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Purification and gene cloning of *Fundulus heteroclitus* hatching enzyme

A hatching enzyme system composed of high choriolytic enzyme and low choriolytic enzyme is conserved between two different teleosts, *Fundulus heteroclitus* and medaka *Oryzias latipes*

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Note
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Two cDNA homologues of medaka hatching enzyme – high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE) – were cloned from *Fundulus heteroclitus* embryos. Amino acid sequences of the mature forms of *Fundulus* HCE (FHCE) and LCE (FLCE) were 77.9% and 63.3% identical to those of medaka HCE and LCE, respectively. In addition, phylogenetic analysis clearly showed that FHCE and FLCE belonged to the clades of HCE and LCE, respectively. Exon–intron structures of *FHCE* and *FLCE* genes were similar to those of medaka *HCE* (intronless) and *LCE* (8-exon-7-intron) genes, respectively. Northern blotting and whole-mount *in situ* hybridization showed that both genes were concurrently expressed in hatching gland cells. Their spatio-temporal expression pattern was basically similar to that of medaka hatching enzyme genes. We separately purified two isoforms of FHCE, FHCE1 and FHCE2, from hatching liquid through gel filtration and cation exchange column chromatography in the HPLC system. The two isoforms, slightly different in molecular weight and in MCA-peptide-cleaving activity, swelled the inner layer of chorion by their limited proteolysis, like the medaka HCE isoforms. In addition, we identified FLCE by TOF-MS. Similar to the medaka LCE, FLCE hardly digested intact chorion. FHCE and FLCE together, when incubated with chorion, rapidly and completely digested the chorion, suggesting their synergistic effect in chorion digestion. Such a cooperative digestion was confirmed by electron microscopic observation. The results suggest that a hatching enzyme system composed of HCE and LCE is conserved between two different teleosts *Fundulus* and medaka.

At hatching of teleost embryos, hatching enzymes are secreted from the embryos to digest their envelope (egg envelope, chorion). The enzymatic properties and gene structures of the hatching enzymes of medaka *Oryzias latipes*, one of the teleosts, have been well studied [1–6]. In conclusion, a view has been

Abbreviations
CBB, Coomassie brilliant blue G; DIG, digoxygenin; FHCE, *Fundulus* high choriolytic enzyme; FLCE, *Fundulus* low choriolytic enzyme; HCE, high choriolytic enzyme; LCE, low choriolytic enzyme; MHCE, medaka high choriolytic enzyme; MLCE, medaka low choriolytic enzyme.

proposed that the hatching enzyme in the medaka is a system consisting of two metal proteases, HCE (high choriolytic enzyme, choriolysin H, EC 3.4.24.67) and LCE (low choriolytic enzyme, choriolysin L, EC 3.4.24.66) [2,7]. HCE partially digests the protein and causes marked swelling of the inner layer of the chorion. LCE hardly affects the intact inner layer, but efficiently digests the inner layer swollen by HCE [3,4]. Thus, HCE and LCE cooperatively digest the chorion [8,9].

In spite of a marked difference in their mode of action toward the chorion, amino acid sequences of the mature enzymes of HCE and LCE are similar to each other with 55% identity. Both enzymes belong to the astacin family of metallo-proteases that includes digestive enzymes such as astacin [10], differentiation factors such as BMP1 [11] and kidney proteases such as meprin [12]. Among all the members of the family, hatching enzymes form one of the orthologous groups [13].

At present, hatching enzyme cDNAs have been isolated from other teleosts such as zebrafish *Danio rerio* [14], masu salmon *Oncorhynchus masou* [14], yellow-tailed damsel *Chrysiptera parasema* [15] and Japanese eel *Anguilla japonica* [16]. Based on molecular phylogenetic analysis, it has been concluded that all the cDNAs are homologous to the medaka *HCE* (*MHCE*) gene. None of the genes homologous to the medaka *LCE* (*MLCE*) have been cloned yet. Whether or not the hatching enzyme of other fish species is an enzyme system consisting of HCE and LCE, as in the case of the medaka, still remains to be studied.

We observed the hatching of *Fundulus heteloclitus* embryos. The environment where the embryos hatch out is quite different between the two fish species, *F. heteroclitus* and medaka: *Fundulus* embryos hatch in estuarine water, madaka embryos in fresh water. *Fundulus* is located at a position closely related to the medaka in morphology-based phylogeny: *Fundulus* and medaka belong to different orders, Cyprinodontiformes and Beloniformes, respectively, but they belong to the same series, Atherinomorpha [17]. *Fundulus* hatching enzyme was partially purified and characterized by DiMichele *et al*. in 1981 [18]. However, the chorion-digesting mechanism of the enzyme remains unclear. In the present study, we cloned cDNAs and genes for *Fundulus* hatching enzymes, purified the enzymes from hatching liquid, and compared their manner of chorion digestion and their enzymatic properties with those of the MHCE and MLCE. We demonstrated that *Fundulus* hatching enzyme is a system consisting of HCE and LCE, similar to that of the medaka.

Results

Fundulus homologues of HCE and LCE

A 170 bp long cDNA fragment was obtained by RT-PCR using degenerate primers designed from amino acid sequences of two active sites conserved in all the astacin family proteases. Seventeen fragments of the PCR product were cloned and subjected to sequence analysis. The nucleotide sequences of all fragments were almost the same, and highly similar to some parts of MHCE cDNA. We considered the fragments as cDNAs for a *Fundulus* homologue of HCE (FHCE).

Because no cDNA homologous to *MLCE* could be obtained using such primers, we designed and synthesized several primers from the sequences similar to that of *MLCE* cDNA. One set of the primers amplified a 140 bp cDNA fragment. The nucleotide sequence of the fragment was more similar to that of *MLCE* (70.7%) than that of *MHCE* (60.6%). We regarded the fragment as a cDNA for *Fundulus* homologue of *LCE* (*FLCE*).

