

Gelatinolytic and anti-trypsin activities in seminal plasma of common carp: relationship to blood, skin mucus and spermatozoa

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Received 29 November 2002; accepted 23 May 2003

Abstract

Proteases and protease inhibitors were detected in the seminal plasma, blood plasma, skin mucus and spermatozoa. Their molecular weights were estimated using SDS-PAGE under non-reducing conditions. The results demonstrate that the two main bands of anti-proteinase activity (APA) detected earlier in common carp seminal plasma with molecular weight of approximately 47 and 58 kDa are also present in other fluids. The intensity of staining was highest in blood and seminal plasma. The intensity in skin mucus was visible, but in a sperm extract it was faint. An additional fast migrating band (30 kDa) was observed only in seminal plasma. Highest APA was found in blood and seminal plasma followed by skin mucus. The activity in sperm extracts was low. The two serine-like proteases with molecular weight of 79 and 189 kDa in seminal and blood plasma have also been found. In skin mucus, protease of 79 kDa was also present. Metalloproteinases with molecular weights of 61 and 69 kDa were found in seminal and blood plasma but metalloproteinases of 44 and 38 kDa were observed only in seminal plasma. Although metalloproteinases, of molecular weight ranging between 61 and 75 kDa, were also visible in a sperm extract, the experimental approach used in this study did not allow unequivocal identification of unique proteases of spermatozoa. Blood plasma contains a serine protease and protease inhibitor not present in seminal plasma. Skin mucus also showed the profile of three unique proteases (two EDTA stimulated and one metalloproteinase). These results indicate that the analysis of proteases and their inhibitors makes it possible to distinguish contamination of milt with either blood or skin mucus. The physiological role of the detected protease-inhibitory system in fish seminal plasma is still unknown. It is possible that the protease inhibitor and proteases, unique for seminal plasma, are involved in a specific function of milt or testis (e.g. the control of spermatogenesis).

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Résumé

Activités inhibitrices de la trypsine et de la viscosité du plasma séminal de la carpe : relations avec le sang, le mucus de la peau et les spermatozoïdes. Protéases et spermatozoïdes inhibiteurs ont été détectés dans le plasma séminal, le plasma sanguin, le mucus de la peau et les spermatozoïdes. Leur poids moléculaires ont été estimés par SDS-PAGE sous conditions non-réduisantes. Les résultats démontrent que les deux principales bandes de l'activité anti-protéinase (APA) détectées auparavant dans le plasma séminal de la carpe commune et d'un poids moléculaire d'environ 47 et 58 kDa sont aussi présentes dans les autres fluides. L'intensité de la coloration était la plus élevée dans le plasma sanguin et séminal. L'intensité du mucus de la peau était visible, mais celle du sperme était faible. Une bande additionnelle de migration rapide (30 kDa) fut observée dans le plasma séminal uniquement. APA la plus élevée a été détectée dans le plasma sanguin et séminal, puis dans le mucus de la peau. L'activité dans les extraits de sperme était faible. Les deux sérine-like protéases d'un poids moléculaire de 79 et 189 kDa ont également été découvertes dans le plasma séminal et sanguin. La protéase de 79 kDa était aussi présente dans le mucus de la peau. Métalloprotéinases d'un poids moléculaire de 61 et 69 kDa ont également été découverte dans le plasma séminal et sanguin mais les métalloprotéinases de 44 et 38 kDa ont été observées seulement dans le plasma séminal. Bien que les métalloprotéinases, d'un poids moléculaire de 61–75 kDa, étaient aussi visibles dans l'extrait de sperme, l'approche expérimentale utilisée dans cette étude ne permet pas une identification certaine d'une protéase spécifique dans les spermatozoïdes. Le plasma sanguin contient soit une sérine protéase ou une protéase inhibitrice non présente dans le plasma séminal. Le mucus de la peau présente aussi le profil de trois uniques protéases (deux stimulées par l'EDTA et une métalloprotéinase). Les résultats indiquent que l'analyse des protéases et de leurs inhibiteurs permet de distinguer une

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contamination de laitance soit avec du sang ou soit avec du mucus de la peau. Le rôle physiologique du système de protéase inhibiteur découvert dans le plasma séminal de poisson est encore inconnu. Il est possible que les inhibiteurs de protéases et les protéases, spécifiques du plasma séminal soient impliqués dans une fonction spécifique de la laitance ou des testicules (entre autre le contrôle de la spermatogenèse).

