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Note

Characterization of protease inhibitors of seminal plasma of cyprinids

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Abstract

Anti-proteinase activity was demonstrated in the seminal plasma of cyprinid fish species (bream, chub, ide, dace, asp, goldfish, roach, common carp) using electrophoretic techniques combined with a detection method based on inhibition of bovine trypsin. We found species-specific protease inhibitors in the seminal plasma of cyprinids. At least three bands of protease inhibitors with different migration rates could be identified by native PAGE. Higher variability was characterized for bands with slower migration rates. Visualization of inhibitors after SDS-PAGE under non-reducing conditions allowed estimation of their molecular weights. Apparent molecular weights were within the range of 51–59 and 47–54 kDa for the bands with slower and moderate migration rates, respectively. The molecular weight of fast migration bands for roach and common carp were estimated to 23 and 30 kDa, respectively. Inhibitors of common carp seminal plasma differed in their affinity toward serine proteases. Three inhibitors in common carp seminal plasma could be visualized using cod and bovine trypsin, but only two inhibitors (of high molecular weight) were recognized with chymotrypsin. There were differences in anti-proteinase activity and seminal plasma protein concentration in relation to the origin of common carp seminal plasma (breeding lines) and time of milt collection (spawning vs. post-spawning season).

Keywords: Seminal plasma; Anti-proteinase activity; Cyprinids

1. Introduction

Protease inhibitors are present in the seminal fluid of many mammalian species, for example human (Fink et al., 1990), bull (Čechova and Fritz, 1976) and boar (Veselsky et al., 1985), and they have been shown to play an important role in male fertility. Protease inhibitors in mammalian seminal fluid have been suggested to protect the reproductive tract and/or sperm from proteolysis by damaged or prematurely acrosome-reacted sperm. They are also involved in regulation the processing or degradation of seminal fluid proteins and coagulation of semen (Kennedy et al., 1982; Straus and Polakoski, 1982; Zheng et al., 1994; He et al., 1999). Protease inhibitors are also present in non-mammalian seminal plasma including fish. These inhibitors belong to main proteins in teleost fish seminal plasma (Ciereszko et al., 2000; Dabrowski and Ciereszko, 1994). Anti-proteinase activity (APA) in seminal plasma of teleost fish exists in species-specific multiple forms and they differ in their affinity toward serine proteases (Ciereszko et al., 1998). This activity correlates with the protein and sperm concentration (Ciereszko et al., 1996). The functions of trypsin inhibitors in the reproduction of male fish is not clear, but it is probably related to protection of spermatozoa from proteolytic attack or to regulation of physiological processes involving proteolysis, for example sperm motility (Cosson and Gagnon, 1988; Inaba and Morisawa, 1991, 1992).

We previously identified two bands of APA in bream seminal plasma (Ciereszko et al., 1998). However, it is not clear if this pattern is representative for other cyprinid fishes, as was indicated for salmonids (Ciereszko et al., 1998). Protease inhibitors of fish seminal plasma have been identified so far by native-polyacrylamide gel electrophoresis (PAGE). This technique allows separation of inhibitors using mainly criteria related to electrical charge of proteins and

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does not enable molecular weight estimation. Better characterization of inhibitors may be also achieved by employing different serine proteases for identification after electrophoresis. In this study, we demonstrate electrophoretic variants of APA in seminal plasma of seven cyprinid species. Electrophoretic profiles of serine protease inhibitors were distinguished by a common pattern of two bands of high molecular weight and one band with low molecular weight. Using SDS–PAGE under non-reducing conditions allowed the molecular weight of these inhibitors to be estimated. Contrary to high molecular weight inhibitors, low molecular weight inhibitor did not inhibit chymotrypsin. Additionally, we found differences in APA and seminal plasma protein concentration in relation to the origin of the common carp (breeding lines) and time of milt collection (spawning vs. post-spawning season).

