Proc. Arthropod. Embryol. Soc. Jpn. (34), 1–9 (1999)

ISSN 1341–1527

Caseinolytic protease in crab hatch water: Preliminary studies of purification, characterization, and its putative role

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Abstract

Zoea larvae of the estuarine terrestrial crab *Sesarma haematocheir* hatch in synchrony with the times of nocturnal high tide. Hatch water (filtered medium into which zoeas have been released) contains a caseinolytic protease. Through three steps of chromatography this protease was partially purified. Caseinolytic activity was eluted by molecular sieve chromatography as two peaks at 50 kDa and 39 kDa. Analysis by SDS–PAGE using a gel containing 0.1% casein indicated that the caseinolytic activity was actually divided into five protein bands with different molecular masses: *i.e.*, three bands between 50 kDa and 60 kDa, a 26-kDa band, and a 20-kDa band. These results may suggest that crab hatch water contains multiple caseinolytic proteases. Transmission electron microscopy showed that the envelope (1.7μm thick) of *S. haematocheir* embryos is composed of three distinct layers: outer (E_1), thick middle (E_2), and thin inner layer (E_3). The first two layers (E_1 and E_2), that are structurally the main components of the envelope, are not morphologically altered during hatching, but the inner layer (E_3) is clearly altered before hatching. Active solutions eluted by molecular sieve chromatography digested the inner layer of the envelope. Alteration of the inner layer may change its permeability before hatching, enabling the embryo to absorb the environmental water and to mechanically break the outer and middle layers from inside.

Introduction

The embryos of decapod crustaceans are encased by a transparent, layered embryonic envelope (other terms: egg capsule or egg case). At oviposition, the outermost layer forms a stalk (funiculus) and is then attached to the ovigerous hairs of the female (Herrick, 1909; Yonge, 1937, 1955; Cheung, 1966; Goudeau and Lachaise, 1983; Fisher and Clark, 1983). The embryos of intertidal and estuarine crabs hatch after a special developmental process called the "hatching program", and this program is under the control of the endogenous clock of each embryo (Saigusa, 1992a, 1993, 1997; Saigusa and Kawagoye, 1997).

Hatching is induced by the digestion of the embryonic envelope. In a variety of animals including echinoderms (Lepage and Gache, 1989; Roe and Lennarz, 1990; Nomura *et al.*, 1991), protochordates (D'Aniello *et al.*, 1997), teleosts (Shoots *et al.*, 1982; Yasumasu *et al.*, 1989a, b, 1992; Yamagami *et al.*, 1992; Yamagami, 1992) and amphibians (Yoshizaki and Katagiri, 1975; Urch and Hedrick, 1981), proteases serve as hatching enzymes. These enzymes are released in hatch water (*i.e.*, the filtered medium in which embryos have hatched).

In an estuarine terrestrial crab, *Sesarma haematocheir*, hatch water contains at least two kinds of active substance: *i.e.*, OHSS (ovigerous-hair stripping substance) and a caseinolytic protease (Saigusa, 1996). The physiology of OHSS has been studied (Saigusa, 1994, 1995), and this substance has recently been purified (Saigusa and Iwasaki, 1999). In contrast, little information is available concerning either the activity or chemistry of the caseinolytic protease. In this study, we partially purified this protease, and analyzed it by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using gels containing 0.1% casein. We also show that

the embryonic envelope is composed of three distinct layers, and that the inner-most layer is digested by the partially purified, active solutions eluted by molecular sieve chromatography.

Materials and Methods

Animals and preparation of hatch water

Hatch water was collected from the females of the estuarine terrestrial crab *S. haematocheir* (akate-gani) inhabiting the thicket along a small estuary at Kasaoka, Okayama Prefecture, Japan. The females release their zoea larvae into the water at the edge of the estuarine stream and the seacoast [for details of the habitat, see Saigusa (1982)]. About the time of full and new moon, many ovigerous females cross a road along the seacoast and estuary to reach the water for 2 h after sunset at 19:00 in summer. Females wait till the times of high water level at the riverside. Hatching in each female occurs in synchrony with the times of nocturnal high water levels. Hatching is also highly synchronized among the embryos carried by the female; all of the embryos may hatch within 30 min in the field. As soon as hatching is complete, the female enters the water to release all the larvae with a fanning behavior of her abdomen: "larval release behavior" (Saigusa, 1982). This behavior is completed within only 4–5 sec.