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Keywords: Proteases; Anti-proteinase activities; Common carp; Seminal plasma

1. Introduction

Proteases and their inhibitors play an important role in numerous physiological processes in body tissues. In mammalian semen, proteases are involved in coagulation and liquefaction, acrosome reaction and fusion of gametes (Lilja et al., 1987; Wilson et al., 1993; Evans, 2001). The activity of these proteases is controlled by their inhibitors. This control is necessary for the protection of spermatozoa and testicular tissues against unwanted proteolysis (Roberts et al., 1995). In blood, some proteases and protease inhibitors are involved in coagulation, fibrinolysis, complement activation and inflammation process. Proteolytic and anti-proteinase activity (APA) have been found in seminal plasma of several teleost fish (Breton et al., 1974; Lahnsteiner et al., 1993a, 1995, 1997, 1998; Huang et al., 1995a, b; Dabrowski and Ciereszko, 1994). The origin and functions of these proteins in seminal plasma is not clear. Some important functions of proteases described for mammalian semen, like coagulation and liquefaction or acrosome reaction are incomparable due to lack of these processes in teleost fish semen. On the other hand roles of trypsin-like proteases and some metalloproteinases presented in mammalian semen may be related to regulation of spermatogenesis through inhibitory-controlled activation of proenzymes and prohormones (Cadel et al., 1995; Paju et al., 2000). Perhaps this function may be fulfilled by teleost fish seminal plasma proteases and their inhibitors.

Loir et al. (1990) indicated the presence of homologous proteins in blood and seminal plasma of rainbow trout. This assumption is corroborated by Ciereszko et al. (1998) who showed the similarity in electrophoretical profiles of inhibitors between seminal and blood plasma of rainbow trout. Some proteins may also be introduced to milt from skin mucous while stripping a fish. In fact, comparable bands of proteolytic activities for seminal plasma and skin mucus were also found in bream (*Abramis brama*) and goldfish (*Carassius auratus*) (Kowalski et al., in press). It is unknown whether these proteins in seminal plasma originate from blood and skin mucus or represent homologous proteins synthesized in male reproductive tract.

Recently, using electrophoresis, three bands of anti-proteolytic activity and numerous bands of gelatinolytic activity in seminal plasma of common carp have been identified (Wojtczak et al., in press; Kowalski et al., in press). This study examines if spermatozoa and blood may be a source of these proteins and sought if potential contaminants, blood and skin mucus may introduce proteases and protease inhibitors to milt. These results demonstrate that seminal and blood

plasma have some common inhibitors and proteases. This suggests that serum-like proteins are produced in the male reproductive tract of common carp or are transferred from blood to milt. Unique proteases and protease inhibitors for semen, blood, and skin mucus were also found. These proteins may be potentially used for the identification of milt contamination.

2. Materials and methods

2.1. Source of semen, blood and skin mucus

Milt, blood and skin mucus of common carp (*Cyprinus carpio*) were obtained from the Experimental Fish Farm Zator (Inland Fisheries Institute, Olsztyn). Samples were transported and stored on ice before analysis. Seminal plasma was obtained by centrifugation ($8000 \times g$, 10 min) within 12 h after sampling. Sperm was washed three times using three volumes (v/w) of 0.7% NaCl solution and centrifuged ($8000 \times g$, 8 min). Supernatants from the first wash were collected. Spermatozoa were homogenized six times in bursts of 5 s at 4°C with an Ultra-Torax homogenizer in six volumes (v/w) of 50 mM Tris-HCl buffer pH 8.0 containing 150 mM NaCl and 1% Triton X-100. After centrifugation ($8000 \times g$, 10 min) supernatants were collected. Skin mucus was diluted using three volume of 0.7% NaCl and vortex during 30 s and after centrifugation ($8000 \times g$, 10 min) supernatants were collected. Blood plasma was obtained by centrifugation ($1500 \times g$, 15 min). Seminal plasma was stored at -70°C until used. Samples of seminal plasma, blood plasma, skin mucus, wash of sperm and spermatozoa homogenate were collected and used in further analysis.

