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Characteristics of sperm acrosin-like activity of paddlefish (*Polyodon spathula* Walbaum)

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Abstract

Spermatozoa of paddlefish and sturgeon fishes (*Acipenseriformes*), unlike teleost fish, have an acrosome. The objectives of this study were to characterize acrosin-like activity of cryopreserved sperm of paddlefish (*Polyodon spathula*) and to test and compare stability of paddlefish acrosin-like activity with that of lake sturgeon and bull spermatozoa. Mean acrosin-like activity of cryopreserved paddlefish sperm was 0.372 ± 0.067 μU/10^6 spermatozoa. This activity was 79% higher in the whole semen than in spermatozoa. Highest activity was recorded at pH 8.0 and 8.5. Triton X-100, zinc ions and 4′-acetamidophenyl 4-guanidinobenzoate (AGB) inhibited the activity. Amidase activity was also inhibited by *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). TLCK at concentrations of 0.1 and 1.0 mM gave a significant decrease in activity of 19 and 61%, respectively. However, TPCK significantly inhibited amidase activity (by 19%) only at concentration 1.0 mM. After acidification and 60 min incubation at 4°C of sperm suspensions only 4% of the activity was retained. A similar phenomenon was observed in the case of lake sturgeon but not bull sperm. These results suggest that trypsin-like activity of Acipenserid fish resembles rather fish trypsin that mammalian one. In frozen–thawed paddlefish sperm a minute chymotrypsin-like activity was also indicated, when GPNA was used as substrate. This activity amounted to 0.0415 ± 0.0138 μU/10^6 spermatozoa and was 18% of total amidase activity. This suggests that chymotrypsin-like activity may also be present in paddlefish spermatozoa. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Acipenserid; Paddlefish; Sturgeon; Milt; Semen; Bull; Spermatozoa; Acrosin

1. Introduction

Paddlefish (*Polyodon spathula*, Walbaum) belongs to the order Acipenseriformes, consisting of families Acipenseridae (25 sturgeon species) and Polyodontidae (only two species: *P. spathula* and Chinese paddlefish, *Psephurus gladius*). Acipenseriformes fish are recognized as the most numerous living 'fossil' fishes because they has existed at least from Lower Jurrasic period (Bemis et al., 1997; Birstein and DeSalle, 1998). Acipenseri-

Abbreviations: AGB, 4′-acetamidophenyl 4-guanidinobenzoate; BAPNA, *N*-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; DMSO, dimethylsulfoxide; GPNA, *N*-glutaryl-L-phenylalanine *p*-nitroanilide; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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formes fish differ greatly from teleost fish in gamete biology and fertilization processes. In this order for example, both sperm with acrosome and eggs with numerous micropyles coexist (Linhart and Kudo, 1997). For this reason, their gametes may represent a transitional stage towards neopterigian conditions of a single micropyle on the egg and no acrosome on the sperm (Jamieson, 1991).

Acrosome of Acipenseriformes fish appears to be active, because acrosome reaction has been described (Cherr and Clark, 1984). The acrosome also has enzymes typical for this sperm structure. Recently, we have documented and characterized amidase activity in lake sturgeon (*Acipenser fulvescens*) and white sturgeon (*Acipenser transmontanus*) spermatozoa (Ciereszko et al., 1994, 1996a). This activity was inhibited by trypsin, but not chymotrypsin inhibitors, indicating the presence of trypsin-like activity in sturgeon spermatozoa. This activity resembles acrosin (EC 3.4.21.10), an enzyme characteristic for the acrosome. Acrosin-like activity shares many characteristics of mammalian acrosin, for example susceptibility to serine proteinase inhibitors and inhibition by zinc ions. However, species-specific characteristics of sturgeon acrosin-like activity, including inhibition by Triton X-100 and high catalytic efficiency at lower temperatures were described by Ciereszko et al. (1994, 1996a). These differences between mammalian and sturgeon acrosins may reflect adaptation of these enzymes to different challenges produced by conditions of internal and external fertilization. Given close relationship between Acipenseridae and Polyodontidae and similarity of their biology of reproduction (Brown and Mims, 1995; Linhart et al., 1995; Cosson and Linhart, 1996; Linhart and Kudo, 1997), the presence of acrosin-like activity in paddlefish spermatozoa may be anticipated.

