HELGOLÄNDER MEERESUNTERSUCHUNGEN
Helgoländer Meeresunters. 49, 295–301 (1995)

Do test cells secrete a hatching enzyme in *Ascidiella aspersa* (Tunicata, Ascidiacea)?

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ABSTRACT: The hatching enzyme of *Ascidiella aspersa* has been characterized as a trypsin-like enzyme. It dissolves the main part of the chorion and renders hatching possible at the end of embryogenesis. In contrast to Knaben's statement, this enzyme is secreted by the embryo itself and not by the test cells.

INTRODUCTION

Both the ascidian oocyte and the developing embryo are surrounded by the egg envelopes, consisting of the follicular epithelium, the test cells, and the interposed acellular chorion. Ever since test cells were first discovered some 100 years ago, their function has remained unclear. They have notably been ascribed a role in nutrition of the oocyte during vitellogenesis (Pérèz, 1954; Kessel & Kemp, 1962; Mancuso, 1965; Reverberi, 1978) or being involved in tunic morphogenesis (Cloney & Cavey, 1982). In *Distaplia occidentalis* (Cavey, 1976) and *Corella inflata* (Cloney & Cavey, 1982), they form minute ornaments, which cover the larval tunic. Finally, Knaben (1936) suggested that at least in *Corella parallelogramma* test cells secrete hatching enzyme. In order to verify this hypothesis, the action of a hatching enzyme was demonstrated, the hatching enzyme was characterized and finally, embryos and test cells were reared separately to determine whether the test cells or the embryos secrete the hatching enzyme.

Since all test cells of *Ascidiella aspersa* are firmly attached to the inner chorion layer until the middle tail-bud stage (Lübbering et al., 1992), dechorionation of early embryonic stages is a neat method to separate the test cells from the embryo.

MATERIAL AND METHODS

Rearing and observation of embryos

Mature specimens of *Ascidiella aspersa* were collected by divers of the Biologische Anstalt Helgoland (BAH) in the vicinity of Helgoland (North Sea) between June and September.

Sperm and oocytes of this self-fertilizing species were obtained separately by

puncturing the gonoducts of collected specimens (for details see Niermann-Kerkenberg & Hofmann, 1989). Fifteen minutes after artificial insemination, the oocytes were transferred to fresh seawater at least three times, in order to remove all excess sperm. The developing embryos were raised in Millipore-filtered seawater at room temperature (22 °C). Living material was observed with a Zeiss photo-microscope equipped with Nomarski differential interference contrast optics and a Contax camera.

Electron microscopy

Tail-bud stage embryos were fixed in 5 % glutaraldehyde buffered in 0.1 M s-collidine (pH 7.4), to which 0.5 % alcian blue was added. They were post-fixed in osmium tetroxide buffered in 0.1 M s-collidine containing 0.8 % $K_3(FeCN)_6$, dehydrated in a graded series of acetone solutions and embedded in Epon 812 (Luft, 1961). Ultrathin sections were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and observed with a Zeiss EM 9 electron microscope.

Characterization of the hatching enzyme

Previous studies have shown that the hatching enzyme of *Ciona intestinalis* and *Halocynthia roretzi* have proteolytic properties (Caggegi et al., 1974; Denucé, 1975; Hoshi & Numakunai, 1981). There was a good chance that the hatching enzyme of *Ascidiella aspersa* would turn out to be a protease. Therefore, neurula stage embryos were incubated in various protease inhibitors (Table 1) of decreasing concentrations (100–0.01 µg/ml). Pepstatin was dissolved in dimethylsulphoxide (DMSO) and then diluted with seawater; the other inhibitors were directly dissolved in filtered seawater.

