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Ascidian sperm chymotrypsin-like enzyme; participation in fertilization

H. Sawada, H. Yokosawa, M. Hoshi[1] and S. Ishii[2]

Faculty of Pharmaceutical Sciences and Institute of Low Temperature Science, Hokkaido University, Sapporo 060 (Japan), August 16, 1982

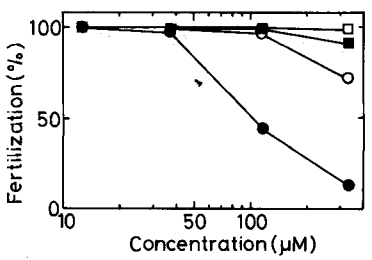
Summary. The action of a chymotrypsin-like enzyme from sperm extract from the ascidian *Halocynthia roretzi* was studied using several substrates. It was found that the most susceptible substrate had the most powerful inhibitory effect on fertilization in this animal. Among the substrates, the order of susceptibility coincided with the order of inhibitory ability, which indicates that the enzyme is involved in the fertilization process.

It has been proposed that sperm acrosin[EC 3.4.21.10], an acrosomal trypsin-like enzyme, plays a key role in the penetration of sperm through the zona pellucida of the ovum of mammals[3]. In marine invertebrates, however, little evidence has been reported[4–6] for the involvement of sperm proteases in fertilization. In a previous paper[7], we reported that chymotrypsin-like and trypsin-like enzymes may be indispensable for the sperm of the ascidian, *Halocynthia roretzi* to penetrate through the egg investment. Now we present further evidence for the involvement in fertilization of a chymotrypsin-like enzyme in sperm extract of the ascidian.

Materials and methods. Preparation of sperm and eggs from the ascidian, *H. roretzi*, type C[8], and the assay of fertilization in the presence or absence of various substrates were described previously[7,9]. Sperm suspension (1.6×10^{10} spermatozoa/ml) stored at −20 °C was thawed and homogenized with a Teflon homogenizer at 0 °C in a equal volume of 0.46 M NaCl-10 mM $CaCl_2$-50 ml $MgCl_2$-10 mM KCl buffered with 50 mM Tris-HCl (pH 8.0). After centrifugation (18,000 × g, 60 min), the resulting supernatant was employed as an enzyme preparation.

Results and discussion. Chymotrypsin-like activity in the enzyme preparation was examined with 4 peptidyl-4-methylcoumaryl-7-amide (MCA) substrates. As shown in the table, succinyl-Leu-Leu-Val-Tyr-MCA (Peptide Institute, Japan) was most susceptible to the enzyme, and acetyl-Ala-Ala-Tyr-MCA (Bachem, Switzerland), succinyl-Ala-Ala-Pro-Phe-MCA (Peptide Institute) and glutaryl-Gly-Gly-Phe-MCA (Bachem) followed in this order. The results suggest that the S_1 site[10] of the enzyme fits into the Tyr residue rather than the Phe residue of the substrate, and the S_2, S_3, or S_4 sites into the residue with a hydrophobic or branched side chain. Succinyl-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity was found to be markedly inhibited with 0.1 mM chymostatin (34% inhibition), 1 mM phenylmethanesulfonyl fluoride (25%) and 1 mM diisopropylphosphorofluoridate (17%), when assayed after preincubation at 25 °C for 30 min, but not significantly with 1 mM EGTA, 0.1 mM leupeptin, 0.1 mM bestatin, 1 mM TPCK, 1 mM TLCK, and 0.01 mM proteinaceous protease inhibitors, such as soybean trypsin inhibitor (Sigma, USA), lima bean trypsin inhibitor (Sigma) and *Streptomyces* subtilisin inhibitor. The activity showed a pH optimum between 8.5 and 9.0. Thus, this enzyme is a chymotrypsin-like protease, different from the acrosin-like enzyme reported previously[9].

Inhibition of fertilization with the substrates used above was then examined (fig.). The results show that succinyl-Leu-Leu-Val-Tyr-MCA, the most susceptible substrate for



Inhibition of fertilization with various substrates. 50 μl of sperm suspension was added to sea water buffered with 50 mM Tris-HCl (pH 8.0) containing in a final volume of 1-ml about 100 eggs in the presence or absence of substrate. After standing for 30 min at about 13 °C, percentage of fertilization was estimated on the basis of chorion elevation. ●, Succinyl-Leu-Leu-Val-Tyr-MCA; ○, acetyl-Ala-Ala-Tyr-MCA; □, succinyl-Ala-Ala-Pro-Phe-MCA; ■, glutaryl-Gly-Gly-Phe-MCA. 7-Amino-4-methylcoumarin, one of the products of hydrolysis, showed no inhibitory activity toward fertilization at the concentration of 0.33 mM.

