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Sperm chymotrypsin-like enzymes of different inhibitor-susceptibility as lysins in ascidians

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Summary. Inhibitory effects of three peptidyl phenylalaninals on fertilization and on chymotrypsin-like enzyme activity of sperm in three species of ascidians were examined. The results suggest that a sperm chymotrypsin-like enzyme is indispensable for the fertilization in each of the ascidians, and that these enzymes have different susceptibilities to inhibitors.
Key words. Fertilization; sperm; chymotrypsin; lysin; ascidian.

It is currently known that spermatozoa penetrate through egg investments with the aid of sperm-bound lytic agents, the lysins. In ascidians, which occupy a phylogenetic position between vertebrates and 'true' invertebrates, sperm proteases are thought to function as lysins, similar to those of mammals[2].

In our previous study of the sperm lysin system of the ascidian, *Halocynthia roretzi,* we reported that two trypsin-like enzymes, acrosin and spermosin, and a chymotrypsin-like enzyme are indispensable for sperm penetration through the vitelline coat of eggs[3–7].

Furthermore, the timing of action of these three proteases in the fertilization of *H. roretzi* has also been demonstrated[8]. In the present report, we attempt to investigate the lysin system of other ascidians and compare the inhibitory effects of three microbial chymotrypsin inhibitors on the fertilization and on the sperm chymotrypsin activity in each of the ascidians.

Materials and methods. Gametes of *H. roretzi* from Mutsu Bay were collected from a pair of gonads with gonaducts as described previously[4], while those of *Ciona savignyi* and *Ascidia ahodori* from Mutsu Bay were collected from the gonaducts with a Pasteur pipette. Eggs (100–200) were incubated for 1–2 min in 1 ml of seawater, filtered and buffered with 10 mM Tris-HCl (pH 8.0), containing various concentrations of protease inhibitors, and then inseminated at the temperature of seawater at which each ascidian spawns, i.e., 13 °C for *H. roretzi,* 15 °C for *C. savignyi,* or 21 °C for *A. ahodori.* The percentage of fertilization in *H. roretzi* was determined at 30 min after insemination on the basis of the expansion of the perivitelline space and again at 2 h on the basis of the first cleavage. Eggs which had undergone either of these reactions were counted as fertilized ones. Fertilization ratios in *C. savignyi* and *A. ahodori* were determined at 2 h and 1 h, respectively, after insemination, on the basis of the first cleavage, since the expansion of the perivitelline space following insemination was not observed in these ascidians.

Spermatozoa stored at −40 °C were thawed, homogenized in 10 vols of artificial seawater (460 mM NaCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 10 mM KCl), buffered with 10 mM Tris-HCl (pH 8.0) using a Teflon homogenizer (1000 rpm, 10 strokes), and stirred for 2 h. After centrifugation (10,000 × g, 30 min), the resulting supernatant was used as an enzyme extract. Chymotrypsin and trypsin activities of the sperm extract were assayed fluorometrically (excitation at 380 nm, emission at 460 nm) at 25 °C in 50 mM Tris-HCl (pH 8.5) containing 10 mM $CaCl_2$ and 0.1 mM bestatin using succinyl (Suc)-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) (Peptide Institute) and t-butyloxycarbonyl (Boc)-Val-Pro-Arg-MCA (Peptide Institute) as substrates for chymotrypsin- and trypsin-like enzymes, respectively. Inhibition of chymotrypsin activity by three microbial chymotrypsin inhibitors was determined by measuring the residual activity after prior incubation with the inhibitors for 30 min. Chymostatin was purchased from the Peptide Institute (Osaka). Leupeptin and bestatin were generous gifts of Dr W. Tanaka of Nippon Kayaku Co. α- and β-MAPI were prepared as described previously[9].