Within 1–2 h after sunset, ovigerous females were collected on the road, disinfected in ice-cold 70–80% ethanol for a few minutes, and then placed individually in plastic cups (10 cm in diameter, 20 cm in height) with no water. These cups with the crabs were brought into the laboratory (50 km from the field) within 1h. The crabs were then placed individually in small plastic cups with lids (5.5 cm in diameter and 5.5 cm in height, or 5.5 cm in diameter and 8 cm in height), each with 9 ml of distilled water. As soon as the larval release had been completed, zoeas were removed with nylon mesh, and then the hatch water was passed through filter paper (No. 2, Toyo Roshi, Japan). The resulting hatch water from five females was pooled in 50 ml plastic bottles (3 cm in diameter, 9 cm in height) and stored at −30℃.

Casein assay

Caseinolytic activity was determined by the method of Zwilling and Neurath (1981). The assay substrate was 1% casein (Ishizu Seiyaku Co., Japan) dissolved in 100 mM Tris–HCl buffer (pH 8.5); the suspension was heated in a boiling water bath for 15–20 min, until the casein was completely solubilized in the buffer. The assay mixture contained 0.2 ml of 100 mM Tris–HCl buffer, 0.4 ml of 1% casein solution, and 0.2 ml of the protease solution (*i.e.*, fractionated hatch water). This mixture was incubated for 30 min at 30℃, and then precipitated by addition of 1.2 ml of 5% trichloroacetic acid (TCA). In control experiments, TCA was added to the casein solution before the addition of protease solution. These incubation mixtures were centrifuged at 18,000 rpm for 20 min, and absorption of the deproteinized supernatant was measured at 280 nm.

Purification of the caseinolytic protease

Crude hatch water frozen and stored in 50 ml bottles was thawed and proteins were precipitated for several hours by addition of ammonium sulfate (80% saturation). After centrifugation at 18,000 rpm for 20 min at 4℃, the precipitate was dissolved in 100 mM Tris–HCl buffer (pH 8.5) (concentrated hatch water). The procedures described below were carried out with a fast protein liquid chromatography system (FPLC, Pharmacia) at 4℃. Protein elution was monitored by measuring absorption at 280 nm.

Step I: Hydrophobic chromatography. In each experiment, the concentrated hatch water was mixed with an equivalent volume of 20 mM Tris–HCl buffer (pH 8.5) containing 2 M $(NH_4)_2SO_4$, and this sample solution was applied to a hydrophobic column (10 ml of Butyl Sepharose 4FF, Pharmacia) pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.5) containing 1 M $(NH_4)_2SO_4$. Proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ at 25 mM/ml at a flow rate of 1.0 ml/min. Caseinolytic activity of each fraction (4 ml) was measured as described above.

Step II: Desalting and ion exchange chromatography. Pooled active fractions (a total volume of 20 ml) eluted from the hydrophobic column (step I) were desalted by a column (C 26/40, Pharmacia) containing 130 ml of Sephadex–G25 SF (Pharmacia) pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.5), at a flow

rate of 3.0 ml/min.

Desalted active solution (a total volume of 30 ml) was applied to an anion-exchange column (MONO–Q HR5/5, prepack column of 0.5×5 cm, Pharmacia) pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.5). Proteins were eluted with two linear gradients of NaCl; 18.75 mM/ml for the first 32 ml (fractions 20–36), and 50 mM/ml for the next 8 ml (37–40) at a flow rate of 1.0 ml/min. The active fractions (a total volume of 10 ml) were pooled and then concentrated to 500 μl by passage through an ultrafiltration membrane (Centricon 10, Amicon Co.).

