

## Sperm require $\beta$ -*N*-acetylglucosaminidase to penetrate through the egg zona pellucida

David J. Miller, Xiaohai Gong and Barry D. Shur\*

Box 117, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

\*Author for correspondence

### SUMMARY

Fertilization in the mouse is initiated by sperm  $\beta$ 1,4-galactosyltransferase (GalTase) binding to terminal *N*-acetylglucosamine residues on the zona pellucida glycoprotein ZP3. Binding of ZP3 induces exocytosis of the sperm acrosome, whose contents are believed to digest a penetration slit in the zona matrix through which sperm reach the egg. As a consequence of acrosomal exocytosis, GalTase is redistributed to the lateral aspect of the sperm head, where its function remains unknown. In this location, GalTase could conceivably impede zona penetration by binding to *N*-acetylglucosamine residues exposed on zona pellucida glycoproteins. Therefore, in this study we investigated the presence and function of acrosomal glycosidases capable of removing the GalTase-binding site from zona pellucida glycoproteins.  $\beta$ -*N*-acetylglucosaminidase was found at very high levels in sperm, being more than 20-fold higher than other glycosidases assayed. The specific isozymic variant was identified as  $\beta$ -hexosaminidase B.  $\beta$ -*N*-acetylglucosaminidase was localized to sperm acrosomes by biochemical and indirect immunofluorescence studies and was released during the acrosome reaction, as expected for an enzyme involved in zona penetration. To

determine if, in fact, acrosomal  $\beta$ -*N*-acetylglucosaminidase facilitated penetration through the zona, an assay was developed using eggs that were rendered incapable of triggering the block to polyspermy. A specific competitive inhibitor of  $\beta$ -*N*-acetylglucosaminidase activity, PUGNAC, inhibited sperm penetration of the zona in a dose-dependent manner, whereas a closely related  $\beta$ -glucosidase inhibitor, PUGLU, had no effect on zona penetration or on  $\beta$ -*N*-acetylglucosaminidase activity. Neither glycosidase inhibitor affected sperm motility or induction of the acrosome reaction. These results demonstrate that  $\beta$ -*N*-acetylglucosaminidase is found in sperm acrosomes and is released during the acrosome reaction, at which time it facilitates sperm penetration through the zona. These results also imply that sperm have developed mechanisms to prevent the formation of stable interactions between surface receptors and their zona pellucida ligands during penetration.

Key words: *N*-acetylglucosaminidase, acrosome, fertilization, galactosyltransferase, hexosaminidase, sperm, zona pellucida, mouse

### INTRODUCTION

The early events of mammalian fertilization involve the initial binding of acrosome-intact sperm to the egg coat, or zona pellucida. Sperm-egg binding induces the acrosome reaction, after which the acrosome-reacted sperm binds transiently to the zona pellucida before penetrating the zona matrix. Presumably, penetration through the zona is facilitated by hydrolytic enzymes released from the acrosome. Following zona penetration, the fertilizing sperm fuses with and activates the egg, triggering the zona block to polyspermy. Although these cellular events are well described in many species, their underlying molecular mechanisms are less well understood (for review, see Yanagimachi, 1988).

In mouse, one of the best understood examples of mammalian fertilization, initial sperm-egg recognition is

mediated by the binding of sperm surface 1,4-galactosyltransferase (GalTase) to terminal *N*-acetylglucosamine residues on ZP3, one of three glycoproteins found in the egg zona pellucida (Miller et al., 1992; Bleil and Wassarman, 1980; Shur, 1991). Other sperm proteins have also been proposed to bind ZP3, including one of  $56 \times 10^6 M_r$  thought to bind ZP3 oligosaccharides (Bleil and Wassarman, 1990) and one of  $95 \times 10^3 M_r$  thought to have intrinsic tyrosine kinase activity stimulated upon zona binding (Leyton and Saling, 1989a). How these other sperm proteins cooperate with GalTase, if at all, to mediate effective sperm-egg binding is not yet known.

Evidence suggests that, after initial binding, ZP3 aggregates a receptor, probably GalTase, which directly or indirectly activates G-protein-dependent exocytosis of the sperm acrosome (acrosome reaction; Florman et al., 1984; Leyton and Saling, 1989b; Macek et al., 1991; Endo et al.,

1988; Miller and Shur, unpublished observations). As a consequence of the acrosome reaction, the area of plasma membrane containing GalTase is lost; however, GalTase is retained on acrosome-reacted sperm by migrating to the lateral surface of the sperm head (Lopez and Shur, 1987).

Currently, it is unclear why GalTase is salvaged during the acrosome reaction, and what function it may serve on acrosome-reacted sperm. Surprisingly, GalTase is no longer able to bind soluble zona glycoproteins after the acrosome reaction, although it is still able to bind non-zona glycoprotein substrates, which may result from differences in substrate affinity following the acrosome reaction (Miller et al., 1992). It is conceivable that GalTase binding to ZP3 oligosaccharides maintains sperm-zona associations during the acrosome reaction, or stabilizes the initial binding of acrosome-reacted sperm to the zona. In any event, the fully acrosome-reacted sperm associates with the zona pellucida by a secondary type of binding thought to involve another zona glycoprotein, ZP2 (Bleil et al., 1988). Some sperm proteins have been proposed as secondary adhesion molecules, but their zona glycoprotein binding specificity remains unclear (Jones et al., 1988; Lathrop et al., 1990). Eventually, the acrosome-reacted sperm penetrates the zona pellucida, presumably relying upon enzymes released from the acrosome (Yanagimachi, 1988). However, with the possible exception of the trypsin-like protease acrosin, there is no clear understanding of how any acrosomal enzyme functions during zona penetration (Anakwe et al., 1991; Urch et al., 1985; Kashiwabara et al., 1990; de Vries et al., 1985).

It is possible that in addition to more generic proteolytic activities, like acrosin, the acrosome may also contain enzymes that specifically prevent stable molecular adhesions between sperm surface receptors and their zona pellucida ligands. In this regard, the presence of GalTase on acrosome-reacted sperm could conceivably impede zona penetration by binding to exposed *N*-acetylglucosamine residues on zona pellucida glycoproteins. Therefore, it seemed possible that the acrosome might release enzymes that would prevent GalTase binding to zona glycoproteins. We specifically wanted to determine whether  $\alpha$ -*N*-acetylglucosaminidase, which is capable of removing the GalTase-binding site in the zona pellucida (Miller et al., 1992), or other glycosidases were released during the acrosome reaction and whether they might function in zona penetration.

Results presented here demonstrate that  $\alpha$ -*N*-acetylglucosaminidase is present in sperm and its activity is at least 20-fold higher than other glycosidases. The enzyme was identified as the isozymic variant  $\beta$ -hexosaminidase B, and was localized to acrosomes by both biochemical and indirect immunofluorescence assays. The function of acrosomal  $\beta$ -*N*-acetylglucosaminidase during zona penetration was assessed by adding a specific inhibitor of  $\beta$ -*N*-acetylglucosaminidase activity, PUGNAC, to eggs as sperm initiated penetration of the zona. PUGNAC inhibited sperm penetration through the zona pellucida without altering sperm motility or the zona-induced acrosome reaction. Another closely related glycosidase inhibitor, which has no inhibitory activity towards  $\beta$ -*N*-acetylglucosaminidase, did not affect zona penetration. Thus, mouse sperm acrosomes

contain high levels of  $\beta$ -*N*-acetylglucosaminidase, which is required for penetration of the zona pellucida.