Results and discussion. Hoshi has described that only the chymotrypsin-like enzyme of spermatozoa seems to be responsible for sperm penetration through egg investments in one order of the ascidians, the Enterogona, whose gonad is unpaired and lies within or behind the loop of the intestine. On the other hand, both chymotrypsin-like and trypsin-like enzymes are required in the other order, the Pleurogona, in which a pair of gonads are in the lateral mantle wall[2]. In *H. roretzi* of the Pleurogona, the fertilization has been demonstrated to be inhibited with chymostatin, a chymotrypsin inhibitor, and leupeptin, a trypsin inhibitor[3]. In this study, we investigated the susceptibility of fertilization to the inhibitors quantitatively, comparing *H. roretzi* (Pleurogona) with *C. savignyi* and *A. ahodori* (Enterogona). The results of the comparison are shown in figure 1. As described previously[3], the fertilization of *H. roretzi* was completely inhibited not only with chymostatin but also with leupeptin, though the effective concentration of the latter was approximately 3-fold higher than that of the former. On the other hand, the fertilization of *C. savignyi* and *A. ahodori* was inhibited only with chymostatin. Leupeptin scarcely inhibited, even at a

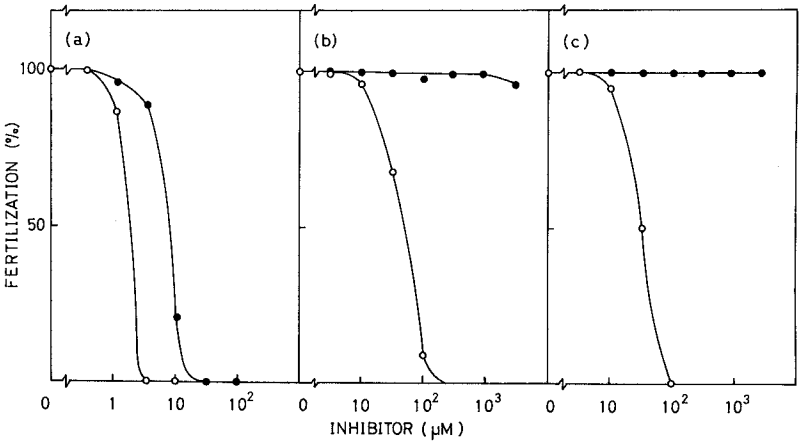

Figure 1. Effects of chymostatin and leupeptin on fertilization of (a) *H. roretzi*, (b) *C. savignyi*, or (c) *A. ahodori*. Eggs (100–200) were incubated for 1–2 min in 1 ml of seawater filtered and buffered with 10 mM Tris-HCl (pH 8.0) containing the inhibitor indicated, and then inseminated. ○, Chymostatin; ●, leupeptin.

concentration approximately 30-fold higher than that of chymostatin.

Furthermore, we compared trypsin activity [Boc-Val-Pro-Arg-MCA (50 μM) hydrolyzing activity that is inhibitable with leupeptin] with chymotrypsin activity [Suc-Leu-Leu-Val-Tyr-MCA (50 μM) hydrolyzing activity inhibitable with chymostatin] in sperm extracts of three species of ascidians. Ratios of trypsin activity to chymotrypsin activity in *H. roretzi*, *C. savignyi*, and *A. ahodori* were 24, 2, and 2, respectively. The high ratio observed in *H. roretzi* seems to be a reflection of the participation of two trypsin-like enzymes, as well as of a chymotrypsin-like enzyme, in the fertilization only of this ascidian[5–7]. Thus, these quantitative analyses support Hoshi's proposal that only a chymotrypsin-like enzyme is involved in sperm penetration in the Enterogona, while both chymotrypsin-like and trypsin-like enzymes are required in the Pleurogona[2]. The difference between the sperm proteases involved in fertilization in the two orders might be due to the structural difference between their vitelline coats, substrates for sperm proteases. Characterization of the vitelline coats would clarify this point.

