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Full Papers

Studies on some enzymes of the toad *(Bufo melanostictus)* testis and their probable role at the time of fertilization

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Summary. Glycosidases like sialidase, β-galactosidase, α-L-fucosidase, N-acetyl hexosaminidase and proteases were detected in toad testis. Neuraminic acid aldolase activity was also detected. The enzyme activities were found to vary as production of spermatozoa varied. All enzymes, except N-acetyl glucosaminidase, were shown to decrease after injection of toad pituitary extract and they were also found to be absent from testis containing no spermatozoa. The glycosidases were found to act on toad oviduct jelly and they may therefore be involved in the degradation of the jelly after fertilization, into smaller bits, which may be utilized as nutrients by the fertilized zygote.
Key words. Bufo melanostictus; glycosidases; testis; pituitary extract; fertilization; oviduct jelly.

Toad oviduct produces jelly which envelops the eggs as they pass through it. Jelly has long been known to be essential for the fertilization of eggs[14, 23, 27]. It is a glycoprotein containing N-acetyl galactosamine, N-acetyl glucosamine, fucose, galactose and N-acetyl neuraminic acid in its carbohydrate moiety. The oligosaccharide moiety is linked to protein through an alkali-labile bond, between serine/threonine of the peptide and N-acetyl-galactosamine of the carbohydrate moiety. A partial structure of this glycoprotein has been proposed by Seshadri and Reddy[22]. It is well known that the testis of many animals contain glycosidases[20, 26]. Although reports are available on acetylcholine esterase[13], ATPase[12] and 5,3-β-hydroxysteroid dehydrogenase[18] activity in amphibian testis, no information is available on glycosidases. It was therefore of interest to investigate the enzymes of toad testis which may be involved in the degradation of jelly after fertilization. This study was also undertaken with a view to using the enzymes as tools in elucidating the structure of toad jelly glycoprotein. The present study reports the presence of the following enzyme activities; neuraminidase; β-galactosidase, α-L-fucosidase, hexosaminidases, proteases, N-acetyl neuraminic acid aldolase and possibly oligosaccharidases, in toad testis.

Materials and methods

Testes were collected from four groups of experimental toads, which were collected from their natural habitat, after anesthetizing the animals with ether. Histological studies were carried out for the selection of the following groups. A) during the breeding season with motile spermatozoa, B) during the nonbreeding season with motile spermatozoa, C) same as group B, but after 24 h of injection of toad pituitary extract (pituitaries collected from five toads, weighing approximately 50 mg, were homogenized in 1.0 ml of holtfreter's solution[27] and centrifuged at 3000 rpm for 3 min. The whole supernatant was injected at once i.p. to each toad. After 24 h, animals were sacrificed and testes were collected), D) during the nonbreeding season without motile spermatozoa.

Toad jelly was prepared according to Srinivasulu Reddy et al.[27]. Rice lectin was isolated as reported by Indravathamma and Seshadri[11].

Chemicals. L-Fucose, N-acetyl neuraminic acid and thiobarbituric acid were obtained from BDH Ltd, England. N-acetyl-D-glucosamine was from Koch Light Laboratories, England. N-acetyl-D-galactosamine, paranitrophenyl (PNP)-α-L-fucopyranoside, PNP-β-D-galactopyranoside, PNP-N-acetyl-β-D-glucosaminide, PNP-N-acetyl-β-D-galactopyranoside, castor bean lectin, Type II, lactate dehydrogenase were from Sigma Chemical Company, USA. Other chemicals used were of analytical grade, bought from commercial sources. All the solvents were distilled before use. Dialysis tubing (Cat. No. 3787H47) was purchased form Arthus H. Thomas Co., USA.

