Timing of action of sperm proteases in ascidian fertilization

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Summary. The timing of action of three sperm proteases, acrosin, spermosin, and a chymotrypsin-like enzyme, in the fertilization of the ascidian, *Halocynthia roretzi*, was examined by adding specific protease inhibitors at various times after insemination. The results indicate that the last two enzymes both function at the early stage of the process of sperm penetration through the egg investment, while acrosin functions at the late stage.
Key words. Fertilization; sperm protease; ascidian; timing.

In mammals, it is at present believed that a sperm trypsin-like protease, acrosin (EC 3.4.21.10), is a lytic agent (lysin) that allows spermatozoa to penetrate the zona pellucida, an investment of the ovum[3-5]. However, the precise timing of action of acrosin or other sperm proteases during the fertilization process has not been well elucidated in any animal.

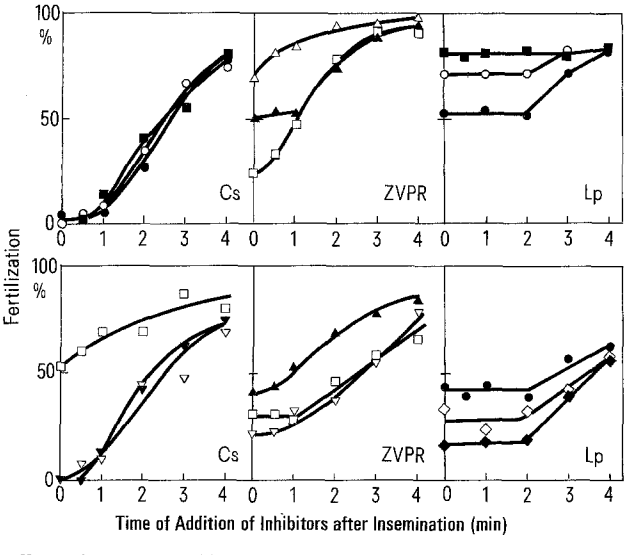
In a previous study of the sperm lysin system of the ascidian, *Halocynthia roretzi*, which occupies a phylogenetic position between vertebrates and 'true' invertebrates, it was demonstrated that both trypsin- and chymotrypsin-inhibitors block fertilization of intact eggs but not of naked (investment-free) ones[6], and that good substrates for either trypsin-like or chymotrypsin-like enzymes in spermatozoa also strongly inhibit fertilization[7,8]. Therefore we have proposed that both the trypsin-like and chymotrypsin-like enzymes are indispensable for ascidian sperm to penetrate the egg investment[7,8]. Furthermore, we have recently found that two types of trypsin-like enzymes, acrosin and spermosin (an unique enzyme with strict substrate-specificity), are present in spermatozoa[9], and proved that not only acrosin but also spermosin participates in fertilization in this animal[10]. In the present report, we attempt to demonstrate the timing of action of these three sperm proteases in ascidian fertilization.

Materials and methods. Preparation of sperm and eggs from the ascidian, *H. roretzi*, type C, was described previously[11]. Eggs (100–200) suspended in 1 ml of seawater buffered with 10 mM Tris-HCl (pH 8.0) were inseminated at 13 °C and the inhibitor solution (12.5–100 µl) was added to the mixture of sperm and eggs at the indicated time. The percentage of fertilization was determined at 1 h after insemination on the basis of the expansion of the perivitelline space and again at 2 h on the basis of the first cleavage[6]. Eggs which had undergone either of these reactions were counted as fertilized ones.

Results and discussion. We followed the time-dependency of the inhibitory effects of leupeptin (acetyl-Leu-Leu-Arg-H), Z-Val-Pro-Arg-H, and chymostatin on fertilization by adding each of the compounds at 0, 0.5, 1, 2, 3, or 4 min after insemination (fig.). We consider that these three compounds are specific inhibitors for acrosin, spermosin, and a chymotrypsin-like enzyme of spermatozoa, respectively, with the following reasons. First, the inhibitor constant of leupeptin for acrosin (Ki = 36 nM) is lower than that for spermosin (Ki = 11 µM)[10]. Second, Z-Val-Pro-Arg-H is the strongest inhibitor toward spermosin (Ki = 97 nM) among ten leupeptin analogues tested, and the inhibitory effect of a low concentration (less than 2.5 µM) of Z-Val-Pro-Arg-H on fertilization is considered to be due to its action on spermosin but not on acrosin (for details, see Sawada et al.[10]). Third, a low concentration of chymostatin strongly inhibits the chymotrypsin-like enzyme partially purified from spermatozoa (Sato et al., unpublished), but shows little effect on the activities of acrosin and spermosin[7].

As shown in the figure, fertilization was suppressed to the lowest level when a fixed amount of every one of the three inhibitors was added at the same time as insemination. The extent of the inhibitory effect on fertilization was observed to be less if chymostatin and Z-Val-Pro-Arg-H were added at 1–2 min after insemination. In the case of leupeptin, however, the level of inhibition was maintained even if the reagent was added at 2 min after insemination under the conditions tested. Reproducible results were obtained with different batches of gametes. Furthermore, the decrease of inhibitory ability showed essentially the same dependency on the addition time irrespective of the concentration used, with each of the inhibitors. Thus, the results prompt us to conclude that the sperm chymotrypsin-like enzyme and spermosin function simultaneously or synergistically at an early stage of the sperm penetration process including sperm binding to the egg investment, while acrosin does so at a late stage of the process. All the inhibitors showed little effect on fertilization when added at 4 min after insemination. Thus, sperm proteases may accomplish their roles within about 4 min during sperm-egg interaction. This result is in accordance with previous reports that the time required for sperm penetration of the egg investment in the case of the ascidian, *Ascidia nigra*[12,13] is 2–5 min. It also supports our previously proposed idea[7,8] that the three sperm proteases are involved in sperm penetration of the egg investment.