Step III: Molecular sieve chromatography (gel filtration). A 500 μl of concentrated active material was further fractionated by a molecular sieve chromatography (Superdex 75, HR10/30, prepack column; Pharmacia) on a column pre-equilibrated with 10 mM Tris–HCl buffer containing 150 mM NaCl (pH 8.5). The elutant was the same as this buffer, and the flow rate was 0.25 ml/min. The marker proteins used to estimate the molecular mass were glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), and trypsin inhibitor (20.1 kDa, Sigma).

SDS–polyacrylamide gel electrophoresis

The protein solution fractionated by molecular sieve chromatography (fractions 8–14) was separately concentrated to 40 μl by ultrafiltration. Concentrated samples were each dissolved in an equivalent volume of buffer consisting of 5% SDS, 10% glycerol and 0.01% bromophenol blue in 125 mM Tris–HCl (pH 6. 8), left at room temperature for 30 min, and then aliquots were subjected to SDS–PAGE according to the method of Laemmli (1970) using 10% gels containing 0.1% casein (Leber and Balkwill, 1997). After electrophoresis, the gels were incubated for 10 h at 28℃ in 10 mM Tris–HCl buffer until casein was digested by the protease, and then stained with 0.05% Coomasie Brilliant Blue (CBB). The markers used were the same as those for molecular sieve chromatography.

Transmission electron microscopy

Embryos at the middle developmental stage were detached from ovigerous females. The embryos were removed from the envelope with a sharp needle; care was taken not to damage the encased embryos to prevent contamination by proteases contained within the embryos. The isolated envelopes were then incubated with the active solution eluted by molecular sieve chromatography for 10 h at room temperature (about 25℃). The incubated envelopes were fixed with 2.5% glutaraldehide dissolved in sodium cacodylate buffer (pH 7.4) for 1.5 h at 4℃, washed with the same buffer, and then post-fixed with 1% OsO_4 dissolved in a mixture of potassium ferrocyanide, 400 mM NaCl and 100 mM sodium cacodylate (pH 7.4) for 1 h at room temperature. After post-fixation, the specimens were dehydrated through a graded series of ethanol and embedded in TAAB resin. Ultrathin sections were cut on a Porter-Blum MT–2 ultramicrotome and collected on copper grids. The sections were stained with 1% uranyl acetate dissolved in 50% ethanol, and then with 0.1% lead citrate, and examined with a Hitachi H500H electron microscope operated at 75 kV.

Results

Chromatography

Concentrated hatch water was subjected to hydrophobic column chromatography, and proteins were eluted with a linear gradient from 1 M to 0 M $(NH_4)_2SO_4$. Each fraction had a volume of 4 ml. The proteolytic activity, as assayed with casein, showed a single peak at 0 M $(NH_4)_2SO_4$ (Fig. 1). After desalting the pooled active fractions (17–21 in Fig. 1), this material (30 ml) was subjected to anion-exchange column chromatography and proteins were eluted with two linear gradients of NaCl. Each fraction had a volume of 2 ml. The proteolytic activity showed a peak at 400–450 mM NaCl (Fig. 2).

The active fractions (29–33) were pooled (10 ml), concentrated to about 500 μl by an ultrafiltration membrane, and were fractionated by the molecular sieve chromatography. As shown in Figure 3A, caseinolytic activity appeared in two peaks; one in fraction 10 and the other in fraction 12. The molecular masses of these two proteases were estimated to be 50 kDa and 39 kDa in comparison with standard proteins.

