Proc. Indian Acad. Sci. (Anim. Sci.), Vol. 97, No. 3, May 1988, pp. 181–187.

Glycosidases in toad (*Bufo melanostictus*) sperm and their role during fertilization

V SRINIVASA RANGAN, N KESHAVA KUMAR and H S SESHADRI*

Department of Studies in Biochemistry, University of Mysore, Manasagangotri, Mysore 570 006, India

MS received 7 September 1987; revised 14 March 1988

Abstract. Sperms were isolated from *Bufo melanostictus* testes, by a simple technique of differential centrifugation. β-Galactosidase, N-acetyl β-D-glucosaminidase, α-L-fucosidase, neuraminidase and acid phosphatase activities were detected in the sperms. All the sugar components of the jelly glycoprotein were released when toad jelly glycoprotein was treated with sperm suspension. These facts suggest that the sperm glycosidases are involved in the penetration of jelly by the sperm at the time of fertilization.

Keywords. *Bufo melanostictus*; glycosidase; sperm; fertilization.

1. Introduction

The eggs of most animals are surrounded by one or more coats or integuments through which a sperm must pass through to fertilize the egg (Rugh 1961; Monroy 1965; Metz 1967; Austin 1968; Piko 1969). They include the vitelline coat and jelly coats in many invertebrates (Wasserman *et al* 1985) and vertebrates (Wolf *et al* 1976) and zona pellucida in mammals (Srivatsava *et al* 1974; Urch *et al* 1985). These coats are composed mostly of glycoproteins with different ultra structures and molecular compositions (Katagiri 1987). The functions of these integuments are multiple such as participation in the process of sperm egg fusion, having specific binding sites for sperm or inducing acrosome reaction and also as a possible block of polyspermy (Gwatkin 1977; Glabe and Vacquier 1978; Suzuki *et al* 1981; Wasserman *et al* 1985).

The egg of an amphibian toad (*Bufo melanostictus*) is surrounded by a vitelline envelop and later on by the jelly which is produced in the oviduct when the egg passes through it. Jelly is an o-glycosidic type of glycoprotein containing N-acetyl glucosamine, N-acetyl galactosamine, fucose, galactose and N-acetyl neuraminic acid as its carbohydrate constituents. A partial structure for the carbohydrate moiety of the jelly has been proposed (Reddy and Seshadri 1978; Seshadri and Reddy 1980). These jelly envelops secreted by the oviduct are found to be indispensible for sperm penetration into eggs, as the removal of jelly layers resulted in the reduced fertilization efficiency (Reddy and Seshadri 1978). However, the exact mechanism by which the jelly coats favour fertilization is not clearly understood (Katagiri 1987).

Unlike most urodels (Katagiri 1987), fertilization in anuran amphibians is external. Fertilizing spermatozoa must pass through several layers of jelly envelops and the vitelline coat before fusing with the egg plasma membrane, a process known as acrosome reaction. Reports are available about the mechanism of passage of sperm

*To whom all correspondences should be addressed.

through the vitelline envelop which reveal the involvement of acrosomal proteases in the lysis of vitelline coat. In amphibians, no reports are available about the mechanism of cleavage of jelly layers. However, the possibility of the sperm containing lytic activities other than proteases in order to accomplish fertilization has been indicated (Katagiri 1987).

Shylaja and Seshadri (1985) have reported the presence of glycosidases such as β-galactosidase, α-L-fucosidase, N-acetyl β-D-hexosaminidase, neuraminidase and also proteases in toad (*B. melanostictus*) testes. These enzyme activities were found to vary with respect to spermatozoa during breeding and non-breeding seasons. The present investigation was carried out with a view to find out whether the glycosidase activities are present in the fertilizing sperms of the toad (*B. melanostictus*) and if present does this sperm uses these enzymes to cleave the egg coat jelly during its passage through it, which is an essential event for the sperm to reach the egg surface.

In this study, we report the isolation of sperms from toad (*B. melanostictus*) testes, the presence of glycosidases in sperms and their involvement in the degradation of the jelly.

