7.0 and similar to that of epididymal sperm α-L-fucosidase. However, unlike the epididymal sperm extracts, the α-L-fucosidase from both testis extracts contained a significant second acidic optimum near 3.0. This second optimum could be due to an additional isoform of fucosidase, or a genetically different fucosidase, which is more lysosome-like in its properties and which is present in one or more type of testicular cell (e.g. sperm, Leydig, Sertoli) but not found in epididymal sperm.

SDS/PAGE analysis of the NaCl-solubilized epididymal sperm extract indicated, as expected, that very little protein was present in this extract. Two faint protein bands with apparent molecular masses of 54 and 50 kDa were found by SDS/PAGE analysis which were very immunoreactive on Western blot analysis with the IgG fraction of anti-fucosidase antibodies, whereas the protein molecular-mass markers were not immunoreactive. In addition, Western analysis of a total sperm homogenate revealed only the presence of the immunoreactive band(s) seen in the NaCl extract, and this band(s) was removed from the NaCl extract by immunoprecipitation with our anti-fucosidase antibodies. These results indicate the specificity of our antibodies for rat sperm fucosidase. The two immunoreactive bands in the NaCl extract had molecular masses very similar to the two subunits of human liver α-L-fucosidase employed in the present study as an authentic standard and positive control, and determined previously to have molecular masses of 56 and 51 kDa [17]. Our results are very similar to most previous results on rat epididymal α-L-fucosidase which have found one or two protein bands with molecular masses of 52 and 54 kDa [20], 47–50 kDa [27] and 54 kDa [26], but unlike the early findings of Carlsen and Pierce [25] who found two subunits of 47 and 60 kDa. In our studies the larger 54 kDa protein could be a precursor to the 50 kDa protein, as was found in studies by Hancock et al. [20] in which a precursor 54 kDa form of rat sperm fucosidase was processed to a mature 52 kDa form of the enzyme. No evidence was found for immunoreactive proteins in our soluble epididymal sperm extract. This was not an unexpected finding since very little of the recovered fucosidase activity was present in this extract.

The overall results of our cellular and biochemical studies provide strong evidence for the presence of a novel, neutral pH optimum α-L-fucosidase which is loosely associated with the plasma membrane of rat epididymal spermatozoa. The soluble and NaCl-solubilized fucosidases were very similar in their properties, and the apparent minor differences observed (e.g. in the isoform profiles) were probably due to the much lower concentration of fucosidase in the soluble extract. The soluble fucosidase probably represents a small percentage of the enzyme which is released from its loose association with the plasma membrane. Although the function of rat sperm fucosidase is unknown, it could be involved in modifying cell-surface carbohydrates during epididymal transit [29] and/or involved in sperm–egg interactions in the female reproductive tract. The latter possibility seems most reasonable because of the specific localization of the fucosidase to the region of the rat sperm head involved in sperm–egg binding and its high enzymic activity on intact epididymal sperm at neutral pH. The neutral pH optimum is consistent with a possible functional role for fucosidase in the ‘neutral environment of the oviduct’ [30]. In addition, a number of previous studies have provided evidence that L-fucose and/or the enzyme α-L-fucosidase may be involved in gamete interactions

in a number of species including the rat. Early studies indicated the presence of fucosyl sites on the vitelline coat of the egg of the ascidian *Ciona intestinalis* [31], and that α-L-fucosidase on the sperm surface might bind to these sites by an enzyme–substrate-type complex [32]. Furthermore, several investigators have demonstrated that L-fucose and/or fucoidan (a polymer of predominantly sulphated L-fucose) can inhibit sperm–egg interactions in several species including guinea pigs [33], boar [34], hamsters [35], mice [36], humans [37–41] and rats [42]. Further studies are necessary to determine whether rat sperm fucosidase is involved in recognizing and/or binding to fucoglycoconjugates on the zona pellucida of rat oocytes.

This research was supported in part by grants from Dirección General de Investigación Científica y Técnica (DGICYT) PB93-1123-C02-01 and SAB95-0074, Madrid (Spain). M.A., I.A. and J.A.M.-M. were supported by fellowships from the Ministerio de Educación y Ciencia (Spain). The collaboration of the Department of Biochemistry of the University of Murcia is gratefully acknowledged.