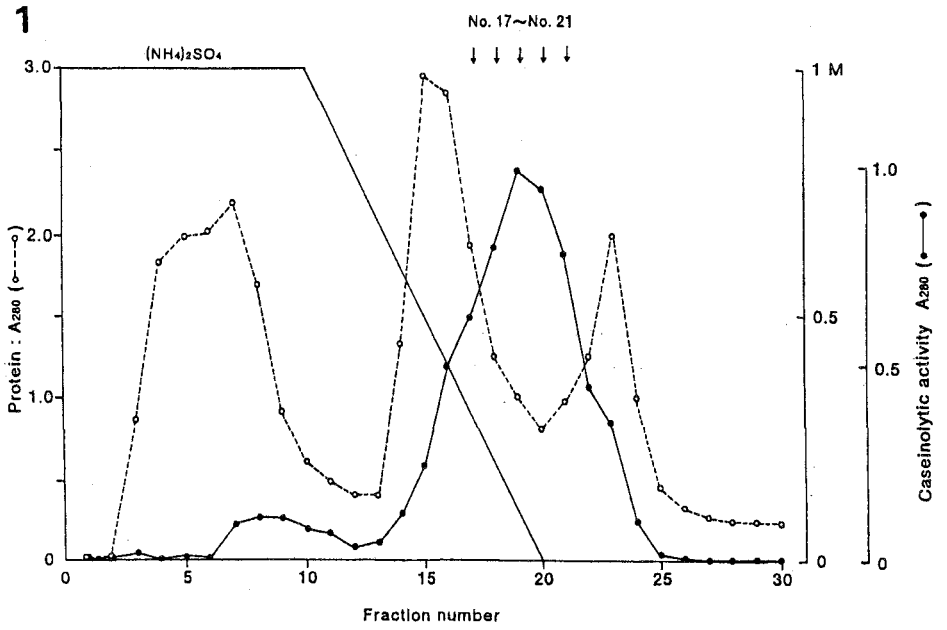

Fig. 1 Fractionation of proteins by hydrophobic chromatography. Concentrated hatch water (20 ml; about 80 females) was applied to the column, and the proteins were eluted with a linear gradient of 25 mM/ml $(NH_4)_2SO_4$. Open circles indicate proteins in each fraction (4 ml). Caseinolytic activity of each fraction is shown by solid circles.

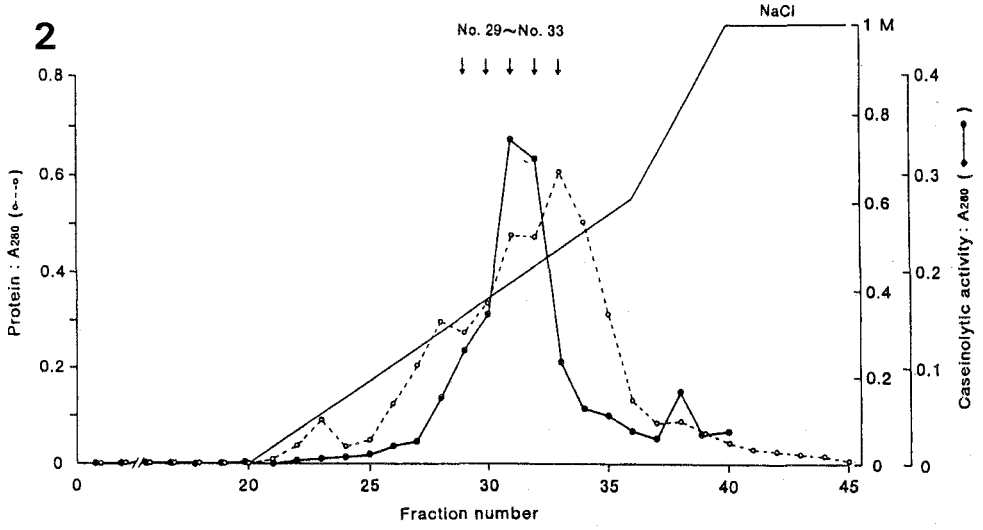

Fig. 2 Fractionation of proteins by anion-exchange chromatography. Active fractions (17–21 in Fig. 1) were desalted, applied to this column, and the proteins (open circles) were eluted by two linear gradients of NaCl. Caseinolytic activity in each fraction (2 ml) is shown by solid circles.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

The fractionated solutions (8–15 in Fig. 3A) were separately concentrated by ultrafiltration to 40 μl, and aliquots were analyzed by SDS–PAGE using gels containing 0.1% casein. After electrophoresis and incubation with buffer, the gels were stained with CBB. The five, unstained, white bands indicated proteolytic