## MATERIALS AND METHODS

### Assays of glycosidase activity

Cauda epididymal sperm were collected from at least two male CF-1 mice and incubated for 60 minutes at 37°C in dmKRBT (120 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 1.2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.6 mM glucose, 1.1 mM sodium pyruvate, 25 mM TAPSO (3-[*N*-tris(hydroxymethyl) methylamino]-2-hydroxy propanesulfonic acid), 18.5 mM sucrose, 6 mg/ml bovine serum albumin (BSA), pH 7.3, to induce capacitation (Neill and Olds-Clarke, 1988). Sperm were centrifuged at 800 *g* and resuspended in dmKRBT three times to remove any contaminating glycosidases from epididymal fluid. To produce populations of primarily acrosome-reacted sperm, capacitated sperm were incubated for an additional 60 minutes in 10  $\mu$ M A23187 added from a stock of 10 mM in DMSO. Activity released from sperm during the acrosome reaction was also measured in the same assay. Detergent extracts of sperm were made by treating washed sperm with 30 mM *n*-octylglucoside for 10 minutes. For enzyme assays, 10<sup>6</sup> sperm equivalents were brought to 100  $\mu$ l in dmKRBT. An equal volume of substrate (2 mM *p*-nitrophenyl  $\alpha$ -L-fucopyranoside, *p*-nitrophenyl  $\alpha$ -D-mannopyranoside, *p*-nitrophenyl  $\alpha$ -D-galactopyranoside, *p*-nitrophenyl  $\alpha$ -D-galactopyranoside, *p*-nitrophenyl  $\alpha$ -D-glucopyranoside, *p*-nitrophenyl  $\alpha$ -D-glucopyranoside, *p*-nitrophenyl *N*-acetyl- $\alpha$ -D-glucosaminide, *p*-nitrophenyl *N*-acetyl- $\alpha$ -D-galactosaminide) in 60 mM trisodium citrate, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM KCl, 1 mg/ml BSA, protease inhibitor cocktail (PIC; Miller et al., 1992), pH 5.0, was added, yielding a final pH of 5.2. The reaction was allowed to proceed for 30 minutes at 37°C and stopped by addition of 1 ml of 1 M Na<sub>2</sub>CO<sub>3</sub>. Absorbance of product was measured at 405 nm ( $E_{405}=18.2$ ).

To ascertain which isoform of the dimeric  $\beta$ -hexosaminidase was present in sperm, cells were washed and 5×10<sup>6</sup> sperm were extracted for 30 minutes on ice with 500  $\mu$ l of lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5 plus PIC). Aliquots (1  $\mu$ l) were incubated in 200  $\mu$ l of citrate phosphate buffer with 2 mM 4-methylumbelliferyl- $\beta$ -D-*N*-acetylglucosaminide to measure total  $\beta$ -hexosaminidase activity or with 2 mM 4-methylumbelliferyl-*N*-acetyl- $\beta$ -D-glucosaminide-6-sulfate, which is cleaved specifically by the  $\beta$ -subunit of  $\beta$ -hexosaminidase A (Ben-Yoseph et al., 1985; Kytzia and Sandhoff, 1985). After 15 or 30 minutes of incubation at 37°C, 3.8 ml of 133 mM glycine, 83 mM NaCO<sub>3</sub> was added to stop the reaction. Fluorescence was measured using excitation at 380 nm and emission at 460 nm (Kaback, 1972).

### Protein assay

Protein was assayed using the BCA assay, according to manufacturer's instructions (Pierce) with BSA as standard.

### Immunolocalization of sperm hexosaminidase

Capacitated sperm or ionophore-treated sperm were washed three times and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 minutes at room temperature. In some cases, sperm were not fixed until after addition of the first antibody. Sperm were washed in PBS and blocked with 0.2 M glycine in PBS for 30 minutes, and then were washed twice in PBS containing 3% BSA and air-dried on glass slides. A 1/100 dilution of normal serum or one of three hexosaminidase antisera was used. Goat antisera were made to human  $\beta$ -hexosaminidase A ( / heterodimer),  $\beta$ -hexosaminidase B ( / homodimer), or the denatured  $\beta$ -subunit found in  $\beta$ -hexosaminidase A (antisera were gifts from

Dr Richard L. Proia, NIH). The antiserum was incubated with sperm in PBS containing 3% BSA for 2 hours. Sperm were washed three times and incubated with 20 µg/ml of biotinylated rabbit anti-goat IgG for 20 minutes (Vector Laboratories, Burlingame, CA). After washing, 5 µg/ml of fluorescein avidin DCS (Vector, cell sorter grade) was added to the sperm for 10 minutes. Sperm were washed, covered with 0.4% *n*-propyl-gallate in 9:1 glycerol:PBS, coverslips were applied and sealed with nail polish. Slides were examined under fluorescence microscopy at 500×.

#### Immunoprecipitation of sperm hexosaminidase activities

Sperm were collected in dmKRBT, washed three times and  $5 \times 10^7$  cells were extracted in 1 ml of lysis buffer on ice for 30 minutes. The extract was centrifuged for 5 minutes at 10,000 *g* and the supernatant was initially treated by addition of 50 µl of normal rabbit serum and 100 µl of rabbit anti-goat IgG coupled to agarose. Following 2 hours at 4°C, the supernatant was removed and 200 µl was incubated with 10 µl of either normal goat serum, goat antiserum to human  $\alpha$ -hexosaminidase A, antiserum to  $\alpha$ -hexosaminidase B, or antiserum to denatured  $\alpha$ -subunit. After 1 hour at 4°C, 20 µl of rabbit anti-goat IgG coupled to agarose was added and samples were incubated for 1 hour at 4°C. Samples were centrifuged and the pellets washed twice and resuspended in 220 µl of lysis buffer. An equal volume of 2 mM *p*-nitrophenyl-*N*-acetylglucosaminide in citrate phosphate (pH 5.0) was added and the samples incubated for 30 minutes at 37°C. Assays were terminated by addition of 1 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> and product was measured by absorbance at 405 nm.

#### Immunoprecipitation of hexosaminidase from <sup>125</sup>I-labelled sperm extracts

Capacitated sperm or capacitated and ionophore-treated sperm ( $3 \times 10^6$ ) were extracted with 300 µl lysis buffer as above (except in this case 50 mM Tris-HCl was replaced with 50 mM sodium phosphate buffer). Approximately 50 µg of protein from each extract was iodinated using 0.5 mCi of Na<sup>125</sup>I and Iodogen-coated tubes (following recommended procedure of Pierce). Free Na<sup>125</sup>I was removed using a desalting column. The specific activity ranged from 0.68 to 0.86 µCi/µg. Radioiodinated sperm proteins (10<sup>7</sup> disintegrations/minute in 1.0 ml of lysis buffer) were initially incubated with 0.1 ml normal goat serum and 0.2 ml rabbit anti-goat IgG-agarose. The remaining supernatant was split into four samples and incubated with 25 µl of either normal goat serum, goat antiserum to human  $\alpha$ -hexosaminidase A, antiserum to  $\alpha$ -hexosaminidase B, or antiserum to denatured  $\alpha$ -subunit in a total volume of 250 µl. Protein was precipitated using anti-goat IgG coupled to agarose, as above. Pellets were washed three times, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and boiled 5 minutes, and the supernatant was loaded onto a 10% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography.

#### Egg penetration assay

A zona pellucida penetration assay was developed from previously published procedures (Boatman et al., 1988; Shur and Hall, 1982). Briefly, oviductal eggs were collected from female CD-1 mice superovulated with i.p. injections of 7.5 U of pregnant mare's serum gonadotropin and after 48 hours, 7.5 U of human chorionic gonadotropin. Females were killed for egg collection 13 hours later. The cumulus cells surrounding the eggs were removed by digestion in 1 mg/ml testicular hyaluronidase for 5 minutes and the eggs placed in droplets of dmKRBT covered with mineral oil.