a) Assay of toad testis enzymes. Testes collected from groups A and D were homogenized in distilled water, centrifuged at 3000 rpm for 2 h at 4 °C, and the supernatant was dialyzed overnight. The dialyzed extracts were again centrifuged and the supernatants analyzed for enzyme activities using both toad jelly and PNP sugar substrates. When toad jelly was used as a substrate, incubation was carried out in dialysis tubing at 37 °C. Dialyzates obtained after 30 min and 90 min of incubation were evaporated to dryness and analyzed for the presence of various constituents of jelly by paper chromatography[27]. Sugars were located by alkaline silver nitrate treatment, sialic acid was visualized by the method of Warren and hexosamines were detected by the Ehrlich reagent, as described in a previous paper[27]. Spots corresponding to all of the sugars present in toad jelly were detected, and also three spots with low mobility probably corresponding to oligosaccharides (OS_1, OS_2, and OS_3).

b) Characterization of oligosaccharides, OS_1, OS_2 and OS_3. Low mol. wt oligosaccharides released after 30 min of incubation were isolated after paper chromatography. Constituent sugars present in OS_1, OS_2 and OS_3 were detected by testing their cross reactivity in agglutination tests using rice lectin (specific for N-acetyl-glucosamine) and castor bean lectin type II (specific for galactose) as described by Lis and Sharon[16].

OS_1 and OS_2 fractions were hydrolyzed with 1 N H_2SO_4 for 4 h at 100 °C. The neutral sugars and amino sugars were separated using Dowex 50 and Dowex 2 columns according to Simkin et al.[25]. Sugars in the neutral fraction of OS_1 and OS_2 were estimated by the Nelson-Somogyi method. Hexosamines were estimated using the Ehrlich reagent[27]. Sugar constituents present in neutral fractions of OS_1 and OS_2 were also identified by paper chromatography.

OS_3 was not studied, as it was not available in sufficient amounts.

Dialyzates obtained after 90 min of incubation were also analyzed for amino acids by two-dimensional paper chromatography and spots were developed by the ninhydrin reagent[30].

c) Colorimetric methods for assay of toad testis enzymes from group A to group C. Sialidase activity was checked using toad jelly glycoprotein as the substrate, by estimating free sialic acid before and after incubation by the method of Aminoff[2].

Neuraminic acid aldolase activity was detected using neuraminic acid as the substrate, by coupling the pyruvate formed to an LDH and NADH system, as described by Comb and Roseman[5]. Galactosidase, fucosidase, N-acetyl-galactosaminidase and N-acetylglucosaminidase assays were performed using appropriate PNP substrates as described by Tausif and Balasubramanian[29].

In one set of experiments, about 50 mg of toad jelly glycoprotein was incubated in a dialysis tube with 10 mg protein of crude testis extract. The reaction was carried out in the presence of toluene at 37 °C and was followed by withdrawing aliquots of 1 ml at regular intervals from the digestion mixture. The latter were heated for 3 min in a boiling water bath and the release of hexosamine was estimated by the method of Reissig et al.[19]. A control experiment was set up in parallel.

Results

a) Assay of testis enzymes in groups A and D. Three oligosaccharide spots OS_1–OS_3 were detected after 30 min

of incubation. Galactose, fucose and N-acetyl hexosamines were located on paper after 90 min of incubation in group A (figs 1A, 1B, 2). In group D, neither oligosaccharides nor free sugars were detected.

b) Composition of oligosaccharides OS_1–OS_3. Inhibition of agglutination by rice lectin was observed only with OS_3. Castor bean lectin activity was inhibited only by OS_1. No inhibition could be observed for either of the lectins with OS_2. Positive reactions for both neutral and amino sugars were obtained with all the three oligosaccharides (OS_1, OS_2 and OS_3). Neutral sugars present in OS_1 and OS_2 are shown in figure 3.

c) Colorimetric enzyme assays. In groups A and B, an increase in free sialic acid was observed in the sample incubated for a short period (45 min) with toad jelly in presence of testis extract, whereas a decrease in sialic acid was observed after prolonged incubation (2 h). In group D, release of sialic acid was not observed. When a crude testis extract from group A animals was incubated with sialic acid, the coupled reaction with LDH gave a de-