2. Materials and methods

2.1. Source of semen

Milt of bream (*Abramis brama*, $n = 5$), chub (*Leuciscus cephalus*, $n = 6$), ide (*Leuciscus idus*, $n = 3$), dace (*Leuciscus leuciscus*, $n = 1$), asp (*Aspius aspius*, $n = 6$), goldfish (*Carasius auratus*, $n = 4$), roach (*Rutilus rutilus*, $n = 5$), was collected from fish raised in hatchery condition in Chair of Lake and River Fisheries (University of Warmia and Mazury in Olsztyn). Milt of common carp (*Cyprinus carpio*) (lines: Japanese ($n = 4$), Hungarian ($n = 9$), Yugoslavian ($n = 8$) and Zator ($n = 11$) was collected twice: during reproductive season (June) and out-of-season (September). These samples were used for comparative studies of APA and protein concentration. Additionally, seminal plasma from Hungarian, Israeli, Gołysz and Zator lines was used for electrophoretic analysis. Milt of all lines of common carp was obtained from Experimental Fish Farm Zator (Inland Fisheries Institute, Olsztyn). Milt was transported and stored on ice before analysis. Seminal plasma was obtained by centrifugation ($8000 \times g$, 10 min) within 12 h after sampling. Seminal plasma was stored at –70 °C until used.

2.2. Gel electrophoresis

Native-PAGE was conducted according to the method of Laemmli (1970) using 10% gel and 5–20% linear gradient gel under non-reducing conditions. The gels were electrophoresed for about 90 min at 200 V in a SE 250 vertical Mighty Small II electrophoresis system (Amersham Biosciences AB, Uppsala, Sweden). The presence of APA in gels was detected using trypsins (bovine and cod) and chymotrypsin according to the method of Uriel and Berges (1968) as described by Ciereszko et al. (1998).

2.3. Estimation of molecular weight

Molecular weights of the inhibitors were estimated by SDS–PAGE on 5–20% linear gradient of acrylamide slab gel run under non-reducing conditions. The pre-stained, low range (Bio-Rad, USA) molecular weight standards (21–110 kDa) were used. The samples were not boiled and dithiotreitol 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 protease inhibitors were estimated with the use of the Kodak 1D program (Eastman Kodak Company, New Haven, USA).

2.4. Assay of inhibitory activity and protein determination of seminal plasma

APA of seminal plasma was evaluated by inhibition of cod trypsin amidase activity (Ciereszko et al., 1994, 1996). Total protein concentrations in the seminal plasma were determined by the method of Lowry et al. (1951).

2.5. Statistical analysis

Data were expressed as means ± S.D. A two-way analysis of variance (ANOVA) and Tukey's multiple comparison test were employed to evaluate the differences between breeding lines of the common carp and time of milt collection. The level of significance was set at 0.05.

3. Results and discussion

3.1. Electrophoretic profiles of APA of seminal plasma

Bovine trypsin allowed detection of APA after PAGE of all cyprinids (Fig. 1a). Up to three bands with different migration rates could be identified. The first zone of bands with the lowest migration rates was present in seminal plasma of all species. Species-specific differences in migration rates of these bands were noticed, for example inhibitors from common carp and chub had fastest and lowest migration rates, respectively. The second zone of bands characterized by moderate migration rates was also detected for all species. Contrary to the first zone we did not find significant species-specific differences in migration rates for this zone. For ide and common carp bands of very fast migration rates were found. The use of linear gradient PAGE (5–20% acrylamide) allowed identification of additional fast migrating bands in bream, chub, asp and roach, which were not recognized by native PAGE (Fig. 1b). We were not able to detect this inhibitor in dace and goldfish. It is not clear at present if it was due to a lack of this inhibitor in seminal plasma of these species or due to its low concentration below detection limit of our staining method. The fast migrating bands of inhibitors were also found in seminal plasma of salmonids (Ciereszko et al., 1998). We also detected a distinct non-specific esterase bands (areas of darker staining) in seminal plasma of all fish species except goldfish and chub