Populations of paddlefish have dramatically declined, both in North America and China, due to anthropogenic influences, such as pollution, loss of spawning grounds and overfishing or illegal fishing (Graham, 1997; Wei et al., 1997). At present, in United States many federal and state hatcheries are propagating paddlefish for restoration and stock enhancement programs. This resulted in stabilization of paddlefish population in 14 states and an increase in three states (Graham, 1997). This species also appeared to be attractive for commercial aquaculture for use as a food (Mims and Shelton, 1998; Semmens and Shelton, 1986). In order to achieve optimization of technologies for gamete handling and fertilization, a better knowledge concerning sperm function is necessary (Brown and Mims, 1995). Knowledge of the acrosome function and biochemistry may be critical for improving short-term and long-term storage of paddlefish spermatozoa.

In this study biochemical properties of acrosin-like activity of paddlefish spermatozoa are described. The specific objectives were: (1) to examine effects of pH, Triton X-100, zinc ions, serine proteinase inhibitors (*N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and 4′-acetamidophenyl 4-guanidinobenzoate (AGB)) on acrosin-like activity; and (2) to test stability of this activity at acid pH in comparison with that of lake sturgeon and bull sperm.

2. Materials and methods

2.1. Reagents

Benzamidine hydrochloride, *N*-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA), dimethylsulfoxide (DMSO), Triton X-100, AGB, TPCK, TLCK and zinc chloride were obtained from Sigma (St. Louis, MO).

2.2. Semen collection and cryopreservation

Paddlefish males were caught in the Ohio River and maintained at the Aquaculture Research Center of Kentucky State University, Frankfort, USA. Stimulation of spermiation and milt collection was performed as previously described (Brown and Mims, 1995). Lake semen sturgeon was obtained as previously described (Ciereszko et al., 1994, 1996a). To protect acrosin-like activity semen was cryopreserved using DMSO-sucrose extender (Ciereszko et al., 1996a,b). Cryopreserved semen of Holstein–Fresian bulls was obtained from Selected Sires Cooperative, Plain City, OH.

2.3. Amidase activity assay

Amidase activity towards BAPNA of frozen–thawed milt was measured according to the

method described by Kennedy et al. (1989) with few modifications. To 0.45 ml 100 mM buffer Tris–HCl, pH 8.5 we added to a such volume of frozen–thawed semen (65–250 μl), to ensure $100–200 \times 10^6$ spermatozoa in the reaction mixture. The volume of the reaction mixture was adjusted to 1 ml with double distilled water. The reaction was started by addition of 0.1 ml BAPNA in DMSO. Samples (control and experimental) were incubated for 6 h at 28°C. A control suspensions employed in the assay contained benzamidine (trypsin and acrosin inhibitor) in the reaction mixture. Absorbance of controls were subtracted from absorbance of experimental solutions. This excluded any errors related to non-specific hydrolysis of BAPNA. One unit (U) of amidase activity was defined as the amount of enzyme that hydrolyzes 1 μM BAPNA/min at 28°C. Activity was expressed in $\mu U/10^6$ spermatozoa, using the formula of Kennedy et al. (1989). During preliminary experiment activity of whole frozen–thawed semen was found to be 79% higher than washed spermatozoa, likely due to leakage of this activity from sperm after freezing–thawing. For this reason, whole frozen-thawed semen was used in these experiments. Amidase activities of sturgeon and bull spermatozoa were measured according to Ciereszko et al. (1994, 1996a) and Kennedy et al. (1989), respectively. Acrosin activity of bull semen was measured in sperm suspension in order to make comparative studies at the same conditions. Chymotrypsin-like activity was measured in above conditions, but GPNA was used instead of BAPNA.

