Acid Glycohydrolases in rat spermatocytes, spermatids and spermatozoa: enzyme activities, biosynthesis and immunolocalization

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Abbreviations: MEM, minimum essential medium; BSA, bovine serum albumin; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PNP, *p*-nitrophenyl; EKRB, enriched Krebs-Ringer bicarbonate

ABSTRACT

Mammalian sperm acrosomes contain several glycohydrolases that are thought to aid in the dispersion and digestion of vestments surrounding the egg. In this study, we have used multiple approaches to examine the origin of acrosome-associated glycohdyrdolases. Mixed spermatogenic cells, prepared from rat testis, were separated by unit gravity sedimentation. The purified germ cells (spermatocytes [SC], round spermatids [RS], and elongated/condensed spermatids [E/CS]) contained several glycohydrolase activities. Metabolic labeling in the cell culture, immunoprecipitation, and autoradiographic approaches revealed that β-D-galactosidase was synthesized in SC and RS in 88/90 kDa forms which undergo processing in a cell-specific manner. Immunohistochemical approaches demonstrated that the enzyme was localized in Golgi membranes/vesicles, and lysosome-like structures in SC and RS, and forming/formed acrosome of E/CS.

INTRODUCTION

Many, if not all, mammalian cells contain a unique class of cytoplasmic (electron dense) organelles termed lysosomes (1, 2). These are membrane limited bag-like structures filled with acid hydrolases, and normally function in intracellular digestion of endogenous and exogenous substrates. Mammalian spermatozoa have no lysosomes, but contain sac-like structures surrounded by inner and outer membranes termed acrosomes (3). The sperm acrosome resembles a cellular lysosome in several ways, including their origin (3,4). The two organelles contain many common enzymes, such as acid glycohydrolases, proteases, esterases, acid phosphatases, and aryl sulphatases (1,3,4). The acrosome contains a host of glycohydrolases with catalytic and immunological properties similar to the enzymes present within the lysosome. These enzymes are exo-glycohydrolases and function by cleaving specific terminal glycosyl residues from glycoproteins and glycolipids (4,5) with a high substrate specificity.

Spermatozoa released from the seminiferous tubules (testis) are terminally differentiated cells with no known synthetic acitivity. Their components are synthesized in the spermatogenic cells during spermatogenesis. Since glycohydrolases are thought to aid in penetration of the zona pellucida, the extracellular coat that surrounds the mammalian egg, it was of interest to quantify these enzymes in purified germ cells and spermatozoa and examine their synthesis and localization. The procedures described below are based on two recent reports from our laboratories (6,7).

MATERIALS AND METHODS

Materials

Adult male Sprague-Dawley rats (8-10 weeks old) were housed in our animal facilities under 16 light: 8 dark conditions with free access to food and water. The animals were either anesthetized or sacrificed by CO_2 asphyxiation. All *p*-nitrophenyl (PNP)-glycopyranoside substrates, gold-labeled (10 nm) anti-rabbit (IgG) goat IgG fraction, fluorescein-labeled affinity-purified anti-rabbit (IgG) goat IgG fraction (1.5 mg protein/ml), DNase I (Type II), trypsin (type III), soybean trypsin inhibitor, and poly-L-lysine were from Sigma Chemical Co. (St. Louis, MO); collagenase (CSL-1) was from Worthington Biochemicals (Freehold,