2. Materials and methods

2.1 *Chemicals*

Para-nitrophenyl derivatives of β-D-galactopyranoside, α-L-fucopyranoside, N-acetyl-β-D-glucosaminide and N-acetyl-β-D-galactosaminide, Fetuin, D-galactose, L-fucose and para-nitrophenyl phosphate were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Dialysis tube (Cat. No. 3787H47) was purchased from Arthur H Thomas Company, Philadelphia, USA. Other chemicals used were of analytical grade. All the solvents were distilled before use.

Locally available mature male toads (*B. melanostictus*) were collected during breeding season from their natural habitat. Testes were collected from the toads after sacrificing the anaesthetised animals.

2.2 *Isolation of sperm*

About 20 g of freshly collected toad (*B. melanostictus*) testes were taken in 5 ml of 0·25 M sucrose solution or DeBoer's solution consisting of 110 mM NaCl, 1·3 mM KCl and 1·3 mM $CaCl_2$ and finely chopped with razor blade to release the sperms. The material was then centrifuged at 80 *g* at 4°C for 10 min using 5CRC Sorvall centrifuge. The debris devoid of sperms, as determined by the microscopic observation was discarded, and the supernatant was again centrifuged at 500 *g* for 15 min at 4°C. The sperms sedimented as a pellet were washed several times with 0·25 M sucrose and later on with ice cold DeBoer's solution until the washings are free from protein and showed no enzyme activity and referred to as sperm suspension. This sperm suspension in DeBoer's solution was screened for various glycosidase activities.

To determine whether these enzymes are membrane bound or cytosolic, the washed sperms were suspended in DeBoer's solution and gently homogenized using a potter Elvehjam blender using 30 strokes per min for 15 min. The homogenate was

centrifuged at 80,000 *g* for 30 min at 4°C. The supernatant was referred to as sperm extract. The sedimented fraction was washed thoroughly with ice cold saline and referred to as sperm membrane pellet. It was suspended in 2 ml of saline. Microscopic observation was carried out to make sure that the sperm extract is not contaminated with membrane fraction. In another experiment, the sperms suspended in DeBoer's solution were homogenized in the presence of 0·2% Triton-X-100 as above and centrifuged at 80,000 *g* for 30 min at 4°C. Both, supernatant and the sedimented sperm membrane pellets, were taken for enzyme assays.

The sperm extract and sperm membrane pellet were dialysed against water and the non diffusible portions were screened for various glycosidase activities.

2.3 *Assay of glycosidases*

β-D-Galactosidase, α-L-fucosidase and N-acetyl-β-D-hexosaminidase activities were assayed using corresponding para-nitrophenyl sugar derivatives as the substrate, according to the method of Alam and Balasubramanian (1978). Neuraminidase activity was assayed using toad jelly glycopeptide (prepared as described by Reddy and Seshadri 1978) or fetuin as the substrate and by estimating the released sialic acid by the method of Aminoff (1961). Acid phosphatase activity was assayed according to the method of Alvarez (1962), using para-nitrophenyl phosphate as the substrate. Protein was estimated by the method of Lowry *et al* (1951) using bovine serum albumin as standard.

2.4 *Action of sperm glycosidases on toad oviduct jelly glycoprotein*

In a dialysis tubing, 20 mg of toad jelly glycoprotein was treated with 2 mg protein of dialysed sperm extract and sperm membrane pellet separately. The reaction was carried out at 30°C by immersing the dialysis tubing in 30 ml of distilled water taken in a beaker. The dialysate obtained after 3 h incubation was evaporated to dryness under vacuum, dissolved in 0·1 ml of distilled water and analysed for the presence of various constituent sugars of jelly glycoprotein by paper chromatography as follows. The dialyzate sample (100 μl) was spotted on Whatman No. 1 paper and chromatographed using Butanol/pyridine/water (6:4:3 v/v) solvent system for 18 h, spots corresponding to different sugars were visualized on the paper using alkaline silver nitrate spraying reagent. Sialic acid was also visualized as described by Warren (1960). Jelly was also treated with sperm suspension under similar conditions.

2.5 *Effect of sulphydryl group modifying reagents on sperm glycosidases*

About 1 ml of sperm suspension (10^6 sperms/ml) in DeBoer's solution was treated with excess of 100 mM iodoacetate or N-ethyl maleimide at 37°C for 30 min according to the method of Colman and Chu (1970). The excess reagent was removed by dialysing the sperm suspension against water. The activity of glycosidases was determined using this sperm suspension, as described earlier.