2.2. Gelatinolytic activity

Electrophoresis was performed using gelatin-containing (0.1% gelatin) polyacrylamide (12.5% acrylamide) gels in the presence of sodium dodecyl sulfate (SDS) under non-reducing conditions (Siegel and Polakoski, 1985). After electrophoresis, the gels were washed at room temperature with 2.5% Triton X-100 for 30 min to remove SDS and then the gels were incubated for 24 h at 37°C in development solution (50 mM Tris-HCl buffer pH 7.5 containing: 200 mM NaCl, 0.02% Triton X-100). After incubation, gels were stained in 0.025% Coomassie Brilliant Blue for 48 h. The stained gels were stored in 2% acetic acid. Areas of proteolysis appear as clear zones against a blue background. For better visualization bands of gelatinolytic activity different dilutions of each

fluid were performed. For dilution 0.7% solution of NaCl was used. The following dilution ratios were used: wash of sperm 1:1, sperm extract 1:1, seminal plasma 1:39, blood plasma 1:49, skin mucus 1:2. From each diluted sample 15 μ l were mixed with sample buffer in 1:1 ratio and 25 μ l were added for wells in the gels. For visualization of specific common carp seminal plasma metalloproteinases, which were not observed in diluted sample, electrophoresis without dilution of seminal plasma was performed.

The effect of the two protease inhibitors, EDTA and benzamidine, on the protease activities in the gelatin-substrate polyacrylamide gels was examined. Samples were electrophoresed in gelatin containing SDS-polyacrylamide gels. After electrophoresis gels were washed in Triton X-100 as described above and then incubated with and without 5 mM CaCl_2 in development buffers containing either 10 mM benzamidine or 5 mM EDTA.

2.3. Anti-proteinase activity

2.3.1. Native-PAGE

Native-polyacrylamide gel electrophoresis (PAGE) was conducted according to the method of Laemmli (1970) using 10% gel and 5–20% linear gradient gel. Approximately 4 μ g of protein of each fluid was applied and the electrophoresis was carried out at 220 V for about 90 min in a SE 250 vertical Mighty Samll II electrophoresis system (Amersham Biosciences AB, Uppsala, Sweden). Following electrophoresis, the slab gels were incubated at 37 °C for 15 min with a fresh solution containing bovine trypsin in 0.1 M 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 appear as unstained bands on a colored background (Uriel and Berges, 1968).

2.3.2. SDS-PAGE

Molecular weights of the inhibitors were estimated under non-reducing conditions by SDS-PAGE using 5–20% linear gradient of acrylamide slab gel. The pre-stained protein standard, low range (Bio-Rad, USA) included: phosphorylase B (110 000), bovine serum albumin (BSA) (90 000), ovalbumin (51 200), carbonic anhydrase (36 000), soybean trypsin inhibitor (29 000) and lysozyme (21 400). The samples were not boiled and DTT was not added to an appropriate buffer. After electrophoresis, the gels were washed with a gentle shaking at room temperature with 2.5% Triton X-100 (two changes) for 30 min to remove SDS and then rinsed with 0.1 M phosphate buffer (pH 7.4). Inhibitors in the gel were stained as described above. Molecular weights of proteases and protease inhibitors were estimated with the use of the Kodak 1D program (Eastman Kodak Company, New Haven, USA).

2.3.3. Assay of inhibitory activity

APA was evaluated by inhibition of cod trypsin amidase activity (Ciereszko et al., 1994, 1996; Browden et al., 1997). APA was expressed as U per l of fluid (U l^{-1}) and U per g of protein (U g^{-1}). One inhibitory unit (U) corresponds to the apparent amount of inhibitor able to block one unit of trypsin activity (defined as hydrolysis of 1 μM of BAPNA min^{-1}).

2.4. Protein determination

Total protein concentrations were determined by the Lowry method (Lowry et al., 1951) using BSA as the standard. The protein levels were expressed as g of protein per l of fluid (g l^{-1}).

2.5. Statistical analysis

Data were expressed as means \pm S.D. A nonparametric Mann–Whitney and Kurskal–Wallis with Dunns post tests were employed to evaluate the differences of APA activity between tested fluids. The level of significance was set at 0.05.

