Purification and Biological Characterization of *N*-Acetyl β-D Glucosaminidase From *Bufo arenarum Spermatozoa*

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ABSTRACT Fertilization in *Bufo arenarum* requires the sperm to penetrate the egg envelopes. The incubation of isolated vitelline envelopes with sperm induces the acrosome reaction, releasing proteases and glycosidases to the media. In the present work *N*-acetyl-β-D-glucosaminidase, β-D-galactosidase, β-D-glucosidase, α-D-mannosidase, α-L-fucosidase, and α-D-glucosidase activities are measured in spermatozoa. *N*-acetyl-β-D-glucosaminidase is the major sperm glycosidase activity assayed. However, *N*-acetyl-β-D-galactosamine show competitive inhibitory effect. The glycosidase pH optimum is 3.5 being inhibited at pHs higher than 7.5. In our study, *N*-acetyl-β-D-glucosaminidase is the only glycosidase that in vitro binds to vitelline envelopes in conditions that resemble natural fertilization media. The isolation of the active enzyme will allow studies of its role in fertilization. The enzyme has been purified in a two-step procedure. After native gel electrophoresis, the activity-stained band was cut out and the eluted enzyme was finally subjected to ConA–sepharose chromatography. In SDS-PAGE, the denatured enzyme migrates as a single band with a molecular mass of 45 kDa. Furthermore, analysis by size-exclusion on HPLC showed a peak of activity at around 45 kDa. Preliminary localization studies showed higher relative activity in the acrosomal content. In addition, 10% of the *N*-acetyl-β-D-glucosaminidase activity was associated with the reacted sperm. By in vitro fertilization assay, it was observed that the inhibition of the enzyme results in the inhibition of fertilization. This last study shows that *N*-acetyl-β-D-glucosaminidase plays an important role in toad fertilization. *Mol. Reprod. Dev. 57:194–203, 2000.* © 2000 Wiley-Liss, Inc.

Key Words: glycosidase; sperm; fertilization jamphibians

INTRODUCTION

Gamete interactions include recognition and sperm penetration through oocyte envelopes. Furthermore, several observations suggest that binding is not the result of a single but of multiple molecular interactions (Litscher and Wassarman, 1993; Foltz, 1995; Snell and White, 1996). It is generally accepted that sperm glycosidases and proteases play relevant roles in sperm–egg coat binding and penetration in different species. Sperm–egg binding induces the acrosome reaction, after which hydrolytic enzymes are released allowing sperm penetration through the egg coats. Among these enzymes, proteases are one of the best characterized (Hoshi et al,. 1981; Iwao and Katagiri, 1982; Cabada et al., 1989; Tesarik et al., 1990).

Certain acrosome glycosidases might be related to sperm penetration in guinea pig and mouse (Miller et al., 1993; Hunnicutt et al., 1996). In sea urchins, sperm glycosidases hydrolyze the glycosidic bonds in the oocyte jelly network (Isaka and Ikemori, 1980). Several lines of evidence suggest that carbohydrate moieties, present on oocyte or sperm surfaces, take part in cellular recognition. In this interaction the presence of enzymes in sperm membranes could be related to recognition phenomena. Among mammals, glycosyltransferases (Miller et al., 1992; Shur et al., 1998) and glycosidases (Tulsiani et al., 1989; Cornwall et al., 1991; Brandelli et al., 1994) have been involved in noncatalytic carbohydrate-binding activities. Sperm surface α-fucosidase (Hoshi, 1986; Hoshi et al., 1994) and *N*-acetyl glucosaminidase mediate sperm–egg binding in ascidians (Godknecht and Honegger, 1991, 1995).

In Anuran species, the sperm have to penetrate the jelly and the vitelline envelopes (hereafter VE) (Katagiri 1987, Hedrick and Nishihara, 1991) to reach the oocyte. Both are composed mainly of glycoproteins (Wolf et al., 1976; Cabada et al., 1978; Arranz et al., 1997). The sperm could penetrate the jelly coat without the lytic activity of the acrosomal proteins (Cabada et al., 1989). Furthermore, in vitro assays showed that a difusible jelly coat protein of *Bufo arenarum* protects the sperm and prevents the acrosome reaction (Arranz and Cabada, 2000). By optical microscopy, it was observed that *Leptodactylus chaquensis* (Raisman and Cabada, 1977) and *Bufo japonicus* (Omata and

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Katagiri, 1996) spermatozoa reach the surface of VE with its acrosome intact. In *Bufo*, the acrosome reaction releases proteases (Raisman et al., 1980) and glycosidases in the media (Shyhara and Seshadri, 1985; Juan and Cabada, unpublished data). The acrosomal proteases are necessary for the sperm to penetrate the VE (Iwao and Katagiri, 1982; Cabada et al., 1989).

On the other hand, in *Xenopus laevis* the block of polyspermy involves the activity of *N*-acetyl glucosaminidase from the cortical granules (Prody et al., 1985). The enzyme could alter the sperm ligand on vitelline envelopes preventing sperm binding. (Hedrick, personal communication).

The aim of this study is to analyze the glycosidases present in *Bufo arenarum* spermatozoa. Six glycosidase activities were detected and *N*-acetyl glucosaminidase was the highest activity measured in sperm. Biological characterization studies and purification of this enzyme were carried out. *N*-acetyl glucosaminidase shows binding to VE immobilized on sepharose, suggesting a potential role in the fertilization process. In addition, the inhibition of the enzyme results in inhibition of fertilization.

MATERIALS AND METHODS

Chemicals

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): protease inhibitors, *p*-nitrophenyl-glycosides, 4-methyl-umbelliferyl-*N*-acetyl glucosamine, cyanogen bromide–activated sepharose CL 4B, phenyl sepharose, Concanavalin A–sepharose, anti-rabbit IgG (whole molecule)–peroxidase conjugate, complete and incomplete Freund's adjuvant, SDS–molecular mass markers and silver satin kit. Nitrocellulose membranes and molecular mass markers were from Bio-Rad (Hercules, CA).

Animals

Bufo arenarum specimens were collected in the neighborhoods of Rosario city, and kept in a moist chamber at 15°C until used.

Spermatozoa Isolation

Spermatozoa were obtained by dislacerating testes in Ringer solution with 10 mM Tris buffer pH 7.6 (hereafter Ringer-Tris) at 4°C. The homogenate was filtered through gauze and the suspension was then centrifuged at 4°C for 10 min at 130 *g* to remove red blood cells and tissue debris. The supernatant was centrifuged at 4°C for 10 min at 650 *g* and the sperm pellet was washed by gentle suspension in Ringer-Tris and centrifuged as above. Sperm pellet was then suspended in the selected buffer as described for each experiment.