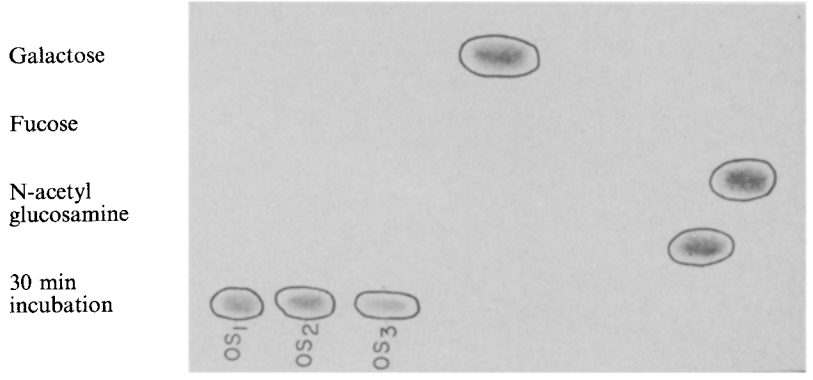

Figure 1A. Paper chromatography of the dialyzate obtained after 30 min of incubation of toad jelly with toad testis extract. Solvent system: butanol/pyridine/water (6/4/3) (v/v). Detection agent: alkaline silver nitrate.

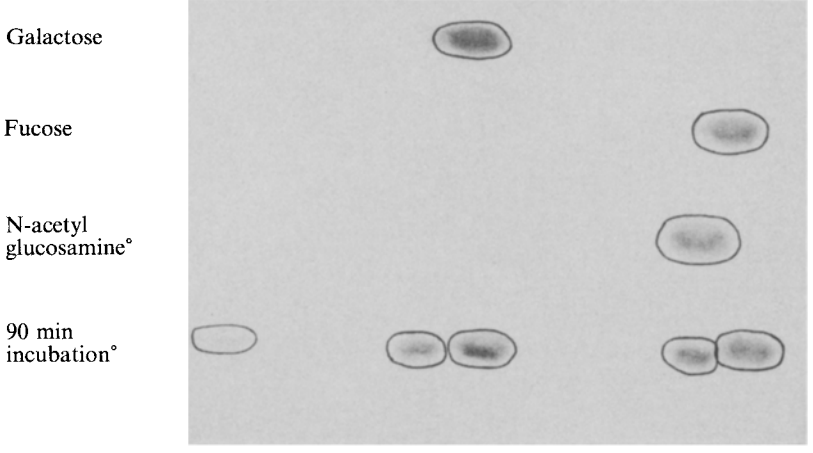

Figure 1B. Paper chromatography of the dialyzate obtained after 90 min of incubation of toad jelly with toad testis extract. Solvent system: butanol/pyridine/water (6/4/3) (v/v). Detection agent: alkaline silver nitrate.

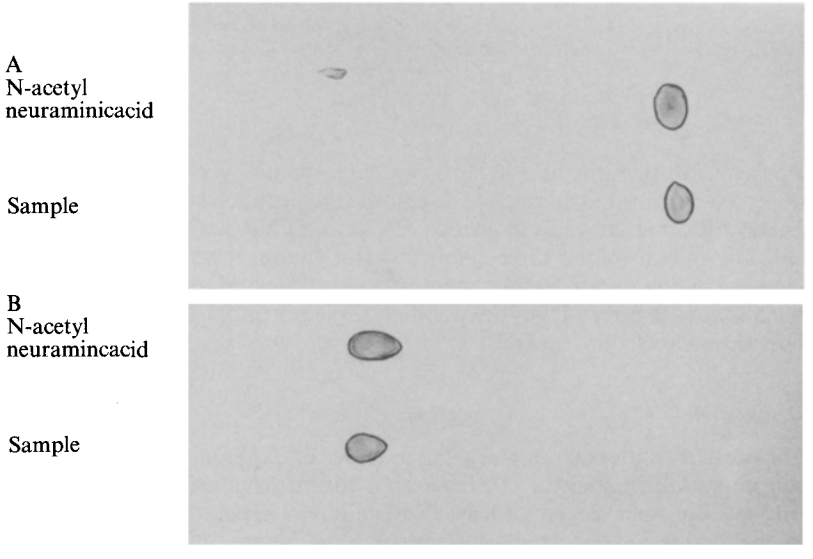

Figure 2. Paper chromatography of N-acetyl neuraminic acid. Solvent systems: *A* butyl acetate/acetic acid/water (3/2/1) (v/v); *B* butanol/propanol/0.1 N HCl (1/2/1) (v/v). Detection agent: by the method of Warren (see text).