3. Results

3.1. Gelatinolytic activity

When the gels were incubated in the absence of calcium ions, two bands of gelatinolytic activity with a molecular weight of 79 and 201 kDa were found in seminal plasma and blood plasma (Fig. 1a). The band of 79 kDa was also observed in skin mucus and wash of sperm. An additional bands of 21 kDa in blood plasma, and 22, 101, and >230 kDa in skin mucus were also detected. These bands were not present in seminal plasma. The homogenization of sperm did not release any gelatinolytic activity.

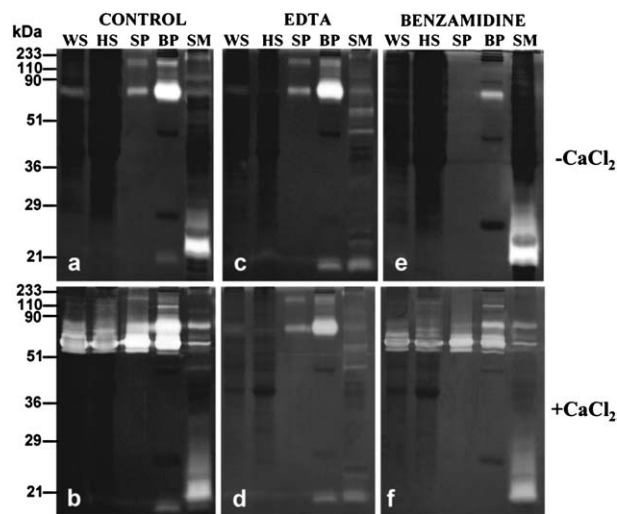


Fig. 1. Effect of benzamidine and EDTA on gelatinolytic protease activities. WS, wash of sperm; HS, homogenized spermatozoa; SP, seminal plasma; BP, blood plasma; SM, skin mucus. Samples were added after appropriate dilution. Gels were incubated in the presence or absence of 5 mM of CaCl_2 .

The addition of CaCl_2 to the development solution resulted in a significant increase in gelatinolytic activity due to an appearance of additional bands (Fig. 1b). These bands included a common band of 71 and 69 kDa detected in all fluids and band of 61 kDa which was present in all fluids, except skin mucus (Fig. 1f). An additional metalloproteinase of 118 kDa in sperm homogenates was also observed. A band of 113 kDa was observed in blood plasma only. The addition of EDTA both in the absence (Fig. 1c) or presence (Fig. 1d) of calcium ions resulted in profiles of gelatinolytic activities of all fluids (except skin mucus) similar to these obtained without any additives in development solution (Fig. 1a). All calcium-activated bands (Fig. 1b) were inhibited by EDTA (Fig. 1d). However, EDTA caused visualization bands of 64 and 49 kDa of skin mucus. A decrease in the activity of 22 kDa band of skin mucus was also observed.

In comparison with the control (development without CaCl_2 , Fig. 1a), benzamidine caused a full inhibition of 201 kDa band of seminal and blood plasma and 79 kDa band of seminal plasma, sperm wash, and skin mucus (Fig. 1e). This latter band was only partially inhibited in blood plasma. Benzamidine fully inhibited bands of 101 and >230 kDa of skin mucus and 21 kDa band of blood plasma. Only 22 kDa band of skin mucus was not affected by benzamidine. When CaCl_2 and benzamidine were present in a development solution, all calcium-stimulated activities were observed (Fig. 1f) as in control (Fig. 1b).

When not diluted samples of common carp seminal plasma were electrophoresed we could observe proteases of molecular weight 44 and 38 kDa (Fig. 2) which were calcium-stimulated and inhibited by EDTA (data not shown), although identification of any other bands was impossible due to high gelatinolytic activity observed as a wide unstained zone. These two bands were not detected in other not diluted fluids (data not shown).

3.2. Anti-proteinase activity

Two common bands of APA in all fluids were detected using native PAGE (Fig. 3). The staining intensity was high-

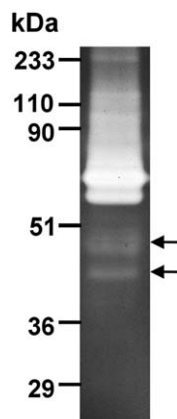


Fig. 2. Gelatinolytic protease activities of common carp seminal plasma (not diluted sample). Arrows indicate metalloproteinases of molecular weight 44 and 38 kDa.

