Adaptive evolution of fish hatching enzyme:

One amino acid substitution results in differential salt dependency of the enzyme

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Abbreviated Title: Environmental adaptation of hatching enzyme

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Abstract

Embryos of medaka *Oryzias latipes* hatch in freshwater, while those of killifish *Fundulus heteroclitus* hatch in brackish water. Medaka and *Fundulus* possess two kinds of hatching enzymes, high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE), which cooperatively digest their egg envelope at the time of hatching. Optimal salinity of medaka HCE was found to be in 0 M NaCl, and the activity was decreased with increased salt concentrations. One of the two *Fundulus* HCEs, FHCE1, showed the highest activity in 0 M NaCl, the other FHCE2 did in 0.125 M NaCl. The results suggest that the salt dependencies of HCEs are well adapted to each salinity at the time of hatching. Different from HCE, LCEs of both species maintained the activity sufficient for egg envelope digestion in various salinities. The difference of amino acid sequence between FHCE1 and FHCE2 was found in only a single site at position 36 (Gly/Arg), suggesting that this single substitution causes the different salt dependency between the two enzymes. Superimposition of FHCE1 and FHCE2 with the 3D-structure model of medaka HCE revealed that position 36 was located on the surface of HCE molecule, far from its active site cleft. The results suggest a hypothesis that position 36 influences salt dependent activity of HCE not with recognition of primary structure around the cleavage site but with recognition of higher ordered structure of egg envelope protein.

Introduction

Hatching, the last dynamic event during egg development, is established by the digestion of egg envelope by a protease called hatching enzyme. Both hatching enzyme and egg envelope protein have been extensively studied in medaka. Medaka possesses two kinds of hatching enzymes, high choriolytic enzyme (HCE; choriolysin H; EC 3.4.24.67) and low choriolytic enzyme (LCE; choriolysin L; EC 3.4.24.66) (Yasumasu et al., 1989a, Yasumasu et al., 1989b). Both of them belong to an astacin metallo-protease family (Yasumasu et al., 1992, Yasumasu et al., 1994). At the time of hatching, the two enzymes cooperatively solubilize the
inner layer of egg envelope: HCE swells the envelope, and then, LCE solubilizes the HCE-swollen envelope completely. Determination of cleavage sites by HCE and LCE on the egg envelope proteins reveals that each enzyme cleaves the specific site on two proteins composing egg envelope (envelope subunit proteins), “ZI-1,2” and “ZI-3”, which possess zona pellucida (ZP) domain, the common structure in all the vertebrate egg envelope proteins. The swelling of the envelope by HCE occurs by cleavage of the N-terminal region of ZI-1,2 and ZI-3, especially by fragmentation of the Pro-Xaa-Yaa repeat region of ZI-1,2 (Yasumasu et al., 2010). The solubilization by LCE is achieved by cleavage at the middle of the ZP domain of ZI-1,2 and upstream of the ZP domain of ZI-3 (Yasumasu et al., 2010).

Recently, hatching enzyme and egg envelope protein of Fundulus were also characterized (Kawaguchi et al., 2005, Kawaguchi et al., 2010). From Fundulus, one LCE (FLCE) and two isoforms of HCE (FHCE1 and FHCE2) were purified through gel filtration and cation exchange column chromatography. FHCE1 and FHCE2 had similar enzymological properties, and both were able to swell the egg envelope. FLCE efficiently solubilized the envelope swollen by FHCE1 and/or FHCE2. The positions of cleavage sites by FHCE1 and 2 (FHCE1/2) and FLCE on Fundulus egg envelope protein were conserved with those of medaka (Kawaguchi et al., 2010).