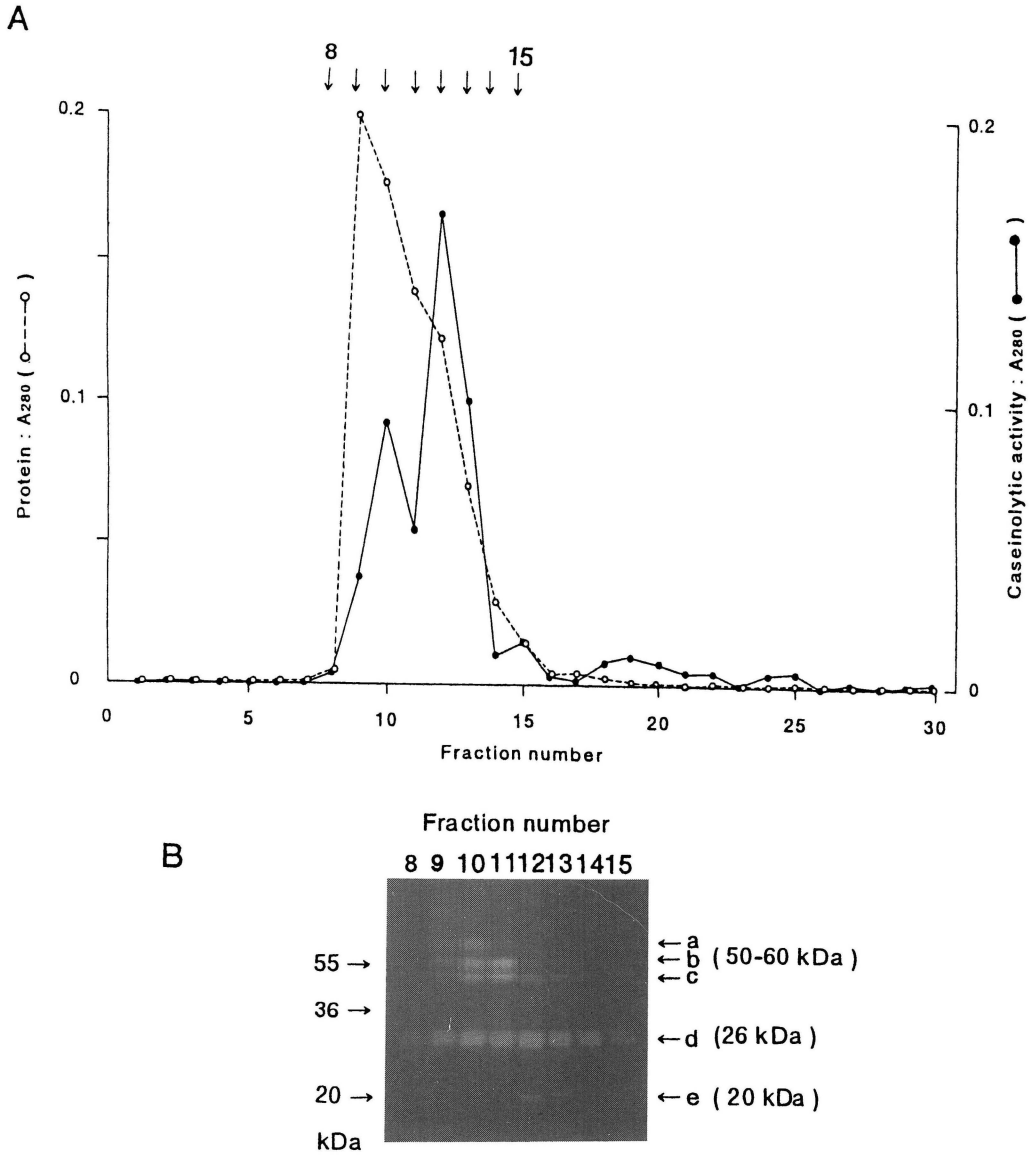

Fig. 3 A. Molecular sieve chromatography. Active fractions (29–33 in Fig. 2) were concentrated, and applied to the molecular sieve column. Open circles and solid circles indicate proteins and caseinolytic activity of each fraction (1 ml), respectively. B. Proteolytic activity of active fractions (Fig. 3A) as analyzed on SDS–PAGE using the gel containing 0.1% casein. Unstained white bands show the presence of caseinolytic proteases. Arrows on the left indicate the molecular masses of marker proteins. Arrows a–e on the right indicate five major bands of protease.

