Proteolytic and anti-proteolytic activity in the seminal plasma of Eurasian perch (*Perca fluviatilis* L.) during the spawning period

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ABSTRACT: The aim of this study was to describe possible changes in protease and anti-protease activity in the seminal plasma of European perch (*Perca fluviatilis* L.) during the spawning time. No significant difference in proteolytic activity was observed between the beginning and the late period of the spawning season in perch. Anti-protease activity significantly increased during the sampling time. Electrophoretic profiles of gelatinolytic activity in the seminal plasma of perch were characterized by four molecular forms, which depend on the presence of Ca^{2+} during incubation. We also found two forms of caseinolytic activity with low molecular weights, which were independent of calcium ions. However, both activities were fully stopped by the chelator of calcium ions (EDTA). In this study, non-typical profiles of gelatinolytic activity were also observed. Profiles of protease activities in the perch seminal plasma are constant during the reproduction season indicating that the regulation of protease activity in seminal plasma occurs via protease inhibitors which are abundant in this fluid. Results concerning electrophoresis revealed at least seven forms of anti-proteases in the seminal plasma of perch. Concluded, anti-proteases comprise a high percentage of all proteins in the seminal plasma of perch, while the increase at the end of spawning season is probably protecting spermatozoa during the spawning time in perch testes.

Keywords: European perch; seminal plasma; protease and anti-protease activity

During the last years, European perch (*Perca fluviatilis* L.) has gained an increasing interest in the freshwater aquaculture sector in Europe (Kestemont and Melard, 2000). Success in fish production principally depends on artificial reproduction methods used in hatcheries and the latter depends on the quality of both male and female gametes. Besides sperm quality parameters and semen osmolality (Alavi et al., 2007, 2010), an important role in determination of fertilization capacity could be played by other components in fish seminal plasma, i.e. proteins. Recently, several protein groups were identified in fish seminal plasma: lipoproteins (Loir et al., 1990), metalloproteases, serine proteases (Kowalski et al., 2003a), transferrin (Wojtczak et al., 2005) and anti-proteases (Ciereszko et al., 2000). Both proteolytic and anti-proteolytic activity has been found in the seminal plasma of several teleost and sturgeon fish species (Dabrowski and Ciereszko, 1994; Ciereszko et al., 1996a, 2000; Lahnsteiner et al., 1997, 1998; Kowalski et al., 2003a).

The role of proteases in fish seminal plasma is not well understood yet. The proposed function of the existent protease-protease inhibitory system in seminal plasma is the protection of spermatozoa against a proteolytic attack during the spawning

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season (Ciereszko et al., 2000). In previous studies it was found out that seminal plasma proteins affect sperm physiology in Nile tilapia (*Oreochromis niloticus* L.) (Mochida et al., 1999) and prolong the viability of rainbow trout (*Oncorhynchus mykiss* Walbaum) spermatozoa, as measured by sperm motility (Lahnsteiner et al., 2004; Lahnsteiner, 2007).

Among the fish examined, perch is characterized by one of the highest values of protein concentration in seminal plasma (Lahnsteiner et al., 1995) which increases during the spawning period (Krol et al., 2006). On the other hand, anti-protease activity and protein concentration decrease during the reproduction season of rainbow trout (Ciereszko et al., 1996b). Mommens et al. (2008) found out that anti-proteolytic activity but not protein concentration decreased during the spermiation period in Atlantic halibut (*Hippoglossus hippoglossus* L.). The previous studies on proteolytic activity in the seminal plasma of teleost fish showed that a major group of proteases were metalloproteases and serine proteases (Kowalski et al., 2003a, 2004), but nothing is known about changes in their activity during the spawning period. We stated that the observation of changes in protease and anti-protease activities in seminal plasma during the reproduction season of European perch could be important for the knowledge of the function of these proteins.

MATERIAL AND METHODS

Fish and milt collection

In both years, perch were collected at the end of March from the Drwęckie Lake located in the Masurian Lake District in northeast Poland (53°42'N; 19°55'E) using gill nets. After catching, fish were transported to the laboratory at the Department of Ichthyology, University of Warmia and Mazury in Olsztyn. In both years, fish were kept under constant thermal conditions at 10 ± 0.5°C. In the first year fish were divided into two experimental groups from which the semen was taken at different times: at the beginning (P1) and at the late period (P2) of the spawning season. In the second year, the two experimental variants were repeated for groups P3 and P4, respectively (Table 1).