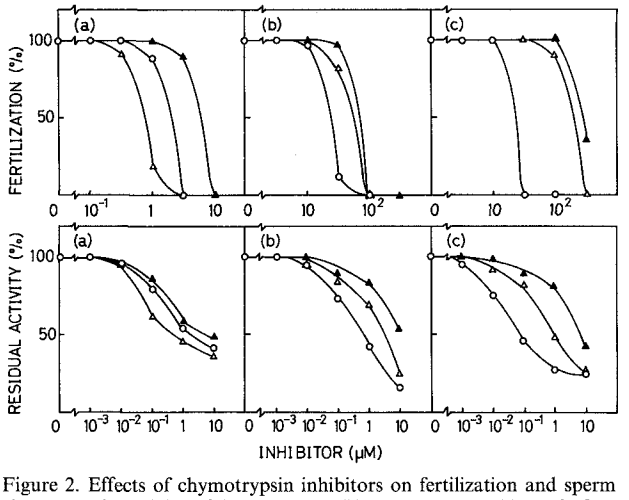

Figure 2. Effects of chymotrypsin inhibitors on fertilization and sperm chymotrypsin activity of (a) *H. roretzi*, (b) *C. savignyi*, or (c) *A. ahodori*. Eggs (100–200) were incubated in 1 ml of seawater filtered and buffered with 10 mM Tris-HCl (pH 8.0) containing the inhibitor indicated, and then inseminated. Chymotrypsin activity toward Suc-Leu-Leu-Val-Tyr-MCA (100 μM) was assayed in sperm extract after prior incubation, with an inhibitor as indicated, for 30 min. ○, Chymostatin; △, α-MAPI; ▲, β-MAPI.

We next investigated effects of chymotrypsin inhibitors on the fertilization and on the sperm chymotrypsin activity in three species of ascidians, to clarify whether a sperm chymotrypsin-like enzyme indeed participates in fertilization of each ascidian and to define the features of the inhibitor involved in recognition by the enzyme. We utilized three Streptomyces inhibitors containing phenylalaninal at the C-termini. One was chymostatin, whose structure is N-[((S)-1-carboxy-2-phenylethyl)carbamoyl]-α-[2-iminohexahydro-4(S)-pyrimidyl]-L-Gly-L-Leu-L-phenylalaninal[10]. The other two inhibitors used were α- and β-MAPI (MAPI stands for microbial alkaline proteinase inhibitor). The former has the structure of N-[(S)-1-carboxy-2-phenylethyl]carbamoyl-L-Arg-L-Val-L-phenylalaninal[11], and the latter is the same except that it has D-phenylalaninal at the C-terminus[12]. The results on the inhibitory effects are shown in figure 2. The fertilization in *H. roretzi* was most susceptible to α-MAPI, followed by chymostatin; β-MAPI was least effective. On the other hand, chymostatin was the strongest fertilization inhibitor in *C. savignyi* and *A. ahodori*; α-MAPI was the next and β-MAPI was the least effective inhibitor. Their inhibitory effects on the chymotrypsin activity in sperm extract are also shown in figure 2. The ranking of the three compounds by their potencies as enzyme inhibitors in each of the three species of ascidians was similar to the ranking of their potencies as fertilization inhibitors in the same ascidian. Thus, these results indicate the participation of sperm chymotrypsin-like enzymes with different inhibitor-susceptibility in the fertilization of three ascidians. In addition, it should be noted that each of the chymotrypsin-like enzymes of the three ascidians is inhibited more strongly when L-phenylalaninal rather than D-phenylalaninal is present at the C-terminus of the inhibitor. Alternatively, several kinds of chymotrypsin-like enzymes with different inhibitor-susceptibility might be present in the sperm of each of the three ascidians, but their proportions would be different in the different species. Purification of the chymotrypsin-like enzymes from sperm would clarify this point. Furthermore, utilization of a purified sperm chymotrypsin-like enzyme preparation would enable us to define its characteristics more precisely and to investigate the mechanism of its digestive action on the vitelline coat.