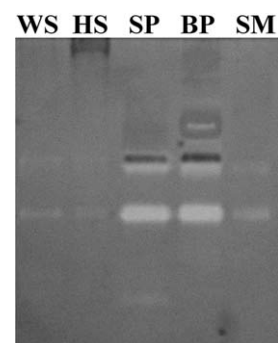


Fig. 3. Electrophorogram (PAGE, 10% acrylamide) of common carp protease inhibitors using bovine trypsin. WS, wash of sperm; HS, homogenized spermatozoa; SP, seminal plasma; BP, blood plasma; SM, skin mucus.

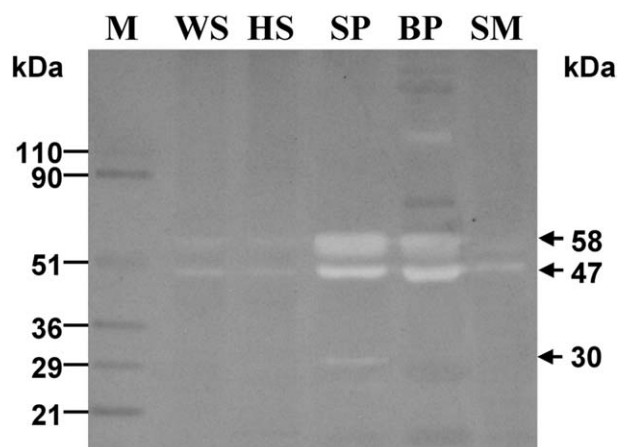


Fig. 4. Electrophorogram (5–20% linear gradient SDS-PAGE) of common carp protease inhibitors using bovine trypsin. WS, wash of sperm; HS, homogenized spermatozoa; SP, seminal plasma; BP, blood plasma; SM, skin mucus; M, molecular weight markers.

est in blood and seminal plasma and lowest in sperm extract. Blood plasma was characterized by the presence of one additional band of low migration rate. A band of high migration rate was unique for seminal plasma. The molecular weights of all inhibitors could be estimated by SDS-PAGE under non-reducing conditions (Fig. 4). The molecular weights of the two common bands were estimated to 58 and 47 kDa; the unique band of blood plasma to 121 kDa, and the unique band of seminal plasma to 30 kDa.

The highest APA expressed per volume was found in blood plasma (Table 1). This activity, expressed per gram of protein, did not differ between blood and seminal plasma. The lowest APA was found in sperm extracts.

4. Discussion

In this work it was found that four proteases described earlier in seminal plasma (Kowalski et al., in press) are common for seminal and blood plasma. Two gelatinolytic proteases of 79 and 201 kDa appeared to belong to serine proteases and two of 61 and 69 kDa belong to metalloproteinases. Gelatinolytic profiles of fish seminal plasma metal-

Table 1
Protein concentration and APA in tested fluids of common carp ($n = 8$). Data are presented as means \pm S.D

Tested fluids	Protein concentration (g l^{-1})	APA	
		U l^{-1}	U g^{-1} Protein
Supernatant	2.02 ± 0.83	12.4 ± 7.8	$5.9 \pm 3.0^{\text{a,b}}$
Homogenized spermatozoa	13.98 ± 5.07	10.8 ± 5.9	$0.8 \pm 0.3^{\text{a}}$
Seminal plasma	3.44 ± 0.72	$584.5 \pm 169.0^{\text{a}}$	$170.2 \pm 35.0^{\text{b,c}}$
Blood plasma	40.79 ± 1.54	$7663.8 \pm 1077.0^{\text{b}}$	$188.2 \pm 27.5^{\text{c}}$
Skin mucus	8.02 ± 1.64	50.2 ± 22.1	$6.5 \pm 3.1^{\text{a,b}}$

Means related to different source of APA with different superscripts within the columns are significantly different ($P < 0.05$).

loproteinases are similar to matrix-metalloproteinases (MMPs) activities observed in epididymal fluid of domestic mammals (Métayer et al., 2002). On the other hand, these authors did not observe serine-like proteases with molecular weights >54 kDa. It is possible that metalloproteinases presented in common carp seminal plasma are similar to MMPs of mammalian epididymal fluid, although serine-like proteases might represent proteins distinctive for fish.