Before semen collection, fish were anesthetized in 2-phenoxyethanol (Sigma-Aldrich®, St. Louis, USA) solution (0.3 ml/l). Milt samples were collected by stripping so as to avoid the water, urine and faeces contamination, and transported on ice (2°C) to the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn. Seminal plasma was obtained by centrifugation (8000 g, 10 min) within 12 h after sampling and stored at –70°C until analysed.

Analytical methods

Proteolytic activity in the seminal plasma of perch was measured with azoalbumin (Sigma-Aldrich®, St. Louis, USA) (1.2% in 0.01M phosphate buffer pH 7.5) as a substrate according to the method described by Bergmeyer (1985). Preliminary enzymatic assays for seminal plasma were done at 30°C and an incubation time of 10 h was used. One unit (U) is defined as the amount of enzyme which catalyses the release of azo dye causing $\Delta A/\Delta t$ = 0.001 min, where A is absorbance and *t* is time in min (Bergmeyer, 1985).

The anti-protease activity of seminal plasma was evaluated by the inhibition of cod trypsin activity (Ciereszko et al., 1994, 1996a; Bowden et al., 1997). Inhibitory activities were expressed as unit per litre (U/l). One inhibitory unit (U) corresponds to the apparent amount of inhibitor able to block one unit of trypsin activity (defined as hydrolysis of 1μM of BAPNA min).

Electrophoresis for proteolytic and anti-proteolytic activity was performed in a SE 250 vertical

Table 1. Description of experimental groups, number of males and time of milt collection

Year of study	Experimental groups	Number of males	Time of milt collection
1st	P1	15	April 12
1st	P2	13	May 10
2nd	P3	15	April 15
2nd	P4	16	May 9

Table 2. Proteinase and anti-proteinase activity of perch seminal plasma

Experimental groups	Protein concentration (g/l)*	Proteinase activity (U/l)	Anti-proteinase activity (U/l)
P1	$4.82^{a} \pm 1.38$	–	$418.66^{a} \pm 187.33$
P2	$6.14^{b} \pm 2.16$	–	$723.98^{b} \pm 421.53$
P3	$3.95^{a} \pm 1.23$	$1.02^{a} \pm 0.35$	$265.55^{a} \pm 96.39$
P4	$5.16^{c} \pm 1.36$	$0.99^{a} \pm 0.23$	$343.78^{a} \pm 187.84$

Results are presented as means in groups ± standard deviation, values in columns with different letter index differ significantly $P < 0.05$

*data of protein concentration was used from Krol et al. (2006)

Mighty Small II electrophoresis system (Amersham Biosciences, AB, Uppsala, Sweden) at 200 V and 40 mA (for two gels). Electrophoresis for proteolytic activity was performed according to the method described by Siegel and Polakoski (1985). Samples of seminal plasma (21 µl) were subjected to electrophoresis in gelatine or casein-containing (0.1% of substrate) polyacrylamide (12% acrylamide) gels in the presence of SDS (sodium dodecyl sulphate) under non-reducing conditions. Following electrophoresis, the gels were washed with 2.5% Triton X-100 at room temperature for 30 min and then the gels were incubated at 37°C for 5 h in a development solution (50mM Tris-HCl buffer pH 7.5 containing: 200mM NaCl, 0.2% Triton X-100). After incubation the gels were stained in 0.025% Coomassie Brilliant Blue for 16 h. Stained gels were stored in 2% acetic acid. The areas of proteolysis appeared as clear zones against a blue background. Molecular mass estimations were done using prestained protein standards (Sigma-Aldrich®, St. Louis, USA): α2-macroglobulin 205 kDa; β-galactosidase 112 kDa; fructose-6-phosphate kinase 87 kDa; pyruvate kinase 69 kDa; fumarase 56 kDa; lactic dehydrogenase 38.5 kDa and triosephosphate isomerase 33.5 kDa. Kodak1D program (Eastman Kodak Company, New Haven, USA) was used for the estimation of molecular masses of proteolytic bands. Anti-proteolytic activity in the seminal plasma of perch was detected according to Uriel and Berges (1968). Following electrophoresis, the gels were incubated at 37°C for 15 min with a fresh solution containing bovine trypsin in 0.1M phosphate buffer (pH 7.4) and then transferred into a solution containing a chromogenic substrate (acetyl-DL-phenylalanine-β-naphthyl ester) for trypsin. Stained gels were stored in 2% acetic acid. The zones possessing inhibitory activity against bovine trypsin appeared as unstained bands on a coloured background.