Crude Sperm Extract Preparation

The sperm pellet, obtained as described above, were freeze-dried and resuspended in distilled water (Cabada et al., 1978) to induce acrosome breakdown. Triton X-100 and EDTA were added to final concentration of 0.1% and 0.5 mM, respectively. The suspension was centrifuged at 45,000 *g* for 20 min at 4°C. The resulting supernatant was named *crude sperm extract*.

Subcellular Fractionation of Spermatozoa

Spermatozoa, isolated as described above, were subjected to acrosome breakdown by incubating in an hypotonic solution, 10% Ringer-Tris solution (Raisman et al., 1980) for 30 min. Then, the suspension was centrifuged for 10 min at 650 *g*. The resulting pellet, enriched in acrosome-reacted sperm (hereafter *reacted sperm*), was placed on ice and the following procedure was performed at 4°C (see Fig. 7A).

The supernatant was centrifuged in a Beckman Instruments Centrifuge at 105,000 *g* for 1 hr. The resulting supernatant was rich in soluble acrosomal proteins and the pellet contained the crude membrane fraction. This pellet was then incubated with 1% Triton X-100 for 30 min to solubilize the membrane proteins.

The reacted sperm was submitted to successive washings and the resulting supernatants were removed by aspiration and saved. The last pellet was incubated in 10% Ringer-Tris with 0.5 M NaCl for 30 min. After centrifugation at 650 *g* for 10 min, the supernatant was enriched in extrinsic proteins. The pellet was treated with 1% Triton X-100 for 30 min, the nonsoluble fraction was separated by centrifugation for 10 min at 12,000 *g*.

Enzyme Assays

N-acetyl glucosaminidase (hereafter NAcGlcase), β-D-Galactosidase (Galase), β-D-glucosidase (β-Glcase), α-D-mannosidase (Manase), α-L-fucosidase (Fucase) and α-D-glucosidase (α-Glcase) activities were assayed using the corresponding *p*-nitrophenyl-glycoside as substrate and measuring the amount of *p*-nitrophenol released. The assay volume was 0.25 ml with final concentrations of 3 mM of the corresponding sugar and 10 mM citrate buffer at pH 3.6 or at the specified pH. The enzyme was added and after incubating for 30 min at 30°C, the reaction was stopped by addition of 0.5 ml of 0.2 M glycine buffer pH 10.4. The released *p*-nitrophenol was determined spectrophotometrically at 400 nm. One unit is defined as the amount of the enzyme that catalyzes the release of 1 μmol of *p*-nitrophenol per hour under the given conditions.

In native gels, the NAcGlcase activity was detected with the substrate 4-methyl-umbelliferyl-*N*-acetylGlucosamine (4-MU-NAcGlc). After electrophoresis, the gel was incubated in 400 mM citrate buffer pH 3.6 for 15 min, the buffer was washed away and then 5 mM of the substrate in 100 mM citrate buffer pH 3.6 was added. The enzymatically active bands were fluorescent under UV illumination (366 nm).

VE Preparation

Oocytes were collected from ovisacs of hormone-stimulated females and dejellied with 1% thioglycolic acid solution (Valz-Gianinet et al., 1991), washed with Ringer-Tris (calcium free) and homogenized in a Potter-

Blumm homogenizer. VE were obtained by filtering the homogenate through a double sheet of 30-mesh nylon screen, and washed several times in cold Ringer-Tris (calcium free). The whole procedure was carried out at 4°C.

VE in 10% Ringer-Tris were solubilized in a water bath at 60°C for about 10 min (Valz-Gianinet et al., 1991) and centrifuged at 3000*g*. The resulting supernatant was stored at −70°C until used.

VE–Sepharose Affinity Chromatography

Solubilized VE, prepared as described above, was dialyzed against 0.1 M $NaHCO_3$ pH 8.3 overnight. VE solution (5 mg/ml total protein in 0.1 M $NaHCO_3$ pH 8.3) was coupled to cyanogen bromide-activated sepharose CL 4B following the manufacturer's instructions. The affinity column (2.5 ml bed volume) was stored at 4°C.

Crude sperm extract (containing 1 mg/ml protein), was applied to VE–sepharose affinity column equilibrated with 10% Ringer-Tris. The column was washed with 20 vol of the same buffer. Bound proteins were eluted with a discontinuous gradient of 0.5, 0.9, 1.5, and 2.0 M NaCl. Fractions of 0.5 ml were collected, dialyzed overnight against 5 mM Tris pH 7.5 and concentrated tenfold by freeze-drying.

Partial Purification of NAcGlcase

Ammonium sulfate was added up to 1 M to the crude sperm extract. After centrifuging, the supernatant was applied to a phenyl–sepharose column previously equilibrated with 1 M ammonium sulfate in 10% Ringer-Tris. The column was washed with a three-step gradient of ammonium sulfate: 1 M in 10% Ringer, 10 mM Tris pH 7.2, and 0.5 M and 50 mM in the same buffer. The bound enzyme was eluted with 40 and 60% ethyleneglycol in the above buffer. These fractions were dialyzed, freeze-dried, and applied to a ConA-sepharose column (20 ml bed volume) equilibrated with ConA buffer (10 mM acetate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1 M NaCl, pH 6.0). After washing with 10 vol of ConA buffer, elution was carried out with 0.5 M methyl-α-D-manopyranoside and 0.5 M NaCl in ConA buffer. Fractions with enzyme activity were dialyzed overnight against 5 mM Tris buffer pH 7.5 and concentrated tenfold by freeze-drying. Aliquots of the different steps of purification with NAcGlcase activity were analyzed by SDS-PAGE.

High-Performance Liquid Chromatography

The partially purified sperm enzyme in 0.2 M phosphate buffer pH 7.0 containing 0.1% Triton X-100 was applied on size-exclusion HPLC. BioSil Sec column (Bio-Rad) was equilibrated with the same buffer at 1 ml/min flow rate during 2 hr. After sample injection, the elution, at a flow rate of 0.5 ml/min, was monitored by measuring the absorbance at 280 nm. To assay for enzyme activity, 1 M citric acid was added up to a final pH 4 to each collected fraction (0.5 ml). The molecular weight was determined by using molecular mass markers: Tyroglobulin (669 kDa), bovine gammaglobulin (158 kDa), chicken ovoalbumin (44 kDa), bovine myoglobin (17 kDa), and cyanocobalamin (1.37 kDa).