To attempt to inactivate cortical granule enzymes, which when released activate the zona block to polyspermy, eggs were treated several ways. Some were stored in a solution of 0.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.75 M MgSO<sub>4</sub>, 0.2 mM ZnCl<sub>2</sub>, 0.1 mg/ml polyvinyl pyrrolidone,

and then equilibrated in dmKRBT and used for a penetration assay as previously reported (Boatman et al., 1988). Alternatively, eggs were incubated for 45 or 90 minutes at 55°C or for 60 minutes at 60°C. Cauda epididymal sperm were collected, capacitated and  $0.5\text{--}2 \times 10^6$  sperm/ml were incubated in 200 µl droplets of dmKRBT with 30–50 eggs under mineral oil in 5% CO<sub>2</sub>. Sperm and eggs were coincubated for 10 minutes at 37°C to allow binding to occur, after which the medium containing unbound sperm was aspirated and replaced with sperm-free medium. This was done three times. In the final step, specific concentrations of glycosidase inhibitors were added from a 10 mM stock. Either PUGNAC [O-(2-acetamido-2-deoxy-D glucopyranosylidene) amino *N*-phenylcarbamate], a competitive  $\alpha$ -*N*-acetylglucosaminidase inhibitor, or the control PUGLU [O-(D-glucopyranosylidene) amino *N*-phenylcarbamate], a  $\beta$ -glucosidase inhibitor (CarboGen, Zurich) were used (Beer and Vasella, 1985). Penetration was allowed to occur for an additional 3.5–6.0 hours before the assay was stopped by addition of sodium azide (final concentration 0.01%).

Eggs were transferred to drops of sperm-free dmKRBT and a fine-pulled glass micropipette was used to remove sperm outside of the zona pellucida. Eggs were fixed in 4% paraformaldehyde in PBS, transferred onto a siliconized glass microscope slide, and washed twice with 50 µl of 0.23% sodium citrate:ethanol (3:1, v/v, 0.75 mg/ml PVP). Extra medium was aspirated, and 50 µl of stain [30 µg Hoechst 33258 per ml 0.23% sodium citrate:ethanol (3:1, v/v, no PVP)] was carefully added to eggs. The slide was placed on a 37°C slide warmer for 6 minutes. The staining solution was aspirated and the eggs on the slide were rinsed three times with 0.23% sodium citrate:ethanol (3:1) and covered with 12 µl of mounting solution (9:1 glycerol:0.23% sodium citrate, 0.4% *n*-propyl gallate). Coverslips were applied and sealed with nail polish, and sperm inside the zona pellucida were counted by fluorescence microscopy at 400×.

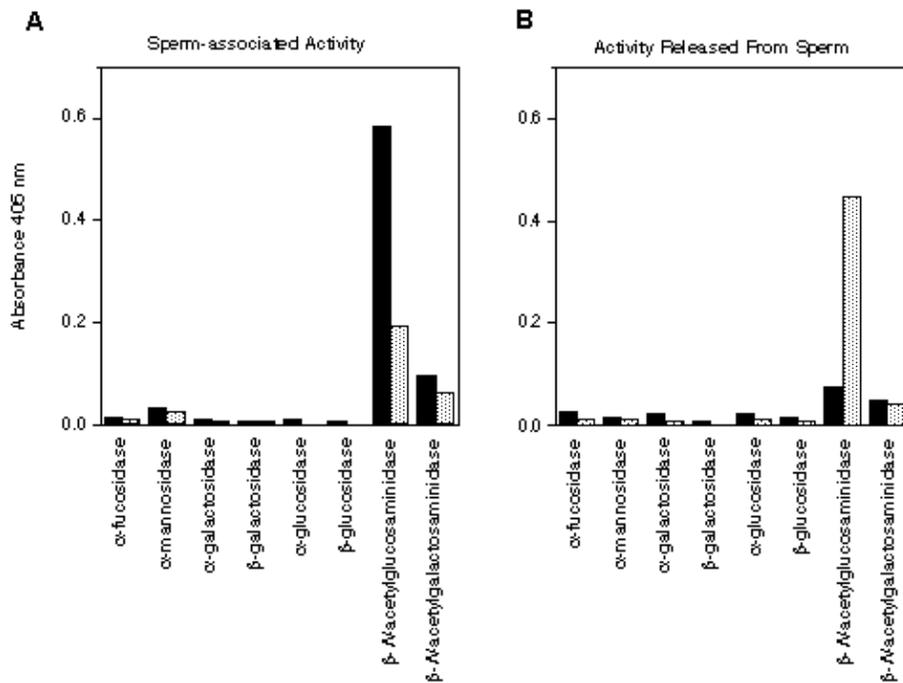
To verify that sperm  $\alpha$ -*N*-acetylglucosaminidase activity was inhibited, 10-µl samples of medium containing sperm were removed after 4 hours of sperm-egg coincubation. An equal volume of 2 mM *p*-nitrophenyl-*N*-acetylglucosaminide in citrate phosphate was added and assays were incubated for 4–10 hours at 37°C.

#### Assay of the zona pellucida-induced acrosome reaction

Sperm ( $10^6$ /ml) were capacitated for 90 minutes and incubated with medium (negative control), 10 µM A23187 (positive control), or 40 µg/ml heat-solubilized whole zonae pellucidae (from ovarian homogenates; Miller et al., 1992; Bleil and Wassarman, 1986). To determine if either PUGNAC or PUGLU inhibited the zona-induced acrosome reaction, 250 µM PUGNAC or PUGLU were added to sperm in the presence of zonae pellucidae or A23187. The total volume was 20 µl. Following a 60-minute incubation at 37°C, sperm were fixed in 4% paraformaldehyde in PBS for 10 minutes. Sperm were washed in 0.1 M ammonium acetate (pH 9.0) and air-dried on microscope slides. Slides were stained in 0.22% Coomassie Blue G-250 in 50% methanol and 10% acetic acid for 2 minutes, rinsed in water and covered with Permount. Sperm were examined at 400×; those with intact acrosomes had an intensely stained acrosomal ridge (Moller et al., 1990).

## RESULTS

Interactions between sperm surface GalTase and ZP3 mediate primary sperm-egg binding in the mouse. After initial gamete binding, ZP3 also induces the acrosome reaction, possibly by aggregating GalTase, resulting in the release of acrosomal enzymes. GalTase is retained on the lateral sperm head following the acrosome reaction, where



**Fig. 1.** *N*-acetylglucosaminidase activity is very high in detergent extracts of cauda epididymal mouse sperm. (A) Sperm were capacitated, washed to remove epididymal fluid glycosidases, and extracted with *n*-octylglucoside. Extracts were incubated with *p*-nitrophenyl-derivatized substrates and product was measured by absorbance at 405 nm (solid bars). An equivalent number of sperm were treated with calcium ionophore (A23187) to induce acrosomal release, after which they were detergent-extracted and assayed (gray bars). (B) Activity released as sperm underwent the acrosome reaction is shown. Sperm treated with ionophore (gray bars) or control sperm (solid bars) were centrifuged and the supernatant was assayed for glycosidase activity. Results are representative of three experiments.

its function remains unknown. We reasoned that after the acrosome reaction, stable binding of GalTase and zona pellucida glycoproteins would impede zona penetration. An acrosomal glycosidase capable of removing or blocking the zona pellucida binding site for GalTase might be involved in enhancing zona penetration. Therefore, the amounts of several glycosidases in mouse sperm were determined.