Egg envelope is known to be permeable to small molecules such as salt, and therefore, perivitelline space, where the hatching enzymes are secreted, is under the same salinity as outer environment. In the present study, we focused on the difference of salinity at hatching between medaka and Fundulus. Medaka is mainly distributed in freshwater (Naruse et al., 1993; Naruse, 1996), although adult fish is occasionally found in brackish water (Miyamoto et al., 1986). The death rate of medaka embryos in brackish water and sea water is higher than that in freshwater (Inoue and Takei, 2002). On the other hand, Fundulus inhabit coastal water (marshes and estuaries) and also freshwater (Nelson, 2006). Adult Fundulus showed wide salinity tolerance (1.7-28.7‰), and their embryos showed no effect on hatching in the
presence of 0-30‰ NaCl (Griffith, 1974; DiMichele and Taylor, 1980). In the present study, we examined salt dependencies of both HCE and LCE, and found that medaka HCE and one of two *Fundulus* HCEs retained the high activity in freshwater, while the other *Fundulus* HCE showed the highest activity in brackish water. In addition, we demonstrated that such different salt dependencies between two *Fundulus* HCEs were caused by a single amino acid substitution. The salt preference of HCE adapts medaka embryos or *Fundulus* embryo to each environmental salinity at the time of hatching.

**Results**

**Hatching rate of medaka and *Fundulus* embryos in various salinities**

We first observed the hatching rate of medaka and *Fundulus* embryos under four different salinities; freshwater (FW), one fourth concentration of seawater (1/4SW), one half concentration of seawater (1/2SW), and seawater (SW). Throughout the experiments, the maximum hatching rate of medaka embryos was around 90%, and that of our *Fundulus* population was around 60%.

As shown in Fig. 1A, the average hatching rate of medaka embryos within 1 h was 73.5% in FW, and was decreased dose-dependently with increased salt concentrations. The rate in SW (35.0%) was about one half of that of FW (Fig. 1A). After 2 h incubation, the rate in FW jumped up to 89.1%. The rates in 1/4SW, 1/2SW and SW were not as high as that in FW. Especially, the difference between the rate in SW and that in FW was statistically significant. The 97.8% of embryos hatched immediately after the embryos cultured in SW were transferred to FW at hatching day (Fig. 1A). This might occur by stimulation of salinity change. We confirmed that the low hatching rate in SW did not result from the developmental delay of embryos.

The hatching rate of *Fundulus* embryos within 1 h was fairly constant in 1/4SW, 1/2SW and SW (62.2% - 70.7%), and was somewhat lower in FW (44.5%) than the others (Fig. 1B).
These values were not changed after 2 h incubation. Summing up the results, medaka embryos have the highest hatching rate in FW, while Fundulus embryos show the constant rate in 1/4SW to SW. Thus, salinity tolerance of medaka and Fundulus embryos was concord well with their salinity of habitats at the time of hatching.

**Optimum salinity of hatching enzyme on its egg envelope digestion**

We determined egg envelope digestion activities of hatching enzymes of both species in various concentrations of NaCl. First, turbidimetric method was employed to examine the egg envelope digestion activity. The egg envelope becomes transparent as the result of its swelling by proteolysis of HCE (Yasumasu et al., 1989a), and therefore, the decrease of turbidity in suspension of the fine fragments of egg envelope is dependent upon the HCE activity (Yamagami, 1973). The activity of medaka HCE (MHCE) toward medaka egg envelope was the highest in 0 M NaCl (224 $\Delta T_{610}$ mg enzyme$^{-1}$ min$^{-1}$), and was remarkably decreased with increase of the salt concentration (Fig. 2A). Most of the activity was not observed in 0.25 M to 0.75 M NaCl.

FHCE1, one of two isoforms of Fundulus HCEs, also showed the highest activity in 0 M NaCl (49 $\Delta T_{610}$ mg enzyme$^{-1}$ min$^{-1}$), and the activity was decreased gradually with increase of the salt concentration (Fig. 2B). The other FHCE2 showed the highest activity in 0.125 M NaCl (50 $\Delta T_{610}$ mg enzyme$^{-1}$ min$^{-1}$), and one half of the activity remained in 0.25 M NaCl.

Next, we determined salt dependency of medaka and Fundulus LCEs, which showed 63% similarity in amino acid sequence of mature enzyme region (Supplemental Figure 1). It is known that LCE efficiently solubilizes the HCE-swollen egg envelope, but not the intact envelope. To examine the egg envelope digestion activity of LCE, the HCE-swollen envelope was used as substrate, and the amounts of peptides liberated from the envelope were measured. The salt dependency curves of both MLCE and FLCE are shown in Fig. 3. The activity of Fundulus LCE was constant in the concentration from 0.125 M to 0.5 M NaCl, and
decreased to one third at 0 M NaCl. On the other hand, the activity of medaka LCE was constant from 0 M to 0.25 M NaCl, and decreased to 63-64% in 0.5 M NaCl. Although the activity of FLCE at 0 M NaCl seems to be low, the swollen egg envelope was efficiently solubilized by FLCE under 0 M to 0.5 M NaCl, when observed in in vitro egg envelope digestion experiments. At the time of hatching, a considerably high concentration of hatching enzymes is considered to be present around the egg envelope, because hatching enzymes are secreted into a narrow perivitelline space. Therefore, we regarded that LCEs maintained sufficient egg envelope digestion activity under various salt concentrations.