Three proteases (61, 69 and 71 kDa) detected in wash and extracts of sperm were similar to metalloproteinases of seminal and blood plasma. For this reason, we assume that these proteases originate from seminal plasma and were adsorbed on the surface of spermatozoa. The experimental approach used in this study did not allow the unequivocal identification of unique proteases of spermatozoa. If present, the only one candidate for such proteases are those stimulated by calcium ions (Fig. 1b, f). However, bands characteristic for sperm extracts were either faint (118 kDa) or present in an area with high gelatinolytic activity (band 74 kDa).

The results indicate that the two main bands of 47 and 58 kDa APA detected earlier in common carp seminal plasma (Wojtczak et al., in press) are also present in other fluids, including blood plasma and skin mucus. This suggests that the inhibitors in semen and skin mucus are related to those of blood. These bands were also present in small amounts in wash of sperm, probably due to their adsorption on the sperm surface. These data suggest that common carp spermatozoa may possess inhibitors on the surface but these inhibitors are rather not present inside spermatozoa. Beside common bands, seminal plasma contained, as described earlier (Wojtczak et al., in press) a unique inhibitor of 30 kDa that was not present in blood, and there was a blood plasma inhibitor of 121 kDa not present in semen.

Blood plasma and skin mucus were characterized by the presence of proteases and proteinase inhibitors that were not present in seminal plasma. This includes low molecular weight proteases, 21 kDa serine protease of blood plasma and skin mucus and 22 kDa protease (inhibited by EDTA, but not stimulated by calcium ions) of skin mucus. Two 49 and 64 kDa proteinases of skin mucus were also unique. These proteases were characterized by the stimulation by EDTA. Blood plasma contained a distinctly high molecular weight inhibitor of 121 kDa. All of these proteins may be good candidates as indicators of milt contamination by blood or skin mucus. Moreover, these results indicate that employing of the analysis of proteases and protease inhibitors make it

possible to distinguish the contamination of milt from either blood or skin mucus.

It was indicated previously that the blood/seminal plasma ratios of APA and protein concentration is very similar (Ciereszko et al., 1998). This ratio was calculated to be 29.7–29.9 and 72.4–85.4 for rainbow trouts of autumn and spring spawning, respectively. In the present work ratio (calculated from data of Table 1) blood/seminal plasma for APA was calculated to 11.8, and ratio blood/seminal plasma for protein concentration to 13.1. Similarity of these ratios is manifested in the similarity of specific activities of APA in blood and seminal plasma. This also was shown for rainbow trout (Ciereszko et al., 1998). These data confirm similarity of these ratios and suggest that this ratio may be characteristic for particular species or populations.

Presence of proteases and protease inhibitors has been described in the blood of teleost fish. Serine protease inhibitors were found in blood plasma and serum of rainbow trout (Ellis and Grisley, 1985; Ciereszko et al., 1998). The presence of these inhibitors was also reported in the blood of salmonids (Zuo and Woo, 1997). The protease inhibitors of the serpin (serine protease inhibitors) family ($\alpha 1$ antitrypsin) with a molecular weight of 62 kDa (Huang et al., 1995a, b) and 55 kDa (Aranishi, 1999a) were identified in blood plasma of common carp. The molecular weights of these inhibitors are similar to those of blood plasma (48 and 57 kDa) described in this study. In blood plasma serine protease with a similar molecular weight (189 kDa) to that of trypsin-like protease from the blood plasma of tilapia (*Oreochromis niloticus*) (170 kDa) described by Inaba et al. (1997) was also detected.

The relationship between blood and seminal plasma proteins was studied by Loir et al. (1990). These authors proved that many rainbow trout seminal proteins are antigenically-related to serum proteins. The similarity of main blood and seminal plasma protease inhibitors was previously reported for rainbow trout (Ciereszko et al., 1998, 2000). This work demonstrates that four proteases and two protease inhibitors of seminal plasma were similar to those of blood plasma. These results strongly suggest that some seminal and blood plasma protease inhibitors are similar in teleost fish, and indicate that this rule also applies to proteases.