NJ). The Sta-Put cell separation apparatus was made by Proscience (Johns Scientific, Toronto, Canada) and consists of a linear gradient marker (2 flasks with double glass outlets), a cell loading chamber with 20 ml syringe attached by silicone tubing to a three way T-bore stopcock (T-valve) and a sedimentation chamber with a micrometering flow valve at the base connected by another tube to the T-valve. A baffle is centered in the bottom of the chamber to avoid aggregation of cells during a high flow rate loading. A fraction collector equipped with a drop counter and a solenoid (to stop the flow during tube changes) is connected by silicone tubing to the micrometering flow valve. A sedimentation chamber of 18 cm diameter can be loaded with 1200 ml of 2-4% BSA gradient and allow the separation of 5-6 x 10^8 germ cells. Seventy micrometer nylon mesh was from Spectrum (Laguna Hills, CA). All electrophoretic reagents were from Bio-Rad Laboratories (Richmond, CA). NCS solubilizer and ^{14}C-labeled standard marker proteins from Amersham. Reagents used for the isolation of testicular germ cells, including stock amino acids (essential and non-essential), L-glutamine, sodium pyruvate, penicillin and streptomycin (100 X) were from GIBCO BRL (Grand Island, NY). Media used for culturing of spermatogenic cells was derived from the protocol of Grootgoed et al. (8) and contained minimum essential medium (MEM, GIBCO), supplemented with 0.4% BSA, non-essential amino acids, antibiotics (100 units penicillin/ml; 0.1 mg streptomycin/ml, 1 µg fungizone/ml), 5 mM sodium lactate, 0.022 g NaHCO$_3$/100 ml, plus leucine, lysine with or without methionine. Vectashield mounting medium and the ABC Vectastain kit were from Vector Laboratories (Burlingame, CA). Glutaraldehyde electron microscopic grade (2%) was from Taab Laboratories (England), and Lowicryl K4M was from PolyScience Inc., (Warrington, PA). Scientiprep-2 was from Fisher Scientific, and photographic film (Biomax MR) used for autoradiography was from Kodak (Rochester, NY). [^{35}S]Methionine (1160 Ci/mmol) was from NEN Research Products (Boston, MA). All other chemicals were obtained commercially and were of the highest purity available.

Purification of β-D-galactosidase and production of antiserum

β-D-galactosidase (97 kDa form) was purified to apparent homogeneity from rat epididymal luminal fluid (9) and used for the production of antiserum by immunizing a female white rabbit by our published procedure (7). Affinity purified polyclonal antibody (IgG fraction) was prepared on a column of immobilized protein G (Pharmacia LKB Biotechnology, Piscataway, NJ) using manufacturer's protocol. The IgG fraction was dialyzed against 50 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl and concentrated (3-4 mg protein/ml) using Centricon 10 micro-concentrator from Amicon (Beverly, MA). The monospecific IgG

fraction was prepared by applying the serum to a column of immobilized β-D-galactosidase (immobilized on Sepharose 4B) equilibrated with 50 mM Tris-HCl buffer, pH 7.5 containing 0.15 M NaCl. The bound γ-globulin was eluted with the above buffer containing 7 M urea, followed by dialysis and concentration as above. The affinity purified and monospecific antibodies were aliquoted, and stored frozen at -75°C. Rat epididymal β-D-galactosidase used as a carrier (unlabeled) enzyme during biosynthetic studies was partially purified from rat epididymis by acid and heat treatment (10). Briefly, epididymal homogenates (10%, w/v) were prepared at 4°C in a solution containing 0.1 M Tris-HCl, pH 7.5, and 0.15 M NaCl by homogenizing for 50 sec with a polytron homogenizer (Type PT 1020 3500; Brinkman Instruments, Westbury, NY) set at position 5. Sodium deoxycholate was added to a final concentration of 0.5% to the vigorously stirred homogenate to solubilize the lysosomal β-D-galactosidase. The pH of the homogenate was lowered to 4.8 by adding 1 M acetic acid (0.06 ml/ml), and the mixture was heat treated by incubation at 56°C for 30 min. Deoxycholate (which precipitated due to the acidic pH) and denatured proteins were removed by centrifugation (25,000 g/30 min). The supernatant was removed by aspiration, assayed for β-D-galactosidase activity using PNP-β-D-galactoside substrate at pH 3.5. The partially purified enzyme (specific activity, 65 units/mg protein) was concentrated to a small volume (~ 2 ml) using a microconcentrator, aliquoted (2 units/aliquot), stored frozen at -20°C, and used as a source of carrier (unlabeled) enzyme.