Test for the site of hatching enzyme secretion

The follicle cells of 30 early tail-bud stage embryos were stripped off. The embryos were dechorionated manually with two sharpened tungsten needles. Embryos and the chorion including their test cells were rinsed several times in seawater, transferred to a siliconised slide and reared separately in a drop of seawater. When the control larvae hatched, the medium was sucked off and tested for the presence of hatching enzyme. As a rapid screening test for trypsin-like enzymes, the following gelatine-digestion-test was used: a small strip of a tri-pack film (Kodakchrome, 25 ASA; not light exposed but developed) was dipped into the medium. Coloration of the water caused by digestion of the emulsion demonstrates protease activity (Gordon et al., 1952).

RESULTS

After a successful insemination, meiosis is completed and the eggs of *Ascidiella aspersa* start with a bilateral cleavage. They go through the blastula, gastrula, neurula and tail-bud stages and eventually hatch as tadpole larvae about 14 h 30 min post-insemination (Niermann-Kerkenberg & Hofmann, 1989).

Light microscopic observations suggest the action of a hatching enzyme in *Ascidiella aspersa*. Until the late tail-bud stage, the chorion surrounds the embryo as a hollow

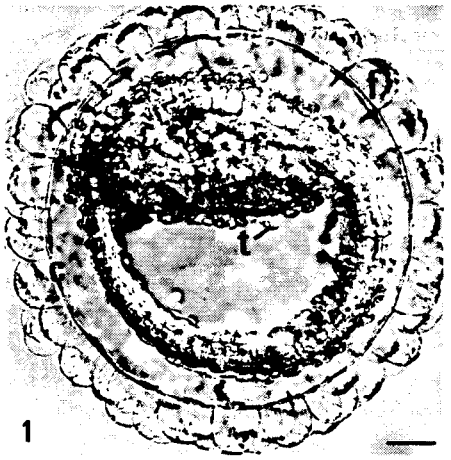
Table 1. Effect of protease inhibitors used

Inhibitor	Directed against:	Not directed against:	References:
Bestatin	aminopeptidase B leucine aminopeptidase	aminopeptidase A trypsin, chymotrypsin, elastase papain pepsin thermolysin	Umezawa (1976a, 1977)
Pepstatin	pepsin cathepsin D renin proctase B pepstanon hydroxypepstatin		Umezawa (1976b, 1977)
Leupeptin	plasmin trypsin kallikrein papain cathepsin B	chymotrypsin	Umezawa (1976b, 1977) Acyagi et al. (1969)
Trypsin inhibitor from bovine lung	trypsin chymotrypsin plasmin kallikrein		Werle (1964)
Trypsin inhibitor from soy bean	trypsin trypsin-like proteases		Vonis Bidlingmeyer (1972)

sphere. The shape of the follicle cells adjusts to the surface of the chorion (Fig. 1). A few minutes before the larva hatches, the chorion seems to disappear. Devoid of the supporting chorion, the follicle cell epithelium falls onto the larva, and the cells assume an ovoid shape (Fig. 2). Electron micrographs confirm these observations. As a matter of fact, the chorion does not disappear completely but the density and the thickness of especially the inner layer decrease considerably. At the two-cell stage, the thickness of the inner chorion layer is about 10–15 µm (Fig. 3). When the larva hatches, the thickness of the inner chorion has diminished to 0.1–0.4 µm (Fig. 4).

In order to characterize the hatching enzyme, neurula stage embryos were reared in seawater to which various protease inhibitors were added. Pepstatin and bestatin effect neither the development nor the hatching of the embryos. In contrast, all inhibitors directed against trypsin-like enzymes (leupeptin, trypsin inhibitor from bovine lung, trypsin inhibitor from soy bean) prevent hatching (Table 2), though these inhibitors do not interfere with the normal development of larvae (Fig. 5) or with the metamorphosis of such larvae inside the chorion (Fig. 6).

