The Journal of Experimental Biology 216, 1609-1615

doi:10.1242/jeb.069716

RESEARCH ARTICLE

Adaptive evolution of fish hatching enzyme: one amino acid substitution results in differential salt dependency of the enzyme

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SUMMARY

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 in 0 mol l^{-1} NaCl, and activity decreased with increasing salt concentrations. One of the two *Fundulus* HCEs, FHCE1, showed the highest activity in 0 mol l^{-1} NaCl, and the other, FHCE2, showed the highest activity in 0.125 mol l^{-1} 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 in amino acid sequence between FHCE1 and FHCE2 was found at 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 3-D 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.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/9/1609/DC1

Key words: adaptation, astacin family, egg envelope, hatching enzyme.

Received 21 February 2012; Accepted 13 January 2013

INTRODUCTION

Hatching, the last dynamic event during egg development, is established by the digestion of the egg envelope by a protease called hatching enzyme. Both hatching enzyme and egg envelope protein have been extensively studied in medaka. Medaka possess 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., 1989b; Yasumasu et al., 1989c). Both of these belong to an astacin metalloprotease family (Yasumasu et al., 1992; Yasumasu et al., 1994). At the time of hatching, the two enzymes cooperatively solubilize the inner layer of the 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 that comprise the egg envelope (envelope subunit proteins), 'ZI-1,2' and 'ZI-3', which possess a 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 in 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).

The egg envelope is known to be permeable to small molecules such as salt, and therefore the perivitelline space, where the hatching enzymes are secreted, is under the same salinity as the outer environment. In the present study, we focused on the difference in salinity at hatching between medaka and *Fundulus*.

Medaka is mainly distributed in freshwater (Naruse et al., 1993; Naruse, 1996), although adult fish are occasionally found in brackish water (Miyamoto et al., 1986). The death rate of medaka embryos in brackish water and seawater is higher than that in freshwater (Inoue and Takei, 2002). However, *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* embryos to each environmental salinity at the time of hatching.

MATERIALS AND METHODS

Hatching rate of medaka and *Fundulus* embryos

Adults of the orange–red variety of Japanese medaka were obtained from a dealer (Koide Fish Farm, Nagoya, Japan), and those of *Fundulus*, originally caught in Massachusetts, were maintained in the laboratory at the National Research Institute of Fisheries Science. Embryos of medaka *Oryzias latipes* and *Fundulus heteroclitus* were collected on the day of fertilization, divided into four batches, and cultured under four conditions: (1) freshwater; (2) one-fourth concentration of artificial seawater (Tetra Japan, Tokyo, Japan); (3) one-half concentration of seawater; and (4) seawater. 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 placed in a small amount of water under the same culture conditions. 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 culture conditions. Photographs of hatched larvae and unhatched embryos were taken every 30 min for 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 by gel filtration and cation exchange column chromatography in an HPLC system, according to procedures described previously (Yasumasu et al., 1989b; Yasumasu et al., 1989c; Kawaguchi et al., 2010).

Estimation of egg envelope digestion activity of HCE

The egg envelope digestion activity of HCE was determined by turbidimetric methods (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 mmol l^{-1} Tris-HCl (pH 8.0), 0–0.75 mol l^{-1} NaCl, egg envelope suspension and purified HCE. The initial turbidity at 610 nm (T_{610}) of the mixture was adjusted to approximately 55% when that of DW was 100%. Increase in transmission caused by the digestion of the fragmented envelopes was monitored for 3 min. The activity was expressed as $\Delta T_{610}\,mg^{-1}$ enzyme 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^{-1}) or FHCE2 (70 μg μl^{-1}) in 50 mmol l^{-1} Tris-HCl buffer (pH 8.0) containing 0 mol l^{-1} NaCl (MHCE) or 0.125 mol l^{-1} NaCl (FHCE2) at 30°C for 30 min. The swollen envelopes were washed with 50 mmol l^{-1} Tris-HCl buffer (pH 8.0) three times, and incubated with purified MLCE or FLCE at 30°C for 15 min in 10 μl of 50 mmol l^{-1} Tris-HCl buffer (pH 8.0) containing 0–0.5 mol l^{-1} NaCl. After 5 min incubation, 2 μl aliquots of the supernatant were collected, and its amount of protein measured at 280 nm absorbance (A_{280}). Egg envelope solubilizing activity was estimated as the amount of protein solubilized from egg envelope at 280 nm in supernatant ($\Delta A_{280}\,\mu g^{-1}$ enzyme min^{-1}).

Cloning of FHCE1 and FHCE2 cDNAs

RT-PCR was performed with RNA of pre-hatching embryos of *Fundulus* using primers designed from 5′- and 3′-UTR (untranslated region) 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 mmol l^{-1} Tris-HCl (pH 8.0) and 3.3 mg ml^{-1} casein, according to the method described previously (Kawaguchi et al., 2010). The CA was expressed as $\Delta A_{280}\,30\,min^{-1}$.