Cloning of FHCE1 and FHCE2 cDNAs

In the previous studies, we purified two HCEs (FHCE1 and 2) from Fundulus hatching liquid, and cloned only one HCE cDNA corresponding to the purified FHCE1 (Kawaguchi et al., 2005). In the present study, in order to clone FHCE2 cDNA, we designed primers from 5′- and 3′-UTR of the FHCE1 cDNA and amplified an 845 bp band by RT-PCR. Two kinds of cDNAs were obtained after sequencing of the eight clones. One of them was identical to the sequence of FHCE1 cDNA. The other was closely similar to FHCE1 cDNA. Three nucleotide substitutions were observed between two cDNAs. Two of them were synonymous, and one was non-synonymous (Gly:GGG→Arg:CGG), resulting in the amino acid substitution at position 36 (Fig. 4A). The molecular weights of their mature enzyme regions were deduced to be 22676.02 and 22775.10, which corresponded to the previously reported m/z values obtained from the purified FHCE1 (22676.5) and FHCE2 (22779.0), respectively (Kawaguchi et al., 2005). Therefore, we assigned the two cDNAs to the FHCE1 and FHCE2 genes.

To confirm whether or not the two cDNAs correspond to the purified FHCE1 and FHCE2, we generated recombinant proteins from cloned cDNAs (rFHCE1 and rFHCE2). Figures 4B and C show the salt dependency curves of rFHCE1 and rFHCE2 toward medaka and Fundulus egg envelopes, together with those of recombinant MHCE (rMHCE). The salt
dependencies of the three recombinant HCEs were fundamentally similar to those of respective purified HCEs (Fig. 2). Therefore, we concluded that the single amino acid substitution at position 36 (Gly/Arg) is responsible for the different salt dependencies of FHCE1 and FHCE2.

3D structure of FHCE1 and FHCE2

We employed 3D-structure of HCE to argue how the different salt dependencies were derived. Recently, we have crystalized MHCE (Kudo et al., 2004), and determined the 3D-structure of MHCE (PDB accession number: 3VTG). The overall 3D-architecture of MHCE has a kidney-like shape (Fig. 5A), which is quite similar to that of astacin, a prototype in astacin metallo-protease family (Bode et al., 1992), and zebrafish hatching enzyme (Okada et al., 2010). As shown in Fig. 4A, the molecule mainly consists of seven \( \beta \)-sheets (\( \beta1-7 \)) and three \( \alpha \)-helices (\( \alpha1-3 \)). On the basis of this X-ray crystallogram of MHCE, we constructed homology models for FHCE1 and FHCE2 (Fig. 5B and C). Comparison of the conformations of the three HCEs revealed that all of them possess similar electric charge of the active site cleft. It is noteworthy that the position 36 residue is located at the middle of an \( \alpha \)-helix (\( \alpha1 \)) in the N-terminal part far from the active site cleft, and its side chain faces the outside of the molecule (Fig. 5). These results suggest that the position 36 residue does not play an important role for the recognition and cleavage of the substrate in the active site cleft.