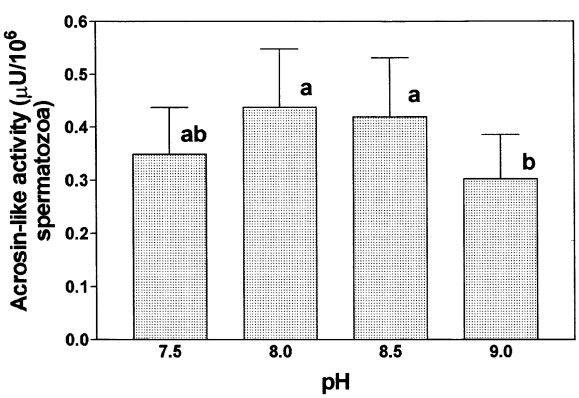

Fig. 1. Effect of pH on acrosin-like activity of paddlefish spermatozoa ($n = 3$). Means followed by different letters are significantly different ($P < 0.05$).

2.4. Effects of pH, Triton X-100, protease inhibitors, Zn^{2+} and acidification on amidase activity

The effect of pH was tested with the use of 50 mM Tris–HCl buffer at pH 7.5, 8.0, 8.5 and 9.0. Triton X-100 and $ZnCl_2$ were dissolved in 50 mM Tris–HCl buffer, pH 8.5. TPCK, TLCK and AGB were dissolved in DMSO. Appropriate amounts of DMSO were added to control tubes (without inhibitors) to ensure the same concentration (16.7%) of this compound in both experimental and control variants. In order to test effects of acidification on acrosin-like activity, three variants were tested: control 1 (no acidification of sperm suspensions), control 2 (acidification with 2% acetic acid and immediate neutralization with 0.5 M Tris–HCl buffer, pH 8.5, and experimental (acidification with 2% acetic acid, pH of suspensions was 2.6). All sperm suspensions were incubated for 1 h at 4°C. After this period experimental suspensions were neutralized with 0.5 M Tris–HCl buffer, pH 8.5, and amidase activity was measured in all samples. Further, this experiment was repeated with cryopreserved lake sturgeon semen (*A. fulvescens*) and bull semen.

2.5. Statistical analysis

Values within figures are presented as means ± S.E.M. One-way analysis of variance was employed to evaluate the differences between treatments. The Tukey test was used for post hoc comparisons.

3. Results

Acrosin-like activity was found in cryopreserved paddlefish spermatozoa. An average activity was 0.372 ± 0.067 $\mu U/10^6$ spermatozoa (range 0.204–0.731 $\mu U/10^6$, $n = 8$). A small, but detectable amount of chymotrypsin-like activity was also found towards GPNA (0.042 ± 0.014 $\mu U/10^6$ spermatozoa) which was only 18.0 ± 6.0% of the activity seen using BAPNA.

Optimum pH of acrosin-like activity was between 8.0 and 8.5 (Fig. 1). Triton X-100 slightly inhibited (by 11%) this activity at 0.01% concentration (Fig. 2). This inhibition was significant (57%, $P < 0.05$) at 0.1%. For this reason, Triton X-100 was omitted from the reaction mixture.

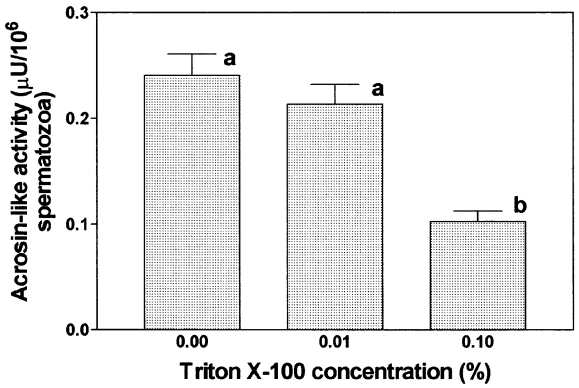

Fig. 2. Effect of Triton X-100 concentration on acrosin activity of paddlefish spermatozoa ($n = 3$). Means followed by different letters are significantly different ($P < 0.05$).