3. Results

Microscopic observation of the washed sperm pellet revealed that it is not contaminated with other testicular cells (figure 1).

β-Galactosidase, α-L-fucosidase, N-acetyl-β-D-glucosaminidase, neuraminidase and acid phosphatase activities were detected in toad (*B. melanostictus*) sperms. The above enzyme activities are found in the sperm extract as well as in the sperm membrane pellet but the specific activity of N-acetyl-β-D-glucosaminidase and neuraminidase was higher in the sperm membrane pellet than in the sperm extract, whereas most of the acid phosphatase activity was found in the sperm pellet (table 1). However, when the sperm suspension was homogenized in the presence of detergents like Triton X-100, all the enzyme activities were found only in the sperm extract.

Figure 1. Isolated and washed sperms from toad (*B. melanostictus*) testes.

Table 1. Glycosidases in toad (*Bufo melanostictus*) sperm.

	Specific activity				
Source	N-acetyl-β-D glucos-aminidase[a]	β-Galacto-sidase[a]	α-L-Fuco-sidase[a]	Acid phos-phatase[a]	Neur-aminidase[b]
Sperm extract	19·5 ± 1·8	13·1 ± 1·0	10·1 ± 1·5	2·0 ± 0·3	4·9 ± 0·8
Sperm membrane pellet	47·1 ± 2·0	5·8 ± 1·0	5·9 ± 1·0	70·7 ± 2·1	12·9 ± 1·3

The values represent the average of 4 separate experiments and expressed as mean ± SD.
[a]nanomol of para-nitrophenol released/mg protein/min.
[b]nanomol of sialic acid released/mg protein/min.

The sperm suspension, sperm membrane pellet and sperm extract hydrolysed the jelly glycoprotein. Silver nitrate positive spots corresponding to galactose, fucose, N-acetyl glucosamine and neuraminic acids were visualised on Whatman No. 1 paper (figure 2), indicating that the sperm glycosidases release the carbohydrate residues present in the jelly glycoprotein.

When the sperm suspension was treated with sulphydryl group modifying reagents, there was complete loss of all the glycosidase activities. This sperm suspension was unable to hydrolyse the jelly glycoprotein, as silver nitrate positive spots corresponding to constituent sugar residues of jelly glycoprotein were absent, after paper chromatography.

4. Discussion

Several methods are available in the literature about the isolation of sperms from mammals (Llanos *et al* 1982; Rahi *et al* 1983), however only little is known about

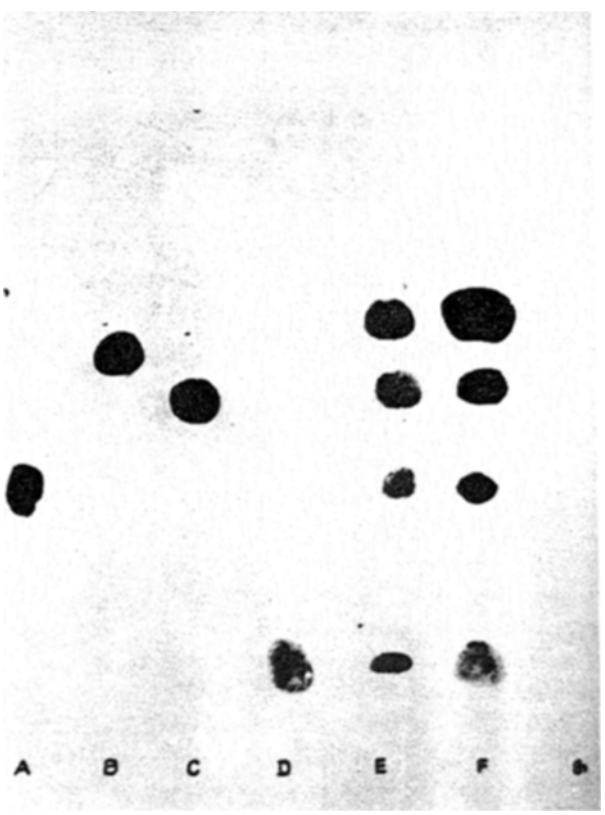

Figure 2. Effect of sperm glycosidases on toad egg coat jelly glycoprotein.