Full-length cDNAs of *FHCE* (1040 bp) and *FLCE* (948 bp) were cloned by the 5′- and 3′-RACE PCR method. Amino acid sequences deduced from their cDNAs are shown in Fig. 1, together with those of Japanese eel (EHCE12), zebrafish (ZHCE1), masu salmon (MsHCE1), MHCE and MLCE. According to the SIGNALP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/), a cleavage site for signal peptidase was predicted at Ala18/Leu19 for FHCE and at Ala20/Tyr21 for FLCE. Based on sequence similarity of MHCE and MLCE, the N terminals of mature enzymes of FHCE and FLCE were predicted to be Asn67 and Thr65, respectively, suggesting that both FHCE and FLCE are synthesized as preproenzyme forms. The mature enzyme portions of FHCE and FLCE were composed of 199 and 204 amino acids, respectively, and their amino acid identity was 51%. Both FHCE and FLCE conserved the two active site consensus sequences HExxHxxGFxHExxRxD (Zn-binding site) and SxMHY (methionine turn) found in all the astacin family proteases. In addition, six cysteine residues were conserved in FHCE and FLCE (Fig. 1).

The amino acid sequence of the mature enzyme portion of FHCE was homologous to that of other hatching enzymes, and the identities were 57.8, 58.3, 59.8 and 77.9% to EHCE12, ZHCE1, MsHCE1 and MHCE, respectively, while FLCE was 63.3% identical in the sequence to MLCE. We constructed a phylogenetic tree using astacin as an outgroup. As shown in Fig. 2, EHCE12, ZHCE1, MsHCE1, MHCE and

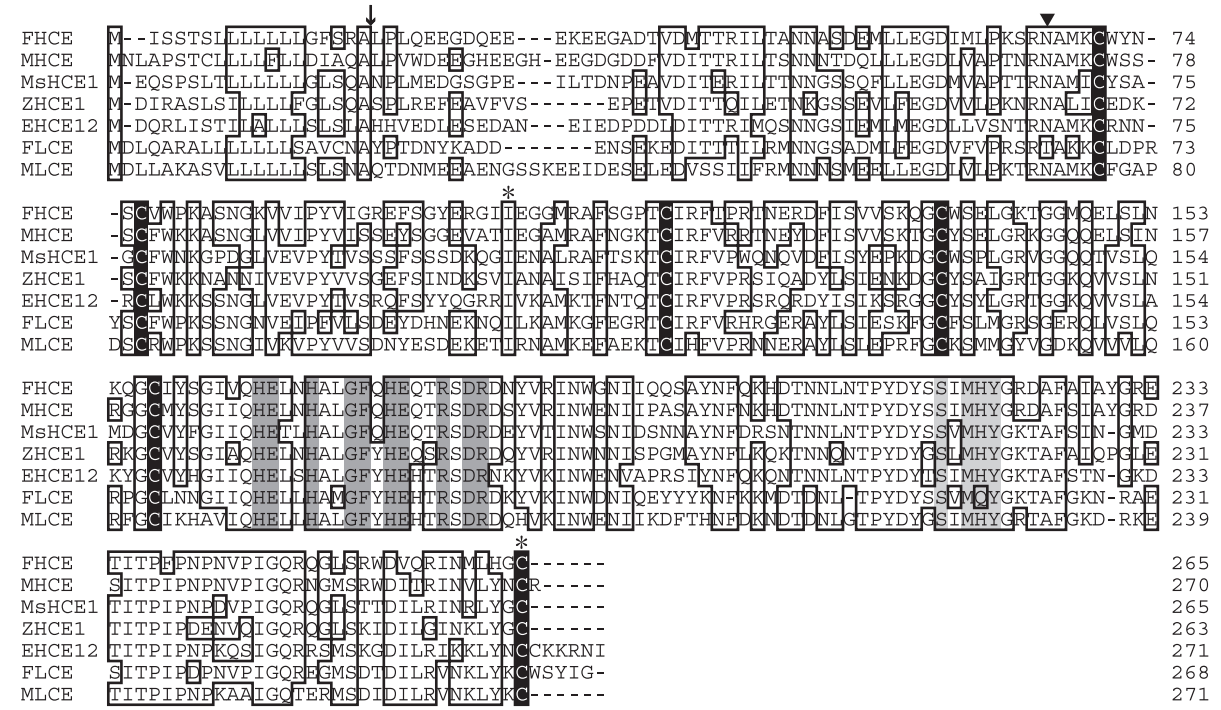

Fig. 1. Multiple sequence alignment of amino acid sequences of *Fundulus* hatching enzymes (FHCE and FLCE), medaka HCE and LCE (MHCE and MLCE), masu salmon HCE (MsHCE1), zebrafish HCE (ZHCE1) and Japanese eel HCE (EHCE12). Arrow and arrowhead indicate putative signal sequence cleavage sites and N terminals of mature enzymes, respectively. Identical residues are boxed. Dashes represent gaps. Two active site consensus sequences of the astacin family protease are indicated in dark and light grey boxes, and conserved cysteine residues are in black boxes. Amino acid sequences from Ile to Cys, indicated by asterisks, were used to construct a phylogenetic tree (Fig. 2). Accession numbers: FHCE, AB210813; FLCE, AB210814; MHCE, M96170; MLCE, M96169; MsHCE1, AB175619; ZHCE1, AB175621; EHCE12, AB071427.

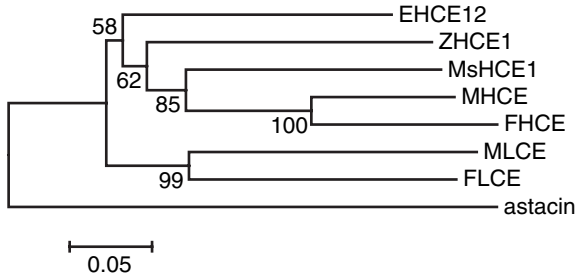

Fig. 2. Phylogenetic tree of amino acid sequences of the mature enzyme portions of teleost hatching enzymes constructed by the neighbor-joining method. Astacin of crayfish *Astacus astacus* was used as an outgroup. Numbers at the nodes represent the bootstrap values with 1000 replications. The scale bar indicates an evolutionary distance of 0.05 amino acid substitutions per site.

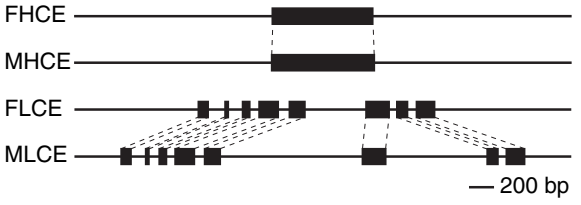

Fig. 3. Exon–intron structures of the *FHCE*, *FLCE*, *MHCE* and *MLCE* genes. The exons and introns are indicated by boxes and solid lines, respectively.