### Sperm acrosomes contain high levels of $\beta$ -*N*-acetylglucosaminidase

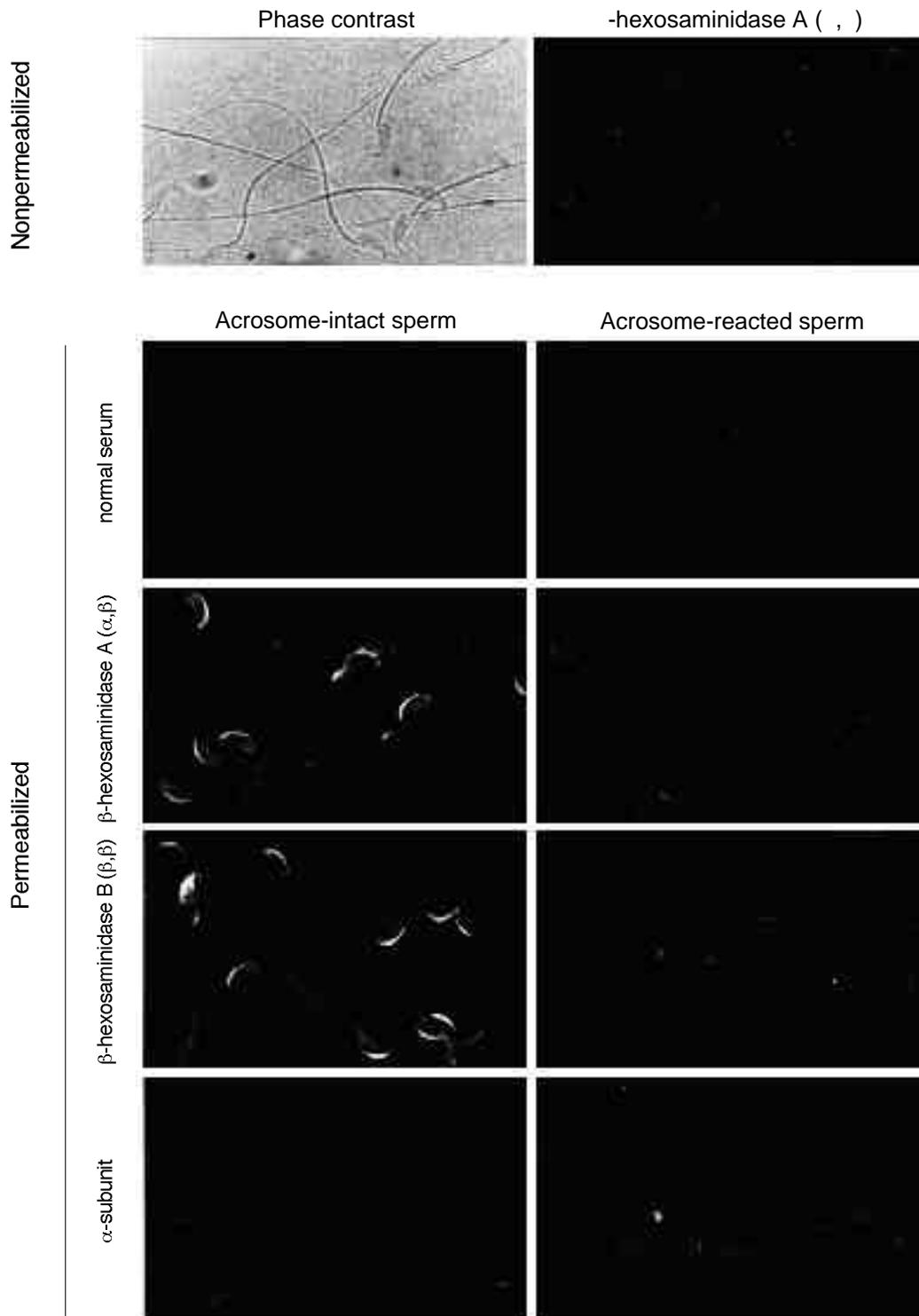
Washed, intact sperm had negligible glycosidase activities, but following detergent solubilization or calcium ionophore treatment to release acrosomes, the activity of several glycosidases was detected. *N*-acetylglucosaminidase activity was sevenfold higher than *N*-acetylgalactosaminidase activity and at least 20-fold higher than other glycosidases (Fig. 1A). It is likely that the activity of *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase can be accounted for by the same enzyme, since most *N*-acetylglucosaminidases also have *N*-acetylgalactosaminidase activity and are referred to as  $\beta$ -hexosaminidases (Beeley, 1985). If sperm were treated with calcium ionophore to reduce the number of sperm with intact acrosomes by 60–75%, there was a corresponding 69% decrease in *N*-acetylglucosaminidase activity associated with sperm, which could be recovered in the soluble material released from the acrosome (Fig. 1B). Control sperm released negligible amounts of *N*-acetylglucosaminidase activity (Fig. 1B). This demonstrated that, of those assayed in sperm,  $\beta$ -*N*-acetylglucosaminidase was the major glycosidase, and that its location was likely the acrosome.

To ascertain if *N*-acetylglucosaminidase was, in fact, localized in acrosomes, indirect immunofluorescence assays were employed using antibodies to human  $\beta$ -hexosaminidase.  $\beta$ -hexosaminidase is found in two major isozyme forms, an  $\alpha/\beta$  heterodimer referred to as  $\beta$ -hex-

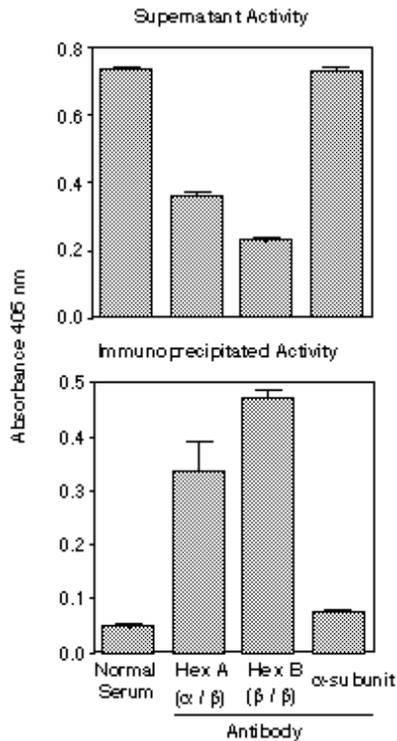
osaminidase A and a  $\beta/\beta$  homodimer referred to as  $\beta$ -hexosaminidase B. Therefore, antibodies to  $\beta$ -hexosaminidase A recognize both  $\beta$ -hexosaminidase A and B as do antibodies to  $\beta$ -hexosaminidase B, due to the subunit common to both isoforms (Proia et al., 1984). However, antibodies to purified  $\beta$ -subunit only recognize  $\beta$ -hexosaminidase A (Proia et al., 1984). None of these antibodies produced immunofluorescent staining when used on live sperm (Fig. 2). However, if sperm were fixed and permeabilized, staining was observed in the acrosomes using antibodies to  $\beta$ -hexosaminidase A and  $\beta$ -hexosaminidase B. This staining was lost as sperm underwent the acrosome reaction. No staining was observed with antibodies to  $\beta$ -subunit or with normal serum, suggesting the  $\beta$ -hexosaminidase found in sperm acrosomes was  $\beta$ -hexosaminidase B.

Since the antibodies used were raised against human  $\beta$ -hexosaminidases, we confirmed that the protein recognized in mouse sperm was  $\beta$ -hexosaminidase (i.e., *N*-acetylglucosaminidase) using immunoprecipitation assays. Detergent extracts of sperm were incubated with normal serum or antibodies to  $\beta$ -hexosaminidase A,  $\beta$ -hexosaminidase B, or  $\beta$ -subunit. Immune complexes were precipitated with rabbit anti-goat IgG-agarose, and the precipitates and supernatants were assayed for *N*-acetylglucosaminidase activity. Antibodies to  $\beta$ -hexosaminidase A and  $\beta$ -hexosaminidase B precipitated up to 70% of the *N*-acetylglucosaminidase activity, but none was precipitated by antibodies to  $\beta$ -subunit (Fig. 3). This demonstrated that the antibodies to human  $\beta$ -hexosaminidases recognized the mouse enzyme and further supports the conclusion that the  $\beta$ -hexosaminidase found in mouse sperm is  $\beta$ -hexosaminidase B.