Isolation of testicular germ cells

The protocol consists of the dissociation of the seminiferous tubules and epithelial cells of the testis followed by separation of mixed germ cells by unit gravity sedimentation on the basis of their size. The method, originally described by Romrell et al. (11), has been extensively used for many laboratory species (12-14) as well as a larger animal (15). The method described here was adopted from a published procedure of O'Brien for mice and rats (16). Rats (~ 8 weeks old) were killed by CO$_2$ asphyxiation. The testis, along with the epididymis and fat pad, was excised and perfused with enriched Krebs-Ringer bicarbonate medium (EKRB) through the testicular artery. The EKRB solution was prepared just before use by mixing stock solutions of EKRB salts, bicarbonate, glutamine-streptomycin-penicillin, essential and non-essential amino acids to a final concentration of 119.4 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO$_3$, 1.2 mM MgSO$_4$, 7 H$_2$O, 1.3 mM CaCl$_2$, 11.1 mM glucose, 1 mM sodium pyruvate, 6 mM sodium L-lactate, 1 mM glutamine, 100 µg streptomycin/ml, 100 U penicillin/ml, 10 ml each of the essential and non-essential amino acid/ liter. The pH was adjusted to 7.0-7.2 by bubbling with a 5% CO$_2$ in air for 5-

10 min.

1. Sequential enzymatic dissociation of seminiferous tubules and epithelial cells

The perfused testes were excised and their contents released by a small incision in the tunica albuginea. Mechanical dispersion of the seminiferous tubules should be avoided since it causes the formation of multinucleated germ cells. The detunicated testes were incubated in siliconised glass or Teflon Erlenmeyer flask in 65 ml of collagenase solution (1 mg/ml EKRB) under 5% CO_2 in air for 20 min at 33°C in a shaking water bath operated at 120 cycles/min. The dispersed seminiferous tubules were isolated by allowing them to sediment for 2-3 min under 5% CO_2 in air and decanting the supernatant. The process was repeated 2-3 times with 10 ml EKRB to stop the reaction and to ensure removal of the dissociated interstitial cells (e.g., Leydig cells, peritubular myoid cells) and blood cells. This step is important for obtaining spermatogenic cells in high purity prior to their separation. The seminiferous tubules were then incubated in 50 ml EKRB containing 0.5 mg trypsin/ml and l µg DNase/ml for 15 min using the above conditions. Cell aggregates which remained after trypsin treatment were dissociated gently by repeated pipetting for 3-5 min with a plastic Pasteur pipet. The reaction was stopped by the addition of 50 ml of EKRB containing 0.5 mg soybean trypsin inhibitor/ml and agitation (3-5 min). The cells were filtered through a 70 µm nylon mesh. The filtered cells were pelleted by centrifugation at 400 g/5 min, and the pelleted cells were washed by suspending in EKRB buffer containing 0.5% BSA and centrifugation as above (2 washes). Finally, the washed cells were suspended in 20-25 ml of EKRB containing 0.5% BSA. The yield of spermatogenic cells prepared from two testes was usually ~ 7.5 to 9 x 10^8 as counted by a hemocytometer on phase contrast microscope. Over 95% of the cells were viable as assessed by the exclusion of trypan blue.

2. Separation of germ cells by unit gravity sedimentation

The cell separation was carried out at 4°C for a total of 4 hours beginning from the loading of the cells and collection of the last fraction. The sedimentation chamber was initially loaded from the bottom through the cell-loading syringe with ~ 50 ml of EKRB to a level just above the baffle. The EKRB should completely fill the tubing leading from the syringe without any air bubbles. An aliquot containing 5-6 x 10^8 germ cells suspended in EKRB supplemented with 0.5% BSA was loaded through the syringe in the sedimentation chamber at a flow rate of 10 ml/min. Immediately after introducing the cell suspension,