The effect of two protease inhibitors, EDTA (which inhibits metalloproteases through chelation of bivalent ions) and serine protease inhibitor Benzamidine, on protease activities in gelatine and casein-substrate polyacrylamide gels was examined. Samples (21 µl) of seminal plasma were electrophoresed in substrates containing SDS-polyacrylamide gels. After electrophoresis the gels were washed in 2.5% Triton X-100 as described above and then incubated with or without 5mM $CaCl_2$ in development buffers containing either 10mM Benzamidine or 5mM EDTA.

Protease and anti-protease activity in seminal plasma was statistically analysed using one-way analysis of variance (ANOVA). The Fisher test was used for post-hoc comparisons with a significant level at $P < 0.05$.

RESULTS

The low quantity of seminal plasma did not enable to perform the analysis of proteolytic activity in the first year of investigation. No significant difference in proteolytic activity was observed between the beginning and the late period of the spawning season in the second year (Table 2). Means values of anti-proteolytic activity in the seminal plasma of perch ranged from 265.55 to 723.98 U/l (Table 2). The average of this activity increased significantly at the late period in comparison with the beginning of the spawning season only in the first year.

Electrophoretic profiles of gelatinolytic activity in the seminal plasma of perch were characterized by four bands with molecular weights approximately 87, 75, 70 and 56 kDa. No difference in bands of ge-