Salt dependencies of HCEs toward small peptidyl and soluble proteinous substrates

Above findings seem to support that the mutation does not affect the recognition of the cleavage site of substrate by the catalytic site of enzyme. Therefore, to determine salt dependency toward small peptides, we first examined substrate specificity of HCEs using 18 kinds of MCA-peptides. As shown in Supplemental Figure 2, the relative MCA-cleavage efficiencies of FHCE1 and FHCE2 were quite similar, suggesting that the mutation at position
36 does not affect substrate specificity of HCEs. Then, cleavage activities of HCEs were examined in 0 M to 0.5 M NaCl, using the best MCA-substrate, Z-Leu-Leu-Glu-MCA. As we expected, all the HCEs showed no significant difference in salt dependency (Fig. 6A). Next, we employed two synthetic peptides including FHCE1/2-cleavage sites (QKQTPS↓YPQQPQ and PSKRPE↓APGVP, where an arrow indicates the cleavage site) and soluble proteinous substrate such as recombinant rFhChgH_ProXY, which was previously synthesized from the entire Pro-Xaa-Yaa repeat region in FhZPB, Fundulus ortholog of medaka ZI-1,2 comprising of 210 amino acid residues (Kawaguchi et al., 2010). As shown in Fig. 6B-D, HCEs showed similar salt dependencies. In conclusion, as far as the small peptidyl and soluble proteinous substrates are used, HCEs do not show different salt dependencies observed from the egg envelope digestion experiment. Therefore, a difference of optimal salinity in HCE activities is detectable only when whole egg envelope is used as substrate, suggesting that the difference is due to HCE’s recognition of higher-ordered structure of the envelope.

Discussion

Medaka embryos hatch in freshwater, and their hatching rate was higher in freshwater than in brackish and sea waters. The egg envelope digestion activity of MHCE was the highest in the freshwater and was decreased with increased salt concentrations. The changes of the activity in 0 M to 0.5 M NaCl well reflected those of the hatching rate in FW to SW, suggesting that hatching enzyme of medaka is well adapted to its salinity at the time of hatching.

On the other hand, Fundulus embryos which hatch in brackish water showed stable hatching rates in freshwater to seawater, although the value in freshwater was relatively low. Interestingly, two isoforms of Fundulus HCE showed different optimal salinities. One of them (FHCE1) had the same optimal salinity as medaka HCE. Dissimilar to medaka HCE, it had
relatively broad adaptability to higher salinity. The other FHCE2 had the highest activity in
0.125 M NaCl, and had broad salt dependency in 0 M to 0.25 M NaCl. Existence of the two
HCEs is considered to ensure the constant hatching rates in a wide range of salinities.
Considering that the salt concentration in brackish region would tend to fluctuate under the
natural conditions, the broad salt dependencies of FHCEs are well adaptable to the salinity at
the time of hatching, and result in the constant hatching rates in various salinities.

Cloning of cDNA and synthesis of recombinant protein revealed that the different
optimal salinities between FHCE1 and FHCE2 were caused by a single amino acid
substitution at position 36 (Gly in FHCE1 and Arg in FHCE2). Many studies have been
reported that even a single non-synonymous substitution affects a protein function such as
enzyme activity and protein-protein interaction (Tracewell and Arnold, 2009). For example,
(i) GAG (Val) to GTG (Glu) substitution in the 6th codon of human β-globin gene results in
sickle cell disease (Ingram, 1956, Ingram, 1957). (ii) Single amino acid substitutions in
carotenoid biosynthetic enzymes alter substrate specificity and reaction selectivity to produce
a variety of carotenoids (Umeno et al., 2005). (iii) Single amino acid change (S84C) of short
wavelength-sensitive pigment 1 (SWS1) is responsible for transformation to UV-sensitive
pigments in birds (Yokoyama et al., 2000). The present study provides one of the good
examples showing that only one non-synonymous substitution causes the environmental
adaptation of the gene.

The amino acid residue at position 36 was Gly in FHCE1, Arg in FHCE2 or Ala in
MHCE. Consequently, FHCE1 and MHCE, having the highest activity in 0 M NaCl, had a
non-charged small residue at position 36, while FHCE2, showing the highest activity in 0.125
M NaCl, had a basic residue at the site. Position 36 is found far from the active site cleft of
HCE (Fig. 6). Several studies have reported that amino acid substitution outside of active site
affects its enzyme activity due to the conformation changes in active site cleft (Somero, 2004;
Fields et al., 2005). In this study, the enzyme activities of both FHCE1 and FHCE2 toward
small peptidyl substrates and soluble substrate were not affected by salt concentrations (Fig. 6). Furthermore, FHCE1 and FHCE2 showed quite similar substrate specificity observed by MCA-substrates (Supplemental Figure 2). These biochemical data do not suggest that the mutation at position 36 alters the structure of active site cleft and influences the recognition of primary structure of cleavage site. However, the different salt dependency of two FHCEs was observed only when egg envelope was used as substrate. HCE has been reported to bind tightly to the egg envelope when acted on the envelope (Yasumasu et al., 1989). In order to digest such solid substrate, HCE would be necessary to recognize 3D-conformation of the substrate in addition to the primary structure around cleavage site. Thus, it is possible that the position 36 residue is one of the residues that affect the accessibility of HCE to the cleavage site on egg envelope protein.