the chamber was filled with 1200 ml of a linear BSA (2-4%) in EKRB at a flow rate of 10 ml/min adjusted with the T-valve and later increased to 40 ml/min. After the gradient loading was completed, the micrometering valve was closed and the cells were allowed to sediment. After 2 h and 40 min at 4°C, fractions (10 ml) were collected in disposable polystyrene tubes (17 x 100 mm) at a rate of 40-42 sec per tube. The tubes were centrifuged (400 g/5 min) and the top 9 ml of the supernatants aspirated, leaving ~ 1 ml in each tube. The presence of cells was confirmed by the appearance of the cell pellet and their quality and quantity assessed by examining cells in every fourth or fifth fraction using phase contrast microscopy (Figs. 1 and 2). Spermatocytes, which are the largest spermatogenic cells, were collected first followed by round spermatids of intermediate size and then condensed/elongated spermatids. The residual bodies were found only in the last few fractions. The fractions rich in spermatocytes, round spermatids, and condensed/elongated spermatids were pooled separately (Fig. 1), washed with EKRB and used for further studies.
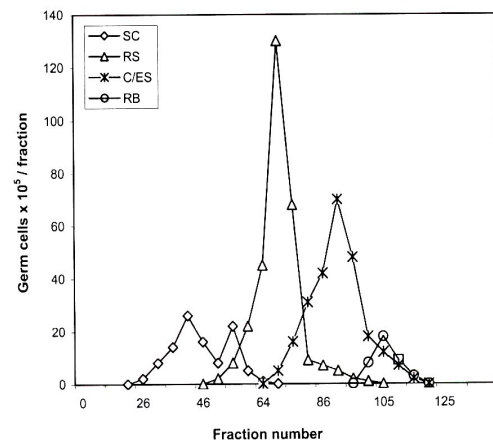

Fig. 1: The sedimentation profile of rat testicular germ cells following unit gravity sedimentation on a linear BSA gradient. Fractions (10 ml) were collected from the bottom and their composition was examined using phase-contrast microscopy. Fractions were pooled as follows: spermatocytes (SC), fractions 25-56; round spermatids (RS), fractions 65-72; condensed/elongated spermatids (C/ES), fractions 84-99. Residual bodies (RB) were present in the last few fractions.

3. Radiolabeling of spermatocytes and round spermatids.

Enriched populations of viable (approximately 95% viable as assessed by the Trypan blue exclusion test)

spermatocytes (>95% pure) and round spermatids (>95% pure) were pooled and 1.5 x 10^7 cells/ml were incubated in 3 ml of methionine-free MEM supplemented with 0.4% BSA, non-essential amino acids, antibiotics (penicillin, 0.1 unit/ml; streptomycin, 0.1 mg/ml), fungizone (1 µg/ml), 5mM sodium lactate as described (7). After 30 min at 34°C, the methionine-depleted cells were centrifuged at 400 g/5 min and the pelleted cells suspended in the above medium containing [^{35}S] methionine (400 µCi/ml) and incubated at 34°C under 5% CO_2 in air. After 30 min of labeling (pulse), the cells were pelleted and washed four times by suspending each time in 2 ml of PBS and centrifugation (400 g/5 min). The washed cells were either frozen (pulse) or suspended in 3 ml of the above medium containing non-radioactive methionine and cultured for pulse-chase studies. At desired time interval (chase), the cells were washed as above and the pelleted cells were frozen.
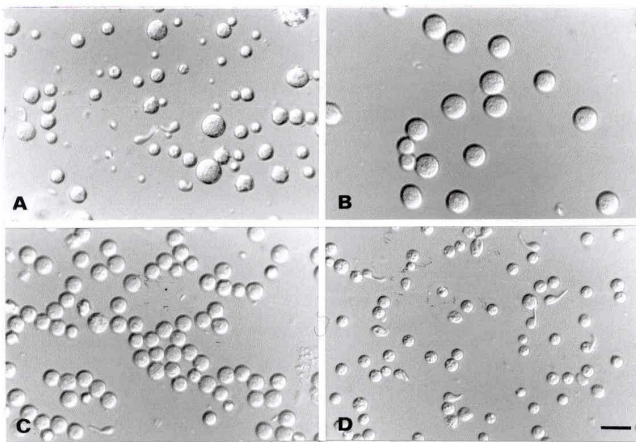
Fig. 2: Microscopic appearance of germ cells prepared from the rat testis. A, mixed germ cells obtained after enzymatic dispersion of the testis; B, spermatocytes; C, round spermatids; and D, condensed/elongated spermatids. Other details are described in Materials and Methods and in the legend to Figure 1. Aliquots of mixed cells (A) or separated cells (B-D) were photographed using Nomarski differential interference contrast optics. Scale bar = 17 µm.