NAcGlcase Antibodies Preparation

NAcGlcase from jack bean (Sigma) was injected into rabbit to obtain antiserum. Rabbit was injected with an antigen solution containing 90 μg of protein. The immunization protocol were carried out according to Harlow and Lane (1988a). Preimmune serum was obtained from the same animal before the first injection.

Purification NAcGlcase From Toad Liver

Five toad livers were homogenized in an Omnimixer with Ringer-Tris containing protease inhibitors (pepstatin, leupeptin, aprotinin) at a final concentration of 1 μg/ml, 1 mM PMSF, and 5 mM EDTA. The homogenate was filtered through gauze and the suspension was then centrifuged at 4°C for 1 hr at 45,000*g*. Proteins in the supernatant were separated on a 10% polyacrylamide native gel. After electrophoresis the activity was detected, as described above, with the substrate 4-MU-NAcGlc under UV. The active fluorescent bands were cut out, transferred to 400 mM citrate buffer pH 4.0, and incubated overnight at 4°C. The sample was centrifuged for 10 min. at 5000*g*. The supernatant was dialyzed, freeze-dried, and applied to a ConA–sepharose column. The chromatography was performed as described above (partial purification). The eluted fractions with detectable enzyme activity were dialyzed overnight against 5 mM Tris buffer pH 7.5 and concentrated tenfold by freeze-drying. The resulting fractions were pooled and analyzed by native and SDS–polyacrylamide gel electrophoresis.

Electrophoresis, Electrotransfer, and Western Blot Analysis

SDS-PAGE was performed on 7–12% polyacrylamide gradient gels or 10% gels according to Laemmli (1970). Native electrophoresis was carried out on 8 or 10% polyacrylamide gel according to Davis (1964). In western blot analysis, electrophoretically separated proteins were transferred to nitrocellulose membranes by the method of Towbin et al. (1979). Immunoreactive proteins were identified using anti-NAcGlcase (1:500 dilution) as a primary antibody or preimmune serum, followed by horseradish peroxidase-labeled goat anti rabbit-IgG (1:1500 dilution) as described by Harlow and Lane (1988b). Peroxidase activity was detected by incubating the membrane in DAB solution (100 μl of 20 mg/ml diaminobenzidine in 0.5 N HCl, 7.5 μl of 30% H_2O_2 in 20 ml final volume of 100 mM Tris buffer pH 7.2).

Jelly Water Preparation

Jelly water was obtained as described (Diaz Fontdevila, 1991). Briefly, strings of *Bufo arenarum* oocytes were removed from the ovisacs and incubated three times in distilled water, each time using a volume equal

to the weight of strings in grams. The media were recovered from the dish, pooled together, and stored at −20°C.

Fertilization Assay

The role of the enzyme was estimated by an *in vitro* fertilization assay. Batches of 40–50 dejellied oocytes in a mixture (1:1) of jelly water and

1. 10% Ringer-Tris (control), or
2. N-acetyl-β-D-Glucosamine (NAcGlc), or N-acetyl-β-D-galactosamine (NAcGal), or α-D-mannose in 10% Ringer-Tris to achieve a final concentration of 0.1 M, or
3. anti-NAcGlcase antibodies or preimmune serum (1:100 dilution) 10% Ringer-Tris, or
4. 0.12 μg of NAcGlcase 10% Ringer-Tris

were inseminated with 25 μl of sperm suspension in 10% Ringer-Tris at a final concentration of 10^6 cells/ml. After 25 min, batches were washed with 10% Ringer-Tris. In all cases the sperm motility was checked by optical microscopy. Oocytes were considered fertilized when subsequent cellular cleavages were normal.

Protein Assays

Protein concentrations were determined according to Lowry and co-workers (1951), using bovine serum albumin as standard.

RESULTS

Glycosidase Activities in the Crude Extract

The activity of several glycosidases was assayed in the crude sperm extract (Table 1). NAcGlcase gave the highest activity tested toward its *p*-nitrophenyl-substrate. It was about fivefold higher than Galase, β-Glcase, and Manase. NAcGalase showed a lower activity (50% of the NAcGlcase activity). Low levels of α-Glcase and Fucase activities were also detected (about 20-fold lower than NAcGlcase).

Affinity Chromatography Using Isolated Vitelline Envelopes

To identify possible sperm proteins involved in sperm–egg interactions, we used solubilized VE bound to sepharose as an affinity matrix. The crude sperm extract was applied to the column and negligible quantities of sperm proteins were retained. NAcGlcase,

TABLE 1. Different Glycosidases Activities (Expressed as U/mg) Were Measured in the Crude Sperm Extract as Described in Materials and Methods

Glycosidase	Activity (U/mg)
N-acetyl glucosaminidase	2.00
β-Galactosidase	0.47
β-Glucosidase	0.36
α-Mannosidase	0.35
α-Fucosidase	0.12
α-Glucosidase	0.10