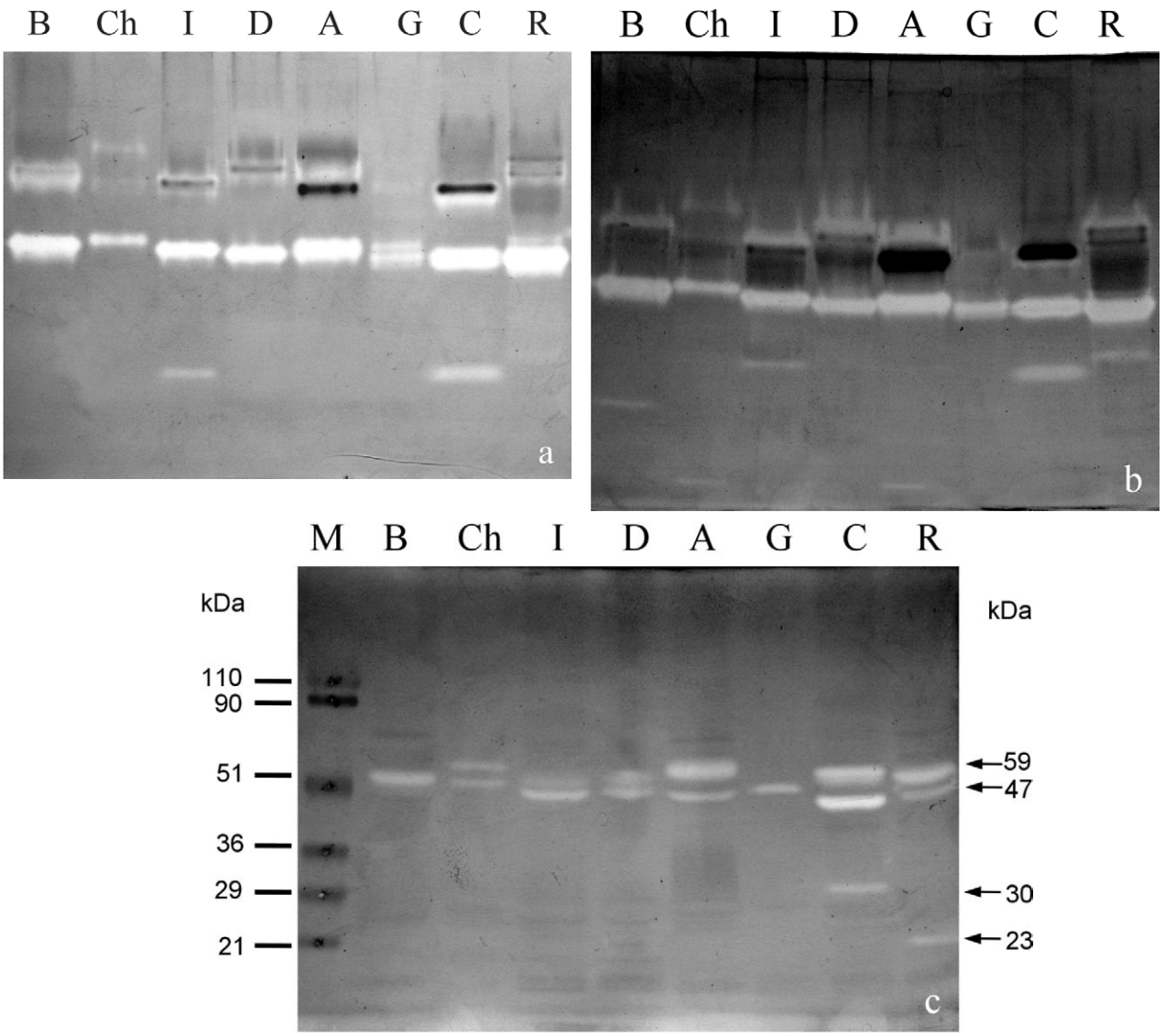

Fig. 1. Electrophorogram of proteinase inhibitors of seminal plasma of cyprinid fish stained using bovine trypsin (a-PAGE, 10% acrylamide, b-PAGE, 5–20% linear gradient of acrylamide, c-SDS-PAGE, 5–20% linear gradient of acrylamide). M-marker of molecular weight, B-bream, Ch-chub, I-ide, D-dace, A-asp, G-goldfish, C-common carp, R-roach.

(Figs. 1 and 2). These bands made visualization of the slow-migrating protease inhibitors difficult due to a similar migration rate of both proteins.

We modified the method of characterization of protease inhibitors in seminal plasma by replacing PAGE with SDS-PAGE. Inhibitory bands could still be visualized after SDS-PAGE although quality of visualization decreased (Fig. 1c). These results indicate that inhibitors of cyprinids seminal plasma are to some extent resistant to denaturation caused by SDS. Visualization of inhibitors after SDS-PAGE allowed us to estimate the molecular weight of some inhibitors. The apparent molecular weights were within the range of 51–59 and 47–54 kDa for the bands of first and second zone, respectively. These molecular weights are within the range 40–100 kDa of most members of the serpin (serine protease inhibitors) family. This assumption is supported by a finding of Huang et al. (1995a, b) who identified a protease inhibitor of the serpin family in carp seminal plasma with a molecular weight of 62 kDa, which resembles mammalian α_1–antitrypsin. We have also recently found a serpin (56 kDa) in seminal plasma of rainbow trout (unpublished data). A high molecular weight and the ability of APA of cyprinids seminal plasma to inhibit activity of bovine trypsin suggest that bands of 47–54 and 51–59 kDa belong to the family of serpins.