In mammals, a number of hypotheses concerning the physiological roles and the timing of action of acrosin in fertilization have been proposed[3,14]. The most feasible role of acrosin is its involvement in sperm penetration through the zona pellucida of the ovum, while other proposed roles of acrosin are its involvement in processes such as acrosome reaction, sperm binding to the zona pellucida, membrane fusion between sperm and eggs, motility of sperm, or decondensation of sperm chromatin after



Effects of protease inhibitors added after insemination on fertilization of the ascidian, *H. roretzi*. The representative results obtained with two different batches of gametes are illustrated in (A) and (B). Inhibitors tested were chymostatin (Cs), Z-Val-Pro-Arg-H (ZVPR), and leupeptin (Lp). Final concentrations of inhibitors examined were 100 (◆), 50 (◇), 25 (●), 12.5 (○), 10 (▼), 6.3 (■), 5 (▽), 2.5 (□), 1.25 (▲), or 0.63 µM (△). The percentages of fertilization in the absence of inhibitors were estimated to be 90–95% and 80–90% in the cases of (A) and (B), respectively.

sperm entry into the egg[3,14]. The reason why such a variety of physiological roles of acrosin have been proposed may be explained by the fact that trypsin inhibitors which are not so specific for acrosin have been used in the studies of mammalian fertilization. In this connection, it is noteworthy that we used the potent cognate inhibitors specific for acrosin, spermosin, and the chymotrypsin-like enzyme of spermatozoa in the present study. By employing three specific inhibitors, we demonstrated for the first time the timing of action of sperm proteases in fertilization in the ascidian. Further studies on morphological events that occur within 4 min after insemination, and on the subcellular localization of the proteases in the spermatozoon passing through the vitelline coat, will be necessary to establish the definite timing of action and the precise role of sperm proteases in fertilization.

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Feulgen-DNA amounts and karyotype lengths of three planarian species of the genus *Dugesia*

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Summary. Genome sizes of the planarians *D. lugubris* (2n = 8), *D. polychroa* (2n = 8) and *D. benazzii* (2n = 16) were evaluated on metaphase plates by measuring both the Feulgen-DNA contents and the karyotype lengths. In the three species, genome sizes are significantly different; this finding rules out the possibility of a karyotype evolution through simple chromosome rearrangements between *D. lugubris* and *D. polychroa*. A different Feulgen-DNA content per unit length of karyotype in the three species studied was also found, which suggests that DNA could be differently packed along metaphase chromosomes.
Key words. Cytotaxonomy; genome size; karyotype length; nuclear DNA amount; planarians.

In the planarian genus *Dugesia*, the '*D. lugubris-polychroa* group' and *D. gonocephala* s.l. are made up of various sibling species, which are reproductively isolated within each group, although they show few morphological differences. The karyological studies and genetic experiments involving breeding, carried out by Benazzi and coworkers over many years, have made it possible to clarify some karyotype relationships among the species belonging to the two groups and to put forward some interesting evolutionary hypotheses (for a review see Benazzi[1,2]).
In order to gain a deeper insight into the karyotype differences in the two groups of the genus, we evaluated the genome sizes of some species. In fact, genome size, as a species specific constant, is a very useful cytological parameter in connection with evolutionary processes[3].
Measurements of genome size have been obtained for several animal groups by means of the microdensitometric and/or microfluorometric evaluation of the DNA content on either interphase nuclei or metaphase plates after cytochemical reactions specific for DNA[4-6]. By means of this methodological approach, we recently demonstrated the genome size constancy of E and F biotypes of *D. lugubris*[7], and supported the hypothesis, for these biotypes, of karyotype evolution through a Robertsonian mechanism, as suggested by Benazzi and Puccinelli[8].
Estimates of genome size were also obtained by linear measurement of the whole karyotype length on metaphase chromosomes[9].

In the present paper, the possible genome size differences among *D. polychroa* (A biotype), *D. lugubris* (E biotype), and *D. benazzii* (diploid biotype) were studied; both the microdensitometric and karyometric methods were applied for the analysis of metaphase plates from regenerative blastemas.
Karyotype characteristics of the different biotypes in the '*D. lugubris-polychroa* group' and in *D. gonocephala* s.l. The European *D. lugubris-polychroa* group comprises four sibling species corresponding to the A–D, E, F, G biotypes established by Benazzi[10]. The A–D biotypes make up an autopolyploid series starting with the A biotype (2n = 8; n = 4); they can interbreed, and correspond to the species *D. polychroa* (O. Schmidt). The E biotype is diploid (2n = 8; n = 4), and the F biotype is again diploid but its chromosome number is 2n = 6, n = 3; the latter should derive from the former mainly through a Robertsonian mechanism[7,8]. Both biotypes were tentatively assigned to *D. lugubris* (O. Schmidt), although Benazzi[2], on the basis of some morphological differences of the copulatory apparatus, and the fact that the two biotypes are genetically isolated, has proposed for the F biotype the specific name *D. nova*. The G biotype is diploid, too, with chromosome number 2n = 8, n = 4; it corresponds to *D. mediterranea*[11].
In *D. gonocephala* s.l., which comprises various species or microspecies widely distributed through the Old World[12], the original karyotype should be the one of *D. gonocephala* s.s. of Central Europe (2n = 16, n = 8). From this karyotype, polysomic and