FHCE formed one clade (HCE clade). Within the HCE clade, EHCE12 first branched off from an ancestor, followed by ZHCE1, MsHCE1, and MHCE or FHCE. Their branching pattern was closely related to the morphology-based phylogeny of fish species proposed by Nelson [17]. On the other hand, MLCE and FLCE also formed one group (LCE clade) outside the HCE clade. This phylogenetic relationship gave evidence that FHCE and FLCE are molecules homologous to MHCE and MLCE, respectively.

We have previously demonstrated that the *MHCE* gene is intronless, while *MLCE* gene is composed of eight exons and seven introns [19]. In the present study, *FHCE* and *FLCE* genes were amplified from genomic DNAs by PCR, and the 862 bp and 1974 bp fragments corresponding to *FHCE* and *FLCE* genes, respectively, were obtained. As shown in Fig. 3, the

FHCE gene was intronless, and the *FLCE* gene had eight exons and seven introns. Thus, the exon–intron structures of *FHCE* and *FLCE* genes were the same as those of *MLCE* and *MHCE* genes, respectively. All splice junctions of the *FLCE* gene were under the GT-AG rule. In addition, the exon–intron boundary and intron phase were conserved in *FLCE* and *MLCE* genes. The results indicate that *FHCE* and *FLCE* genes are highly homologous to *MHCE* and *MLCE* genes, respectively.

Expression of *FHCE* and *FLCE* genes

Gene expression of *FHCE* and *FLCE* was analysed by northern blotting (Fig. 4). Digoxygenin (DIG)-labelled DNA probes synthesized from full-length cDNAs were hybridized with total RNA from several stage embryos. Each *FHCE* and *FLCE* probe was hybridized with 0.9 kb RNAs, and their sizes were consistent with the sizes of respective cDNAs (1040 bp for *FHCE*, 948 bp for *FLCE*). Both transcripts increased in amount during the developmental period from stage 21 to 25, and decreased thereafter. The *FHCE* signals were much stronger than those of *FLCE* at all the stages examined, suggesting that *FHCE* mRNA is much more abundant than *FLCE* mRNA, as in the case of medaka. It has been reported that the expression of the *MHCE* gene is four times greater than that of the *MLCE* gene [2,7]. In addition, as shown later, the amount of FHCE protein in hatching liquid was much more abundant than that of FLCE. These results suggest that the relative amount of HCE and LCE expression is conserved between *Fundulus* and medaka.

Whole-mount *in situ* hybridization using antisense RNA probes for *FHCE* and *FLCE* genes reveals distribution of cells expressing hatching enzyme gene transcripts in developing *Fundulus* embryos. In stage 19 embryos, *FLCE* gene transcripts were detected in a U-shaped cell mass at the anterior end of the forebrain (Fig. 5D). This cell mass is considered to be homologous to 'pillow' of medaka and zebrafish embryos [14,20,21]. In stage 21 embryos, the FHCE-expressing cells were located between two eye rudiments (Fig. 5A). At stage 25, the strong expression of *FHCE* and *FLCE* genes was found in branchial arches (Fig. 5B and E). From stage 29 (Fig. 5C and F) to the prehatching stage, the signals for *FHCE* and *FLCE* were observed in the restricted regions of pharyngeal cavity and the periphery of the mouth. Although the *FLCE* signals were weaker in intensity than those of *FHCE*, the developmental expression patterns of the *FLCE* gene were the same as those of *FHCE* gene. Signals from sense RNA probe were not observed in any embryo.

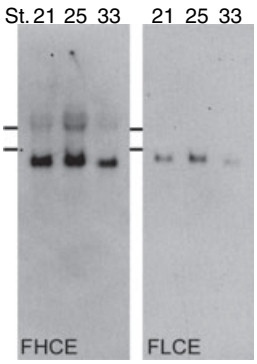

Fig. 4. Northern blot analysis of the expression of *FHCE* and *FLCE* gene. Numbers at the top show the developmental stages. Bars indicate the positions of 18 S and 26 S rRNA.

In medaka embryos, it has been reported that hatching gland cells differentiate at the anterior end of the hypoblast layer in late gastrula embryos. After that, the hatching gland cells at the front of the head rudiment, called 'pillow', migrate to the branchial arches during organogenesis [22,23]. Before the hatching stage, the hatching gland cells migrate anterior accompanied with morphogenesis of lower jaw, and are distributed around the inner wall of the pharyngeal cavity and gill. Although the expression of *Fundulus* hatching enzyme gene was not observed in the inner wall of the pharyngeal cavity but only in the gill and periphery of the mouth, the spatio-temporal expression pattern of the *FHCE* and *FLCE* genes basically resembles that of medaka hatching enzyme gene [24].

Isolation and enzymological characterization of FHCE and FLCE

To investigate enzymological properties and mode of choriolytic action of FHCE and FLCE, *Fundulus* hatching enzymes were isolated from hatching liquid. Figure 6A shows a Toyopearl HW-50S column chromatogram. A large amount of chorion protein was eluted just after the void volume. Fractions having proteolytic or caseinolytic activity were divided into two, a minor peak just after the peak of chorion protein (fraction I) and a major peak near the bed volume (fraction II). These two fractions were separately subjected to cation exchange column chromatography in the HPLC system. About a half of the protein in fraction I was adsorbed to the column, and eluted as a sharp single peak (fraction I-a) (Fig. 6B). Fraction I-a contained three proteins that could be detected by SDS/PAGE. Their molecular sizes were estimated at 32, 29 and 24 kDa. On the other hand, almost all