To characterize further the  $\beta$ -hexosaminidase isoform in mouse sperm, sperm proteins were immunoprecipitated and characterized by polyacrylamide gel electrophoresis. Detergent extracts of acrosome-intact sperm were radioiodinated and incubated with normal serum or antibodies to  $\beta$ -

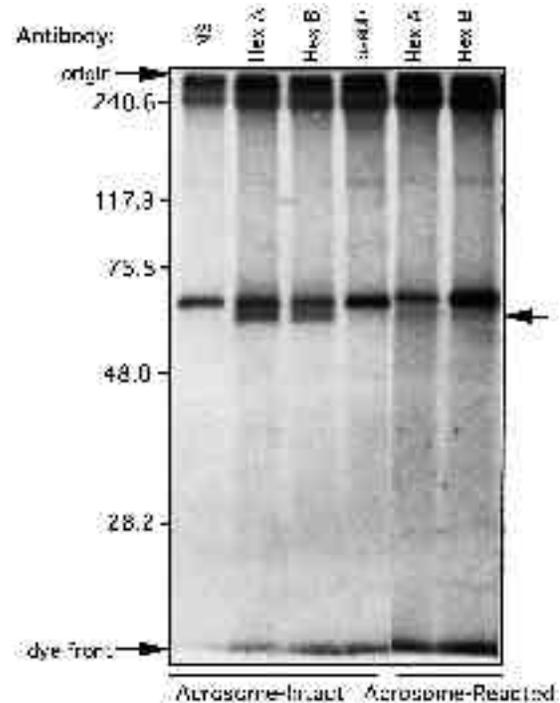


**Fig. 2.** Immunostaining of sperm with antibodies to  $\alpha$ -hexosaminidase demonstrates that the enzyme is found in sperm acrosomes. For reference, a phase contrast micrograph of mouse sperm is shown. Live, non-permeabilized sperm did not stain with any of the antibodies, including antisera raised against human  $\alpha$ -hexosaminidase A ( / ) (shown),  $\alpha$ -hexosaminidase B ( / ) and purified  $\alpha$ -subunit. However, if sperm were fixed with paraformaldehyde, permeabilized with Triton X-100, and then stained, the acrosomal region was found to contain  $\alpha$ -hexosaminidase using antibodies to  $\alpha$ -hexosaminidase A and  $\alpha$ -hexosaminidase B. No immunoreactivity was detectable using antibodies to  $\alpha$ -subunit. This suggested that the  $\alpha$ -N-acetylglucosaminidase activity present in sperm was  $\alpha$ -hexosaminidase B, the / homodimer. Following the acrosome reaction, immunofluorescence with antibodies to either  $\alpha$ -hexosaminidase A or  $\alpha$ -hexosaminidase B was lost. Results are representative of four experiments.



**Fig. 3.** Antibodies to human  $\alpha$ -hexosaminidase A ( / ) and  $\beta$ -hexosaminidase B ( / ) immunoprecipitate mouse  $\alpha$ -*N*-acetylglucosaminidase activity. Detergent extracts of mouse sperm were incubated with normal serum or immune serum and immune complexes were precipitated by addition of rabbit anti-goat IgG coupled to agarose. Both the washed precipitate and the supernatant were assayed for  $\alpha$ -*N*-acetylglucosaminidase activity. Results are averages of four experiments and bars indicate standard errors. Antibodies to human  $\alpha$ -hexosaminidase A and  $\beta$ -hexosaminidase B precipitated activity, but antibodies to  $\alpha$ -subunit did not. Thus, antibodies to human  $\alpha$ -hexosaminidase cross-reacted with the mouse sperm enzyme.

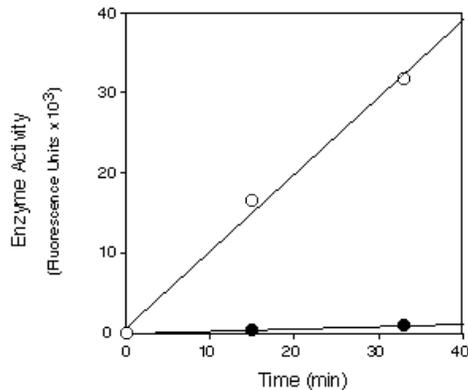
hexosaminidase A,  $\beta$ -hexosaminidase B, or  $\alpha$ -subunit. The complexes were precipitated with rabbit anti-goat IgG-agarose and the molecular mass of the precipitated protein estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antibodies to  $\alpha$ -hexosaminidase A and  $\beta$ -hexosaminidase B precipitated bands of equivalent size ( $63 \times 10^3$ ; Fig. 4), a relative molecular mass similar to that reported for  $\alpha$ -hexosaminidases (Bapat et al., 1988; Beccari et al., 1992; Mahuran, 1991; Neote et al., 1990). If both  $\alpha$ -hexosaminidase A and  $\beta$ -hexosaminidase B were present in sperm, one would expect antibodies to each isoform to immunoprecipitate two different proteins representing each isoform. This is due to the fact that the two different isoforms usually have different molecular masses and antibodies to each isoform cross react with the  $\alpha$ -subunit common to both forms. However, this was not the case, since only a  $63 \times 10^3 M_r$  band was specifically immunoprecipitated. More significantly, no specific radiolabeled band was immunoprecipitated using antibodies against  $\alpha$ -subunit (Fig. 4), even though these antibodies effectively immunoprecipitate the  $\alpha$ -subunit of hexosaminidase A in other cells



**Fig. 4.** Mouse sperm  $\alpha$ -hexosaminidase migrates as a  $63 \times 10^3 M_r$  protein following immunoprecipitation. Detergent extracts of mouse sperm were radioiodinated and incubated with goat antibodies to human  $\alpha$ -hexosaminidase A ( / ),  $\beta$ -hexosaminidase B ( / ) or  $\alpha$ -subunit. Immune complexes were precipitated with rabbit anti-goat IgG coupled to agarose and characterized by SDS-polyacrylamide electrophoresis using a 10% acrylamide gel. Molecular mass standards are indicated on the left ( $\times 10^{-3}$ ) and the arrow on the right indicates the specifically immunoprecipitated  $63 \times 10^3 M_r$  band. Bands of approx.  $66 \times 10^3 M_r$  and  $>250 \times 10^3 M_r$  were nonspecifically immunoprecipitated in all lanes. Results are representative of three experiments.

(Proia et al., 1984). Finally, if sperm were treated with calcium ionophore to stimulate acrosome reactions, there was a loss in the  $63 \times 10^3 M_r$  band that corresponded to the loss in  $\alpha$ -*N*-acetylglucosaminidase activity after the acrosome reaction (Fig. 1). These studies further suggest that the primary isoform found in sperm is  $\beta$ -hexosaminidase B, which has an apparent relative molecular mass of  $63 \times 10^3$ .