Using SDS-PAGE, we were also able to estimate the molecular weight of fast migration bands for roach (23 kDa) and common carp (30 kDa). Fast migrating inhibitors of cyprinids seminal plasma have a higher molecular weight than many protease inhibitors of mammalian semen (5–13 kDa). A small (12 kDa) serine protease inhibitor called secretory leukocyte protease inhibitor (SLPI) or antileukoproteases (ALP) is present in mammalian seminal plasma and may be secreted by leukocytes (Ohlsson et al., 1995; Tomee et al., 1998). Homologous to mammalian antileuko-

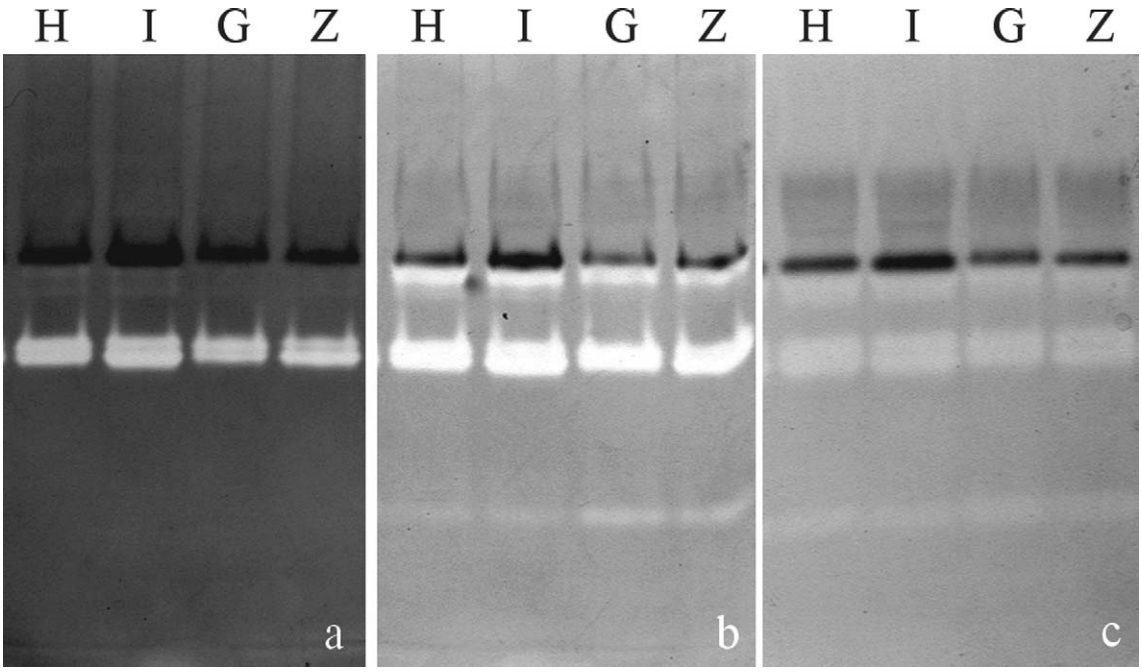

Fig. 2. Electrophorograms (PAGE, 10% acrylamide) of proteinase inhibitors of common carp seminal plasma of four breeding lines (H-Hungarian, I-Israeli, G-Golysz, Z-Zator) stained using chymotrypsin (a), bovine trypsin (b) and cod trypsin (c).

proteases is a group of trout ovulatory proteins (53, 39, 29 and 24 kDa) called TOPs that are produced by the ovary and secreted into the coelomic fluid to act as protease inhibitors following ovulation (Garczynski and Goetz, 1997; Coffman and Goetz, 1998). The molecular weight (23–30 kDa) of fast migrating trypsin inhibitors of cyprinids described in this study are within the range (24–53 kDa) described for TOPs. This suggests that these inhibitors may be homologous to SLPI. Further studies directed to isolation and characterization of these inhibitors should provide more information necessary for their classification.