To test Knaben's (1936) hypothesis, early tail-bud stage embryos were dechorionated manually. At this stage, dechorionation produces embryos totally devoid of test cells. The latter are attached to the chorion. Embryos and the chorion carrying test cells were kept separately. At the time when control larvae hatched, the medium was tested for the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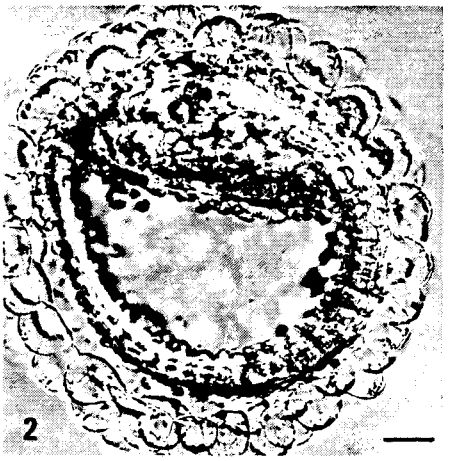
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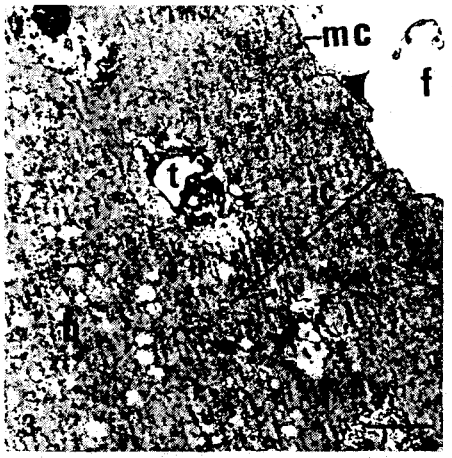
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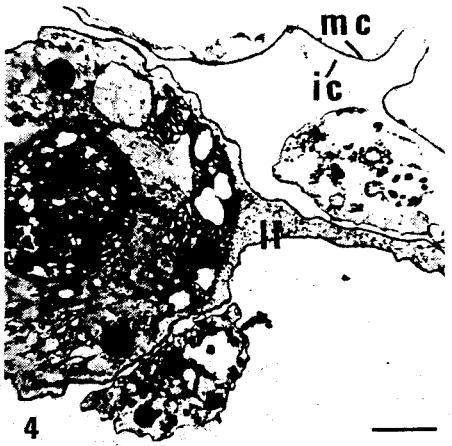
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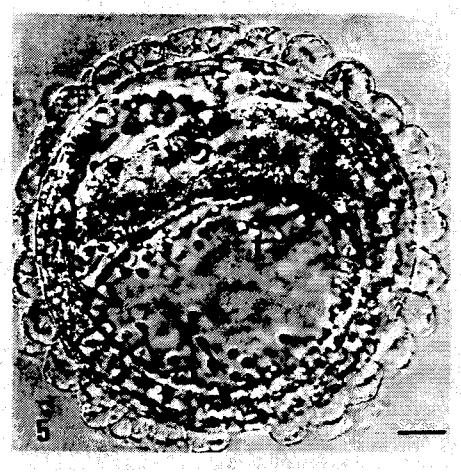
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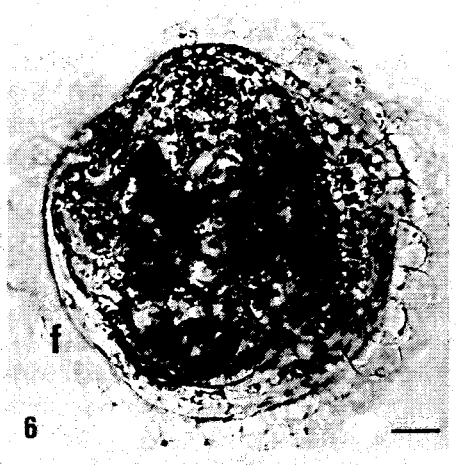
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Table 2. Influence of various protease inhibitors on the hatching of *Ascidiella aspersa* larvae. +: normal hatching; (+): delayed hatching; −: no hatching

Inhibitor in sea water	100 µg/ml	10 µg/ml	1 µg/ml	0.1 µg/ml	0.01 µg/ml
Pepstatin	+	+	+	+	+
Bestatin	+	+	+	+	+
Leupeptin	−	−	−	(+)	+
Trypsin inhibitor from bovine lung	−	−	−	−	+
Trypsin inhibitor from soy bean	−	−	−	(+)	+

presence of hatching enzyme. The seawater in which the embryos were reared clearly contained a trypsin-like enzyme, whereas no proteolytic activity could be demonstrated for the seawater in which the chorion and the accompanying test cells were reared.