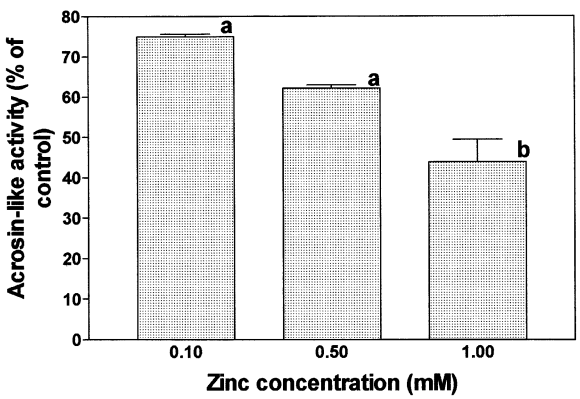

Fig. 3. Effect of zinc concentration on acrosin-like activity of paddlefish spermatozoa ($n = 3$). Means followed by different letters are significantly different ($P < 0.05$).

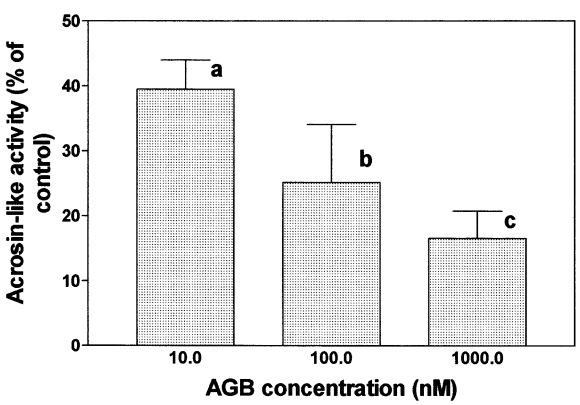

Fig. 4. Inhibition of paddlefish sperm acrosin activity by AGB ($n = 3$). Means followed by different letters, with the inhibitor treatment, are significantly different ($P < 0.05$).

Zinc inhibited the acrosin-like activity, and reduced it by about 15, 38 and 56% in the presence of 0.1, 0.5 and 1.0 mM $ZnCl_2$, respectively (Fig. 3). AGB appeared to be a very effective inhibitor of the activity (Fig. 4). This inhibitor at 10 nM concentration reduced the trypsin-like activity by about 60%. Both TLCK and TPCK reduced amidase activity (Fig. 5). This inhibition was more effective in case of TLCK. However, at 1 mM TPCK concentration a significant ($P < 0.05$) inhibition was also observed (approximately 19%).

Acidification of sperm suspensions caused almost a total inhibition (by 96%) of acrosin-like activity of paddlefish sperm (Fig. 6A). Sturgeon acrosin was also significantly (64%, $P < 0.05$) inhibited (Fig. 6B). On the other hand, acrosin activity of bull spermatozoa remained unchanged after acidification (Fig. 6C). It should be noted that acrosin-like activity of paddlefish sperm was about 10-times lower than that of sturgeon sperm. Acrosin activity of bull spermatozoa was 370 and 35-times higher than that of paddlefish and sturgeon sperm, respectively.