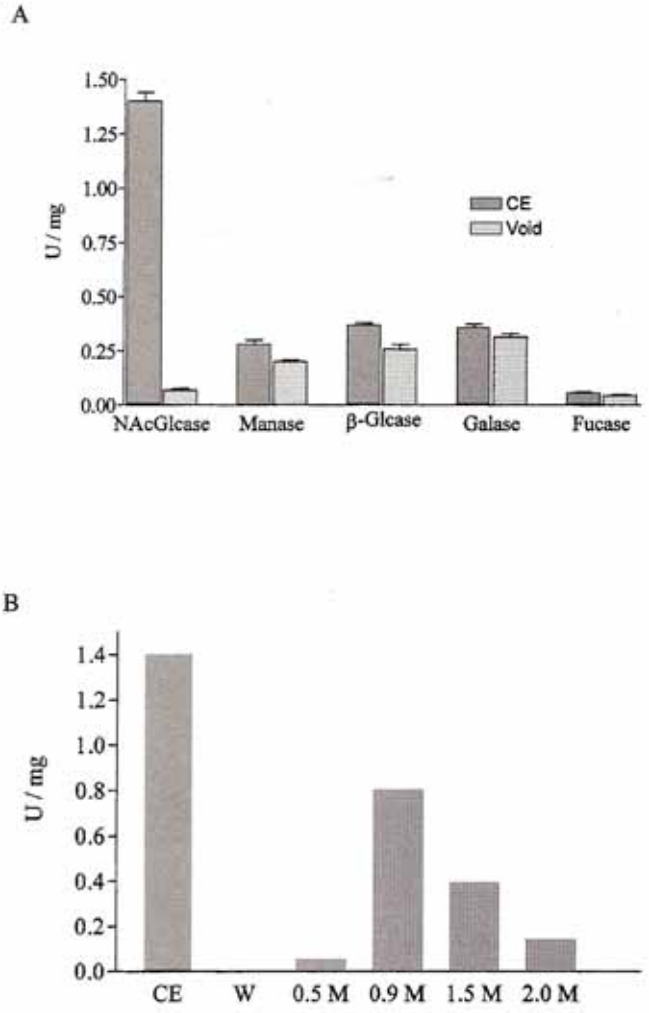

Fig. 1. Glycosidase activities after VE-Sepharose affinity chromatography. **A.** 1 mg of crude sperm extract (CE) was incubated with VE covalently bound to sepharose as described in the Materials and Methods. The enzyme activities (expressed as U/mg) of acrosomal glycosidases were measured in the CE and fractions unbound to the matrix (void). Error bars represent the standard error of the mean (SEM) of four independent experiments. **B.** After washing the column (W corresponds to wash fraction), bound proteins were eluted with discontinuous gradients of NaCl solutions (0.5 M, 0.9 M, 1.5 M and 2 M, respectively). NAcGlcase activity (expressed as U/mg) was measured in each fraction as described in Materials and Methods.

Galase, Manase and Fucase activities were measured in the crude extract and void fractions. NAcGlcase activity was found to be highly diminished in the void fraction. No variation was observed for the other glycosidase activities (Fig. 1A). Bound proteins were eluted with a discontinuous gradient of NaCl, and NAcGlcase activity was detected between 0.9 and 1.5 M NaCl (Fig. 1B). Similar results were obtained when the same matrix was reused in at least five successive chromatographies. Therefore, we can assume that NAcGlcase ligand remains bound to the matrix. These data show that the enzyme interacts with VE in the experimental conditions, showing, however, no

catalytic activity toward the sepharose-bound VE glycoproteins.

NAcGlcase Properties

Partial purification. The purification procedure was carried out starting with 8 mg of total protein in the crude sperm extract. The extraction was improved by the addition of 0.1% of Triton which resulted in the solubilization of most in the pelleted sperm suspension. NAcGlcase was bound to a ConA–sepharose column, showing that it was mannosylated, as described for most lysosomal enzymes. In addition, it was also strongly bound to a phenyl–sepharose column as up to 60% ethylenglicol must be added for complete elution of the enzyme. This result suggests that sperm NAcGlcase has a high content of hydrophobic amino acids. As it was shown by SDS-PAGE, four major bands were detected in the last purification step (Fig. 2) with apparent molecular mass of 130, 94, 45, and 29 kDa.

This partially purified enzyme fraction was used to carry out biochemical characterization analysis. As several glycosidases need cations for their activities, possible cation requirements for NAcGlcase were studied. Neither EDTA nor metal ions such as Ba^{2+}, Mg^{2+},Ca^{2+}, Mn^{2+}, and Zn^{2+} had significant effect on the enzyme activity (data not shown).

Effect of pH. Enzyme activity was measured at different pHs by incubating the NAcGlcase with *p*-nitrophenyl NAcGlc. The enzyme showed maximum activity at pH 3.5 with a smaller peak at pH 5.0, and

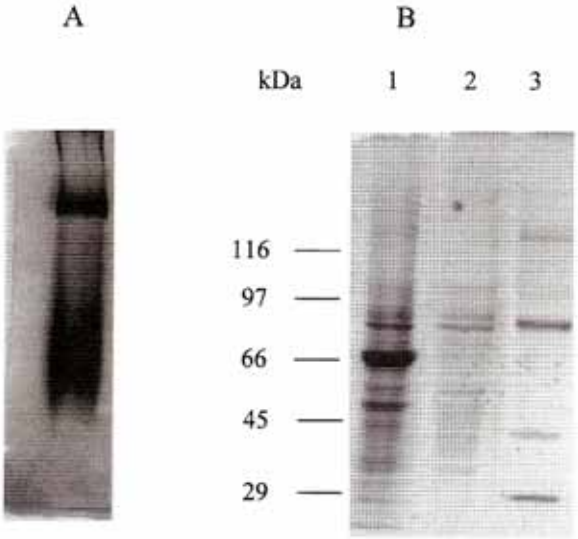

Fig. 2. Electrophoretic analysis of sperm NAcGlcase. **A.** Native electrophoresis. The partially purified fraction (5 µg of total protein) was run on a native gel (10% polyacrylamide). Two bands are detectable after performing the partial purification procedure as described in the Materials and Methods. **B.** SDS-PAGE. Fractions of different purification steps were run on SDS-PAGE (10% polyacrylamide). Lane 1, crude sperm extract (20 µg of total protein); Lane 2, fraction bound to Phenyl–sepharose column (5 µg), and Lane 3, fraction bound to ConA–sepharose (5 µg). Molecular mass markers are displayed on the left of the gel (numbers indicate the molecular mass in kDa). Both gels were silver stained.

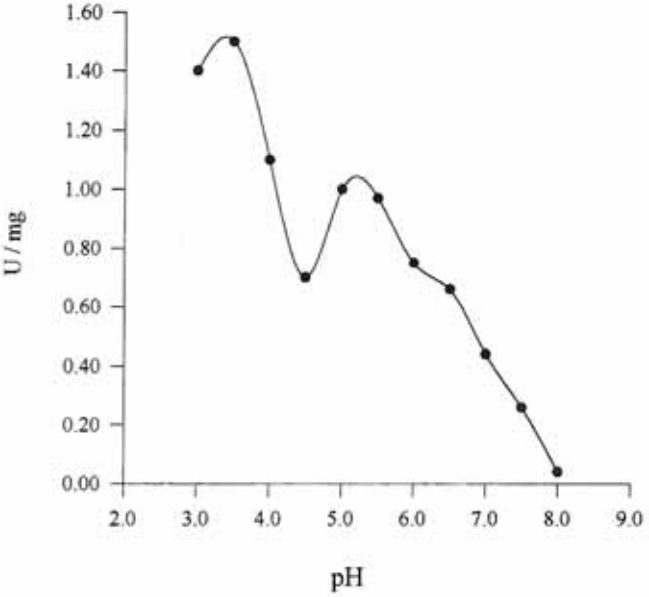

Fig. 3. pH dependence of NAcGlcase activity. Equal aliquots of the partially purified enzyme were added to *p*-nitrophenyl NAcGlc 3 mM in citrate–phosphate buffer (Mc Ilvaine, 1921) at the indicated pHs. The enzyme activity (expressed as U/mg) was assayed as described in the Materials and Methods.

was inhibited at pHs higher than 7.5 (Fig. 3). However, when the enzyme was incubated at pHs higher than 8.0 during an hour and then the activity measured at pH 5.0, no differences were observed between treated and controls, which have been incubated at pH 5.0 for the same period. Similar results were obtained when the glycosidase was incubated overnight at pH 8.8 at 4°C, and the activity measured at pH 5.0. These results show that the enzyme was not denatured at high pH, although its activity was inhibited.