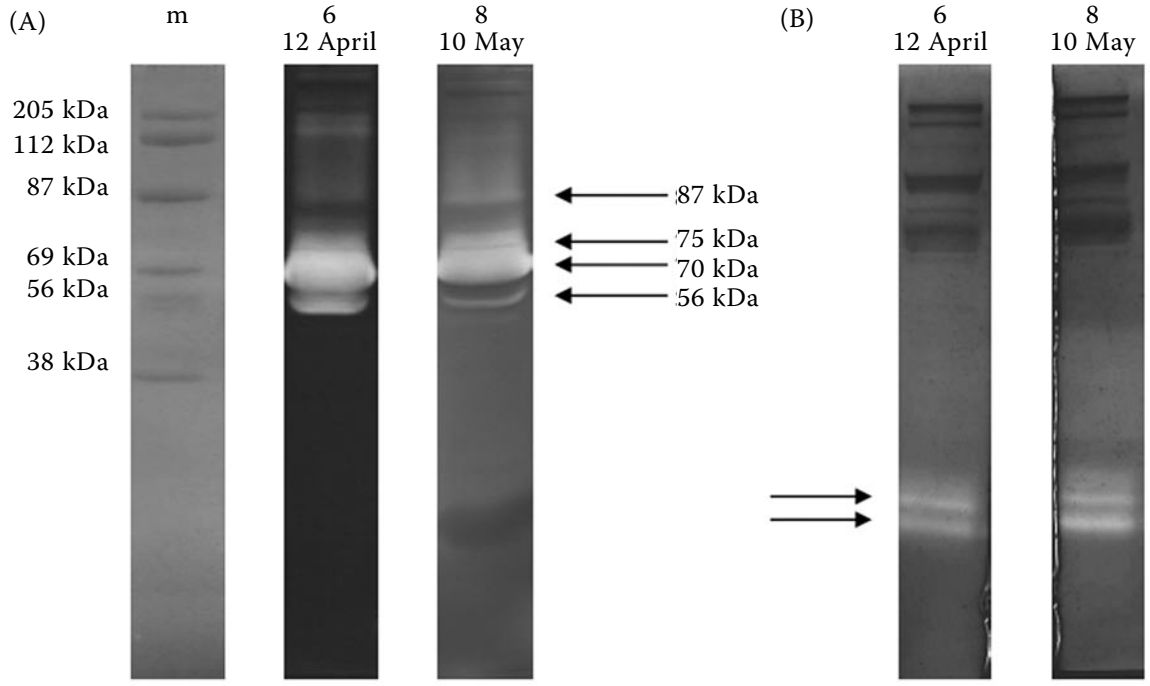

Figure 1. Electrophoretic profiles of gelatinolytic (A) and caseinolytic (B) protease activities in perch seminal plasma. Each profile characterizes protease activities in seminal plasma of one male, which were appointed to serial number and date of collecting milt; m = standards of molecular masses

latinolytic activity was observed between the beginning and the late period of the spawning season in either year (Figure 1A). We also found two forms of caseinolytic activity in the seminal plasma of perch. Both forms had low molecular weights and were not detected in gelatinolytic activity (Figure 1B). In two cases non-typical profiles of gelatinolytic activity were observed which were characterized by present additional bands of protease activity with low molecular weights (Figure 2).

The effect of metalloprotease and serine protease inhibitors was examined in the gelatine and casein-substrate polyacrylamide gels. All gelatinolytic activities in the seminal plasma of perch depended on the presence of Ca^{2+} during incubation (Figure 3A, D) in contrast to bands of caseinolytic activities which were independent of calcium ions. (Figure 4A, D). Both gelatinolytic (Figure 3B, E) and caseinolytic (Figure 4B, E) activity was fully stopped after incubation in EDTA. The addition of Benzamidine to the incubation buffer without $CaCl_2$ caused a full inhibition of all gelatinolytic activities (Figure 3F) contrary to the situation when Benzamidine was added to the incubation buffer with $CaCl_2$ (Figure 3C). Bands of protease activity in the casein-substrate polyacrylamide gels were

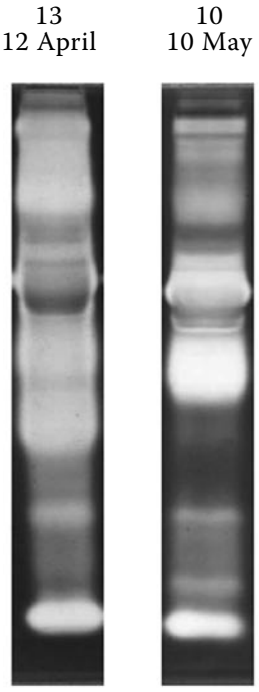

Figure 2. Non-typical electrophoretic profiles of gelatinolytic protease activities in perch seminal plasma. Each profile characterizes gelatinolytic protease activities in seminal plasma of one male, which were appointed to serial number and date of collecting milt

