A chymotrypsin-like protease involves in motility of sperm in salmonid fish

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ABSTRACT

Ten kinds of protease inhibitors were examined for their effects on motility of the demembranated sperm of chum salmon. Among them, aprotinin, *N*-tosyl-phenylalanyl-chloromethane (TPCK) and chymostatin showed strong inhibitory effects on the sperm motility. The inhibitory effects of these three protease inhibitors were observed only when the demembranated sperm were reactivated with relatively high concentration of ATP (>0.5 mM). The results suggest that a chymotrypsin-like protease involves in sperm motility in an ATP-dependent manner.

Sperm proteases have so far been investigated in two aspects. First, fertilization and the acrosome reaction of sperm have been shown to be inhibited by some protease inhibitors, indicating that proteases involve in acrosome reaction at fertilization (7, 8). Actually, some kinds of protease, such as acrosin (14), spermosin (16) and proteasome (multicatalytic proteinase) (9, 15), have been isolated from the sperm of sea urchins, tunicates or mammals.

Second, Gagnon and his co-workers have demonstrated that some trypsin inhibitors and substrates inhibit the sperm motility in mammals, carp and sea urchin (3, 4). However, the protease responsible for the control of sperm motility have not yet been isolated.

Teleost fish sperm are devoid of apparent acrosomal structure (1, 12). Therefore, it seems advantageous to use teleost sperm for investigating the role of proteases in the control of sperm motility. We show here using sperm from chum salmon that a chymotrypsin-like protease is likely to involve in sperm motility. Furthermore, the action of the protease appears to depend on the concentration of ATP.

Abbreviations: TPCK, *N*-tosyl-phenylalanyl-chloromethane; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*- α -tosyl-lysyl-chloromethane; STI, soybean trypsin inhibitor

The semen of chum salmon (*Oncorhynchus keta*) was collected by inserting a pipette into sperm duct. Sperm were demembranated with 0.04% Triton X-100 solution and the demembranated sperm were reactivated with 0.5 mM ATP in the presence of several protease inhibitors (Table 1). Among ten kinds of protease inhibitors, aprotinin (20 μ M), a high molecular weight serine protease inhibitor, exerted the most potent inhibitory effect on the motility of demembranated sperm. Sperm motility was completely blocked by aprotinin even at 1 μ M. *N*-tosyl-phenylalanyl-chloromethane (TPCK) and chymostatin, chymotrypsin-like serine protease inhibitors, also significantly reduced the percentage of motile sperm. The 50% reduction in sperm motility was observed with approximately 0.2 μ M aprotinin, 40 μ M TPCK or 80 μ M chymostatin.

An irreversible serine protease inhibitor, phenylmethanesulfonyl fluoride (PMSF), also reduced the percentage of motile sperm to a certain extent. Other trypsin-like serine protease inhibitors [leupeptin, *N*- α -tosyl-lysyl-chloromethane (TLCK), antipain and soybean trypsin inhibitor (STI)], a cysteine protease inhibitor (E-64) and an aspartic protease inhibitor (pepstatin) showed 50% inhibition at the most.

We then examined the inhibitory effect of protease inhibitors upon changing the ATP concentration in reactivation medium (Fig. 1). Although the beat frequency of sperm flagella increased with the

Table 1 *Effects of Protease Inhibitors on the Motility of Demembranated Sperm*^a

Inhibitor	Concentration (μ M)	Relative motility (%) ^b
—	—	100
Leupeptin	100	81
TLCK	100	54
Antipain	100	51
STI	1,000	71
Aprotinin	20	0
PMSF	1,000	37
TPCK	100	5
Chymostatin	100	11
E-64	10	71
Pepstatin	1	57

^aDemembranation and reactivation of sperm were performed according to the method previously described (6) with some modifications. A small volume of semen ($\sim 10 \mu$ l) was added to 200 μ l of extraction medium (0.15 M KCl, 2 mM MgCl₂, 0.5 mM EDTA, 2 mM Tris-HCl, 0.5 mM DTT and 0.04% Triton X-100, pH 8.0), and kept on ice for 30 sec. Then 40 μ l of the suspension was mixed with 1 ml of a reactivation medium (0.15 M KCl, 2 mM MgCl₂, 1 mM EGTA, 20 mM Tris-HCl, 0.5 mM DTT, 2% polyethylene glycol (average M_r 20,000), 100 μ M cAMP and 0.5 mM ATP, pH 8.0). Sperm motility was recorded on videotape via TV camera attached to the microscope with dark-field illumination. The percentage of motile sperm was counted. Protease inhibitors were dissolved in water, methanol or dimethyl sulfoxide. The final concentration of dimethyl sulfoxide or methanol in the reactivation medium was less than 1%. At this concentration, methanol or dimethylsulfoxide had no effect on sperm motility. ^bThe percentage of motile sperm was expressed taking the motility in the absence of inhibitors as 100%. Each value represents the mean of at least three different experiments.

Fig. 1 Effects of ATP concentration on inhibition of the motility of demembranated sperm by protease inhibitors. Demembranated sperm were reactivated with various concentrations of ATP in the absence ($\circ$), and presence of protease inhibitors: 2 μ M aprotinin ($\bullet$); 100 μ M TPCK (Δ); 100 μ M chymostatin ($\blacktriangle$). Sperm motility was expressed in relative value by defining the motility in the presence of 0.05 mM ATP and in the absence of protease inhibitor as 100%.

increase of ATP concentration, the percentage of motile sperm changed only slightly. The inhibitory effects of three protease inhibitors (aprotinin, TPCK, chymostatin) were slight when the demembranated sperm were reactivated with relatively low concentration of ATP (<0.2 mM); the inhibitory effects were clearly observed with ATP at concentrations higher than 0.5 mM.

Gagnon and his co-workers have previously reported that some protease inhibitors including aprotinin, *N*- α -benzyloxycarbonyl-lys-thiobenzyl ester (BLT) and tosyl-gly-pro-arg-*p*-nitroanilide (Chromozym TH) inhibited the motility of demembranated sperm in mammals, carp and sea urchin. They concluded that a serine protease with lys- and arg-ester bond specificity involves in the control of sperm motility in these species (3, 4). The present study suggests that a chymotrypsin-like protease, rather than a trypsin-like protease, involves in motility of sperm in chum salmon.

The effect of protease inhibitors was dependent on the concentration of ATP. This suggests that the regulation of sperm motility by protease occurs at

high concentration of ATP. It is possible that the sliding of microtubules by dynein ATPase is regulated by protease under the condition of high concentration of ATP. However, chum salmon sperm remain immotile even though they retain high concentration of ATP (3–4 mM) (2). It is well known that the cAMP-dependent protein phosphorylation of a protein is involved in the initiation of sperm motility in salmonid fish (10, 11). The addition of ATP and trypsin to the demembrated sperm of salmonid fish caused the initiation of sperm motility even in the absence of cAMP (13). Therefore, it is also possible that protease may act on the sperm axoneme of chum salmon as one of the factors which enable immotile sperm to initiate motility.

Recently, multicatalytic proteinases (proteasomes) (17) have been isolated from ascidian and sea urchin sperm and the possible participation of these enzymes in acrosome reaction has been suggested (9, 15). On the other hand, the chymotrypsin-like activity of the proteasome isolated from rabbit muscle was shown to be highly activated (~12-fold) in the presence of 2 mM ATP (5). Therefore, it is possible that the chymotrypsin-like activity responsible for the regulation of sperm motility is that of a proteasome. The isolation and characterization of the protease are now in progress in our laboratory.

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