4. Extraction, immunoprecipitation, and SDS-PAGE of [^{35}S]methionine-labeled β-D-galactosidase.

The frozen cells were suspended in a small volume (0.2 ml) of ice-cold 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and a protease inhibitor mixture (2 µg antipain/ml, 0.1% aprotinin, 10 µg benzamidine/ml, 1 µg chymostatin/ml, 1 µg leupeptin/ml, and 1 µg pepstatin/ml) as described (17).

The mixture was sonicated (3 x 10 second bursts on ice) in a Fisher sonicator set at speed 40, and centrifuged at 105,000 g/30 min. Supernatant was removed by aspiration and mixed with 2 units of the partially purified β-D-galactosidase carrier (see above), and 60 µg of affinity-purified immune or preimmune IgG. Samples were incubated at 34°C for 30 min followed by overnight incubation at 4°C with gentle rocking. Following these incubations, the immunoprecipitates were collected by centrifugation (8000 g/10 min). The immune/preimmune precipitates were washed by suspending in 0.1 M Tris-HCl buffer, pH 7.5, 0.15 M NaCl, and 1% SDS and centrifugation as above. The washed residues were mixed in Laemmli buffer containing 4 M urea, boiled in a water bath (100°C/5 min), centrifuged in a microfuge, and the soluble peptides were electrophoresed on 7% polyacrylamide gels (SDS-PAGE) under reduced conditions (18). Gels were exposed to Biomax MR film, and the radioactive bands were revealed by processing after 1 week. Intensifying screens can be used to shorten the processing time.

In pulse-chase studies, after radiographic exposure, individual bands were cut from the gel and then placed in 1 ml of NCS solubilizer (diluted 9:1 with water) at room temperature overnight. Scintiprep-2 in toluene (10 ml of 4%, v/v) was then added and radioactivity was determined after 48 h at room temperature using liquid scintillation spectroscopy.

Isolation of spermatozoa

Following median laparotomy, the epididymis trimmed of adipose tissue was taken with the whole vas deferens and placed in a Petri dish. A small catheter (plastic tubing), connected to a 10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) and 25 mM benzamidine was introduced into the vas deferens. The duct was then ligated around the catheter with a surgical thread and the proximal cauda was excised with a scissor. The flow rate of the buffer was adjusted by a pump to remove the cauda spermatozoa by retrograde flushing through the vas deferens. The fluid was centrifuged at 500 g/10 min, and the pelleted spermatozoa were suspended in desired buffer and used for immunocytochemistry or enzyme assay.

Immunolocalization of β-D-galactosidase

The β-D-galactosidase was immunolocalized using monospecific antibody (experimental) or preimmune IgG or immune (monospecific) IgG preabsorbed with a five-fold excess of purified β-galactosidase (negative controls) by three different protocols:

1. Preparation and immunostaining of testicular

sections

After rats were anesthetized, the tissues were cleared of blood by perfusion through the left ventricle with a saline solution (0.9% NaCl) before they were perfused with Bouin's fixative (75% saturated picric acid, 20% formol and 5% glacial acetic acid). The fixed testes were cut and immersed for 20 h in the same fixative after which the tissue pieces were dehydrated in graded ethanol and embedded in paraffin before thin sections (~5 µm) were prepared. The sections were freed from paraffin and hydrated through graded ethanol to PBS, pH 7.4. Endogenous peroxidase activity was quenched by incubation (30 min) in 0.03% H_2O_2 in methanol. Nonspecific binding sites were blocked (30 min) with 10% normal goat serum (NGS) in PBS. The sections were then incubated (1 h at 37°C) with preimmune/monospecific IgG (5 µg protein/ml of 10% NGS in PBS) in a humidified chamber, washed (3 x 10 min) in PBS before incubation (1 h at 20°C) with a 1/200 (v/v) diluted goat anti-rabbit IgG. The sections were washed as before and incubated (30 min) in avidin-biotin horseradish peroxidase complex according to the ABC kit protocol. The antigen was visualized by revealing the peroxidase activity with 0.05% diaminobenzidine tetrahydrochloride in Tris-HC1 0.2M, pH 7.5 and 0.02% H_2O_2. After washing in distilled water to stop the reaction, the sections were dehydrated and mounted.