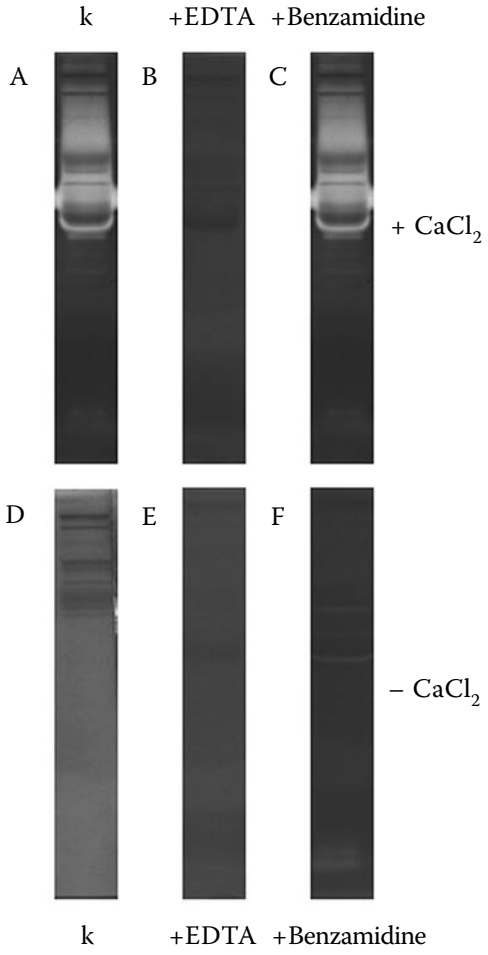

Figure 3. The effect of EDTA and Benzamidine in the absence and presence of Ca ions on gelatinolytic protease activities in seminal plasma of perch. Control with (A) and without (D) $CaCl_2$, incubation in EDTA with (B) and without (E) $CaCl_2$, incubation in Benzamidine with (C) and without (F) $CaCl_2$

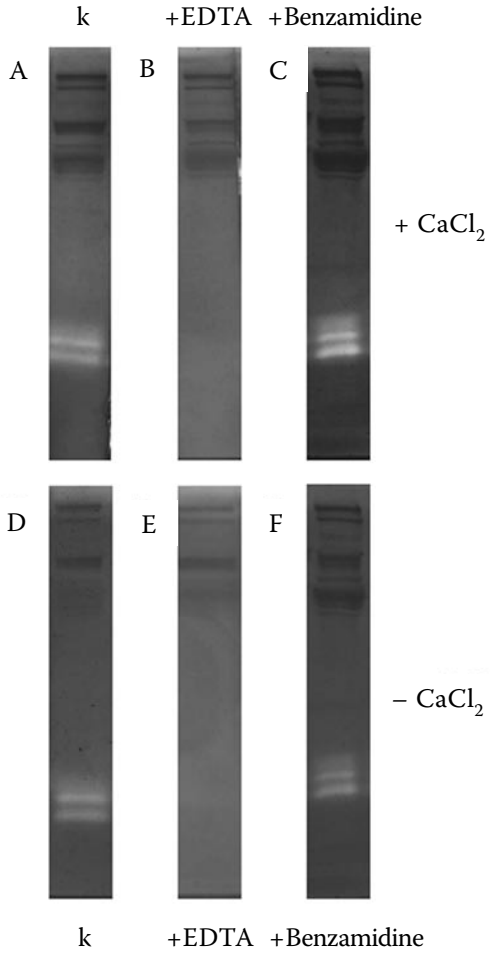

Figure 4. The effect of EDTA and Benzamidine in the absence and presence of Ca ions on caseinolytic protease activities in seminal plasma of perch. Control with (A) and without (D) $CaCl_2$, incubation in EDTA with (B) and without (E) $CaCl_2$, incubation in Benzamidine with (C) and without (F) $CaCl_2$

not affected by Benzamidine independently of the presence or absence of $CaCl_2$ during incubation (Figure 4C, F).

At least seven bands of protease inhibitors with different migration rates were found out (Figure 5). No difference in the bands of anti-protease activity was observed between the beginning and the late period of the spawning season in either year.

DISCUSSION

The present data confirm results obtained by Kowalski et al. (2003a) concerning the presence of proteolytic activity in perch seminal plasma. However, the very low value of such activity indicates that conventional methods for detecting protease activity in seminal plasma have a limited use, which is caused by the abundant presence of protease inhibitor in this fluid.

The physiological functions of these enzymes and other protein components of teleost sperm are not well understood and the available data have mostly been obtained from mammals (Li et al., 2009). Mammalian semen contains numerous proteolytic enzymes, e.g. cysteine, serine and metalloproteases, which are involved in many physiological functions in semen. Some of them are involved in coagulation and liquefaction of semen and proteolysis of seminal proteins (Wilson et al., 1993). Protease activity is also involved in the motility of fish spermatozoa. Serine protease with Lys- and Arg-ester bond spe-