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Presence of giant mitochondria during cerebellar ontogenesis in reptiles[1]

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Summary. Giant mitochondria were observed in the perikarya and dendrites of Purkinje cells in the developing cerebellar cortex of the lizard *Gallotia galloti* at several stages previous to hatching. Such mitochondria are absent form the adult cerebellum.
Key words. Purkinje cells; cerebellar ontogeny; giant mitochondria; reptiles.

The ultrastructural embryonic development of the cerebellum has scarcely been studied in lower vertebrates. However, this subject has been approached in teleostei[2, 3], in the frog[4], in the lizard[5], in the chick[6, 7], in the rat[8–11], in the mouse[12], in the hamster[13] and in man[14].

The development of the cerebellar cortex of *Gallotia galloti* occurs during the embryonic period. At hatching the cerebellar cortex is morphologically nearly mature; most of the proliferative external granular layer, of which only a few cellular clusters remain, has disappeared. At this stage the cortex shows its three characteristic layers; molecular, Purkinje cell and inner granular layer. In the depth of the granular layer there is a non-stratified ependyma in continuity with the rhombencephalic fourth ventricle. The adult cerebellum of *Gallotia* is anatomically a single plate, without apparent subdivisions, and is joined to the brainstem by the basal pedunculi.

In this work we describe the presence, distribution and morphology of giant mitochondria in the developing reptilian cerebellum.

Materials and methods. The embryos of *Gallotia galloti* (Reptilia: Lacertidae) were collected in the field and classified according to the developmental table of *Lacerta vivipara*[15] and were sacrificed immediately. They were fixed by immersion or by cardiac perfusion, in 5% glutaraldehyde buffered with Millonig solution at pH 7.3. They were postfixed in 2% osmium tetroxide buffered in the same solution, dehydrated in acetone and embedded in araldite. Ultrathin sections were stained with lead citrate and examined in a Hitachi H-300 electron microscope.

Results and discussion. In the embryonic stages 39, 40 and hatching of *Gallotia galloti,* the Purkinje cells are aligned in a single layer. The giant mitochondria have been observed in the perikarya and in the main stem dendrites (in the molecular layer neuropil) in all the Purkinje cells. In these stages they are not observed in any other cerebellar cell type. In earlier embryonic stages or in postnatal or adult lizards giant mitochondria are not detectable (figs 1 and 2).

The giant mitochondria are always observed in smaller numbers than the normal-sized mitochondria.

The morphological characteristics of the giant mitochondria are as follows. They are five to seven times larger than normal mitochondria; their shape is spherical, oval or occasionally almost triangular in the dendritic branches; they show abundant cristae which are sometimes radially arranged. Electron dense granules are observed in the mitochondrial matrix, as in normal mitochondria (fig. 1).

In the same embryonic stages in which giant mitochondria appear, we have observed small protrusions in the Purkinje cell body. They have a thin pedicula and their distal portion is thicker and piriform or ovally shaped. They are longer than the somatic spines which are typical in the mature cell.

In the later embryonic stages, near the hatching stage, the progressive disappearance of the external granular layer in the *Gallotia* cerebellum occurs. This disappearance coincides with an increase in the development of the distal dendritic tree of the Purkinje cells. Moreover, this period of development shows a progressive increase in the number of synapses, mostly in the superficial half of the molecular layer. This corresponds to the strata where dendritic spines and also dendritic arborizations are most abundant. A great number of these synapses are established between the en passant and terminal boutons of the parallel fibers of the granular cells and the dentritic spines of the Purkinje cells.

The large mitochondria are present only in the Purkinje cells of the lizard and the chick[7]. Its transitory presence coincides with the establishment of a great number of synapses by the Purkinje cell and the start of an accelerated myelination in the lizard's cerebellar cortex. Although this temporal coincidence does not offer conclusive evidence, we think that there is a relation between the presence of these mitochondria and the physiological maturation of the cerebellar cortex. But we have no evidence of its exact role in cellular metabolism, and more data are necessary to elucidate this event.

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