Definitive identification of the sperm  $\alpha$ -hexosaminidase isoform was obtained using a sulfated substrate that is cleaved specifically by the  $\alpha$ -subunit of  $\alpha$ -hexosaminidase A and, therefore, can be used to distinguish between  $\alpha$ -hexosaminidases A and B (Ben-Yoseph et al., 1985; Kytzia and Sandhoff, 1985). Detergent extracts of mouse sperm were incubated with 4-methylumbelliferyl-*D*-*N*-acetylglucosaminide or 4-methylumbelliferyl-*D*-*N*-acetylglucosaminide-6-sulfate. Although the unsulfated substrate, which is cleaved by both  $\alpha$ -hexosaminidases, was cleaved by the sperm enzyme, the sulfated substrate, which is cleaved by the  $\alpha$ -subunit found only in  $\alpha$ -hexosaminidase A, was not (Fig. 5). This confirms that mouse sperm contain only  $\beta$ -hexosaminidase B. The sperm enzyme was unusual



**Fig. 5.** Mouse sperm contain only the  $\alpha$ -hexosaminidase B isoform. Detergent extracts of washed mouse sperm were incubated with 4-methylumbelliferyl- $\alpha$ -D-*N*-acetylglucosaminide (open circles) or with 4-methylumbelliferyl- $\alpha$ -D-*N*-acetylglucosaminide-6-sulfate (closed circles). The latter substrate is cleaved only by  $\alpha$ -hexosaminidase A. The mouse enzyme hydrolyzed the unsulfated but not the sulfated substrate, demonstrating that the isoform found on mouse sperm was  $\alpha$ -hexosaminidase B. Results are representative of three experiments performed in triplicate.

since it was completely inactivated by heating at 52°C for 2 hours (data not shown), unlike the serum and somatic cell  $\alpha$ -hexosaminidase B (Kaback, 1972). It is noteworthy that other sperm enzymes have also been reported to be particularly heat-labile (Shur and Neely, 1988), which may relate to the lower temperature required for spermatogenesis.

#### Acrosomal $\beta$ -*N*-acetylglucosaminidase is required for zona pellucida penetration

Since  $\beta$ -*N*-acetylglucosaminidase is found at high levels in sperm acrosomes and is released during the acrosome reaction, and because it is capable of removing the GalTase binding site from the zona pellucida (Miller et al., 1992), it could be involved in sperm penetration of the zona pellucida. To test this possibility, we developed an assay to measure penetration of the zona pellucida by sperm. Since the endpoint is the number of sperm penetrating the zona pellucida, the most sensitive and quantitative assays would allow many sperm to penetrate the zona. However, after the fertilizing sperm activates the egg, the zona block to polyspermy ensues, preventing additional sperm from binding and penetrating the zona pellucida. Therefore, several methods to inactivate the block to polyspermy were tried on eggs and these eggs were incubated with sperm for 3.5-6.0 hours in zona penetration assays.

The highest rates of penetration were achieved using eggs that had been heated at 55°C for 45-90 minutes to inactivate the cortical granule enzymes responsible for the zona block to polyspermy (Table 1; Fig. 6). This treatment did not affect initial sperm binding, since sperm bound to eggs at normal levels (50-75 sperm/egg), in agreement with previous work demonstrating that the sperm binding activity of the zona pellucida is heat stable up to approx. 80°C at which point the zona solubilizes (Bleil and Wassarman, 1980; Shur and Hall, 1982). Penetration rates did not differ if sperm were allowed to penetrate the zona for 3.5-6.0 hours. The tem-

**Table 1. Methods of treating eggs for zona pellucida penetration assays**

Methods	Number of sperm penetrating zona pellucida/egg ( $\pm$ s.e.m.)
Normal unfertilized eggs*	2.47 $\pm$ 0.39
Heated eggs (55°C, 45 minutes)†	49.25 $\pm$ 6.51
Heated eggs (55°C, 90 minutes)‡	69.20 $\pm$ 5.96
Heated eggs (60°C, 60 minutes)§	2.20 $\pm$ 0.35
Salt-stored eggs¶	0.00 $\pm$ 0.01
Two-cell embryos**	0.43 $\pm$ 0.12

Several methods of treating eggs to inactivate the block to polyspermy were attempted. After treatment of eggs, capacitated sperm were added to eggs for 10 minutes and then unbound sperm were removed and bound sperm were allowed to penetrate the zona pellucida for 4 hours. Experiments were done in duplicate or triplicate using approximately 40 eggs per droplet.

\*Unfertilized eggs were collected and incubated with  $1.0 \times 10^6$  sperm/ml.

†Eggs were collected, heated 45 minutes at 55°C and incubated with  $2.0 \times 10^6$  sperm/ml.

‡Eggs were collected, heated 90 minutes at 55°C and incubated with  $2.0 \times 10^6$  sperm/ml.

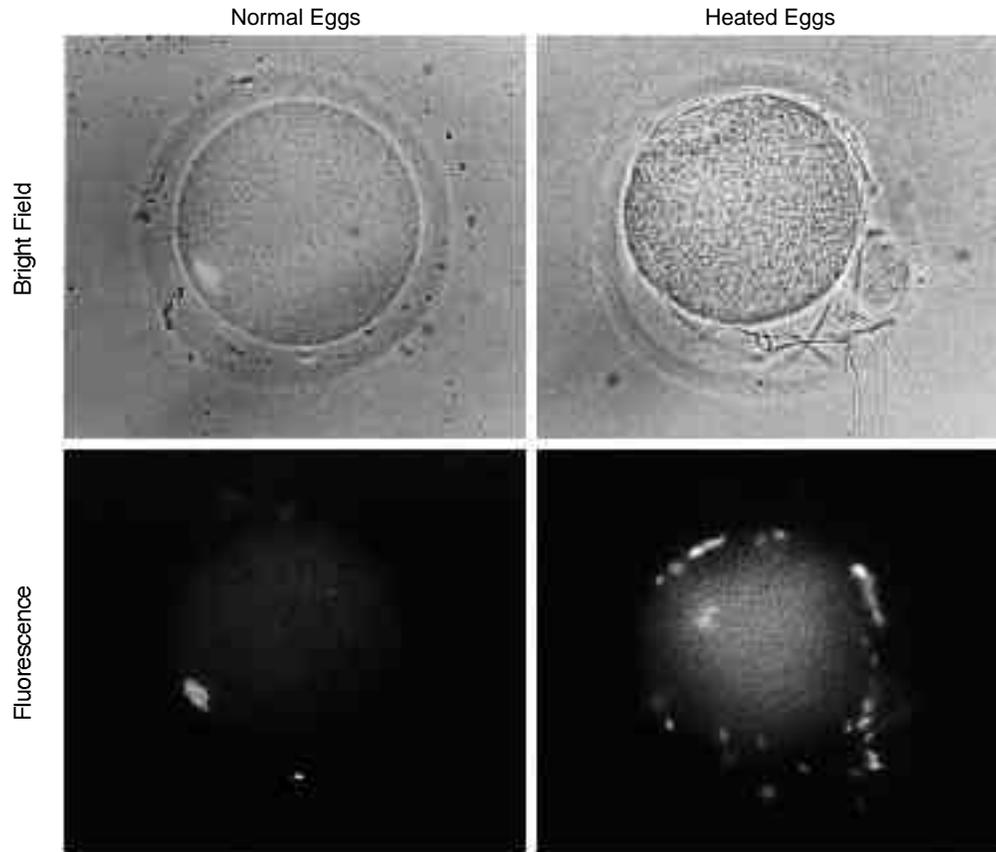
§Eggs were collected, heated 60 minutes at 60°C and incubated with  $1.0 \times 10^6$  sperm/ml.

¶Eggs were collected, stored in high salt and incubated with  $1.0 \times 10^6$  sperm/ml.