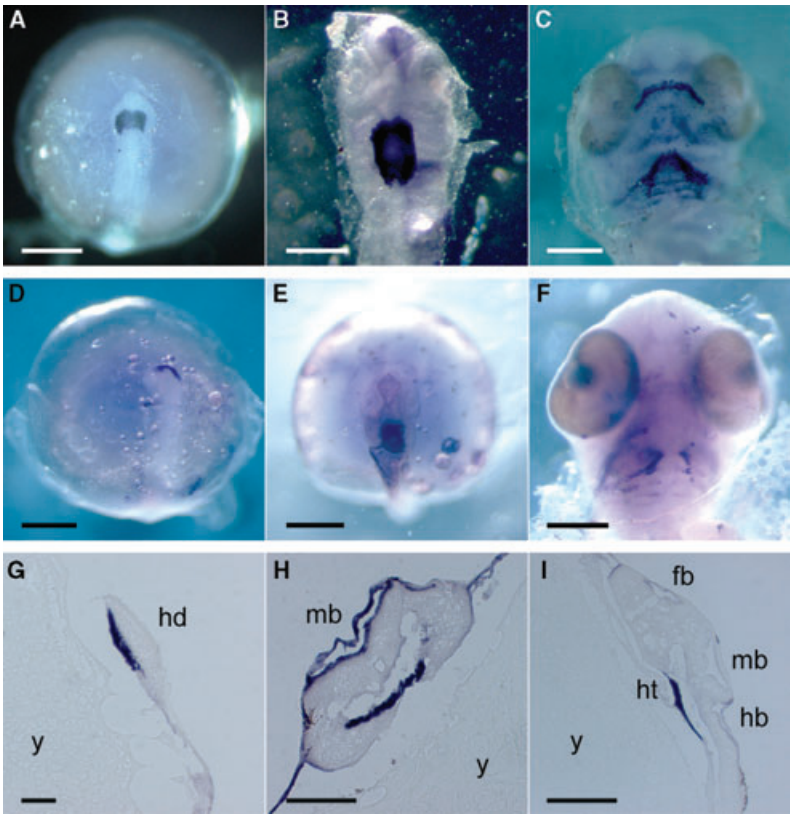

Fig. 5. *FHCE* and *FLCE* gene expression in *Fundulus* embryos detected by whole-mount *in situ* hybridization with FHCE (A–C) and FLCE (D–F) RNA probes. (A, D, E) Ventral views, scale bar, 500 μm. (B, C, F) Dorsal views, scale bar, 250 μm. (A, D) Stage 19–21 embryos. (B, E) Stage 25 embryos. (C, F) Stage 29 embryos. Yolk was removed before performing the colour reaction except stage 19–21 embryos. Sections were made from hybridized embryos with the FHCE (G and H) and FLCE probe (I). (G and I) Sagittal sections of stage 21 embryos. (H) Transverse section of stage 25 embryo. Scale bars, 100 μm. y, Yolk; hd, head; fb, forebrain; mb, midbrain; hb, hindbrain; ht, heart.

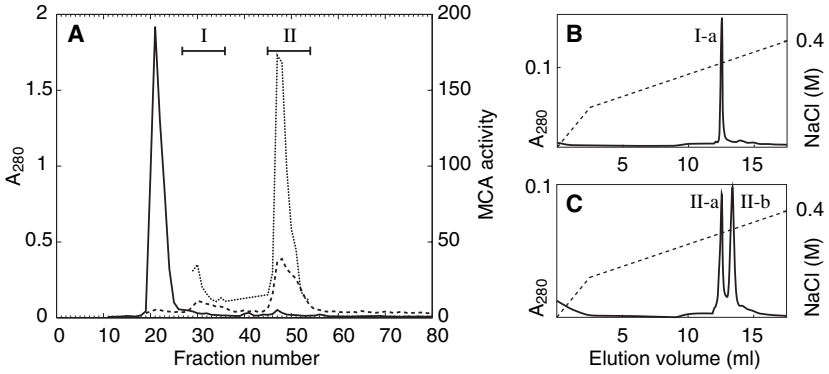

Fig. 6. Purification of *Fundulus* hatching enzymes. (A) Elution pattern of hatching liquid by Toyopearl HW-50S column chromatography. Solid line, absorbance at 280 nm; dashed line, caseinolytic activity; dotted line, MCA-peptide cleaving activity. (B) Elution pattern of fraction I by cation exchange HPLC with a linear gradient from 0 to 400 mM NaCl. (C) Elution pattern of fraction II by cation exchange HPLC with a gradient from 0 to 400 mM NaCl.

proteins in fraction II were adsorbed to the column and fractionated into two peaks (fraction II-a and II-b) (Fig. 6C). The fraction II-a and II-b exhibited a single band on SDS/PAGE, and their electrophoretic mobility was almost the same (Fig. 7). Their molecular mass was estimated at 24 kDa.

Specific caseinolytic activity of fraction I-a was 0.33 ΔA_{280}·min^{-1}·mg protein^{-1} and considerably lower than others, while the activities of fraction II-a and II-b were 4.68 and 4.37 ΔA_{280}·min^{-1}·mg protein^{-1}, respectively, about two-thirds to that of MHCE-1 (7.03) and MHCE-2 (6.67) [3,4].

Amino acid sequencing revealed that the sequences from the N terminus of the 24 kDa proteins in fraction I-a and II-b were NAMKCWYNSCVXPKA and NAMKCWYNSCV, respectively, and that both sequences matched completely to the N-terminal sequence predicted from FHCE cDNA, suggesting that the 24 kDa proteins in fraction I-a and II-b are FHCE.

TOF-MS showed that fractions II-a and II-b exhibited two single peaks of *m/z* 22 676.5 and 22 779.0, respectively (Fig. 8). The former was almost the same as the molecular mass calculated from FHCE cDNA

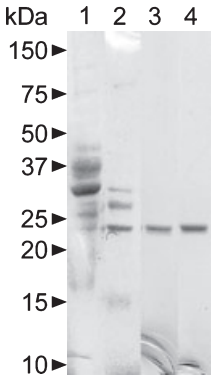

Fig. 7. SDS/PAGE. (1) *Fundulus* hatching liquid (2) fraction I-a (3) fraction II-a (4) fraction II-b. Numbers on the left refer to the sizes of molecular markers.

(22 637), the latter was slightly but significantly larger than that. The results suggest the existence of two isoforms of FHCE having a minor difference in molecular weight. We designated the proteins in fraction II-a and II-b as FHCE1 and FHCE2, respectively.

The previous study showed that MLCE is eluted by Toyopearl HW-50S column chromatography just after the peak containing a large amount of chorion protein [4]. This position where MLCE was eluted was consistent with the position of fraction I in the present study. The TOF-MS analysis showed the existence of several protein peaks in fraction I-a. We focused on a major peak (*m/z*, 22 676.5) and a minor peak (*m/z*, 23 739.1) as shown in Fig. 8. The molecular mass of the major peak was the same as that of FHCE1 in fraction II-a, while that of the minor peak was almost the same as that predicted from FLCE cDNA (23751). It is reasonable to conclude that fraction I-a contains FLCE in addition to FHCE1. This is the first identification of a molecule homologous to LCE in fishes other than medaka.

Because isolation of FLCE was difficult, fraction I-a was used in a later investigation to examine the effect of FLCE on chorion digestion.