Materials and Methods

Hatching rate of medaka and Fundulus embryos

Adults of medaka were obtained from dealer, and those of Fundulus, originally caught in Massachusetts, were laboratory maintained at National Research Institute of Fisheries Science. Embryos of medaka Oryzias latipes and Fundulus heteroclitus were collected at the day of fertilization, divided into 4 batches, and cultured in 4 conditions: (1) freshwater; (2) one fourth concentration of artificial seawater (Tetra Japan, Tokyo, Japan); (3) a half concentration of the sea water; (4) sea water. The embryos were cultured in a shaking incubator at 28 °C for medaka and 25 °C for Fundulus. For medaka hatching, the embryos immediately before hatch were taken into a small amount of water under the same as culture condition. For Fundulus hatching, the embryos immediately before hatch were taken out of the water, allowed to stand in air for 20 min to induce the hatching, and transferred into a small amount of water under the same as culture condition. Photographs of hatched larvae and unhatched embryos were taken every 30 min until 2 h, and then, hatching rates were calculated.
Purification of medaka and Fundulus hatching enzymes

Medaka hatching enzymes (MHCE and MLCE) and Fundulus hatching enzymes (FHCE1, FHCE2 and FLCE) were purified from hatching liquid through gel filtration and cation exchange column chromatography in HPLC system according to the procedures described previously (Yasumasu et al., 1989a, Yasumasu et al., 1989b, Kawaguchi et al., 2010).

Estimation of egg envelope digestion activity of HCE

The egg envelope digestion activity of HCE was determined by turbidimetric method (Yamagami, 1973). The isolated egg envelopes of medaka or Fundulus were minced into fine fragments, and suspended in distilled water (DW). The suspension was allowed to stand overnight to remove rough fragments, and the supernatant containing fine fragments was used as substrate. The enzyme reaction was carried out in 1 mL of a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0 to 0.75 M NaCl, egg envelope suspension and purified HCE. The initial turbidity at 610 nm (T_{610}) of the mixture was adjusted to about 55% when that of DW was to 100%. Increase in transmission caused by the digestion of the fragmented envelopes was monitored for 3 min. The activity was expressed as $\Delta T_{610}$ mg enzyme$^{-1}$ min$^{-1}$.

Estimation of egg envelope digestion activity of LCE

Twelve isolated envelopes of medaka or Fundulus were pre-incubated with purified MHCE (25 μg/μL) or FHCE2 (70 μg/μL) in 50 mM Tris-HCl buffer (pH 8.0) containing 0 M NaCl (MHCE) or 0.125 M (FHCE2) at 30 ºC for 30 min. The swollen envelopes were washed with 50 mM Tris-HCl buffer (pH 8.0) three times, and incubated with purified MLCE or FLCE at 30 ºC for 15 min in 10 μL of 50 mM Tris-HCl buffer (pH 8.0) containing 0 to 0.5 M NaCl. After 5 min incubation, 2 μL aliquots of the supernatant were collected, and its protein amount was measured absorbance at 280 nm. Egg envelope solubilizing activity was
estimated by an amount of protein solubilized from egg envelope as the value of 280 nm in supernatant (ΔABS$_{280}$ μg enzyme$^{-1}$ min$^{-1}$).

**Cloning of FHCE1 and FHCE2 cDNAs**

RT-PCR was performed from RNA of pre-hatching embryos of *Fundulus* using primers designed from 5’- and 3’-UTR of previously cloned FHCE1 cDNA (Kawaguchi et al., 2005).