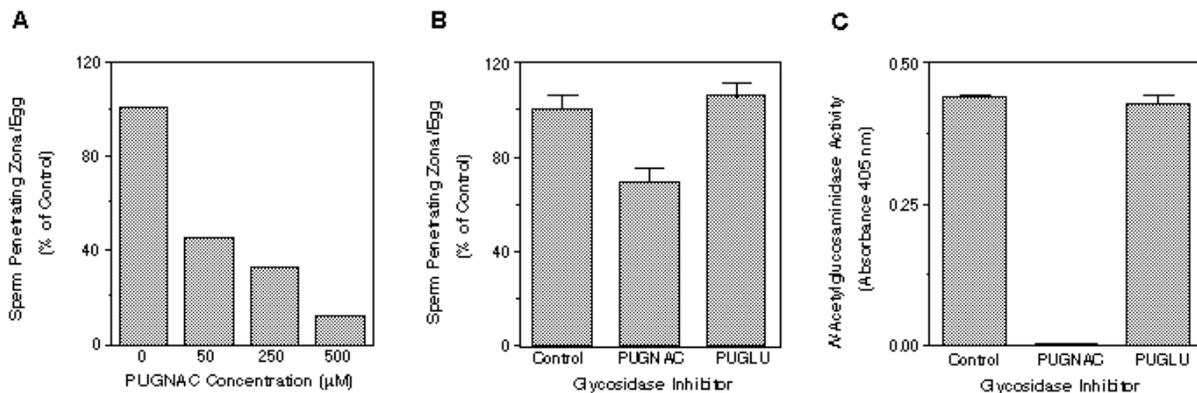
\*\*Fertilized eggs were flushed from oviducts of mated females.

perature of heat-inactivation was found to be critical, since raising the temperature to 60°C reduced penetration rates, likely due to heat-induced structural changes in the zona pellucida that prevented penetration (Table 1). Storing eggs in high salt also produced low penetration rates (Table 1), unlike that described previously for hamster eggs (Boatman et al., 1988). In all cases penetration of two-cell fertilized eggs was low (<1 sperm/egg).

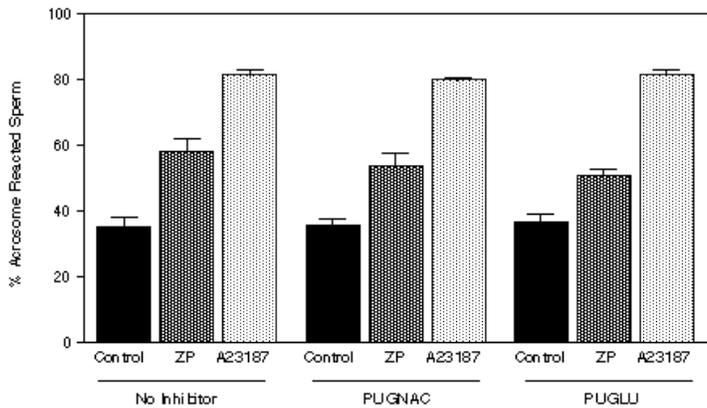
To determine the function of  $\beta$ -*N*-acetylglucosaminidase during zona penetration, the specific competitive inhibitor, PUGNAC, was used. Preliminary experiments indicated that 10  $\mu$ M PUGNAC inhibited over 90% of sperm  $\beta$ -*N*-acetylglucosaminidase activity, but when sperm were combined with eggs in penetration assays, 20-fold more PUGNAC was required to achieve this same degree of inhibition of  $\beta$ -*N*-acetylglucosaminidase activity, possibly because the inhibitor was adsorbed by eggs, mineral oil, or plastic surfaces ( $ID_{50}$  for sperm enzyme = 1  $\mu$ M when assayed using fresh medium;  $ID_{50}$  for sperm enzyme = 20  $\mu$ M when assayed using medium from sperm-egg coin incubations). Therefore, PUGNAC was added to eggs with bound sperm at concentrations required to inhibit sperm enzyme activity and 4 hours were allowed for sperm penetration of the zona. PUGNAC inhibited zona penetration in a dose-dependent manner; 500  $\mu$ M PUGNAC (effective concentration = 25  $\mu$ M) reduced zona penetration by nearly 90% (Fig. 7A). Similar concentrations of PUGNAC are also required to inhibit ascidian fertilization (Godknecht and Honegger, 1991). To verify that  $\beta$ -*N*-acetylglucosaminidase was inhibited, aliquots of sperm-egg coin incubation medium that contained sperm were removed and assayed for  $\beta$ -*N*-acetylglucosaminidase activity. Concentrations of PUGNAC greater than 250  $\mu$ M (effective concentration = 12.5  $\mu$ M) inhibited  $\beta$ -*N*-acetylglucosaminidase activity by 99% (Fig. 7C).



**Fig. 6.** Heat-inactivation of egg cortical granule enzymes allows eggs to be penetrated by many sperm. Unfertilized eggs were heated for 90 minutes at 55°C to inactivate the block to polyspermy and were incubated with capacitated sperm for 10 minutes to allow binding to occur. Unbound sperm were removed and eggs were incubated 4 hours to allow bound sperm to penetrate through the zona pellucida. Sperm on the outside of the zona were removed using a micropipette and sperm inside the zona were stained with Hoescht 33342 and counted. In the left panel is an untreated egg showing one penetrating sperm at the bottom of the egg. Egg chomatin is also stained, as seen in the lower left portion of the egg. In the right panel is a heat-inactivated egg with approx. 30 penetrating sperm.



**Fig. 7.** Specific inhibition of  $\alpha$ -*N*-acetylglucosaminidase activity reduces sperm penetration of the zona pellucida. (A) Sperm were capacitated and incubated with heat-inactivated eggs for 10 minutes. After binding, unbound sperm were diluted out with medium and several concentrations of the competitive  $\alpha$ -*N*-acetylglucosaminidase inhibitor PUGNAC was added. Following 4 hours to allow sperm to penetrate eggs, sperm on the outside of the zona were removed and sperm inside the zona were counted. Assays were performed on 40 eggs per droplet in duplicate droplets. The range between duplicate assays was 10-15%. (B) To verify that the inhibition by PUGNAC was due to its effect on  $\alpha$ -*N*-acetylglucosaminidase, equal concentrations (250 μM) of PUGNAC and the control PUGLU (a  $\beta$ -glucosidase inhibitor) were tested in zona penetration assays. Although PUGNAC inhibited zona penetration, PUGLU had no effect. Results are averages of three experiments performed in triplicate droplets using 40 eggs per droplet. Bars indicate standard errors. (C) Samples of medium containing sperm from sperm-eggs droplets were removed and *p*-nitrophenyl *N*-acetylglucosaminide was added to verify that  $\alpha$ -*N*-acetylglucosaminidase activity was inhibited. These assays indicated that 250 μM PUGNAC (12.5 μM effective concentration) inhibited  $\alpha$ -*N*-acetylglucosaminidase activity in zona penetration assays, whereas equal concentrations of PUGLU did not. Bars indicate standard errors.



**Fig. 8.** Glycosidase inhibitors do not affect the zona pellucida- or ionophore-induced acrosome reaction. To determine whether inhibition of zona penetration by PUGNAC was due to inhibition of the zona-induced acrosome reaction, sperm were capacitated and incubated with 250  $\mu$ M PUGNAC and PUGLU. To induce acrosome reactions, solubilized zonae pellucidae glycoproteins or A23187 were added. After 60 minutes, sperm were fixed and stained with Coomassie Blue G-250 to detect acrosomes. Results are averages of two experiments performed in triplicate; 100 sperm were scored in each triplicate. Bars indicate standard errors.

To determine if the inhibition of zona penetration by PUGNAC was specifically due to its inhibition of  $\alpha$ -*N*-acetylglucosaminidase, a control using PUGLU, a chemically related  $\beta$ -glucosidase inhibitor, was tested. PUGLU and PUGNAC are prepared similarly and would be expected to contain the same impurities, if any (Beer and Vasella, 1985). As expected, PUGLU inhibited  $\beta$ -glucosidase activity (data not shown) but not  $\alpha$ -*N*-acetylglucosaminidase (Fig. 7C). Although 250  $\mu$ M PUGNAC inhibited zona penetration, equivalent concentrations of PUGLU did not (Fig. 7B).