Substrate specificities of FHCE1, FHCE2 and MHCE were examined using various MCA-peptides. Their relative MCA-peptide cleaving activities were somewhat different from each other as shown in Fig. 9. The best substrate for both FHCE1 and FHCE2 was Suc-Ala-Pro-Ala-MCA. The second one is different between the two; Boc-Val-Pro-Arg-MCA for FHCE1, Suc-Leu-Leu-Val-Tyr-MCA for FHCE2. FHCE2 showed a low activity to Suc-Ile-Ile-Trp-MCA, while FHCE1 showed no activity to this substrate.

MHCE cleaved almost the same MCA substrates as FHCE1 and FHCE2 did. However, their cleaving efficiency was considerably different from each other: the best substrate for FHCE1 and FHCE2 was Suc-Ala-Pro-Ala-MCA as described earlier, whereas that of MHCE was Suc-Leu-Leu-Val-Tyr-MCA. Figure 10 shows the pH dependency of the MCA-peptide-cleaving activity of FHCE1, FHCE2 and MHCE. Optimum pH of the activity of both FHCE1 and FHCE2 was 7.0, the same as that of MHCE.

Because of difficulty of isolation, the MCA cleaving activity of FLCE was not examined in this study.

To investigate chorion digesting activity, the FHCE and/or FLCE fractions were incubated with chorion fragments, and amounts of peptides liberated from the fragments were measured. FHCE1 or FHCE2 moderately digested the chorion as shown in Fig. 11. Specific activities of FHCE1 and FHCE2 in such choriolysis were 6.48 and 10.3 ΔA_{595} per 60 min·mg protein^{-1}, respectively, showing that the activity of FHCE2 was slightly higher than that of FHCE1. When the FLCE fraction, fraction I-a, was incubated with chorion, the activity was low. When the mixture of FHCE1 or FHCE2 and the FLCE fraction was incubated with

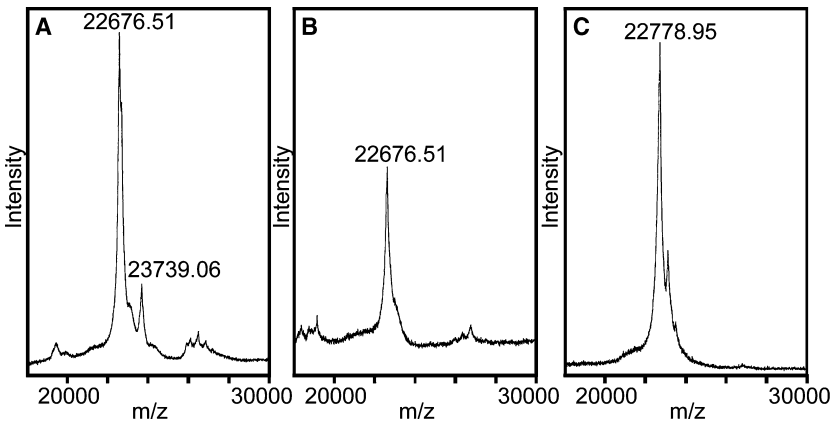

Fig. 8. Part of the TOF-MS spectrogram of fraction I-a (A), II-a (B) and II-b (C).

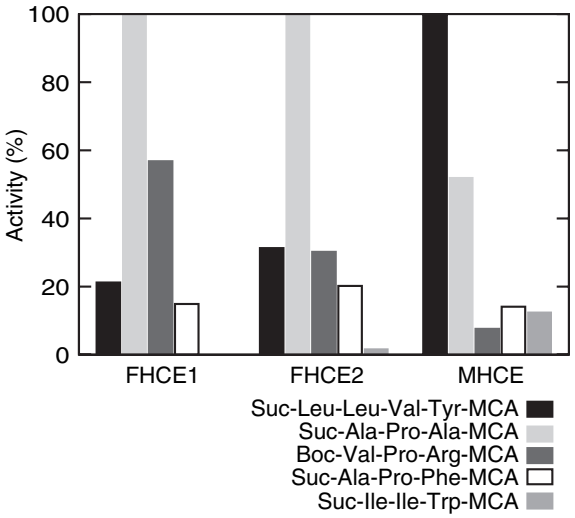

Fig. 9. Substrate specificity of FHCE1, FHCE2 or MHCE examined with several MCA-peptides. Activity is expressed as percent of the activity to the best substrate; Suc-Ala-Pro-Ala-MCA for FHCE1 and FHCE2, Suc-Leu-Leu-Val-Tyr-MCA for MHCE.

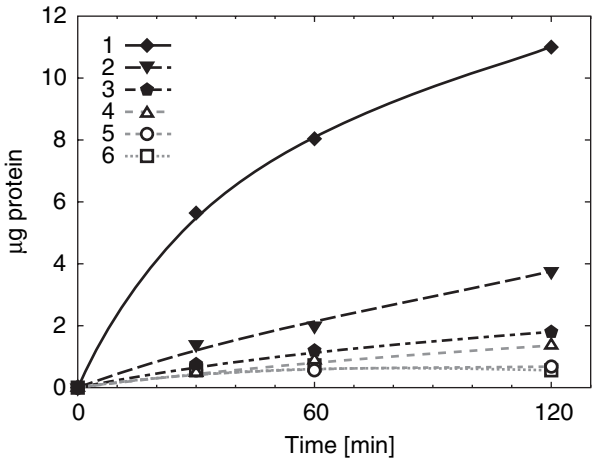

Fig. 11. Time course of solubilization of chorion by FHCE1, FHCE2 and/or FLCE fraction. Amount of protein solubilized in 25 μL supernatant was plotted vs. reaction time (min). 1, FHCE2 2.1 μg + FLCE 0.8 μg; 2, FHCE1 1.3 μg + FLCE 0.8 μg; 3, FHCE1 0.7 μg + FHCE2 1.1 μg; 4, FHCE2 2.1 μg; 5, FHCE1 1.3 μg; 6, FLCE 0.8 μg.