Substrate specificity of chymotrypsin-like enzyme in sperm extract

Substrate P_5 P_4 P_3 P_2 P_1 P_1'	Activity ($\times 10^{-11}$ mU/spermatozoon)	% Activity
Suc-Leu-Leu-Val-Tyr-MCA	6.3	100
Ac- Ala-Ala-Tyr-MCA	1.7	27
Suc-Ala- Ala-Pro-Phe-MCA	0.40	6
Glt-Gly-Gly-Phe-MCA	0.13	2

Enzymatic activity was measured by fluorophotometrical determination (excitation, 380 nm; emission, 460 nm) of the generation of 7-amino-4-methylcoumarin at 25 °C in the mixture of 0.1 mM substrate solution (0.5 ml) in 50 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ and the enzyme solution (20 μl). One unit of activity is defined as the amount of enzyme that hydrolyzes 1 μmole of the substrate per min. The number of spermatozoa was counted under a microscope using a hemacytometer. Abbreviations used are: Suc, succinyl; Ac, acetyl; Glt, glutaryl; MCA, 4-methylcoumaryl-7-amide.

the enzyme, is the strongest inhibitor for fertilization. The next most effective inhibitor was acetyl-Ala-Ala-Tyr-MCA, the order being the same as in the case of substrate susceptibility (table). Furthermore, our preliminary experiments indicate that the extent of inhibition with the specific substrate is considerably diminished in the fertilization of naked eggs (egg-investment-free eggs), as previously reported[7] in the inhibition with chymostatin. Thus, chymotrypsin-like enzyme, as well as acrosin-like enzyme[9], plays an important role in fertilization of the ascidian, *H. roretzi,* especially in sperm-penetration of egg investments.

In ascidians, a typical acrosome has not been observed at the apex of the spermatozoon[11], or it is very small[12] in contrast with that of mammals[3] or sea urchins. Therefore, subcellular localization of the chymotrypsin-like enzyme is an important problem in connection with its physiological role in fertilization. We have recently demonstrated the presence of an acrosin-like enzyme at the mitochondrial portion and the apex of the spermatozoon of this ascidian by a histochemical procedure with dansyl-leucylargininal, a specific fluorescent inhibitor of the acrosin-like enzyme (Sawada et al., in preparation). The chymotrypsin-like enzyme may also be localized at this region(s) and may exhibit a cooperative action with the acrosin-like enzyme. Recently, a chymotrypsin-like enzyme has been reported as a lysin, that is a vitelline layer(coat) lytic enzyme, in sea urchins[5,6] and frogs[13]. The existence of such an enzyme has not been proved, however, in spermatozoa of the mammal, although a chymotrypsin-like enzyme, seminin, has been reported in seminal plasma[14]. On the other hand, it has been debated whether acrosin is a real zona-lysin or not, because a purified preparation of acrosin has little or no activity of solubilizing zona pellucida[14] and zona-penetration of sperm is not inhibited with trypsin inhibitors[15]. The involvement of some chymotrypsin-like enzyme in the sperm-penetration through egg investments, therefore, might be a general feature applicable from sea urchins to mammals.

Our investigations on the physiological roles of both the chymotrypsin-like and the acrosin-like enzymes in ascidian sperm, as well as their purification, are now in progress.

1 Present address: Faculty of Science, Nagoya University, Nagoya (Japan).

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Evaluation of aromatic tetrahydropyranyl ethers as feeding deterrents for the striped cucumber beetle, *Acalymma vittatum* (F.)[1]

D.K. Reed and M. Jacobson[2]

USDA-ARS, Fruit and Vegetable Insects Research Laboratory, Vincennes (Indiana 47591, USA) and Biologically Active Natural Products Laboratory, Beltsville (Maryland, USA), August 24, 1982

Summary. Laboratory tests with striped cucumber beetle, *Acalymma vittatum,* adults indicated that a number of aromatic tetrahydropyranyl ethers were promising as antifeedants at dosage rates of 0.1 and 0.5%.

In recent years, increasing attention has been given to plant chemicals as sources of insect feeding deterrents. Some of the complexities of this research were reported by Schoonhoven[3]. Plant phenolics were shown to possess such activity when entomologists at the USDA Insects Affecting Man and Animals Research Laboratory in Gainesville, Florida conducted tests early in 1980 with a number of naturally occurring and synthetic phenols and their derivatives. These tests showed that the tetrahydropyranyl (THP) ethers of 2-methoxyphenol, 4-methoxyphenol, 2,3-dimethoxyphenol, and 3,4-methylenedioxyphenol were repellent to adult yellow fever mosquito, *Aedes aegypti* (L.), and the malaria mosquito, *Anopheles quadrimaculatus* Say[4]. The THP ether of 2,3-dimethoxyphenol also protected the arms of human subjects to which the compound had been applied from bites by a saltmarsh mosquito, *Aedes taeniorhynchus* (Wiedemann). The Army Environmental Hygiene Agency subsequently showed that these compounds had low topical hazard potential when tested on rats, rabbits, and guinea-pigs. These results and previous results reported by Reed et