Effect of temperature. NAcGlcase showed a conventional thermal dependence. Increasing activity was detected with increasing temperature up to 55°C (Fig. 4). Although the enzyme activity was not detected at low temperatures, the enzyme seems to be stable as it could be stored at 0–4°C for several days without appreciable loss of activity.

Molecular mass. The molecular mass was estimated by size-exclusion on HPLC. An aliquot of a partially purified enzyme containing 0.1% Triton X-100, was submitted to fractionation, and the enzyme activity was assayed troughout the chromatographic run. As can be observed in Fig. 5, a peak of the enzyme activity was detected at 45–50 kDa. However, when the chromatography was carried out without Triton X-100, two peaks of activity were detected at approximately 190–200 kDa and 45–50 kDa, respectively (data not shown).

Kinetic characterization of NAcGlcase. The enzyme was tested for its catalytic properties as a function of substrate concentration. The K_m for *p*-nitrophenyl β-D-*N*-acetylglucosaminide was 0.59 mM. Inhibition of the hydrolysis reaction by different sugars was also

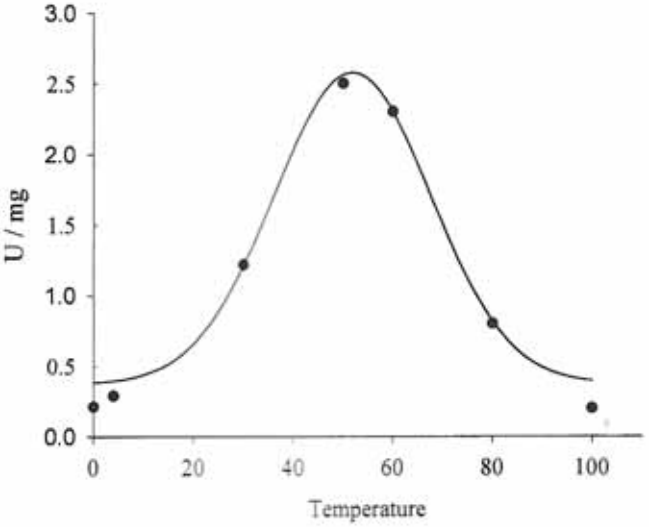

Fig. 4. Influence of temperature on NAcGlcase activity. The reaction mixtures (containing partially purified enzyme and *p*-nitrophenyl NAcGlc 3 mM in 10 mM citrate buffer pH 3.6) were incubated for 30 min at the indicated temperature. After stopping the reaction, the released *p*-nitrophenol was determined as described in the Materials and Methods. NAcGlcase activity was expressed as U/mg.

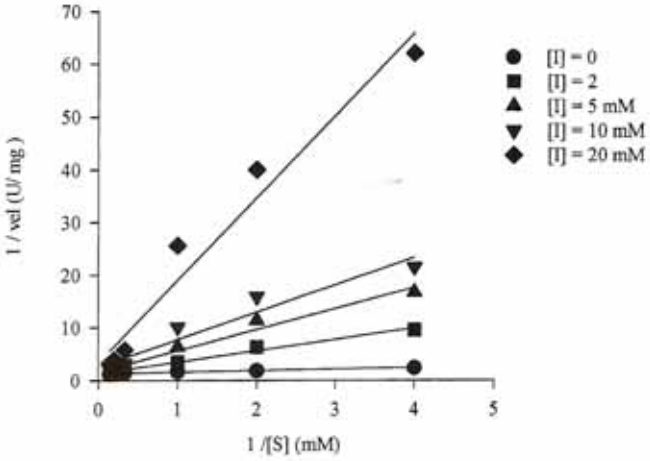

Fig. 6. Double reciprocal (Lineweaver–Burk) plot to illustrate competitive inhibition of the sperm NAcGlcase by NAcGalactosamine. The enzyme activity of the partially purified enzyme was assayed in the presence of different NAcGal concentrations. The reaction was started by addition of 0.25, 0.5, 1.0, 3.0, 5.0 or 7.0 mM of the substrate *p*-nitrophenyl NAcGlc. [I] represents the inhibitor concentration.

carried out. *N*-acetyl galactosamine was more effective than NAcGlc to inhibit the enzyme activity. A double-reciprocal plot of velocity versus substrate concentration in the presence of various NAcGal concentrations shows a classical competitive inhibition with a $K_i = 3.2 \pm 0.3$ mM (Fig. 6).

Subcellular Localization

In order to study enzyme distribution, the activity was measured in different subcellular fractions. After inducing the acrosomal breakdown, most NAcGlcase activity was readily detected in the soluble fraction enriched in acrosomal content.

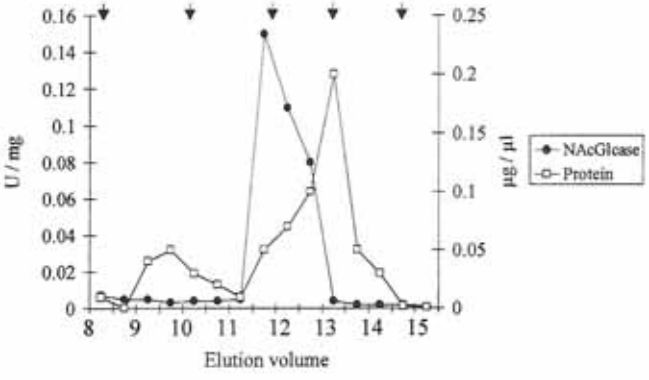

Fig. 5. Size-exclusion on HPLC of sperm NAcGlcase. Partially purified enzyme (0.1 mg of total protein) in 200 mM phosphate buffer pH 7 containing 0.1% of Triton X-100, was loaded on BioSil Sec column. Fractions of 0.5 ml were collected. Each fraction was analyzed for NAcGlcase activity (●) and protein (□). Arrows show the elution volume of molecular mass markers, from left to right: 669, 158, 44, 17, and 1.35 kDa.