activity.

As shown in Figure 3B caseinolytic activity was actually divided into three components, three bands with 50–60 kDa, a 26-kDa band and a 20-kDa. The 26-kDa protein band appeared in all fractions.

Morphology of the embryonic envelope, and digestion of this envelope with caseinolytic protease

Embryos of *S. haematocheir* are incubated by the female for one month before hatching. The embryonic envelope was 1.7μm thick in the middle and late stages of development, and was composed of three distinct layers [previously called L_1, L_2 and L_3, see Saigusa (1997)] (Fig. 4). The outer layer (E_1) is 0.3 μm thick;

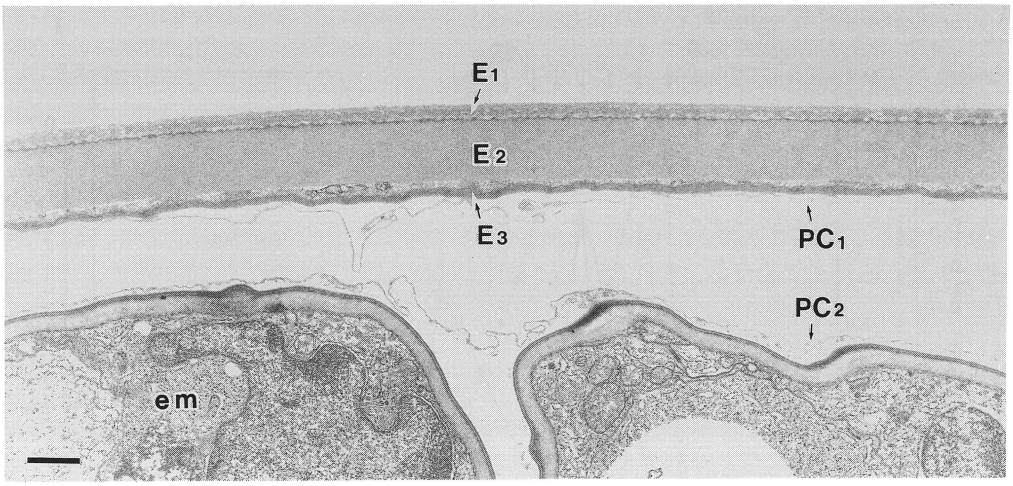

Fig. 4 Fine structure of the embryonic envelope of *S. haematocheir* 1 day before hatching. The envelope consists of the outer layer (E_1), the thickest middle layer (E_2) and the inner layer (E_3). em: embryo, PC_1, $_2$: zoeal cuticles. Scale = 1μm.

it covered not only the surface of the envelope, but also formed the funiculus (the stalk binding the embryos to the ovigerous hairs), and was the "coat" wrapping the hairs (see Saigusa, 1994).

The thickest middle layer (E_2) was present under the outer layer (E_1). Neither this (E_2) nor the inner layer (E_3) were seen in either the funiculus or investment coat. E_2 was 1.2 μm thick, and was uniform in structure (Fig. 4). The inner (E_3) layer was thin (0.2 μm thick) and electron-dense (Fig. 4). E_3 was often separated from the middle layer, and fine granular materials sometimes filled the space between E_2 and E_3 (such as shown here in the control experiment, see below). In such cases, the envelope was much thicker than 1.7μm. The outer surface of the inner layer was delineated with a thin membranous structure.