3.2. Affinities of common carp inhibitors towards chymotrypsin and bovine and cod trypsin

Cod and bovine trypsin allowed the identification of three APA bands in common carp (Fig. 2). The quality of visualization decreased when cod trypsin was used. The fast migrating band could not be recognized by chymotrypsin and the low migrating band was stained faintly. Staining of the band with moderate migration rates using chymotrypsin suggests that this band contains two forms migrating close to each other. Our previous data confirm the usefulness of bovine and cod trypsin and chymotrypsin for estimating APA in fish seminal plasma. Using bovine trypsin allows the detection of three protease inhibitors in seminal plasma of salmonids. However, two additional protease inhibitors can be identified using fish (Atlantic cod *Gadus morhua*) trypsin (Ciereszko et al., 1998). It indicates that using both trypsins allows the detection the maximal number of antitrypsin bands in salmonid seminal plasma. These data suggest that protease inhibitors of salmonid and cyprinid fish differ in affinity toward serine proteases. Bovine trypsin appeared to be most useful for the detection of inhibitors of cyprinids seminal plasma due to the good quality of zymograms. No differences in migration rates of inhibitors among breeding lines (Japanese, Hungarian, Yugoslavian and Zator) were observed.

3.3. APA and protein concentration in seminal plasma collected during spawning and post-spawning season

There were not significant differences in APA and protein concentration in seminal plasma among Hungarian, Yugoslavian and Zator lines in both spawning and post-spawning seasons (Table 1). On the other hand, APA and protein concentration were significantly lower in seminal plasma of the Japanese line. For this reason it appears that APA and protein concentration may reflect genetic differences among lines of common carp. Protein concentration in seminal plasma of Hungarian, Yugoslavian and Zator collected in September was significantly lower in comparison with samples collected in June. Low APA in seminal plasma collected in September was found only for the Zator line. These results agree with our data indicating that APA (as well as protein concentration) in rainbow trout seminal plasma decrease at the end of the reproductive season (Ciereszko et al., 1996). Low levels of these parameters suggest that the secretion ability of sperm duct during post-spawning season is lower than during the spawning season.

4. Conclusion

In summary, this work indicates the presence of species-specific protease inhibitors in the seminal plasma of cyprinids.

Table 1
Anti-proteinase activity (APA) and protein concentration in seminal plasma of four breeding lines (Japanese, Hungarian, Yugoslavian, Zator) of common carp collected during spawning (June) and post-spawning (September) season; data are presented as means ± S.D.

		Japanese	Hungarian	Yugoslavian	Zator
Protein concentration (mg ml^{-1})	June	1.747 ± 0.471 [a]	3.804 ± 0.993 [b]	3.620 ± 1.056 [b]	3.720 ±0.749 [b]
	September	1.477 ± 0.157 [a]	2.149 ± 0.287 [b,*]	2.364 ± 0.493 [b,*]	2.268 ±0.403 [b,*]
APA (U l^{-1})	June	228.2 ± 100.6 [a]	523.3 ± 134.7 [b]	479.2 ± 170.9 [b]	599.1 ±168.0 [b]
	September	204.0 ± 62.1 [a]	403.1 ± 119.2 [b]	452.2 ± 129.3 [b]	429.0 ±104.2 [b,*]

Means, related to different breeding lines of common carp, with different superscripts within the lines are significantly different ($p < 0.05$).
* Different from June ($p < 0.05$).

Protease inhibitors differ both in electrophoretic profiles and in affinity toward proteolytic enzymes. APA and protein concentration may be indicators of genetic differences among lines of common carp. These parameters may be useful for characterization of carp milt collected during different periods.

Acknowledgements

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References

Čechova,, D., Fritz, H., 1976. Characterization of the proteinase inhibitors from bull seminal plasma and spermatozoa. Hoppe Seylers Z. Physiol. Chem. 357, 401–408.

Ciereszko, A., Dabrowski, K., Lin, F., Doroshov, S.I., 1994. Identification of trypsin-like activity in sturgeon spermatozoa. J. Exp. Zool. 268, 486–491.

Ciereszko, A., Liu, L., Dabrowski, K., 1996. Effects of season and dietary ascorbic acid on some biochemical characteristics of rainbow trout (*Oncorhynchus mykiss*) semen. Fish Physiol. Biochem. 15, 1–10.