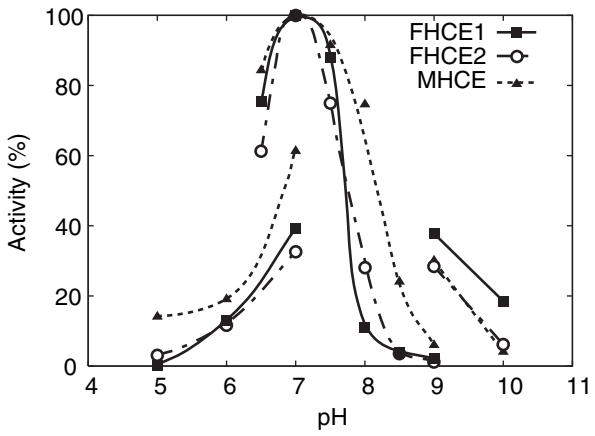

Fig. 10. pH dependency of proteolytic activity of FHCE1, FHCE2 or MHCE. Maleic acid buffer pH 5–7, Tris/HCl buffer pH 6.5–9 and bicarbonate buffer pH 9–10 at the final concentration of 50 mM were used.

the chorion, however, the solubilized peptides increased in amount as compared with the case of incubation of FHCE1 or FHCE2 alone. The combined treatment of FHCE1 and FHCE2 did not show such a synergistic effect. The result clearly shows that FHCE and FLCE cooperatively and synergistically digest chorion as do MHCE and MLCE.

Finally, we observed changes in the fine structures of *Fundulus* embryo chorion by electron microscopy. Intact chorion (Fig. 12A) had two layers – a thin outer layer consisting of electron-dense materials and a thick inner layer showing a lamellar structure – as found in the medaka chorion [25]. When FHCE2 was incubated with such isolated chorion, the inner layer of the chorion was swollen. Such a structural feature of the swollen chorion was similar to that of the medaka chorion swollen by MHCE alone (data not shown). In the present observation, some fibrillar structures were found just beneath the outer layer as shown in Fig. 12B. When the fraction I-a (FLCE fraction) alone was incubated with the chorion, no significant change in the chorion structure was observed (data not shown). When the mixture of FHCE2 and the FLCE fraction was incubated with the chorion, however, the inner layer of chorion was completely solubilized, and only the outer layer and some fragmented structures remained undigested (Fig. 12C). Digestion of the chorion in such a manner by the mixture was quite similar to that in natural hatching of *Fundulus* (Fig. 12D) and medaka embryos [4].

Discussion

From *Fundulus heteroclitus*, we cloned two cDNAs and genes for astacin family proteases homologous to hatching enzyme. Comparison and phylogenetic analysis of amino acid sequences clearly showed that the cloned cDNAs and genes were *Fundulus* homologues of *HCE* (*FHCE*) and *LCE* (*FLCE*), respectively. Northern blot analysis and whole-mount *in situ* hybridization revealed that the genes for *FHCE* and *FLCE* were concurrently expressed in hatching gland cells,

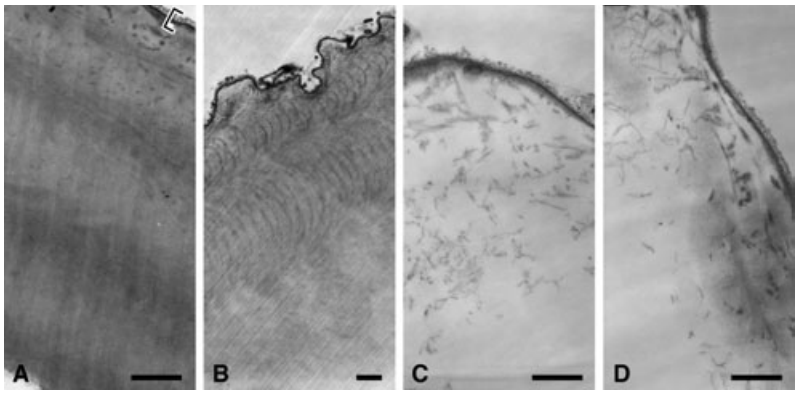

Fig. 12. Electron microscopic observation of solubilization of chorion by FHCE2 and/or FLCE fraction. Small fragments of the isolated chorion were incubated in 5 μL of 50 mM Tris/HCl pH 8.0 at 30 °C with or without enzymes. (A) Chorion was incubated in buffer only. (B) Chorion was incubated with purified FHCE2 alone. Fibrillar structures underneath the outer layer were magnified. (C) Chorion was incubated with purified FHCE2 and FLCE fractions. (D) Chorion after the natural hatching of *Fundulus* embryos. Outer layer is indicated by a bar in (A). Scale bars, 0.1 μm (A, C, and D) and 0.25 μm (B).

and their spatio-temporal expression patterns were similar to those of medaka *HCE* and *LCE* as reported previously [20]. The results suggest that the regulation of *HCE* and *LCE* gene expression is conserved between the two fish species, *Fundulus* and medaka.

As described earlier, the environment where embryos hatch out is quite different in the two fish species: *Fundulus* embryos hatch in estuarine water, medaka embryos in fresh water. The optimum ionic strength of choriolytic activity of *Fundulus* hatching enzyme has been reported to be around 0.2 M for NaCl [18], whereas medaka hatching enzyme is scarcely active in such high ionic solution (data not shown), suggesting that the characters of the two enzymes adapt well to the environment surrounding the respective embryos. A structural similarity of hatching enzyme genes of the two fish species shows that their salt adaptation does not result from a change of large molecular structures such as domain structure but from substitutions of amino acids involved in salt dependency of their choriolytic activity.

We purified two types of HCE, FHCE1 and FHCE2, from hatching liquid of *Fundulus* embryos. Although substrate specificity examined using MCA substrate and molecular masses of the two were slightly different from each other, both swelled the inner layer of chorion, and not completely but partially digested the inner layer, due to their limited proteolysis as with MHCE. A similar result has been reported that two types of *MHCE* cDNA, *MHCE21* and *MHCE23*, were cloned from a cDNA library of medaka embryos, and identity of amino acid sequences of their mature enzyme forms was 95% [19]. This suggests that *FHCE* genes are multicopy genes, similar to the *MHCE* genes.

As shown by SDS/PAGE and TOF-MS, fraction I-a from *Fundulus* hatching liquid contained a large amount of FHCE1 in addition to a small amount of FLCE. Compared with isolated FHCE1 (fraction II-a), the HCE activity of FHCE1 in this fraction was severely suppressed: its specific caseinolytic activity was 10 times lower, and its choriolytic activity was also very low as shown in Fig. 11. In addition, when fraction I-a was incubated with intact chorion, any morphological change of the chorion was not observed. FHCE1 in this fraction, not isolated FHCE1, is considered to bind tightly to the final products as found in MHCE [3]. The suppression of the HCE activity of FHCE1 may be due to such complex formation. Thus, fraction I-a exhibits only the LCE activity such as inaccessibility to intact chorion, digestion of chorion swollen by active FHCE1 or 2, and synergistic choriolytic activity in the treatment of intact chorion with fraction I-a combined with active FHCE1 or 2.