DISCUSSION

As Berrill (1929) already described, light microscopic observations indicate that in ascidians a hatching enzyme dissolves the chorion and thus allows hatching. Electron microscopic studies confirm these observations. The chorion of *Ascidiella aspersa* consists of three layers (Lübbering et al., 1992). The outer layer is thin and composed of fine fibrils. The rigid middle sheet appears homogeneous and electron-dense. The inner layer is composed of granular and fibrillar elements loosely embedded in an electron-lucent

Figs 1–6. *Ascidiella aspersa*

Fig. 1. Late tail-bud stage, about 30 min before hatching. The chorion is clearly visible. Scale bar: 50 µm

Fig. 2. Larva, a few minutes before hatching. The chorion seems to have disappeared. Scale bar: 50 µm

Fig. 3. Transmission electron micrograph of a two-cell stage. The chorion of *Ascidiella aspersa* is three-layered, but on this micrograph with low magnification, the outer chorion layer is not visible. The inner chorion layer is about 15 µm thick, the middle chorion layer is only seen as a fine, electron dense ribbon. Scale bar: 5 µm

Fig. 4. Transmission electron micrograph of a larva's tail a few minutes before hatching. The tail of the larva is sectioned transversely. The thickness and the density of the inner chorion layer have decreased considerably. Scale bar: 5 µm

Fig. 5. Inhibitor-treated larva still confined to the egg envelopes two hours after hatching of control larvae. From neurula stage onwards it was reared in trypsin inhibitor from bovine lung (10 µg/ml). Scale bar: 50 µm

Fig. 6. Inhibitor-treated post-larval ascidian eight hours after hatching of control larvae. Rearing in bovine lung trypsin inhibitor (10 µg/ml) and continued confinement to the egg envelope had not prevented metamorphosis. Scale bar: 50 µm

Abbreviations: b: blastocyte; c: chorion; cd: chorda dorsalis; f: follicle cell; ic: inner chorion layer; mc: middle chorion layer; lf: larval fin; t: test cell

matrix. The thickness and the density of especially the inner chorion layer decreases considerably during *Ascidiella aspersa* embryogenesis, due to the action of the hatching enzyme.

Proteolytic hatching enzymes were already found in other ascidian species (Berrill, 1929; Caggegi et al., 1974; Denucé, 1975; Hoshi & Numakunai, 1981). Berrill (1929) demonstrated that the hatching protease is not species-specific. Caggegi and co-workers (1974) have shown that the hatching enzyme of *Ciona intestinalis* has proteolytic as well as glycolytic properties.

In the present study, using various types of protease inhibitors, the hatching enzyme could be characterized in *Ascidiella aspersa*. Embryos that were reared in seawater to which various trypsin inhibitors were added, did not hatch. Other protease inhibitors had no influence on hatching. The embryos developed normally and the larvae were even capable of metamorphosing inside the chorion. Therefore, a toxic secondary effect of the inhibitors can be ruled out. Working with *Halocynthia roretzi*, Hoshi & Numakunai (1981) obtained similar results. Denucé (1975) attributed to the hatching enzyme of *Ciona intestinalis* the characteristics of serine proteases, a group of enzymes that also comprises trypsin and trypsin-like enzymes.

Knaben (1936) alleged that test cells of *Corella parallelogramma* secrete hatching enzyme. In order to verify his hypothesis, embryos and test cells were reared separately. Our results clearly disprove Knaben's hypothesis: in *Ascidiella aspersa*, the embryos, and not the test cells, secrete the hatching enzyme.

Acknowledgements. Thanks are due to all staff members at the BAH host laboratories for their help. The authors are grateful to Prof. G. Goffinet (Université de Liège, Belgium) for critically reading the manuscript.

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