Forward primer: 5’-GAGTCTCCACATCGCCCCTGAAG-3’
Reverse primer: 5’-CATTTTCATTGATCTTTTACATTG-3’

**Caseinolytic activity**

The caseinolytic activity (CA) of hatching enzyme was measured using a 750 μL reaction mixture consisting of 83 mM Tris-HCl (pH 8.0), 3.3 mg/mL of casein, according to the method described previously (Kawaguchi et al., 2010). The CA was expressed as ΔABS$_{280}$·30 min$^{-1}$.

**Construction of recombinant proteins**

The mature enzyme regions of FHCE1 or FHCE2 were amplified from their full-length cDNA using primers designed to contain suitable restriction enzyme sites (BamHI and NdeI) at their 5’ regions, and the primer sequences were partially optimized for codons preferred to *Escherichia coli*. The sequences are as follows: F: 5’-CATATGAACGCGATGAAATGCTGGTATAACAGCTGCGTGTGGCCGAAAG-3’ and R: 5’-GGATCCCTTAGCAGGCCATGCAGCATGTATTGCGCTGCACATCCCAGC-3’. For MHCE, MHCE cDNA previously cloned (Y asumasu et al., 1992) was used as template for PCR. After digestion with the BamHI and NdeI, the fragments were inserted into pET3c vector.

The FHCE1/pET3c, FHCE2/pET3c and MHCE/pET3c plasmids were transformed into *E.
coli BL21 (DE3) pLysE strain cells. The cells were cultivated at 37 °C in 250 mL of LB medium with 0.1 mg/mL carbenicillin and 25 μg/mL chloramphenicol. Protein expression was induced with 1 mM IPTG when an absorbance at 600 nm reached to 0.6. After 4 h induction, the cells were harvested by centrifugation at 5800 g for 10 min. The cells were suspended in 10 mL of 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and were frozen at -20 °C. After incubation at 37 °C for 2 h, the cells were disrupted by sonication, and centrifuged at 12300 g for 10 min. The precipitate was suspended in 10 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 5% Triton X-100 and 1 mM EDTA), sonicated and centrifuged (12300 g for 10 min). After the procedures were repeated three times, the inclusion bodies thus obtained were dissolved into denaturing buffer (50 mM Tris-HCl pH 8.0, 8 M Urea, 0.1 M 2-mercaptoethanol and 1 mM EDTA) at a protein concentration of 20 mg/mL. After centrifugation (18500 g for 10 min), the supernatant was added to a refolding buffer (50 mM Tris-HCl pH 8.0, 0.8 M L(+)-arginine hydrochloride, 1 mM glutathione, 0.1 mM oxidized glutathione and 5 μM ZnSO₄) to be a final concentration of 20 μg/mL protein, and incubated at 4 °C for 2 days. The protein solution was dialyzed once against 25 mM Tris-HCl buffer (pH 8.0) with 5 μM ZnSO₄ and five times against the same buffer without ZnSO₄. The folding mixture was loaded onto an S-Sepharose column equilibrated with 25 mM Tris-HCl (pH 8.0), and eluted once with the same buffer containing 400 mM NaCl.

Because the refolding efficiencies of the recombinant proteins were different from sample to sample, the amount of active enzyme in the sample was estimated with CA and the activities of the enzymes were normalized by CA.

**Proteolytic activity of HCEs**

(i) 4-methylcoumaryl-7-amide (MCA)-peptide cleavage activity

A 50 μL reaction mixture containing 100 μM MCA-peptide (Peptide Institute, Inc., Osaka, Japan), 50 mM Tris-HCl buffer (pH 8.0), 0 to 0.75 M NaCl and enzyme was incubated...
at 30 °C for 30 min. The MCA-peptide cleavage activity was measured according to the
method described previously (Kawaguchi et al., 2008).

(ii) synthetic peptide cleavage activity

A 40 μL reaction mixture containing 100 μM of peptide, 50 mM Tris-HCl (pH 8), 0 to
0.75 M NaCl and an appropriate amount of enzyme was incubated for 30 min at 30 °C. The
activity was measured according to the method described previously (Kawaguchi et al.,
2010).