In control experiments, the isolated envelope was incubated with 10 mM Tris–HCl buffer (pH 8.5) for 10 h at room temperature. As shown in Figure 5A, the morphological structures of the three layers were not different from those of the intact envelope (Fig. 4). Active solutions (9 and 10, and 12 and 13 in Fig. 3A) did not alter the upper two layers, but clearly digested the inner thin layer (E_3). Digestion was more clearly induced by fractions 9 and 10 than by 12 and 13 (Fig. 5B, C).

Discussion

Hatch water of the estuarine crab *S. haematocheir* contains caseinolytic protease. This protease was partially purified through three steps of chromatography. Caseinolytic activity was eluted as two peaks in the final step. Caseinolytic activity appeared as five protein bands on the SDS–PAGE gels containing 0.1% casein.

The embryonic envelopes of many animals are digested by proteases (hatching enzymes) upon hatching (Yamagami, 1988, 1996). For example, Yasumasu *et al.* (1989a, b) found that the medaka hatching enzyme actually consists of two kinds of protease with different molecular masses and biochemical properties. High choriolytic enzyme (HCE, 24 kDa) causes swelling of the embryonic envelope as a result of proteolysis. Low choriolytic enzyme (LCE, 25.5 kDa), in contrast, digests the envelope only weakly. Marked choriolysis is induced only by the cooperative actions of HCE and LCE.

In sea urchin embryos, hatching enzyme may be first secreted as a single protease, but it is converted to forms with smaller molecular weight *in vitro*. In *Paracentrotus lividus*, for example, it is released as a 57-kDa form, but cleaved by autolysis to a 30-kDa form that can no longer digest the fertilization envelope (Lepage and Gashe, 1989). In *Strongylocentrotus purpuratus*, it is initially secreted as a 57-kDa protease, but during purification it seems to be converted to a 33-kDa form, which still maintains some proteolytic activity, by autolysis or digestion by other proteases contained in the hatching liquid (Roe and Lennarz,