Sperm penetration through the zona pellucida could conceivably be impeded by inhibition of sperm motility or the zona-induced acrosome reaction. However, sperm incubated with PUGNAC and PUGLU had motility that was indistinguishable from controls at all concentrations tested (10–500  $\mu$ M). To verify that the acrosome reaction proceeded normally in the presence of PUGNAC and PUGLU, sperm were capacitated and incubated with solubilized zona pellucida glycoproteins or with calcium ionophore. Neither PUGNAC nor PUGLU affected the induction of the acrosome reaction by zona glycoproteins or ionophore (Fig. 8). Therefore, the ability of PUGNAC to inhibit zona penetration was due to its inhibition of  $\alpha$ -*N*-acetylglucosaminidase and was not due to inhibition of sperm motility or the acrosome reaction.

## DISCUSSION

Although the acrosome is thought to contain a variety of enzymes, the function of these enzymes is not understood, with the possible exception of acrosin (Anakwe et al., 1991; Urch et al., 1985; Kashiwabara et al., 1990; de Vries et al., 1985; Liu and Baker, 1993; Llanos et al., 1993). Studies presented here investigated the presence and function of acrosomal glycosidases capable of removing the zona pellucida binding site for sperm GalTase.  $\alpha$ -*N*-acetylglucosaminidase activity was found in sperm acrosomes at very high levels, at least 20-fold higher than other glycosidases. The isozyme found in sperm was  $\beta$ -hexosaminidase B, the / homodimer. The enzyme was released during the acrosome reaction, at which time it facilitates sperm penetration through the zona matrix, as shown by the ability of PUGNAC to reduce zona penetration by competitively inhibiting acrosomal  $\alpha$ -*N*-acetylglucosaminidase activity.

It is unclear why zona penetration was somewhat less sensitive to PUGNAC concentration than was sperm  $\alpha$ -*N*-acetylglucosaminidase activity, in that concentrations of PUGNAC that inhibited enzyme activity towards *p*-nitrophenyl substrates by 99% (250  $\mu$ M) only inhibited penetration by approx. 70%. This may simply result from a higher affinity of the acrosomal glycosidase for zona pellucida glycoprotein substrates, relative to low molecular mass *p*-nitrophenyl substrates, such that more PUGNAC would be required to inhibit activity towards glycoproteins. Furthermore, even under conditions when  $\alpha$ -*N*-acetylglucosaminidase is completely inhibited by PUGNAC, acrosin activity may still enable partial zona penetration.

In any event, it is clear from these results that acrosomal  $\alpha$ -*N*-acetylglucosaminidase is required for efficient penetration through the zona pellucida. It is less clear, however, how acrosomal  $\alpha$ -*N*-acetylglucosaminidase activity affects the ability of sperm GalTase to interact with zona glycoprotein substrates. The most attractive hypothesis is that acrosomal  $\alpha$ -*N*-acetylglucosaminidase facilitates zona penetration by removing or masking *N*-acetylglucosamine residues on zona glycoproteins that could potentially interact with GalTase on acrosome-reacted sperm, and therefore impede zona penetration. However, earlier studies showed that after the acrosome reaction, GalTase's affinity for soluble zona glycoprotein substrates is reduced, although sperm GalTase is still active towards non-zona glycoproteins (Miller et al., 1992). Since sperm retain GalTase during the acrosome reaction, rather than release it with its associated plasma membrane, it is probable that GalTase serves some function during or after the acrosome reaction (Lopez and Shur, 1987). The most likely scenario at this time is that GalTase binds ZP3 initiating primary binding, which leads to aggregation of GalTase and induction of the acrosome reaction (Macek et al., 1991). During the acrosome reaction, the sperm remains associated with the zona matrix by redistributing GalTase to the lateral sperm surface, still complexed with ZP3 oligosaccharides. However, this transient binding is of such short duration and/or low affinity that it cannot be assayed using soluble zona glycoproteins (Miller et al., 1992) or allow acrosome-reacted sperm to initiate binding to the zona (Wassarman, 1988). Thus, the acrosomal contents are exposed without releasing the sperm from the zona surface. As a result, the fully acrosome-reacted sperm is adherent to the zona by its lateral surface,

where it engages in secondary binding to other zona glycoproteins using previously identified sperm components (Jones et al., 1988; Lathrop et al., 1990). At this point, zona penetration begins and acrosomal *N*-acetylglucosaminidase may be necessary to prevent GalTase from reassociating with exposed *N*-acetylglucosamine residues, which would impede either the initiation or progression of zona penetration.

Acrosomal *N*-acetylglucosaminidase may function catalytically during zona penetration to remove terminal *N*-acetylglucosamine residues, or rather, may function in a lectin-like fashion to mask terminal *N*-acetylglucosamine residues, thereby blocking GalTase from binding zona glycoproteins (Goldstein et al., 1980). Like most lysosomal-type enzymes, acrosomal *N*-acetylglucosaminidase has high activity at acidic pH but low activity at pH 7.4 when assayed using *p*-nitrophenyl substrates (Farooqui and Srivastava, 1980; Majumber and Turkington, 1974; and data not shown). Because the zona penetration assays were performed at neutral pH, the *N*-acetylglucosaminidase could be simply binding and masking terminal *N*-acetylglucosamine residues. However, since the acrosome is an acidic vesicle, the pH of the microenvironment surrounding the released acrosome may be sufficiently acidic to allow catalytic activity and subsequent removal of terminal *N*-acetylglucosamine residues from zona glycoproteins. If a portion of *N*-acetylglucosaminidase is retained on acrosome-reacted sperm, as are some other acrosomal proteins (Kopečný and Flechon, 1981), it may act catalytically throughout sperm penetration of the zona, preventing GalTase from interacting with exposed *N*-acetylglucosamine residues.

It is not likely, however, that acrosomal *N*-acetylglucosaminidase activity contributes to dissolution of the zona matrix, as acrosin may, since treatment of intact zona with *N*-acetylglucosaminidase alone does not affect overall zona integrity (Farooqui and Srivastava, 1980). Furthermore, if *N*-acetylglucosaminidase functioned in digesting the zona matrix, other glycosidases would probably also be involved, since the zona has several different terminal monosaccharides (Mori et al., 1991). However, as shown here, *N*-acetylglucosaminidase activity is much higher than other glycosidases assayed.

It is probably not coincidental that the acrosome contains very high concentrations of *N*-acetylglucosaminidase and that the zona pellucida contains glycoproteins that require terminal *N*-acetylglucosamine residues for sperm receptor activity (Miller et al., 1992). These two observations emphasize the importance of this particular terminal monosaccharide for zona function and the apparent requirement to regulate tightly its availability. Egg coat glycoproteins with terminal *N*-acetylglucosamine residues are important in binding sperm in other species as well. In animals ranging from *Xenopus laevis* to ascidians, *N*-acetylglucosaminidase has been proposed to function in the block to polyspermy, since it is released by eggs at activation and inactivates sperm receptors on eggs (Lambert, 1989; Prody et al., 1985).

Traditionally, glycosidases are thought of as intracellular enzymes that degrade oligosaccharides on glycoconjugates destined for the endocytic/lysosomal pathway. However, it

has been suggested previously that glycosidases can also be secreted extracellularly, where they may facilitate tumor cell metastasis in concert with protease activities (Liotta et al., 1986). In an analogous fashion, we show in this study that acrosomal *N*-acetylglucosaminidase is released after initial sperm-egg binding and facilitates sperm penetration through the zona pellucida matrix, presumably by removing the binding site for a sperm adhesion molecule on the egg coat.

The authors are indebted to Dr. Richard L. Proia (National Institutes of Health) for providing antisera to  $\alpha$ -hexosaminidase A,  $\alpha$ -hexosaminidase B and  $\beta$ -subunit as well as for helpful discussions and to members of our laboratory for critical review of the manuscript. This work was supported by research grant HD23479 to B. D. S., by NRSA HD07418 to D. J. M. and by Cancer Center Support grant CA16672 from the National Institutes of Health.

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