4. Discussion

The presence of trypsin-like activity in paddlefish spermatozoa corresponded with the presence of an acrosome in the order of Acipenseriformes, which includes more than 20 species of sturgeons and two species of paddlefish worldwide. It is believed that sperm acrosome of Acipenserid fishes is active and important for fertilization, despite the presence of micropyles in

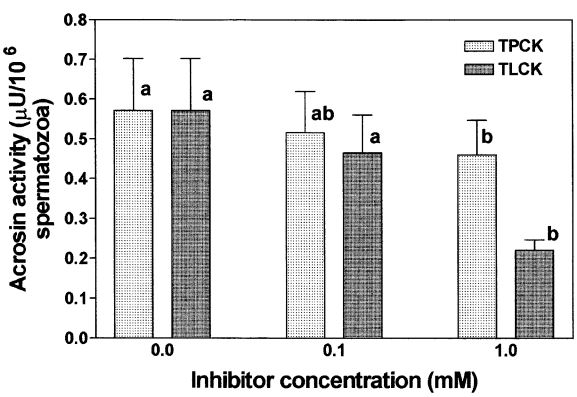

Fig. 5. Effects of TPCK and TLCK on acrosin-like activity of paddlefish spermatozoa ($n = 3$). Means followed by different letters, within the inhibitor treatment, are significantly different ($P < 0.05$).

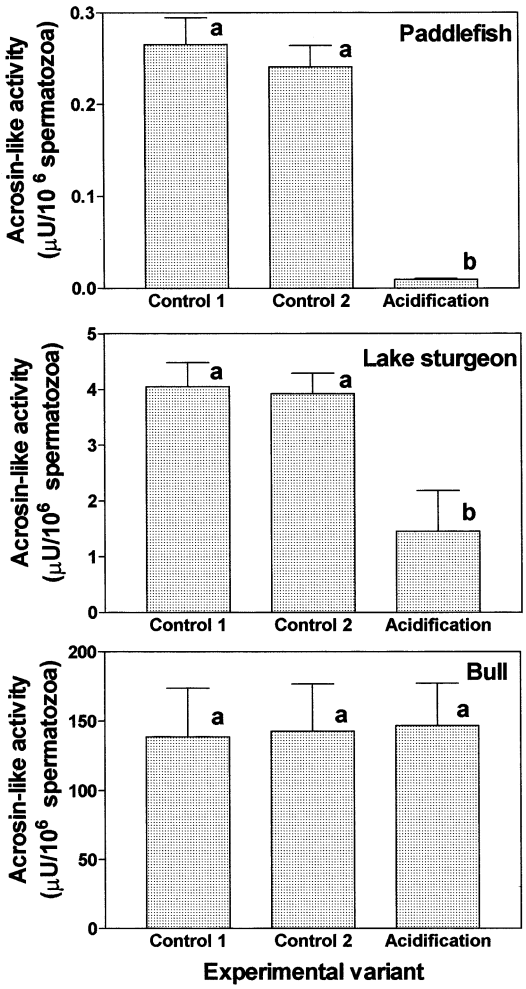

Fig. 6. Effect of acidification with 2% acetic acid on acrosin-like activity of paddlefish, lake sturgeon and bull spermatozoa ($n = 3$). Control 1 (no acidification of sperm suspensions), control 2 (acidification with 2% acetic acid and immediate neutralization with 0.5 M). Tris–HCl buffer, pH 8.5, and experimental (acidification with 2% acetic acid). Means followed by different letters are significantly different ($P < 0.05$).

eggs and usage of them by spermatozoa as a route for fertilization (Cherr and Clark, 1984). In view of earlier results (Ciereszko et al., 1994, 1996a), indicating the presence of acrosin-like activity in spermatozoa of lake and white sturgeons, the presence of this activity in paddlefish sperm was anticipated. The main characteristics of paddlefish acrosin appeared to be very similar to that of sturgeon acrosin, due to similar effects of pH, trypsin inhibitors, and zinc ions on amidase activity. However, it is now clear that there are species-specific characteristics of acrosin among Acipenserid fishes. For example, acrosin-like activity of paddlefish sperm was 10 and 4-times lower than that of white sturgeon and lake sturgeon, respectively (Ciereszko et al., 1994, 1996a). The results also suggested that unlike in lake sturgeon sperm, a chymotrypsin-like activity (like in mammalian sperm Morales et al., 1994), may be present in paddlefish sperm. This suggestion is based on the ability of paddlefish sperm to hydrolyze GPNA and inhibition of amidase activity by TPCK. It remains to be established, how species-specific differences in quality and quantity of sperm proteolytic enzymes of particular Acipenserid species are related to their reproductive biology.