The isolated egg coat jelly was treated with toad sperm suspension and sperm membrane pellet separately and incubated in a dialysis tubing at 37°C for 3 h. The dialysate was collected after 3 h and evaporated to dryness in vacuo and analysed for jelly constituents by paper chromatography. A, Galactose; B, fucose; C, N-acetyl glucosamine; D, neuraminic acid; E, jelly treated with sperm membrane pellet; F, jelly treated with sperm suspension; G, control.

isolation of sperms from amphibians. In amphibians, the sperms are stored in testes which contain several specialized tissues and ejected only during spawning to accomplish fertilization. The fact that they have no specially differentiated organ like epididymis, as in higher organisms to store the spermatozoa; poses problems in isolation of sperms from amphibians. The present investigation describes a method for the isolation of sperms from toad (*B. melanostictus*) testes, adopting a simple technique of differential centrifugation which is very efficient and reproducible. Microscopic observation of the sperm suspension clearly showed that the method adopted for the isolation of sperms ensures that the sperm suspension is not contaminated with other cells present in the testes. This procedure can be adopted to isolate the sperms from the testes of other species of amphibians especially frogs.

There are many reports on the presence of glycosidases in mammalian sperm and their involvement in facilitating the fertilization (Allison and Hartree 1970; Srivatsava *et al* 1974), whereas no such reports are available in case of amphibians. However, there are evidences for acrosomal localization of proteases involved in the lysis of vitelline coat (Penn and Gredhill 1972; Elinson 1974; Iwao and Katagiri 1982), atleast in some species of amphibians. The involvement of certain lytic factors which are present in the sperm, for the degradation of jelly coat and the nature of these lytic factors are not clearly understood. The results of our experiments clearly demonstrate the presence of glycosidases such as β-galactosidase α-L-fucosidase, N-acetyl glucosaminidase and neuraminidase in toad sperms. This indicates that sperms contain a set of glycosidases required to breakdown the carbohydrate moieties of toad egg coat jelly glycoprotein.

When the toad egg coat jelly glycoprotein was treated with the toad sperm suspension galactose, fucose, N-acetyl glucosamine and sialic acid were released from the jelly glycoprotein indicating that the sperm glycosidases can use jelly glycoprotein as substrate. The sequential action of these sperm exo-glycosidases results in the dissolution of the jelly layer. The glycosidase activities were found both in sperm extract and sperm membrane pellet though their specific activities varied (table 1). However, when the sperm suspension was homogenized in the presence of detergents like Triton X 100, all the enzyme activities were found only in sperm extract which clearly indicates that these enzymes are bound to the sperm membrane. This fact indicates that the fertilizing sperm uses these jelly digesting enzymes during their passage through the jelly envelop. The presence of acid phosphatase activity in mammalian sperm is well known, though its role in fertilization is yet to be understood. In toad sperm also acid phosphatase activity was detected and from the present knowledge, its importance in facilitating the fertilization is not clear.

Treatment of the sperm suspension with the sulphydryl group modifying reagents like iodoacetate and N-ethyl maleimide resulted in the complete loss of glycosidases activity. This sperm suspension could not degrade jelly glycoprotein establishing that the jelly could be degraded only by these glycosidases during the passage of sperm through the jelly.

Thus, the present investigation clearly demonstrates that the sperm glycosidases are responsible for cleavage of the carbohydrate portion of jelly during its passage through the jelly in order to meet the egg.

Acknowledgements

The authors are thankful to Ms M Shylaja, Central Food Technological Research Institute, Mysore for her help and suggestions. The authors also acknowledge the

financial assistance from the University Grants Commission and Council of Scientific and Industrial Research, New Delhi.