The similarity of blood and seminal plasma proteases and protease inhibitors suggests that these proteins in seminal plasma may originate from blood. On the other hand, a protease inhibitor (121 kDa), serine proteases of 21 kDa, and

metalloproteinase of 113 kDa that are present in blood but not seminal plasma were also detected. This suggests that the blood–testis barrier is selective and does prohibit passing the above mentioned protease inhibitor and proteases to the reproductive tract. It is also possible, however, that these proteins are transferred to the testis but are inactivated during passage of semen to spermatic duct. It should be stressed that serum-like proteins may also be produced in the testis (Mather et al., 1983). The data presented herein indicate that one protease inhibitor (30 kDa) and two metalloproteinases (38 and 44 kDa) are unique for seminal plasma and were likely produced in the reproductive system as it was reported for aminopeptidase (Lahnsteiner et al., 1993b, 1994). The similarity of coagulation factor VII (proteases) from zebrafish and β antitrombin from Atlantic salmon to related coagulation factors from mammalian blood were already confirmed (Sheehan et al., 2001; Andersen et al., 2000). Moreover it is known that blood coagulant activity is present in human semen (Fernandez et al., 1997). Perhaps some of described herein proteases and protease inhibitors common for blood may be a part of coagulation pathway. If it is correct, it suggests presence of coagulation pathway in fish semen. Summing up, our results indicate that some proteases and protease inhibitors are produced in the reproductive tract and some can be potentially transferred from blood.

The presence of protease inhibitors and proteases in the skin mucus was confirmed. Among proteins described in skin mucus are trypsin-like enzyme, cathepsin (cystein proteases) and their low and high molecular weight inhibitors (cystatins) and metalloproteinase (Hjelmeland, 1983; Yamashita and Konagaya, 1991, 1996; Aranishi and Nakane, 1997a, b; Aranishi, 1999b). Role of skin mucus proteases are related to bacteriolysis and liberation of antimicrobial peptides (Cho et al., 2002). Protease inhibitors may play an important role in protection of the host against microorganisms due to their ability to inhibition the growth of fish pathogenic bacteria and viruses (Björck et al., 1989; Ylönen et al., 1999). The presence of trypsin inhibitors in skin mucus of common carp is reported here. These inhibitors are related to those found in blood and seminal plasma. Presence of such protease inhibitors in skin mucus and their relationship to blood protease inhibitors have already been postulated by Hjelmeland (1983). It is possible that physiological role of skin mucus anti-trypsin is related to controlling of activity of trypsin-like protease described by Hjelmeland (1983). Data presented herein strongly suggest presence of such protease in skin mucus of common carp (21 kDa) and demonstrate similarity to that from blood plasma. Serine protease of 79 kDa and metalloproteinase of 69 kDa, common for seminal and blood plasma, were also visible in the skin mucus. Two proteases stimulated by EDTA which were unique for skin mucus were also found. Such stimulation was observed by Aranishi and Nakane (1997a, b) for eel epidermal proteases (up to 152%). Metalloproteinase of low molecular weight (22 kDa) was detected only in skin mucus. Summing up, our data indicate that some of proteases and two protease inhibi-

tors of skin mucus are related to blood and some of proteases are unique for skin mucus.

In conclusion, seminal plasma, blood plasma and skin mucus of common carp show similar profiles of gelatinolytic and anti-proteinase activities. It is possible that the protease-inhibitory system detected in seminal plasma may be related to the system present in other body fluids (e.g. blood). The proteases and their inhibitors of spermatozoa likely originate from seminal plasma. Their functions in milt are unknown at present. Unique for a seminal plasma 30 kDa inhibitor, and the two metalloproteinases were also identified. Their functions may be related to specific function of milt or testis. Some proteases and protease inhibitors of blood and skin mucus may be used as an indicator of contamination of milt by proteases and their inhibitors.

Acknowledgements

This work was supported by State Committee for Scientific Research, Project 5 P06D 026 19. We thank H. Karol and D. Kubiak for excellent technical assistance.

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