2. Indirect immunofluorescence

This method was used to examine the binding of monospecific or preimmune IgG to the isolated spermatogenic cells and cauda epididymal spermatozoa. The cells were suspended in PBS/1% BSA and placed onto poly-L-lysine coated coverslips. The cells were allowed to adhere to the coverslips by keeping them in a humidified chamber for 15 min at 20°C. After two washes in PBS/0.1% BSA, the adherent cells were either included with IgG (intact cells) or permeabilized in methanol (15 min at -20°C) before interaction with the IgG. Drops of monospecific/preimmune (5 µg protein/ml PBS/0.1% BSA) were placed on top of the cells and the coverslips kept in a humidified chamber (60 min at 20°C). After three washes in PBS/0.1% BSA, the cells were incubated in a dark humidified chamber (60 min at 20°C) with 1/100 diluted secondary antibody (FITC-labeled anti-rabbit goat IgG). Following this incubation, the cells were washed in PBS, mounted with vectashield, sealed with nail polish, and observed under phase-contrast epifluorescence illumination or under a confocal microscope using Nomarski differential interference contrast optics.

3. Immunoelectron microscopy

After rats were anesthesized, the tissues were cleared of blood and fixed as above by perfusion through the left ventricle for 10 min with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Following this fixation, each testis and cauda epididymis was removed, cut into small pieces, and immersed in the above fixative for 50 min at room temperature. The tissues were dehydrated in graded solutions of dimethylformamide (20 min each) and progressively infiltrated under agitation with Lowicryl K4M according to Altmann et al. (19). Samples were then transferred to 6 mm diameter gelatin capsules filled with Lowicryl and closed. Polymerization was done for 4 days under indirect UV-light at 4°C and for one day at 20°C. The capsule holder was placed at 30-40 cm from the UV source. All instructions for use of Lowicryl K4M were furnished by the supplier. Ultrathin sections were mounted on uncoated nickel grids and floated for 30 min on a drop of 20 mM Tris-HCl buffered saline (pH 7.8) containing 0.15% BSA (TBS-BSA) to block non-specific binding sites and 0.04% glycine to reduce the free aldehyde groups. The sections were immediately incubated (2 hours at 20°C) with monospecific/preimmune IgG (5 µg protein/ml TBS-BSA). After being washed in TBS-BSA, the sections were incubated with gold-labeled secondary antibody at a dilution of 1:20 (v/v) in the above Tris-BSA buffer. The sections were stained with uranyl acetate (20 min at 20°C) before electron microscopic observation.

Enzyme assay

Acid glycohydrolases were assayed by measuring the hydrolysis of PNP-glycopyranoside substrate at their optimum pH as below.

All enzymes were assayed by incubating the purified germ cells (~ 1 x 10^6 cells/tube), appropriate substrate and Triton X-100 (0.2%, v/v) in a total volume of 0.5 ml at their optimum pH as follows: α-D-mannosidase, 5 mM PNP α-D-mannoside, 0.1 M sodium acetate buffer, pH 4.4; N-acetyl β-D-glucosaminidase, 5 mM PNP-β-N-acetylglucosaminide, 0.1 M sodium acetate buffer, pH 4.5; β-D-glucuronidase, 1 mM PNP-β-D-glucuronide, 0.1 M sodium acetate buffer, pH 5.0; α-L-fucosidase, 5 mM PNP-α-L-fucoside, 0.1 M sodium acetate buffer, pH 5.0; and β-D-galactosidase, 5 mM PNP-β-D-galactoside, 0.1 M sodium citrate buffer, pH 3.5. After incubation for 1 h at 37°C, the reaction was stopped by the addition of the glycine buffer containing 0.133 M glycine, 0.067M NaCl, 0.083 M Na_2CO_3 adjusted to pH 10.7 with 1 M NaOH (20). The amount of p-nitrophenol released was estimated by measuring the absorbance of the sample at 400 nm, and comparing with standard curves. Enzyme or substrate controls were incubated concurrently and the corresponding readings were subtracted from that obtained with complete enzyme system (enzyme and substrate). One unit is the

amount of enzyme that catalyzes the release of 1 µmol *p*-nitrophenol per h at 37°C.