The result that fraction I-a contained a small amount of FLCE was consistent with a previous result that the content of MLCE in hatching liquid of medaka embryos was about 10 times lower than that of MHCE [4]. Fraction I-a containing a small amount of FLCE caused a synergistic effect on chorion digestion when applied to the chorion together with FHCE1 or FHCE2. This resembles a previous result on chorion digestion of hatching enzymes in medaka: a considerably large amount of MHCE is required for efficient swelling of chorion, whereas a small amount of MLCE completely solubilizes the swollen chorion, that is, at least 6 μg of MHCE and 0.5 μg of MLCE are required to completely solubilize 10 mg of chorion [4]. The results suggest that

a hatching enzyme system composed of two enzymes, HCE and LCE, is conserved between *Fundulus* and medaka, not only in molecular structure but also in mode of action toward the chorion.

We incubated medaka chorion in *Fundulus* hatching liquid. The *Fundulus* enzyme swelled the medaka chorion. Thus, FHCE is able to swell medaka chorion. However, FLCE could not solubilize the swollen chorion of medaka. MHCE also swelled *Fundulus* chorion, whereas MLCE could not efficiently solubilize the swollen chorion of *Fundulus* (data not shown). Thus, HCEs showed cross-species digestion of chorion, but LCEs did not. A difference of such species specificity between the enzymes might be established by their adaptation to changes of chorion proteins induced by mutations or amino acid substitutions during evolution.

Electron microscopic observation in the present study showed a similarity of gross morphology of *Fundulus* and medaka chorion, that is, the chorion consists of two layers, outer and inner layer. The inner layer is of a multilamellar structure digested by hatching enzyme. Furthermore, it is well known that the inner layer of medaka chorion is composed of at least two ZP-domain-containing proteins ZI-1,2 and ZI-3 [26–29]. Their precursors – called choriogenin – are produced from spawning female liver [29]. Recently, an analysis using differential display has identified a cDNA homologous to one of the medaka choriogenin cDNAs from *Fundulus heteroclitus* liver (accession number, CV575998), suggesting that the *Fundulus* chorion is also constituted by ZP-domain-containing proteins as is the medaka chorion. Sites of chorion proteins cleaved by hatching enzyme might also be mutated during evolution.

The identity of amino acid sequences between FHCE and MHCE (77.9%) was higher than that between FLCE and MLCE (63.3%). Thus, the mutation rate of LCE was much higher than that of HCE. A difference of evolutionary mutation rate between HCE and LCE might explain the results of the cross-species digestion experiment: LCE, having the higher mutation rate, did not show the cross-species digestion. These results are interesting in coevolution of hatching enzyme and chorion. To further understand such coevolution, cloning of *HCE* and *LCE* genes of other fish species and the elucidation of their mode of action toward chorion protein containing ZP domains should be carried out as the next study.

Experimental procedures

Embryos of *F. heteroclitus* were collected every day and cultured in tap water at 25 °C. On day 8 of culture, the embryos were taken out of the water, allowed to stand in air for 15 min ('dry stimulation' or 'air-incubation' to induce the embryo hatching), and then transferred into a small amount of a medium consisting of 0.23 M NaCl, 5 mM KCl, 5 mM $CaCl_2$, 18 mM $MgCl_2$, 8.8 mM $MgSO_4$, 10 mM H_3BO_4, 6.8 mM NaOH and 2 mM $NaHCO_3$. When more than 50% of the embryos hatched out (about 30 min later), the medium called hatching liquid was filtered, and stored at 4 °C. Developmental stages were determined according to the criteria proposed by Armstrong and Child [15].

Cloning of *Fundulus* homologues of HCE and LCE cDNAs

Total RNA was extracted from stage 20 embryos, and subjected to RT-PCR (Qiagen, Valencia, CA, USA) to obtain cDNA fragments for HCE and LCE. For HCE, degenerate primers were designed from the consensus sequences of two active site regions of the astacin family proteases, HExxHxxGFxHExxRxDR and YDYxSxSxMHY [30]. For LCE, several primer sets were generated from nucleotide sequences of medaka LCE. The nucleotide sequences of upstream and downstream primers by which *Fundulus* LCE fragment was successfully amplified were 5′-TGCATG CTCTGGGTTTCTAC-3′ and 5′-GTCATACGGGGTG CCCAGATT-3′, respectively. PCR was performed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and followed at 72 °C for 5 min. The fragments thus obtained were inserted into a pGEM-T easy vector (Promega, Madison, WI, USA). Nucleotide sequences of the fragments were determined by a 377 DNA sequencer (ABI, Foster City, CA, USA) using a Big Dye Cycle Sequencing Kit. The cloning of full-length cDNAs was performed by the 5′- and 3′-RACE method using the 5′ RACE system (Invitrogen, Carlsbad, CA, USA) and an Advantage cDNA PCR kit (Clontech, Mountain View, CA, USA).

Phylogenetic analysis

A multiple sequence alignment was performed using the CLUSTAL W program [31]. A tree was constructed using the amino acid sequences of mature enzymes according to the neighbor-joining method in the program MEGA2 version 2.1 [32]. Astacin, one of the members of the astacin family proteases, was used as an outgroup. The reliability of the tree was assessed by a bootstrap analysis with 1000 replicates.

Gene amplification

Genomic DNA was extracted from the liver of an adult *F. heteroclitus*. PCR amplification was performed with LA *Taq* polymerase (Takara, Tokyo, Japan). The primers were

designed and synthesized from nucleotide sequences of the 5′- and 3′ portions of full-length cDNAs.