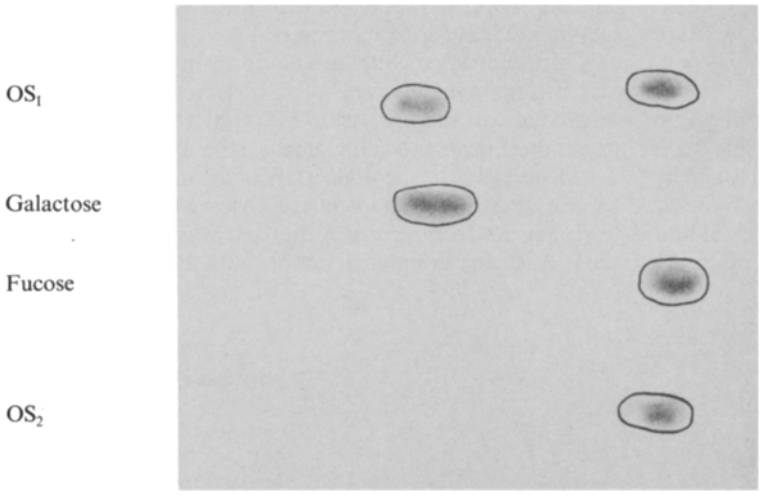

Figure 3. Paper chromatography of neutral sugars of OS_1 and OS_2. Solvent system: butanol/pyridine/water (6/4/3) (v/v). Detection agent: alkaline silver nitrate.

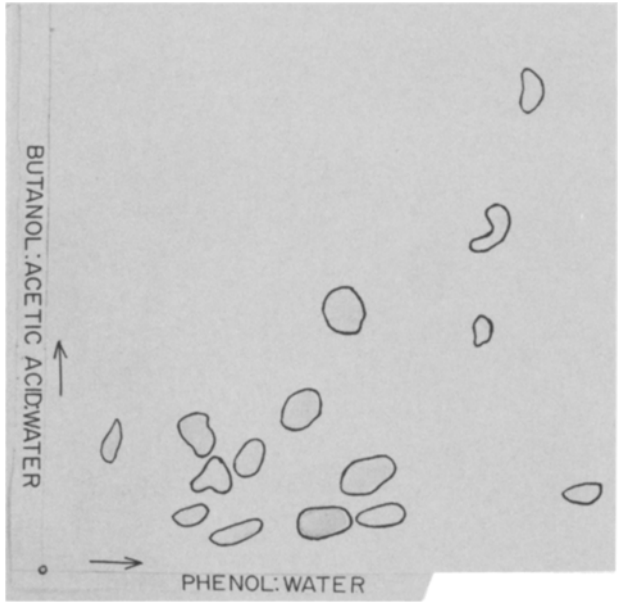

Figure 4. Two-dimensional paper chromatography of amino acids, after incubating jelly with toad testis extract. Solvent systems: 1st direction: butanol/acetic/water (4/1/5) (v/v); 2nd direction: phenol saturated with water. Detection agent: ninhydrin (see text).

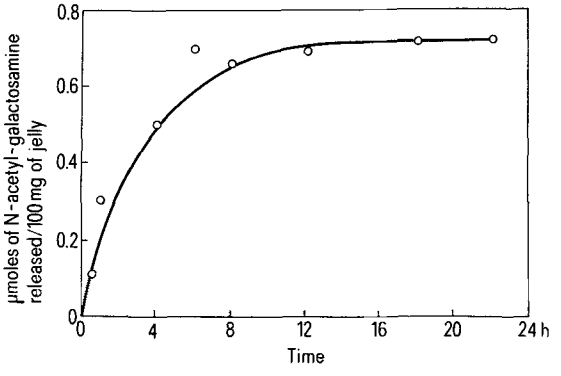

Figure 5. Ehrlich's reagent color during the incubation of jelly with toad testis extract (see text for details).