The glycosidase activity was still present in the reacted sperm. The enzyme appears to be an intrinsic membrane protein, since treating this suspension with 0.5 M NaCl and 5 mM EDTA did not release the enzyme as no NAcGlcase activity was detected in the supernatant. The addition of 1% Triton X-100 to the last pellet resulted in a complete extraction of the activity (Fig. 7B). In contrast, no glycosidase activity was detected when this last pellet was extracted with a detergent-free buffer. These results show that a fraction of the enzyme, approximately 20% of the total activity, was detected in the reacted sperm, suggesting that it is associated with the sperm membranes.

Purification of NAcGlcase

In order to better understand the role of this glycosidase in the fertilization process, its purification was carried out. Liver NAcGlcase was used in the purification protocol due to limiting amounts of the sperm enzyme. Anti-NAcGlcase antibodies, as described above, specifically recognized the same band in samples from both sources. Furthermore, liver and sperm enzymes showed maximal activity at pH 3.6 and 55°C. They were both strongly bound to phenyl–sepharose and ConA-–sepharose columns. These results indicate that liver and sperm NAcGlcase have identical biochemical characteristics.

The purification procedure was carried out starting with 70 mg of total protein in the liver homogenate. After native PAGE, run at pH 8.8, the enzyme activity was detected when assayed at pH 3.6. These data are in good correlation with those shown above, as no modification was found in the activity of the enzyme previously incubated at pH 8.8.

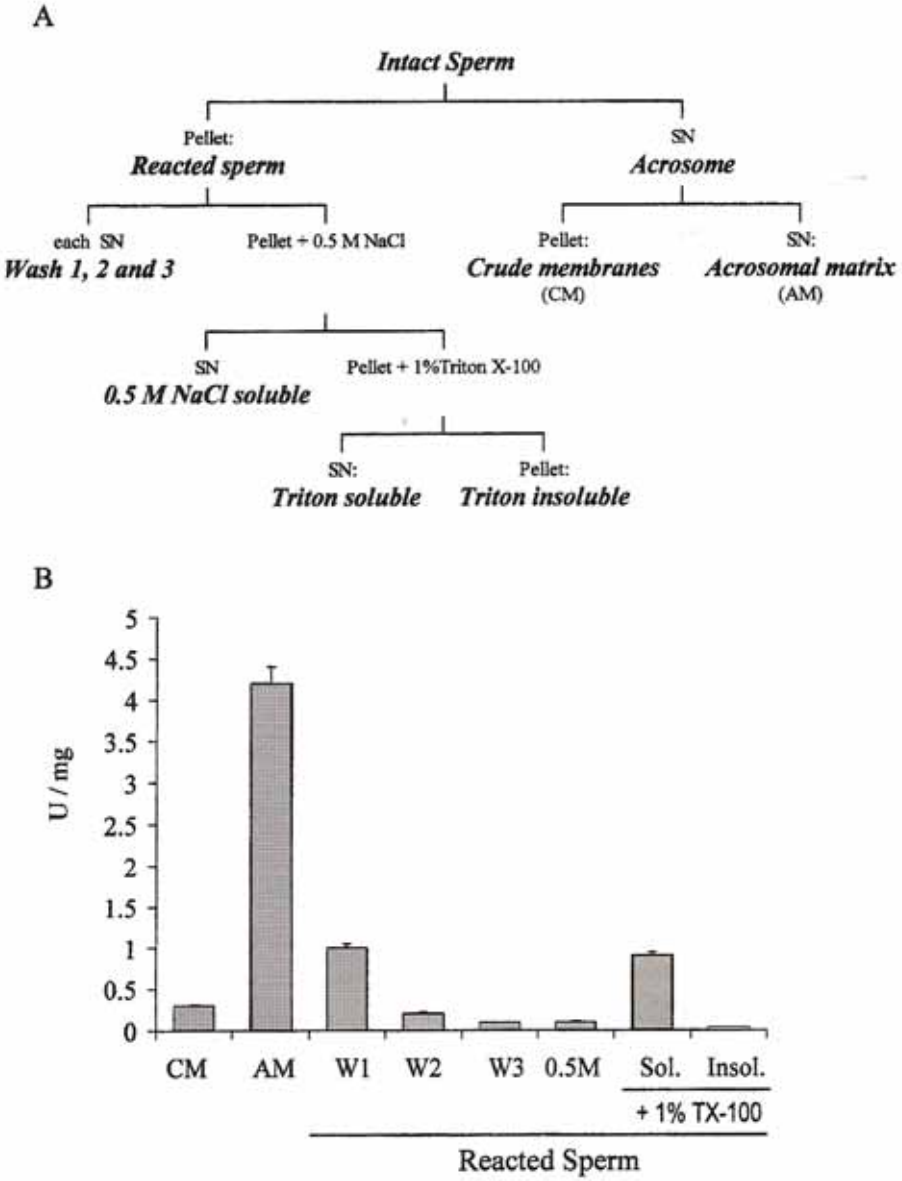

Fig. 7. Subcellular fractionation of the spermatozoa. **A.** The diagram indicates the different subcellular fractions obtained from the intact sperm pellet as described in the Materials and Methods. **B.** Aliquots of different subcellular fractions were incubated with *p*-nitrophenyl NAcGlc 3 mM in 10 mM citrate buffer pH 3.6 and NAcGlcase activity (expressed as U/mg) was assayed as described in the Materials and Methods. The enzyme activity in the following fractions are shown: CM corresponds to crude membrane fraction and AM to the acrosomal matrix. The subsequent samples were obtained from the reacted sperm. W1, W2 and W3 were the first, second and third wash fractions respectively. 0.5 M NaCl corresponds to proteins extracted with this salt solution. After treating with 1% Triton X-100, soluble and insoluble fractions were obtained.

Liver NAcGlcase was retained in ConA affinity chromatography, indicating that it contains mannose residues. The purified enzyme was resolved in a silver stained band on native gel (Fig. 8). The enzyme appeared to dissociate into a band of approximately 45 kDa on SDS-PAGE. This band was also recognized by anti-NAcGlcase antibodies.