Northern blot analysis

A digoxigenin-labelled DNA probe was synthesized with a PCR DIG probe synthesis kit (Roche, Indianapolis, IN, USA) using *FHCE* and *FLCE* cDNA as templates. In this study, *Fundulus* homologues of *HCE* and *LCE* were named *FHCE* and *FLCE*, respectively. Equal amounts of total RNA (10 μg) extracted from embryos at stage 21, 25 and 33 were electrophoresed on a 1% (v/v) formaldehyde/agarose gel, and transferred to nylon membrane (Hybond N^+, Amersham, Piscataway, NJ, USA). Hybridization was performed in DIG Easy Hyb (Roche) at 37 °C overnight. The membrane was washed twice with 2 × standard NaCl/Cit–0.1% (w/v) SDS for 5 min at room temperature, and three times with 1 × NaCl/Cit–0.1% (v/v) SDS for 15 min at 60 °C. The filter was incubated with 0.2% (w/v) blocking reagent and 0.1% (v/v) Tween-20 in NaCl/P_i for 30 min at room temperature, and with 1 : 5000 alkaline phosphatase-conjugated anti-digoxigenin Igs in the same buffer for 1 h. After that, the filter was incubated in a substrate solution consisting of 1% disodium 3-[4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl] phenylphosphate (CSPD), 0.1% (v/v) diethanolamine and 1 mM $MgCl_2$ for 5 min, and exposed to scientific imaging film (Kodak), in the dark.

Whole-mount *in situ* hybridization

Stage 19–21, 25, 26–28, 29, 30 and 31–32 embryos were fixed with 4% (v/v) paraformaldehyde in NaCl/P_i at 4 °C overnight, and stored in 100% methanol at −20 °C. After chorions were removed, the embryos were washed for 3 × 5 min in PBST [NaCl/P_i containing 0.1% (v/v) Tween-20], and prehybridized in a hybridization buffer consisting of 50% (v/v) formamide, 5 × NaCl/Cit, 0.1% (v/v) Tween 20, 50 μg·mL^{-1} tRNA and 50 μg·mL^{-1} heparin for 1 h at 55 °C. Hybridization was performed overnight at 55 °C in the hybridization buffer with DIG-labelled antisense or sense RNA probe for FHCE or FLCE. After four 30-min washes with a solution of 50% (v/v) formamide, 2 × NaCl/Cit and 0.1% (v/v) Tween (NaCl/CitT) at 68 °C, the embryos were incubated for 2 × 15 min in 2 × NaCl/CitT at 68 °C, washed for 3 × 20 min in 0.2 × NaCl/CitT at 68 °C, and transferred to PBST at room temperature. The embryos were incubated for 90 min with 1% (w/v) blocking reagent and 0.1% (v/v) Tween-20 in NaCl/P_i, and then with anti-DIG Igs (1 : 8000) in PBST at 4 °C overnight. After 6 × 30-min washes in PBST, the embryos were incubated in a staining buffer consisting of 100 mM Tris/HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% (v/v) Tween 20 for 10 min, and stained with NBT/BCIP. After the staining reaction, the embryos were washed with NaCl/P_i.

Purification of hatching enzyme

All procedures, except for HPLC, were performed at 0–4 °C. Ammonium sulfate powder was added to about 100 mL of hatching liquid derived from about 5000 embryos (60% saturation). The precipitate was collected by centrifugation and dissolved in 15 mL 50 mM bicarbonate buffer pH 10.2. After dialysis against 50 mM bicarbonate buffer, the clear solution was applied to a Toyopearl HW-50SF (TOSOH Inc., Tokyo, Japan) column equilibrated with 50 mM bicarbonate buffer pH 10.2. The fractions having proteolytic activity were collected and dialysed against 20 mM Tris/HCl buffer pH 7.5. The samples were subjected to a Source 15S column for HPLC system (Gilson, Middleton, WI, USA) and eluted with a linear gradient of 0–400 mM NaCl in 10 mM Tris/HCl buffer pH 7.5.

SDS/PAGE

To evaluate the purity of enzyme SDS/PAGE was carried out by the method of Laemmli using a 12.5% gel [33]. The gel was stained with Coomassie brilliant blue G (CBB).

Determination of amino acid sequences of the N-terminal regions of FHCE1 and FHCE2

Sample was analysed by SDS/PAGE, and electrically blotted onto polyvinylidene difluoride membrane. After staining with CBB, the band portion of FHCE1 was cut out and subjected to an amino acid sequencing (Procise 491HT amino acid sequencer, Applied Biosystems, Foster City, CA, USA). The purified FHCE2 was dotted onto the membrane and applied to the sequencer.

TOF/MS

The HPLC fractions containing 2 μg of protein were desalted through C_{18} reversed-phase chromatography, and subjected to AXIMA-CFR plus mass spectrometer (Shimadzu, Kyoto, Japan). The matrix, α-cyano-4-hydroxycinnamic acid, was dissolved in a reaction solution containing equivolumes of acetonitrile and 0.1% (v/v) trifluoroacetic acid.

Estimation of proteolytic activity

The proteolytic activity of hatching enzyme was measured using a 0.5 mL reaction mixture consisting of 83 mM Tris/HCl pH 8.0, 3.3 mg·mL^{-1} casein and the enzymes. Incubation was performed for 60 min at 30 °C. After the reaction was stopped by adding 500 μL of 20% (v/v)

perchloric acid, the mixture was allowed to stand in an ice-cold water bath for 10 min, and centrifuged at 18 500 ***g*** for 5 min at 4 °C. Absorbance of the supernatant was measured at 280 nm. The activity was expressed as ΔA_{280} per 60 min.

Estimation of MCA-peptide-cleaving activity

MCA peptides, peptidyl-4-methylcoumaryl-7-amides (Peptide Institute, Inc., Osaka, Japan), were used for evaluating substrate specificity of the purified hatching enzyme. A 0.5-mL reaction mixture containing 100 μM MCA peptide, 50 mM Tris/HCl buffer pH 8.0 and the enzyme was incubated at 30 °C for 60 min. After the reaction was stopped by adding 1 mL 20% (v/v) acetic acid, the fluorescence was measured with a Hitachi 204 fluorescence spectrophotometer (Tokyo, Japan) at 380 nm (excitation) and 460 nm (emission).

Determination of protein content

Amount of protein was determined by the Bradford method [34] using BSA as standard.

Estimation of choriolytic activity

Choriolytic activity was determined with a reaction mixture consisting of 0.25 M NaCl, 10 mM Tris/HCl buffer pH 7.5, chorion fragments as substrate and the enzyme. Incubation was performed at 30 °C for 60 min. Two microlitres of the supernatant were added to 100 μL Bradford reagent, and then the absorbance at 595 nm was determined.

Electron microscopy

Chorion was immersed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4 °C overnight. After rinsing in the buffer, the chorion was postfixed with 1% (w/v) osmium tetroxide in the same buffer, dehydrated in acetone, and embedded in epoxy resin.

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