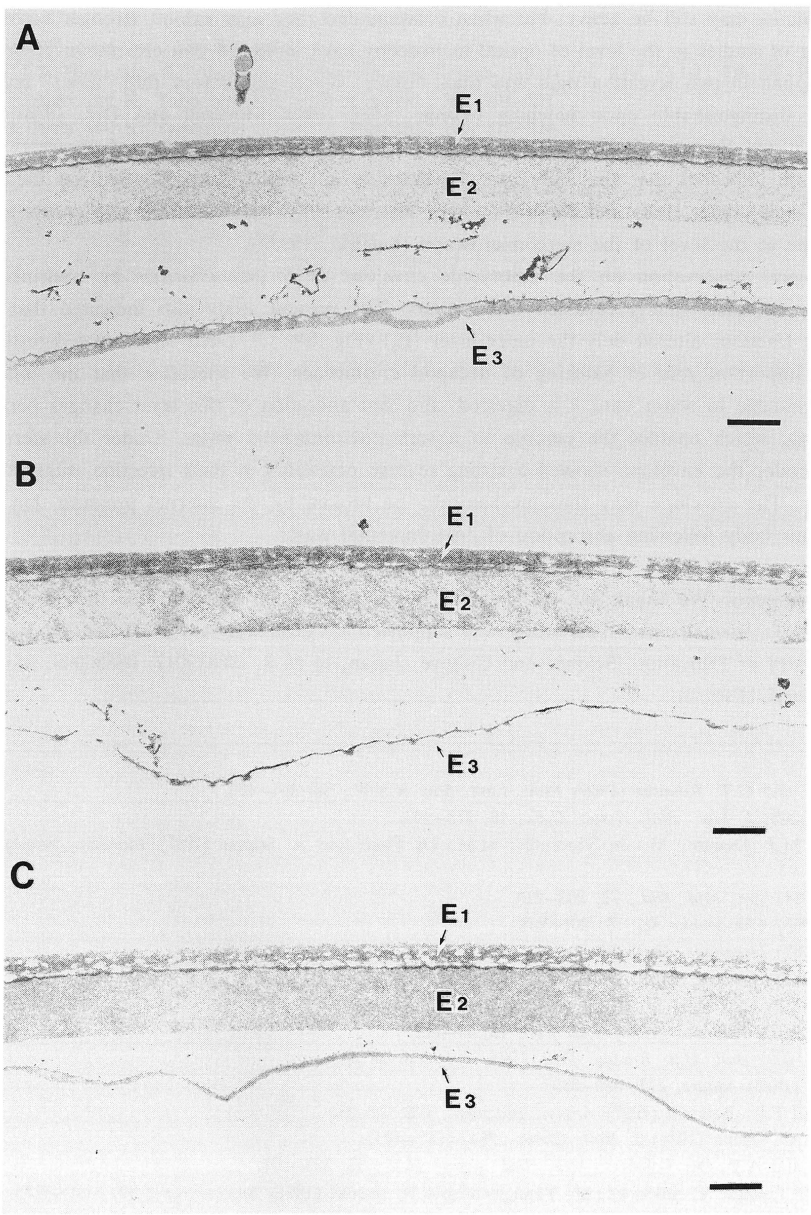

Fig. 5 Digestion of the inner layer (E_3) by partially purified preparation of caseinolytic proteases. A. Control experiment. Isolated embryonic envelope incubated in Tris–HCl buffer. B. Isolated embryonic envelope incubated with active fractions 9 and 10 (Fig. 3A). C. Isolated embryonic envelope incubated with active fractions 12 and 13 (Fig. 3A). Scale = 1 μm.

1990).

In *S. haematocheir*, caseinolytic activity was eluted with two peaks on molecular sieve chromatography; one peak at 50 kDa and the other at 39 kDa (Fig. 3A). When the peak fractions were subjected to SDS–PAGE using gels containing casein, caseinolytic activity was detected as five bands (Fig. 3B), three bands (a–c) between 50 kDa and 60 kDa, a 26-kDa band (d) and a 20-kDa band (e). At the moment we can only speculate as to the nature of the caseinolytic enzyme protein. The *S. haematocheir* caseinolytic protease may be in multiple forms, each of which, in turn, may be composed of multiple subunits.

Individual subunits may still be active, but when compounded they may exhibit stronger activity.

A number of studies at the level of optical microscopy have indicated that crustacean embryos including crabs are wrapped in two layers: a rigid and thick "outer" layer, and a very thin "inner" layer, and these structures are distinguishable upon hatching (Yonge, 1937, 1946; Marshall and Orr, 1954; Davis, 1964, 1965; Anderson and Rossiter, 1969; Trotman *et al.*, 1980). In addition, a number of observations under the stereomicroscope indicated that the embryonic envelope is not digested but is ruptured mechanically [for reviews, see Davis (1968, 1981) and Saigusa (1997)]. Hatching of *S. haematocheir* also occurs as a breakage of the envelope at the level of the stereomicroscope (Saigusa, 1992b).

Morphological observation on the embryonic envelope of *S. haematocheir* by transmission electron microscopy, in contrast, clearly revealed three layers. The present study also indicated that the partially purified active fractions altered only the inner layer (E_3) (Fig. 5B, C). Such small-scale digestion, however, may play an important role in hatching of decapod crustaceans. We speculate that the inner layer (E_3) remains impermeable to water until it is digested, and that alteration of this layer changes permeability just before hatching, which enables the embryo to absorb environmental water. Under the stereomicroscope, the embryos under the envelope showed a strong reverse peristalsis in their intestine, suggesting uptake of ambient water. The envelope may thus mechanically be broken by the internal pressure that is caused by swelling of their body following absorption of environmental water.

Acknowledgments: We thank Mr. H. Iwasaki, K. Oishi and A. Ikumoto for their help in collecting hatch water of *S. haematocheir*. This study was supported by Grants-in-Aid for Scientific Research (C)(2) from the Ministry of Education, Science and Culture, Japan, to M.S. (06839017, 08833009, Marine Biology; 10836014, Natural History).

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