References

Alam T and Balasubramanian A S 1978 Purification and properties of two forms of β-galactosidase from monkey brain; *J. Neurochem.* **30** 1199–1202

Allison A C and Hartree E F 1970 Lysosomal enzymes in the acrosome and their possible role in fertilization; *J. Reprod. Fertil.* **21** 501–515

Alvarez E F 1962 The kinetics and mechanism of the hydrolysis of phosphoric acid esters by potato acid phosphatase; *Biochim. Biophys. Acta* **59** 663–672

Aminoff D 1961 Methods for quantitative estimation of N-acetyl neuraminic acid and their application to hydrolyzates of Sialo mucoids; *Biochem. J.* **81** 384–392

Austin C R 1968 *Ultrastructure of fertilization* (New York: Academic Press)

Colman R F and Chu R 1970 The role of sulphydryl groups in the catalytic function of ICDH. Effect of NEM on kinetic properties; *J. Biol. Chem.* **245** 601–607

Elinson R P 1974 A comparative examination of amphibian sperm proteolytic activity; *Biol. Reprod.* **11** 406–412

Glabe C and Vaquier V D 1978 Egg surface glycoprotein receptor for Sea urchin sperm binding; *Proc. Natl. Acad. Sci. USA* **75** 881-885

Gwatkin R B L 1977 *Fertilization mechanisms in man and mammals* (New York: Plenum Press)

Iwao Y and Katagiri C 1982 Properties of the Vitelline coat lysin from toad sperm; *J. Exp. Zool.* **219** 87–95

Katagiri C 1987 Role of oviducal secretions in mediating gamete fusion in anuran amphibians; *Zool. Sci.* **4** 1–14

Llanos M L, Lui C W and Meizel S 1982 Studies of phospholipase A_2 related to the hamster sperm acrosome reaction; *J. Exp. Zool.* **221** 107–117

Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951 Protein measurement with the Folin phenol reagent; *J. Biol. Chem.* **193** 265–275

Metz C B 1967 Gamete surface components and their role in fertilization; *Fertilization* **1** 163–236

Monroy A 1965 *Chemistry and physiology of fertilization* (New York: Academic Press)

Penn A and Gredhill B L 1972 Acrosomal proteolytic activity of amphibian sperm: A direct demonstration; *Exp. Cell. Res.* **74** 285–288

Piko L 1969 Gamete structure and sperm entry in mammals; *Fertilization* **2** 325–403

Rahi M, Sheikhnejade G and Srivatsava P N 1983 Isolation of inner acrosomal nuclear membrane complex from rabbit spermatozoa; *Gamete Res.* **7** 215–226

Reddy M S and Seshadri H S 1978 Studies on toad (*Bufo melanostictus*) egg coat jelly glycoprotein: Part I. Isolation, chemical composition and biological role; *Indian J. Biochem. Biophys.* **15** 465–470

Rugh G H 1961 *The frog; its reproduction and development* (New York: McGraw Hill)

Seshadri H S and Reddy M S 1980 Studies on the toad egg coat jelly glycoproteins: Part II. Isolation of glycopeptide and investigation of structure of carbohydrate moiety; *Indian J. Biochem. Biophys.* **17** 24–31

Shylaja M and Seshadri H S 1985 Studies on some enzymes of the toad (*Bufo melanostictus*) testis and their probable role at the time of fertilization; *Experientia* **41** 1113–1118

Srivatsava P N, Munnel J F, Yang C H and Foley C W 1974 Sequential release of acrosomal membranes and acrosomal enzymes of Ram spermatozoa; *J. Reprod. Fertil.* **36** 363–372

Suzuki N, Nomura K, Ohtake H and Isaka S 1981 Purification and the primary structure of sperm-activating peptides from the jelly coat of sea urchin eggs; *Biochem. Biophys. Res. Commun.* **90** 1238–1244

Urch U A, Wardrip N J and Hedrick J L 1985 Limited and specific proteolysis of the zona pellucida by Acrosin; *J. Exp. Zool.* **233** 479–483

Warren L 1960 Thiobarbituric acid spray reagent for deoxy sugars and sialic acids; *Nature (London)* **186** 237

Wasserman P M, Florman H M and Gerve J M 1985 In Biology of fertilization (New York: Academic Press) **2** 341–412

Wolf D P, Nishihara T, West D M, Wyrick R F and Hedrick J L 1976 Isolation, physicochemical properties and the macromolecular composition of the vitelline and fertilization envelops from *X. laevis* eggs; *Biochemistry* **15** 3671-3678