Anti-NAcGlcase Antibodies Specificity

Polyclonal antibodies against commercial plant NAcGlcase were prepared. By Western blotting analysis, it was seen that these antibodies reacted with a single band in Amphibian's sperm crude extract and liver homogenate. The band had an apparent molecular weight of 45 kDa in both cases. The antiserum obtained proved to be specific to this protein since it was not seen in controls immunoblots using preimmune rabbit serum (Fig. 9). In addition, after native electrophoresis the immunoreactive band had identical electrophoretic mobility to the active band visualized by activity-staining using 4-MU-NAcGlc (data not shown). These results indicate that antibodies recognize the toad enzyme present in both spermatozoa and liver.

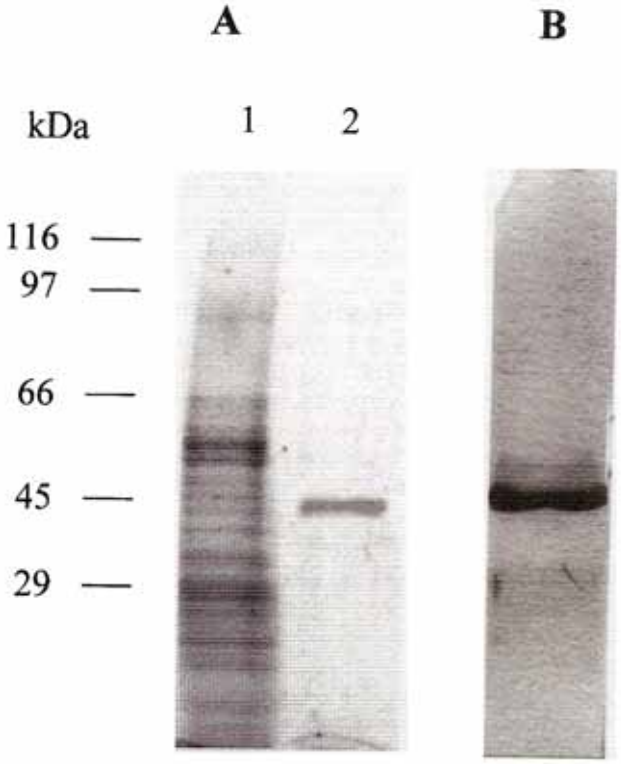

Fig. 8. Analysis of purified NAcGlcase. **A.** SDS-PAGE, on a 10% polyacrylamide, was carried out and the gel was silver stained. A single band of 45 kDa is detectable after performing the purification procedure as described in the Materials and Methods. Lane 1: protein profile for liver homogenate (20 μg). Lane 2: Purified NAcGlcase (2 μg). Molecular mass markers are displayed on the left of the gel (numbers indicate the molecular mass in kDa). **B.** Western blot analysis. After SDS-PAGE, purified NAcGlcase (2 μg) was transferred to nitrocellulose membranes and detected with anti-NAcGlcase antibodies as described in the Materials and Methods.

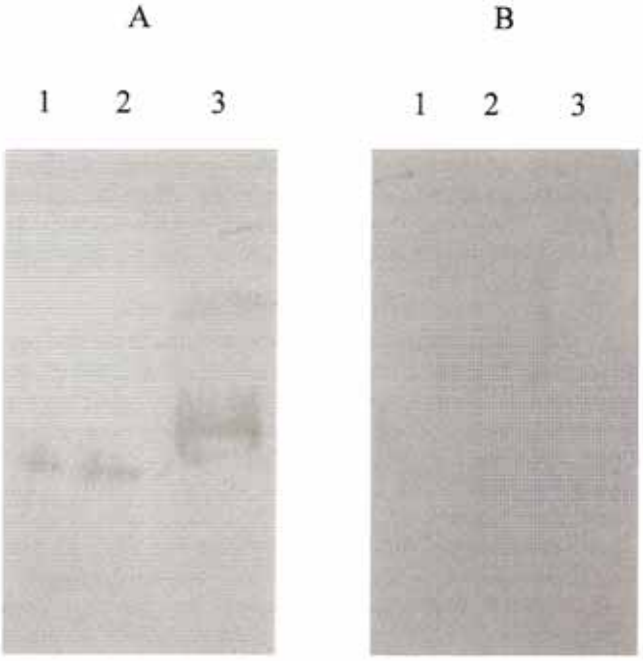

Fig. 9. Anti-NAcGlcase antibodies specificity. Western blot analysis showing antiserum specificity. Proteins were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Immunodetection was performed using anti-NAcGlcase antibodies (**A**) or preimmune serum (**B**) as described in the Materials and Methods. Lane 1: crude sperm extract (40 μg), Lane 2: purified liver NAcGlcase (0.5 μg), Lane 3: jack bean NAcGlcase (0.5 μg).

Fertilization

To test whether the NAcGlcase is directly involved in fertilization, initial studies were undertaken. NAcGlc and NAcGal have previously been shown to inhibit the enzyme activity and presumably these sugars would compete with the glycoconjugate on the vitelline envelope for the sperm NAcGlcase. On the other hand, anti-NAcGlcase antibodies or the purified enzyme were tested for their ability to block fertilization.

The fertilization rate was between 70–80% in controls, where fertilization was performed in saline solution (Fig. 10). In contrast, fertilization of intact eggs was reduced to 1% by the addition of anti-NAcGlcase antibodies or the purified enzyme. Moreover, only 2% of the oocytes were fertilized in the presence of 0.1 M of NAcGlc or NAcGal.

Appropriate controls to demonstrate antibody specificity were carried out with preimmune serum at the same concentration and the fertilization rate was between 65–80%. In addition, inactivating the purified enzyme by heating or the presence of 0.1 M of mannose in the fertilization media failed to inhibit fertilization.