The role of acrosin-like activity in reproductive biology of Acipenserid fish may be related to the function of male gametes and fertilization process. Acrosin may participate in acrosome reaction (as in mammals, De Jonge et al., 1989) and penetration of egg envelopes. The latter function of acrosin as a sperm lysine is puzzling, considering presence of egg's micropyles. However, in Acipenserid fish a delay in fertilization is possible, due to prolonged viability of sturgeon gametes in freshwater. Therefore there may be a need for sperm lysin action, because longer contact of oocytes with water may cause obstruction of micropyle by jelly coat. This hypothesis proposed by S.I. Doroshov (S.I. Doroshov, personal information) may explain why acrosin-like activity was retained on spermatozoan of Acipenserid fish, despite the presence of micropyles in egg.

Despite many similarities between trypsin-like activity of Acipenserid fish and mammalian acrosin, there are substantial differences between these activities. As was pointed out before (Ciereszko et al., 1996a) these differences include Triton X-100 effect (which is stimulatory at low concentrations toward mammalian acrosin (Kennedy et al., 1989; Glogowski et al., 1998) and effect of temperature on acrosin activity (sturgeon enzyme is more active in lower temperatures). Results from this study indicate that the stability of acrosin at low pH may be one of fundamental differences between fish and mammalian sperm proteolytic enzymes. Fish acrosins, unlike mammalian enzymes, are inactivated at low pH. Therefore, it has to be emphasized, that similar differences exist between fish and mammalian proteolytic enzymes, including trypsins (Asgeirsson et al., 1989; Raae and Walther, 1989; Asgeirsson and Bjarnason, 1991; Kristjansson,

1991; Bjarnason et al., 1993; Outzen et al., 1996). It is possible, that evolution of sperm proteinases might be similar to the evolution of other proteolytic enzymes. In general it is assumed that structure of proteinases of ectothermic organisms (including fish) is better suited for catalysis at lower temperatures than those of endothermic organisms (mammals). A molecular basis for this phenomenon may be related to a loose-fit tertiary polypeptide structure of fish enzymes which would enhance ability of these enzymes to lower the activation energy (Low and Somero, 1974).

Studies of acrosome enzymes may be crucial for development of optimal cryopreservation procedure of Acipenserid milt. This task is important in view of the fact, that many of these species have become threatened or endangered. According to the authors' knowledge, consistent cryopreservation technologies are not available at the present. Brown and Mims (1999) were the first to cryopreserve paddlefish milt and obtained hatched larvae. However, good motility rate did not give similar fertilization and hatching rate, indicating possible structure damage to the frozen-thawed spermatozoa of paddlefish. It seems that it is rather easy to preserve sperm motility by cryopreservation (Ciereszko et al., 1996b). An excellent sperm motility was also observed in the samples of cryopreserved paddlefish milt (data not shown). On the other hand, usually sperm fertilizing ability of such sperm is poor, despite good preservation of motility apparatus. It suggests that critical injuries to sperm cells due to cryopreservation process might be done to other sperm structures, including the acrosome. Earlier results indicated that sturgeon acrosin-like activity is very susceptible to freezing–thawing. This activity may be well preserved when a cryoprotectant (DMSO) is used. On the other hand, it has to be tested in the future, if this activity can leak from spermatozoa, indicating damage to acrosome. The potential usage of acrosin-like activity for this purpose is supported by data indicating, that only 29% of this activity was present in spermatozoa from the frozen–thawed samples, which might be a result of damage to sperm acrosome.

Similar phenomenon of leakage of enzymes due to sperm injury was also observed for other enzymes of teleost fish (Ciereszko and Dabrowski, 1994; Glogowski et al., 1996; Lahnsteiner et al., 1996).

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