REFERENCES

1 Johnson, S. W. and Alhadeff, J. A. (1991) Comp. Biochem. Physiol. **99B**, 479–488
2 Alhadeff, J. A. (1981) in Lysosomes and Lysosomal Storage Diseases (Lowden, J. A. and Callahan, J. W., eds.), pp. 299–314, Raven Press, New York
3 Willems, P. J., Gatti, R., Darby, J. K., Romeo, G., Durand, P., Dumon, J. E. and O'Brien, J. S. (1991) Am. J. Med. Genet. **38**, 111–131
4 Phillips, D. M. and Shalgi, R. M. (1980) J. Exp. Zool. **213**, 1–8
5 Oko, R. (1988) Biol. Reprod. **39**, 169–182
6 Tulsiani, D. R. P., Skudlarek, M. D. and Orgebin-Crist, M.-C. (1989) J. Cell Biol. **109**, 1257–1267
7 Mercer, E. H. and Birbeck, M. S. C. (1972) Electron Microscopy, Blackwell Scientific Publications, London
8 Zimmermann, H. (1992) Biochem. J. **285**, 345–365
9 Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. and Candia, O. A. (1979) Anal. Biochem. **100**, 95–97
10 Andrews-Smith, G. L. and Alhadeff, J. A. (1982) Biochim. Biophys. Acta **715**, 90–96
11 Johnson, S. W. and Alhadeff, J. A. (1990) Comp. Biochem. Physiol. **97B**, 713–717
12 Avilès, M., Martinez-Menárguez, J. A., Castells, M. T., Madrid, J. F. and Ballesta, J. (1994) Anat. Record **239**, 137–149
13 Frens, G. (1993) Nature (Phys. Sci.) **241**, 20–22
14 Geoghegan, W. D. and Ackerman, G. A. (1977) J. Histochem. Cytochem. **25**, 1187–1200
15 Carlemalm, E., Garavito, M. and Villiger, W. (1982) J. Microsc. (Oxford) **126**, 123–143
16 Alhadeff, J. A., Miller, A. L., Wenaas, H., Vedvick, T. and O'Brien, J. S. (1975) J. Biol. Chem. **250**, 7106–7113
17 Johnson, S. W., Piesecki, S., Wang, R. F., Damjanov, I. and Alhadeff, J. A. (1992) Biochem. J. **282**, 829–834
18 Martinez-Menárguez, J. A., Geuze, H. J. and Ballesta, J. (1996) Eur. J. Cell Biol., in the press
19 Yanagimachi, R. (1994) in The Physiology of Reproduction (Knobil, E. and Neill, J. D., eds.), pp. 189–317, Raven Press, New York
20 Hancock, L. W., Raab, L. S. and Aronson, Jr., N. N. (1993) Biol. Reprod. **48**, 1228–1238
21 Hopfer, R. L. and Alhadeff, J. A. (1985) Biochem. J. **229**, 679–685
22 Tulsiani, D. R. P., NagDas, S. K., Skudlarek, M. D. and Orgebin-Crist, M.-C. (1995) Dev. Biol. **167**, 584–596
23 Opheim, D. J. and Touster, O. (1977) J. Biol. Chem. **252**, 739–743
24 Bosmann, H. B. and Hemsworth, B. A. (1971) Biochim. Biophys. Acta **242**, 152–171
25 Carlsen, R. B. and Pierce, J. G. (1972) J. Biol. Chem. **247**, 23–32
26 Wright, K., Northcote, D. H. and Davey, R. M. (1976) Carbohydr. Res. **47**, 141–150
27 Leray, G., Philouze, V., Blayau, M. and Prodhomme, C. (1986) IRCS Med. Sci. **14**, 792
28 Shoarinejad, F. and Alhadeff, J. A. (1993) Comp. Biochem. Physiol. **105B**, 523–528
29 Hall, J. C. and Killian, G. J. (1987) Biol. Reprod. **36**, 709–718
30 Skudlarek, M. D., Tulsiani, D. R. P., NagDas, S. K. and Orgebin-Crist, M.-C. (1993) Biol. Reprod. **49**, 204–213
31 DeSantis, R., Pinto, M. R., Cotelli, F., Rosati, F., Monroy, A. and D'Alessio, G. (1983) Exp. Cell Res. **148**, 508–513
32 Hoshi, M., DeSantis, R., Pinto, M. R., Cotelli, F. and Rosati, F. (1983) in The Sperm Cell (Andre, J., ed.), pp. 107–110, R. Martinus Nijhoff Publishers, The Hague
33 Huang, T. T. F. and Yanagimachi, R. (1984) Exp. Cell Res. **153**, 363–373
34 Sanz, L., Calvette, J. J., Mann, K., Gabius, H.-J. and Topfer-Petersen, E. (1993) Mol. Reprod. Dev. **35**, 37–43
35 Ahuja, K. K. (1982) Exp. Cell Res. **140**, 353–362
36 Boldt, J., Howe, A. M., Parkerson, J. B., Gunter, L. E. and Kuehn, E. (1989) Biol. Reprod. **40**, 887–896
37 Huang, T. T. F., Ohzu, E. and Yanagimachi, R. (1982) Gamete Res. **5**, 355–361
38 Jones, R. (1989) Hum. Reprod. **4**, 550–557
39 Oehninger, S. C., Clark, G. F., Fulgham, D., Blackmore, P. F., Mahony, M. C., Acosta, A. A. and Hodgen, G. D. (1992) J. Androl. **13**, 519–525
40 Tesarik, J., Mendoza, C., Ramirez, J.-P. and Moos, J. (1993) Fert. Steril. **60**, 344–350
41 Lucas, H., Bercegeay, S., Le Pendu, J., Jean, M., Mirallie, S. and Barriere, P. A. (1994) Hum. Reprod. **9**, 1532–1538
42 Shalgi, R., Matityahu, A. and Nebel, L. (1986) Biol. Reprod. **34**, 446–452

Received 9 January 1996/11 April 1996; accepted 15 May 1996