RESULTS AND DISCUSSION

Highly enriched populations of rat testicular germ cells were prepared by sequential enzymatic dissociation of rat testis. The treatment apparently preserves morphological and biochemical integrity of the dissociated germ cells (12,13) which were purified by sedimentation based on their size. Three critical steps during cell separation are worth mentioning. First, loading of the mixed germ cells to the sedimentation chamber and the formation of a linear BSA gradient are important steps, and should be followed as described in the Materials and Methods. Second, the

sedimentation time is very critical and should be established in a few preliminary runs. Finally, any vibrations to the sedimentation chamber during cell separation should be avoided to get reproducible results.

The purified diploid (spermatocytes) and haploid (round and elongated/condensed spermatids) cells were found to contain all five acid glycohydrolase activities (Table 1), a result suggesting that sperm-associated enzymes are first expressed in the diploid cells. The addition of a detergent (Triton X-100) in the assay mixture was to ensure that the cells were lyzed and that the PNP-substrates were in contact with the active site of the intracellular enzyme. In the absence of detergent, the enzyme activities were 50-70% of the values reported in Table 1.

TABLE 1.   Glycohydrolase Activities In Rat Testicular Germ Cells And Spermatozoa[1]

Enzyme[2]	Cells (milli units/10^6 Cells)			
	SC	RS	C/ES	SP[3]
β-N-Acetylglucosaminidase	31.0 ± 2.7	7.4 ± 0.2	4.3 ± 0.6	220.8 ± 12.4
β-D-Galactosidase	12.4 ± 2.9	6.6 ± 0.4	3.7 ± 0.6	43.5 ± 4.4
β-D-Glucuronidase	1.6 ± 0.2	0.8 ± 0.1	0.5 ± 0.1	2.4 ± 0.2
α-D-Mannosidase	15.3 ± 1.1	7.5 ± 0.5	4.3 ± 0.2	18.1 ± 1.0
α-L-Fucosidase	44.8 ± 2.7	51.1 ± 3.0	21.5 ± 0.4	ND

[1]The germ cells were prepared from rat testis, and spermatozoa from the distal cauda epididymidis as described under Materials and Methods. Fractions rich in spermatocytes (SC), round spermatids (RS), condensed/elongated spermatids (C/ES) were pooled (see Fig. 1) and centrifuged (400 g/5 min). The pelleted cells were suspended in PBS (approximately 100 x 10^6 cells/ml) and used for enzyme assay. Values are average of four separate experiments in triplicate with ± standard deviation (SD).

[2]All enzymes were assayed using PNP-glycoside substrates.

[3]Values reported for spermatozoa (SP) were calculated from a previous report (22).

The radiolabeling of cells in culture, and immunoprecipitation of the [35S]methionine-labeled β-D-galactosidase studies demonstrated that the enzyme was synthesized in spermatocytes and round spermatids in high-molecular weight precursor forms (90/88 kDa) which undergo processing to lower molecular weight mature forms in a cell-specific manner. The net result is the formation of predominantly 64- and 62- kDa forms in spermatocytes and round spermatids, respectively. The protocol reported in

this article can be adopted to study synthesis and processing of other proteins/glycoproteins in cell culture or to assess the effect of specific agents on spermatogenesis. For reproducible results, it is important to use highly specific antibody (preferably affinity purified IgG fraction). The antibody should first be titrated against a carrier antigen to establish the ratio of antibody needed for quantitative immunoprecipitation of a known amount of the unlabeled protein. Since the newly-synthesized radiolabeled antigen

contributes very little protein/glycoprotein, the addition of a carrier antigen is highly recommended to ensure that the immune/preimmune precipitate forms a visible pellet during washing procedure.