Enzyme activities in testes collected from different groups of toads

Enzymes	µmoles of PNP released/mg protein/h		
	Group A*	Group B*	Group C*
3-Galactosidase	6.05(3)	6.05(3)	3.1(2)
α-L-Fucosidase	4.00(3)	3.94(3)	1.2(2)
N-Acetyl-β-D-Galactosaminidase	6.50(3)	6.35(3)	3.2(2)
N-Acetyl-β-D-Glucosaminidase	8.50(4)	8.58(3)	25.53(3)

Numerals in parentheses indicate the number of experiments. *See text for details.

crease in absorption at 340 nm. No such change was observed in group D animals. The results of colorimetric assays for other enzymes in groups A–C using PNP-sugars, are shown in the table. As shown in figure 5, an increase in color of Ehrlich reagent was also observed in the aliquots removed from the nondialyzable portion, as time increased from 1 to 24 h.

Discussion

The present studies demonstrate the presence of oligosaccharidases which could cleave the carbohydrate group of jelly glycoprotein to give at least three oligosaccharides, after short periods of incubation, as shown in figure 1A. OS_1 was found to contain galactose, fucose and N-acetyl galactosamine while OS_2 contains only fucose and N-acetyl galactosamine. Both neutral sugars and hexosamines were present in OS_3. Since each oligosaccharide fraction differs from others in its composition, it is possible that there are oligosaccharidases which cleave at different positions of the oligosaccharide chain.

Absence of free neutral and amino sugars after 30 min of incubation and the presence of all these jelly constituents after 90 min of incubation indicates the successive action of several glycosidases, namely neuraminidase, β-galactosidase and α-L-fucosidase, N-acetyl glucosaminidase and N-acetyl galactosaminidase on jelly glycoprotein. The presence of all these activities was also confirmed by

colorimetric assays using PNP sugars as substrates (table).

The increase in free sialic acid content in the incubated sample is accounted for by the presence of sialidase in the testis extract. It was found to be absent in group D. Decrease in sialic acid content after prolonged incubation in the sample, suggests the possible presence of an enzyme which cleaves sialic acid. This was confirmed by the identification of neuraminic acid aldolase, which cleaved sialic acid to pyruvate and N-acetyl-D-mannosamine.

The results presented in figure 5 indicate the presence of an enzyme which cleaves the bond between a peptide and an N-acetyl galactosamine in carbohydrate chain. A similar type of increase was observed by Seshadri and Reddy[22], after treatment of jelly with sodium hydroxide and sodium borohydride. Oligosaccharides from the native jelly glycoprotein with N-acetyl galactosamine residues at the reducing end, attached to a peptide moiety, have in fact been isolated after sodium hydroxide and sodium borohydride treatment, purified and analyzed by us[24]. This enzyme may be similar to Endo-H activity, with respect to N-glycosidically linked glycoproteins[28, 31]. Further work to isolate and purify this enzyme is in progress. Release of all the amino acids present in jelly, as observed in the experiment illustrated in figure 4, indicates the presence of proteases.

The possibility that the activity of glycosidases may be synchronized with the reproductive periods in toads was indicated by the studies of these activities in the testes obtained under different conditions, as described in the table.

It has been reported that 'Spermatozoa remain active in the testis of frog throughout the entire period of hibernation and are liberated only under the influence of sex stimulating hormones during the early spring'[21]. Our findings are consistent with the above statement and show that there is a synchronization between the various enzyme activities and the presence or absence of spermatozoa in testis. There was no change in specific activity of enzymes in testis of group A and group B. Group C testis were almost devoid of motile sperms and a decrease in enzyme activity was noticed. However, N-acetyl glucosaminidase activity increases, for which no explanation can be offered at present.

Sialidase and neuramine acid aldolase activities appeared to decrease in testes of group C and were found to be absent in group D. (Srinivasa Rangan and Seshadri, unpublished; Keshava Kumar and Seshadri, unpublished). The presence of neuraminic acid aldolase has been reported earlier in microorganisms[5, 7], and in mammalian brain tissues[3]. However, there are no reports of its presence in the reproductive organs of amphibians. The presence of sialic acid has been reported in reproductive organs of various animals[6, 9]. We have also found sialic acid in toad testis. Hence, it is interesting to note the presence of both sialidase and neuraminic acid aldolase activities and their changes in different groups. The significance of this finding is under study in our laboratory.