(iii) rFhChgH_ProXY cleavage activity

Recombinant protein rFhChgH_ProXY was designed to possess His tag at the C-terminus
(Kawaguchi et al., 2010). The digests that lost His tag by the cleavage of its Pro-Xaa-Yaa
region by HCE are passed through a Ni column, while the undigested rFhChgH_ProXY is
adsorbed to the column. The protein amount of the runoff fraction was determined to evaluate
the cleavage activity of HCE. A 100 μL reaction mixture consisting of 6 mg/mL
rFhChgH_ProXY, 0 to 0.75 M NaCl and an appropriate amount of enzyme in 50 mM
Tris-HCl (pH 8) was incubated at 30 °C for 30 min. The reaction mixture was directly applied
to a Ni-NTA Superflow (QIAGEN, Valencia, CA, USA) column, which was previously
equilibrated with PBS. The unadsorbed proteins were eluted with PBS, and monitored by
absorbance at 280 nm (ABS280). As control, the reaction mixture without incubation was
applied to the column. Activity was determined as the observed ABS280 minus the control
value and expressed as ΔABS280.

Homology modeling of FHCE1 and FHCE2

The 3D structure of MHCE was used as template (PDB accession number: 3VTG) to
build homology model of FHCE1 and FHCE2 on the program Modeller (Sali et al., 1993).
Ten models were obtained and the model having minimum DOPE score was selected as the
best model. Then, the selected model was assessed by “evaluate_model” script in the program
to confirm that the obtained model was a reasonable model.

Acknowledgment

The present study was supported in part by a Grant-in-Aid for JSPS Fellows to MK.

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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. *FEBS J.* 272, 4315-4326.


**Figure Legends**

**Figure 1  Hatching rate of medaka and *Fundulus* embryos in different salinities**

Hatching rates of medaka (A) and *Fundulus* (B) embryos were observed after 60 min (gray bar) and 120 min incubation (black bar). The embryos were cultured in freshwater (FW), one fourth concentration of seawater (1/4SW), one half concentration of seawater (1/2SW), or seawater (SW). The hatched bar indicates the hatching rate after 120 min incubation of medaka embryos, which were cultured in SW and transferred to FW at the day of hatching (SW→FW in A). The white bar indicates the rates of the medaka embryos continuously cultured in SW. Bars indicate standard error (n=3), and asterisks indicate statistically significant changes (P<0.05).

**Figure 2  Salt dependency of HCE activity in egg envelope digestion**

Optimal salinities of purified MHCE, FHCE1 and FHCE2 were determined using medaka (A) and *Fundulus* egg envelopes (B) as substrate, respectively. Activities were determined by turbidimetry. Bars indicate standard error (n=3).

**Figure 3  Salt dependency of LCE antivity in egg envelope digestion**

Optimal salinities of purified MLCE and FLCE were determined using medaka and *Fundulus* egg envelopes as substrate, respectively. Bars indicate standard error (n=3).

**Figure 4  Amino acid sequences of HCEs and their recombinant proteins**

(A) Alignment of amino acid sequences, deduced from FHCE1, FHCE2 and MHCE cDNAs.
Identical residues are boxed. Triangle indicates the N-terminus of mature enzyme. Position 36 is shaded in black where the numbering system is started from the N-terminus of the mature enzyme (shown as 1 on the triangle). Consensus sequences of metal binding site HExxHxxGFxHExxRxDR and methionine turn SxMHY are shaded in gray. The zinc ligands and the catalytic glutamic acid (Bode et al., 1992) are shown with boldface. Spirals and arrows represent α-helix and β-sheet, respectively, on the basis of the 3D-structure of MHCE.

Accession numbers: MHCE, NM_001201498; FHCE, AB210813. The salt dependencies of recombinant HCEs (rMHCE, rFHCE1 and rFHCE2) were determined by turbidimetry using medaka (B) and Fundulus egg envelopes (C) as substrate. Bars indicate standard error (n=3).

**Figure 5** 3D-structures of MHCE, FHCE1 and FHCE2.

Crystal structure of MHCE (A), and homology models of FHCE1 (B) and FHCE2 (C) constructed from the 3D-structure of MHCE. Amino acid residue at position 36 is highlighted with light blue for basic amino acid residue (Arg for FHCE2) or gray for non-charged residues (Ala for MHCE and Gly for FHCE1). Red and dark blue denote acidic and basic residues, respectively. Yellow shows the zinc ligands and the catalytic glutamic acid, whose residues are shown with boldface in Fig. 4A.