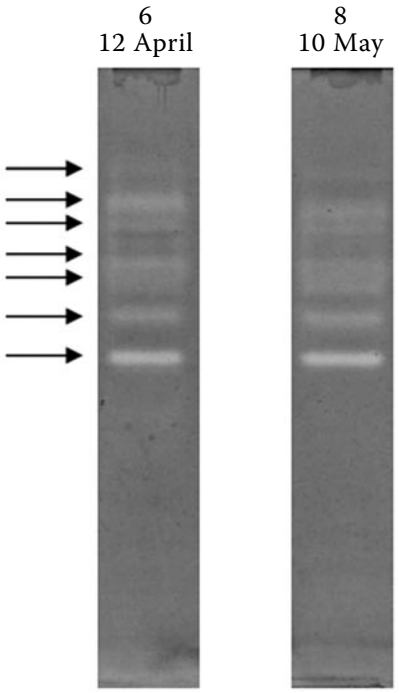

Figure 5. Electrophoretic profiles of anti-proteolitic activities in perch seminal plasma. Each profile characterizes anti-protease activities in seminal plasma of one male, which were appointed to serial number and date of collecting milt. The estimated molecular forms of the anti-protease activities are given on the left side of the figure

cificity is involved in the control of sperm motility in carp (*Cyprinus carpio* L.) (Cosson and Gagnon, 1988). They also concluded that the motility of common carp spermatozoa is inhibited by serine protease inhibitors. Moreover, proteases were purified and found to be localized along the sperm flagella of salmonid fish, which could probably regulate flagellar motility (Inaba et al., 1993). The lack of significant differences in proteolytic activity in perch seminal plasma between the beginning and the late period of the spawning season in this species could result from anti-proteolytic activity which increased during the same time. The increase of anti-protease activity and protein concentration (Krol et al., 2006) in seminal plasma during the reproduction season in perch suggests the existence of the protease-protease inhibitory system which protects spermatozoa against proteolysis during spermiation.

The profiles of gelatinolytic activity revealed four bands in the seminal plasma of perch without individual differences in the number of bands. Similar results were obtained by Kowalski et al. (2003a) where the profiles of gelatinolytic activity of seminal plasma from perch were very constant contrary to gelatinolytic activity in the seminal plasma of other teleost species e.g. pike (*Esox lucius* L.), which showed high individual variability. Large individual differences in bands of proteolytic activity in the seminal plasma of Atlantic halibut were also reported (Mommens et al., 2008). In two cases non-typical profiles of gelatinolytic activity were observed which was characterized by the presence of additional bands of protease activity. Perhaps it could be related to milt contamination by blood or slime which was not monitored visually during the sperm collection (Wlasow et al., 1999; Ciereszko et al., 2004). Or the blood plasma and skin mucus contained proteases that were not present in the fish seminal plasma (Kowalski et al., 2003b). Additionally we found two common bands of caseinolytic activity with low molecular masses that confirmed the existence of substrate specificity of proteolytic enzymes in the seminal plasma of fish. Both gelatinolytic and caseinolytic proteinase activities were previously found in the seminal plasma of humans and rats (Wilson et al., 1992, 1993).

Gelatinolytic activity in the seminal plasma of 10 teleost fish species including perch was described for the first time by Kowalski et al. (2004). These authors suggested that metalloproteases and serine proteases were major gelatinolytic proteases in the seminal plasma of fish. In our work we found out only gelatinolytic activities in the perch seminal plasma that appeared to be metalloproteases on the basis of their stimulation by calcium ions and inhibition by EDTA. Gelatinolytic activities inhibited by the serine protease inhibitor Benzamidine were not found out. Contrary to gelatinolytic activities two common bands of caseinolytic activity were independent of the presence or absence of $CaCl_2$ during incubation, but they were fully stopped after incubation in EDTA and they were not affected by Benzamidine either. This suggests that their activity might be related to other than calcium bivalent ions.

The mean values of anti-protease activity in the perch seminal plasma slightly increased at the end of the spawning season contrary to the observation during the reproduction season of rainbow trout (Ciereszko et al., 1996b) and Atlantic halibut (Mommens et al., 2008) where such activity significantly decreased. Results concerning the separation of anti-proteases after electrophoresis revealed seven bands of this activity in the seminal plasma of perch. It was remarkably more than had been reported in other teleost species (2 – 3 bands). These

observations suggest that anti-proteases comprise a high percentage of all proteins in the seminal plasma of perch and play a major role in protecting spermatozoa against an auto-proteolytic attack during the spawning season in perch testes.

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