We also examined the stage-specific localization of β-galactosidase during spermatogenesis and followed the successive formation of the acrosome during the cap and elongation phases of spermiogenesis. This was attempted using light and electron microscopic approaches in three different protocols. Data obtained from these approaches allow us to conclude that β-galactosidase is first seen in the late spermatocytes (pachytene spermatocytes). The immunopositive reaction was confined by light microscopy to granules dispersed in the cytoplasm around the nucleus (see Fig. 4 in reference 7). The presence of the enzyme in these cells is in agreement with the enzymatic assay data presented in Table 1 and the biosynthetic and processing studies with isolated spermatocytes. In round spermatids, the Golgi apparatus and the acrosomal vesicle (stages 2-3) or cap-like structure (stages 5-7) seen at one pole of the cell showed an intense immunopositive reaction in paraffin sections and isolated cells. As spermiogenesis continues, the reaction was seen over an enlarging cap-like structure extending from the base of the nucleus. It becomes confined to a distinct sickle-shaped structure which was also seen in testicular spermatozoa (Fig. 3).
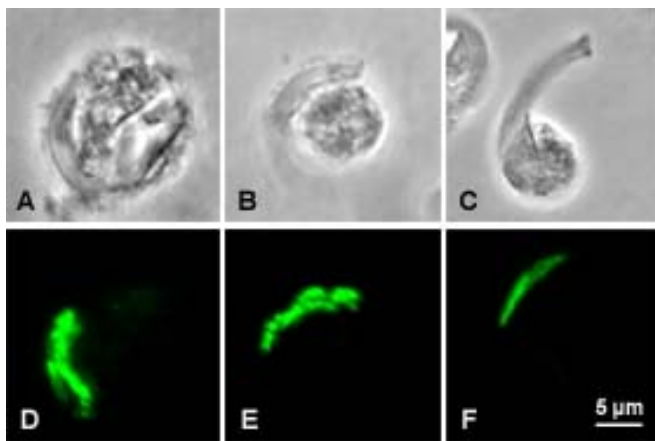
Fig. 3: Confocal micrographs showing immunolocalization of β-galactosidase in the forming acrosome during spermiogenesis in the rat. The testicular germ cells were prepared and immunostained using anti-β-galactosidase (monospecific IgG, 5 µg protein/ml) as primary antibody and FITC-labeled anti-rabbit goat IgG as secondary antibody as described in Materials and Methods.

Various phases of acrosome formation were photographed with a confocal microscope using Nomarski differential interference contrast optics (A-C) and immunofluorescence (D-F). Note the presence of intense fluorescence in the forming acrosome of steps 9 (A & D), 12 (B & E) and 15 (C & F) elongated spermatids. The absence of a flagellum is due to its loss during preparation of the spermatogenic cells by enzymatic disruption of the testis.

At the ultrastructural level, the enzyme was visualized by the presence of gold particles in the Golgi apparatus, Golgi-associated vesicles and lysosome-like structures present within the spermatocytes and early round spermatids. The immunolabeling was obvious in the proacrosomal granules present in the trans-Golgi region, the acrosomal vesicles, and the head cap of round spermatids. By following the immunolocalization of β-galactosidase during the elongation and maturation phases of spermiogenesis, the gold particles were seen in the head cap of elongated spermatids and became confined to the acrosomal cap of maturing spermatids and spermatozoa (see Fig. 6 in reference 7). This localization is similar to our published reports with β-glucuronidase (21), another exoglycohydrolase. The distribution of the two enzymes showed the successive formation of the acrosome during the progressive transformation of the spermatids into spermatozoa with fully developed acrosome. Thus, glycohydrolases can be used as markers to examine acrosome formation.

It is important to emphasize that immunocytochemical approaches are qualitative and detect the antigen when the local concentration is reasonably high. Thus a high titer antibody is needed to detect low amounts of glycohydrolases present in the germ cells. In addition, the preservation of the antigenic site(s) during tissue (cell) fixation and processing of the tissue is an important factor which may have an influence on the immunopositive/immunonegative results. It is highly recommended to establish the optimal conditions of antibody concentration and incubation time to determine non-specific background staining. Also, samples (tissue sections or isolated cells) should be incubated in the presence of preimmune and/or immune IgG preabsorbed with an excess of purified enzyme to establish negative staining (negative controls).

ACKNOWLEDGEMENTS

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