**Figure 6** Salt dependency of HCE toward peptidyl and soluble proteinous substrates

Salt dependency of rMHCE, rFHCE1 and rFHCE2 activity was examined using Z-Leu-Leu-Glu-MCA (A), synthetic peptides (B and C) and recombinant protein, rFhChgH_ProXY (D). The peptide sequences are shown at the top of the figures, and arrows in the sequences indicate cleavage sites. Bars indicate standard error (n=3).

**Supplemental Figure 1** Alignment of amino acid sequence of FLCE and MLCE

Identical residues are boxed. Triangle indicates the N-terminus of mature enzyme. Consensus
sequences of metal binding site HExxHxxGFxHExxRxDR and methionine turn SxMHy are shaded in gray. Accession number: FLCE, AB210814; MHCE, NM_001104822.

Supplemental Figure 2  Substrate specificity of HCE toward MCA-peptides
Substrate specificity of rMHCE, rFHCE1 and rFHCE2 was examined with eighteen kinds of MCA-peptides. Sequences of the MCA-peptides are shown at the right. Activity to each substrate is expressed as percent of the best substrate, Z-Leu-Leu-Glu-MCA.
Fig 1

(A) medaka

(B) Fundulus

hatching rate (%)
Fig 3

Kawaguchi et al
Fig. 4

Kawaguchi et al.

(A) 

(B) medaka egg envelope

(C) Fundulus egg envelope

\[
\begin{align*}
\text{FHCE1} & : \\
& -\text{SSTLLLSSLLGFSLPPCGLDP-EEGEDEEYEEGADTVVMTTRTILNADNLNL-56} \\
\text{FHCE2} & : \\
& -\text{SSTLLLSSLLGFSLPPCGLDP-EEGEDEEYEEGADTVVMTTRTILNADNLNL-56} \\
\text{MHCE} & : \\
& -\text{NLLAPSTRUCTURALHELIXWDEGEGELDDILNGTSTNMTILLL-60}
\end{align*}
\]

\[
\begin{align*}
\text{FHCE1} & : \\
& -\text{EDILMKPSRNAMCKNWCYNVCVPPASNGKVIVVIPYVIGRELFSGSGPTC-116} \\
\text{FHCE2} & : \\
& -\text{EDILMKPSRNAMCKNWCYNVCVPPASNGKVIVVIPYVIGRELFSGSGPTC-116} \\
\text{MHCE} & : \\
& -\text{EGILVAHNRANMKCWSNSCARGKNCGTVVIPSYSSCSVAcEIELAFSSTFNC-120}
\end{align*}
\]

\[
\begin{align*}
\text{FHCE1} & : \\
& -\text{ERFPRTRNDFIVSVVSGCGWSELCGETREMQLPELNSKQGCIYSGIVCHELNHALGEPHE-176} \\
\text{FHCE2} & : \\
& -\text{ERFPRTRNDFIVSVVSGCGWSELCGETREMQLPELNSKQGCIYSGIVCHELNHALGEPHE-176} \\
\text{MHCE} & : \\
& -\text{ERFPRTRNDFIVSVVSGCGWSELCGETREMQLPELNSKQGCIYSGIVCHELNHALGEPHE-180}
\end{align*}
\]

\[
\begin{align*}
\text{FHCE1} & : \\
& -\text{TCSDRMNYVRINNQIQQSAYNFQYDKTTNNLNPYDSSSIMVYCRDFAIAAYGRET-236} \\
\text{FHCE2} & : \\
& -\text{TCSDRMNYVRINNQIQQSAYNFQYDKTTNNLNPYDSSSIMVYCRDFAIAAYGRET-236} \\
\text{MHCE} & : \\
& -\text{TCSDRMNYVRINNQIQQSAYNFQYDKTTNNLNPYDSSSIMVYCRDFAIAAYGRET-240}
\end{align*}
\]

\[
\begin{align*}
\text{FHCE1} & : \\
& -\text{FFPNPNVUIGQGQLRSDVQHTIMMLHGC-265} \\
\text{FHCE2} & : \\
& -\text{FFPNPNVUIGQGQLRSDVQHTIMMLHGC-265} \\
\text{MHCE} & : \\
& -\text{FFPNPNVUIGQGQLRSDVQHTIMMLHGC-270}
\end{align*}
\]