To confirm that the observed inhibition of fertilization by NAcGlc or NAcGal is not due to the effect of these sugars on the sperm motility, microscopy observations were carried out. Spermatozoa were incubated with 0.1 M NAcGlc or NAcGal and the presence of

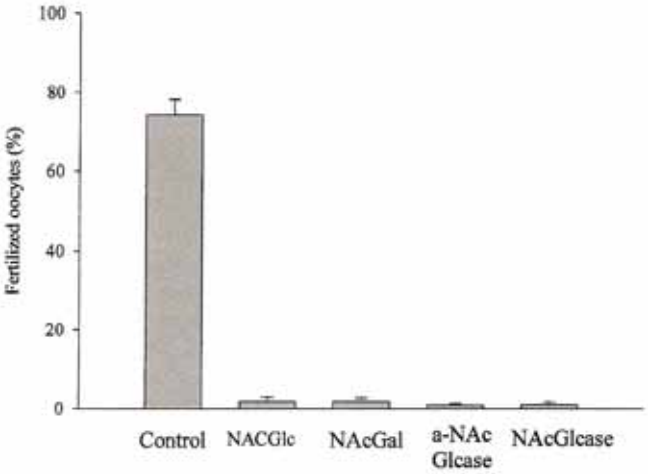

Fig. 10. Fertilization assay. Dejellied oocytes were inseminated with 25 μl of sperm suspension (10^6 cells/ml) in different media containing: 10% Ringer-Tris (Control), 0.1 M NAcGlc, 0.1 M NAcGal, anti-NAcGlcase antibodies (1:100 dilution) or 0.12 μg of NAcGlcase as described in the Materials and Methods. All data represent the percentage of fertilized oocytes. Error bars represent the SEM of eight independent experiments where at least 50 oocytes were evaluated in each one.

motile spermatozoa was determined over 25 min by optical microscopy. The percentage of spermatozoa that were motile after incubation with the monosaccharides was not different from control spermatozoa in the absence of sugars. These studies suggest that the inhibition of fertilization in the presence of these sugars is not due to their nonspecific effect on sperm motility. These results are a direct evidence that the NAcGlcase plays an important role in toad fertilization.

DISCUSSION

Fertilization requires the sperm to penetrate the egg envelopes. The acrosome reaction in Amphibians occurs near vitelline envelopes of the oocyte releasing proteases and glycosidases in the media. The presence of these glycosidases opens a field for research and speculation of their role in Amphibian fertilization since these enzymes are reported to be involved in sperm binding as well as in penetrating through the oocyte envelopes in phylogenetically distant animals.

NAcGlcase, Galase, β-Glcase, and manase activities were detected in the crude sperm extract. Low levels of α-Glcase and fucase activities were also measured (about 20-fold lower than NAcGlcase). NAcGalase, presented relatively less activity than NAcGlcase. However, both activities can be accounted for the same enzyme, since most NAcGlcase also have NAcGalase activity (Beeley, 1985).

NAcGlcase is the major glycosidase activity detected in *Bufo arenarum* sperm. The enzyme binds to VE in vitro, suggesting a potential role in the fertilization process. Furthermore, in the amphibian *Xenopus laevis*, removing NAcGlc residues from the VE reduces fertilization (Prody et al., 1985). Based on these observations the characterization of the enzyme has become an interesting aim.

The purification procedure of the enzyme from liver homogenate is a relatively fast method. The loss of activity that was observed in the successive dialysis and concentration processes were considerably reduced in this two-step protocol. Under reducing conditions, the purified enzyme resulted in one band with an apparent molecular mass of 45 kDa on SDS-PAGE. In our work, the use of liver NAcGlcase was an advantage since large amounts of starting material could be obtained. Liver and sperm enzymes seem to be similar since both have identical pH and temperature optimum, and show similar chromatographic behavior on phenyl–sepharose and ConA columns. Moreover, the antibody against commercial plant NAcGlcase has been prepared and it showed reactivity with the toad liver and sperm glycosidase. By immunoblots, using these antibodies, we observed a band with an apparent molecular mass of 45 kDa under reducing conditions in both liver and sperm homogenates. Furthermore, analysis by size-exclusion on HPLC in the presence of 0.1% of Triton, showed a peak activity at around 45 kDa. However, the other peak of activity was detected at approximately 190 kDa when the chromatography was carried out without Triton. These results suggest that the enzyme might prevail as a tetramer constituted by 45 kDa subunits, assembled by hydrophobic interactions.

Studies of the sperm NAcGlcase showed that neither EDTA nor metal ions had significant effect on the enzyme activity. As many other lysosomal enzymes, NAcGlcase has an acidic pH optimum and was retained in ConA affinity chromatography, indicating that it contains mannose residues.

The described kinetic properties show a K_m of the enzyme for *p*-nitrophenyl NAcGlc of 0.59 mM. These values are comparable to those of NAcGlcase isolated from jack bean (Li and Li, 1970) and *Xenopus laevis* (Prody et al., 1985) in which K_m was 0.64 mM. In addition, NAcGlc and NAcGal are potent inhibitors of the enzyme activity in all cases.

The acrosome reaction releases the acrosomal enzymes and exposes the inner acrosomal membrane. In our work, NAcGlcase is the most important glycosidase activity in the acrosomal matrix and the reacted sperm retains lower levels of NAcGlcase activity. As the enzyme strongly binds to an hydrophobic interaction matrix (phenyl–sepharose), it may have an important hydrophobic domain. Our results show that NAcGlcase could be found as both soluble and associated with the sperm membranes and it appears to be an integral protein.

Several sperm membrane–bound glycosidases have been reported to play a role in gamete interaction. NAcGlcase is the only acrosomal glycosidase from *Bufo arenarum* that binds to homologous VE. As the binding conditions were low osmolarity and pH 7.6, which resemble the fertilization media, the NAcGlcase–VE complex formed in vitro would be rather stable because the glycosidase has an acidic pH optimum and the rate of hydrolysis in alkaline pH is low. Therefore, the binding to VE could be maintained at the alkaline pH of fertilization media. However, the possibility cannot be ruled out that in vivo, since the acrosome is an acidic vesicle, the pH of the microenvironment surrounding the released acrosome may be acidic enough to allow for NAcGlcase catalytic activity. Mammalian NAcGlcase present in the acrosome has been proposed to function catalytically in mouse (Miller et al., 1993) and in pig (Takada et al., 1994).

The involvement of the enzyme in fertilization was tested by in vitro assays. We have shown that when the NAcGlcase is inhibited by the addition of anti-NAcGlcase antibodies, the fertilization rate is highly reduced. This result could be due to blocking of the sperm enzyme. On the other hand, inclusion of NAcGlc or NAcGal in the assay reduced the rate of fertilized eggs. Since these two sugars have inhibitory effect on the sperm NAcGlcase, the above result could be due to inhibition of the glycosidase. Finally, treatment of the intact eggs with the purified NAcGlcase caused nearly complete inhibition of fertilization. Collectively, these studies suggest that the sperm NAcGlcase may have a role in fertilization. Further studies are needed to understand its role in vivo.

Although the biological function of NAcGlcase remains unknown, it may be significant that the enzyme interacts in vitro with the oocyte. Moreover, the inhibition of the enzyme results in the inhibition of fertilization, showing that the NAcGlcase plays an indispensable role in toad fertilization.

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