Ciereszko, A., Piros, B., Dabrowski, K., Kucharczyk, D., Łuczyński, M.J., Dobosz, S., Glogowski, J., 1998. Serine proteinase inhibitors of seminal plasma of teleost fish: distribution of activity, electrophoretic profiles and relation to proteinase inhibitors of blood. J. Fish Biol. 53, 1292–1305.

Ciereszko, A., Kwaśnik, M., Dabrowski, K., Piros, B., Glogowski, J., 2000. Chromatographic separation of trypsin-inhibitory activity of rainbow trout blood and seminal plasma. Fish Shellfish Immunol. 10, 91–94.

Coffman, M.A., Goetz, F.W., 1998. Trout ovulatory proteins are partially responsible for the anti-proteolytic activity found in trout coelomic fluid. Biol. Reprod. 59, 497–502.

Cosson, M.-P., Gagnon, C., 1988. Protease inhibitor and substrates block motility and microtubule sliding of sea urchin and carp spermatozoa. Cell Motil. Cytoskel. 10, 518–527.

Dabrowski, K., Ciereszko, A., 1994. Proteinase inhibitor(s) in seminal plasma of teleost fish. J. Fish Biol. 45, 801–809.

Fink, E., Hehlein-Fink, C., Eulitz, M., 1990. Amino acid sequence elucidation of human acrosin-trypsin inhibitor (HUSI-II) reveals that Kazal-type proteinase inhibitors are structurally related to beta-subunits of glycoprotein hormones. FEBS Lett. 270, 222–224.

Garczynski, M.A., Goetz, F.W., 1997. Molecular characterization of a ribonucleic acid transcript that is highly up-regulated at the time of ovulation in the brook trout (*Salvelinus fontinalis*) ovary. Biol. Reprod. 57, 856–864.

He, S., Lin, Y.L., Liu, Y.X., 1999. Functionally inactive protein C inhibitor in seminal plasma may be associated with infertility. Mol. Hum. Reprod. 5, 513–519.

Huang, C.J., Chen, C., Chen, H.J., Huang, F.L., Chang, G.D., 1995a. A protease inhibitor of the serpin family is a major protein in carp perimeningeal fluid: I. Protein purification and characterization. J. Neurochem. 64, 1715–1720.

Huang, C.J., Lee, M.S., Huang, F.L., Chang, G.D., 1995b. A protease inhibitor of the serpin family is a major protein in carp perimeningeal fluid: II. cDNA cloning, sequence analysis, and *Escherichia coli* expression. J. Neurochem. 64, 1721–1727.

Inaba, K., Morisawa, M., 1991. A chymotrypsin-like protease involves in motility of sperm in salmonid fish. Biomed. Res. 12, 435–437.

Inaba, K., Morisawa, M., 1992. Chymotrypsin-like protease activity associated with demembranated sperm of chum salmon. Biol. Cell 76, 329–333.

Kennedy, W.P., Swift, A.M., Parrish, R.F., Polakoski, K.L., 1982. Proacrosin conversion inhibitor. Purification and initial characterization of a boar sperm protein which prevents the conversion of proacrosin into acrosin. J. Biol. Chem. 257, 3095–3099.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 277, 680–685.

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

Ohlsson, K., Bjartell, A., Lilja, H., 1995. Secretory leucocyte protease inhibitor in the male genital tract: PSA-induced proteolytic processing in human semen and tissue localization. J. Androl. 16, 64–74.

Straus, J.W., Polakoski, K.L., 1982. Acrosin inhibition. Comparisons of membrane-associated and -solubilized enzyme. J. Biol. Chem. 257, 7962–7964.

Tomee, J.F., Koeter, G.H., Hiemstra, P.S., Kauffman, H.F., 1998. Secretory leukoprotease inhibitor: a native antimicrobial protein presenting a new therapeutic option? Thorax 53, 114–116.

Uriel, J., Berges, J., 1968. Characterization of natural inhibitors of trypsin and chymotrypsin by electrophoresis in acrylamide-agarose gels. Nature 218, 578–580.

Veselsky, L., Jonáková, V., Čechova,, D., 1985. A Kunitz type of proteinase inhibitor isolated from boar seminal vesicle fluid. Andrologia 17, 352–358.

Zheng, X., Geiger, M., Ecke, S., Bielek, E., Donner, P., Eberspacher, U., Schleuning, W.D., Binder, B.R., 1994. Inhibition of acrosin by protein C inhibitor and localization of protein C inhibitor to spermatozoa. Am. J. Physiol. Cell Physiol. 267, 466–472.