Presence of enzyme activities in testis with spermatozoa and absence of these in testis without spermatozoa indicate, though not directly, that these jelly-degrading enzymes (glycosidases and proteases) play a role at the time of fertilization. They may be instrumental in cleaving the large molecular weight jelly into smaller bits. This could be the mechanism of cleavage of jelly layers in toads, which helps the embryo to escape from the jelly at the time of hatching. The studies suggest that dissolution of jelly layers is not merely by a combination of osmotic forces and the elongation of the embryo as described by Kobayashi[15] and Carroll[4], but that an enzymatic mechanism is also involved in the degradation. Although the interaction of sperm and jelly has been described, its exact mechanism is not clear. Despite the knowledge of the chemical nature of the jelly layers at the time of fertilization[33, 27], the fate of the jelly after fertilization is not fully understood. Experiments were undertaken by Hedrick to study the role of jelly[10] and investigate its utilization as an energy source during development. Results obtained were not consistent with earlier finding[17], and Hedrick suggested that jelly is not utilized as a nutrient. This point could not be proved, owing to experimental limitations.

The present experimental results enable suggestions to be made about the mechanism of cleavage of the jelly and its fate after fertilization. The testis enzymes may act on jelly and, as reported by Umbert and Hedrick[32], hatching enzyme detected in the embryo may be involved in the process of dissolution of the fertilization envelope, providing contact of the embryo with the degraded jelly constituents, and thus assisting the absorption of the jelly constituents for embryonic development. This suggestion is not consistent with the finding of Hedrick[9] but it supports the suggestion of Nace et al.[17] that, in *R. pipiens,* macromolecules secreted by the upper portion of the oviduct enter the oocyte. When a more complete understanding of these enzyme properties has been reached, it will be possible to investigate the exact physiological role played by the enzymes using studies of the incorporation of jelly constituents into the oocyte, in the presence and absence of these enzymes. These studies may also give validity to the proteolytic hypothesis mentioned by Gusseck[8].

The presence of sialidase may also be significant as it may be involved in the mechanism of blockage of polyspermy and initiation of jelly degradation. Removal of sialic acid inhibits fertilization[27] and also decreases the swelling capacity of jelly. After fertilization, it is possible that sialidase which accompanies the spermatozoa removes sialic acid and enables other glycosidases to remove other carbohydrates by sequential degradation, and thus helps the fertilized zygote to hatch out.

An investigation designed to increase our understanding of the mechanism of action, control of activity and importance of these enzymes during the hatching process is in progress.

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A redox cycling model for the action of β-adrenoceptor agonists[1,2]

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Summary. A cyclic redox mechanism for the action of β-adrenoceptor agonists is proposed. It has the following features: a) β-adrenoceptor agonists act by 'reductive activation' of the β-adrenoceptor (R); b) the redox state of R is reciprocally coupled to the redox state of the guanine nucleotide binding protein (G); c) binding of GTP to G reverses the agonist-induced alteration of the redox states of R and G; d) according to a specific version of the model the activation process involves a disulfide-thiol interchange reaction which leads to a GTP-revertible cross-linking of R and G by a disulfide bond. The way in which desensitization events may interfere with the proposed redox cycle is discussed.
Key words. β-Adrenoceptor; guanine nucleotide binding protein; redox cycle; disulfide-thiol interchange; desensitization.

Introduction

The catecholamines (adrenaline, noradrenaline, dopamine) represent an important class of neurotransmitters and hormones in the animal kingdom and in man. Their physiological and biochemical effects are quite diverse and often opposite (e.g. vasoconstriction or vasodilation, depolarization or hyperpolarization, stimulation or inhibition of adenylate cyclase). Furthermore, pharmacological and biochemical research has revealed the existence of at least six subtypes of catecholamine receptors: two α-adrenoceptors (α_1 and α_2)[9,18,71], two β-adreno-