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 (*Bam*HI and *Nde*I) at their 5′ regions, and the primer sequences were partially optimized for codons preferred to *Escherichia coli*. The sequences are as follows: F: 5′-CATATGAACGCGATGAAATGCTGGTATAACAGCT-GCGTGTGGCCGAAAG-3′, and R: 5′-GGATCCTTAGC-AGCCATGCAGCATGTTAATGCGCTGCACATCCCAGC-3′.

For MHCE, MHCE cDNA cloned previously (Yasumasu et al., 1992) was used as template for PCR. After digestion with *Bam*HI and *Nde*I, 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 lysogeny broth (LB) medium with 0.1 mg ml^{-1} carbenicillin and 25 μg ml^{-1} chloramphenicol. Protein expression was induced with 1 mmol l^{-1} isopropylthio-β-galactoside (IPTG) when an absorbance at 600 nm reached 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 mmol l^{-1} Tris-HCl (pH 8.0) and 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), and frozen at −20°C. After incubation at 37°C for 2 h, the cells were disrupted by sonication, and centrifuged at 12,300 ***g*** for 10 min. The precipitate was suspended in 10 ml of lysis buffer [50 mmol l^{-1} Tris-HCl (pH 8), 5% Triton X-100 and 1 mmol l^{-1} EDTA], sonicated and centrifuged (12,300 ***g*** for 10 min). After the procedures were repeated three times, the inclusion bodies thus obtained were dissolved in denaturing buffer [50 mmol l^{-1} Tris-HCl (pH 8.0), 8 mol l^{-1} urea, 0.1 mol l^{-1} 2-mercaptoethanol and 1 mmol l^{-1} EDTA] at a protein concentration of 20 mg ml^{-1}. After centrifugation (18,500 ***g*** for 10 min), the supernatant was added to a refolding buffer [50 mmol l^{-1} Tris-HCl (pH 8.0), 0.8 mol l^{-1} L(+)-arginine hydrochloride, 1 mmol l^{-1} glutathione, 0.1 mmol l^{-1} oxidized glutathione and 5 μmol l^{-1} $ZnSO_4$] to a final concentration of 20 μg ml^{-1} protein, and incubated at 4°C

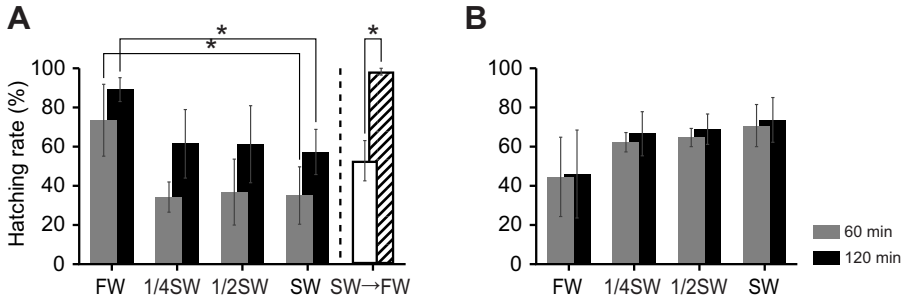

Fig. 1. Hatching rate of medaka and *Fundulus* embryos in different salinities. Hatching rates of medaka (A) and *Fundulus* (B) embryos were observed after 60 min (gray bars) and 120 min incubation (black bars). The embryos were cultured in freshwater (FW), one-fourth concentration of seawater (1/4SW), 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 on 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); *statistically significant changes (P<0.05).

for 2 days. The protein solution was dialyzed once against 25 mmol l^{-1} Tris-HCl buffer (pH 8.0) with 5 μmol l^{-1} $ZnSO_4$ and five times against the same buffer without $ZnSO_4$. The folding mixture was loaded onto an S-Sepharose column equilibrated with 25 mmol l^{-1} Tris-HCl (pH 8.0), and eluted once with the same buffer containing 400 mmol l^{-1} 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

4-Methylcoumaryl-7-amide (MCA) peptide cleavage activity

A 50 μl reaction mixture containing 100 μmol l^{-1} MCA peptide (Peptide Institute, Osaka, Japan), 50 mmol l^{-1} Tris-HCl buffer (pH 8.0), 0–0.75 mol l^{-1} 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).

Synthetic peptide cleavage activity

A 40 μl reaction mixture containing 100 μmol l^{-1} peptide, 50 mmol l^{-1} Tris-HCl (pH 8), 0–0.75 mol l^{-1} 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).

rFhChgH_ProXY cleavage activity

Recombinant protein rFhChgH_ProXY was designed to possess a His tag at the C-terminus (Kawaguchi et al., 2010). The digests that lost the His tag by cleavage of its Pro-Xaa-Yaa region by HCE were passed through a Ni column, while the undigested rFhChgH_ProXY was adsorbed to the column. The amount of protein in the run-off fraction was determined to evaluate the cleavage activity of HCE. A 100 μl of reaction mixture consisting of 6 mg ml^{-1} rFhChgH_ProXY, 0–0.75 mol l^{-1} NaCl and an appropriate amount of enzyme in 50 mmol l^{-1} 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 phosphate-buffered saline (PBS). The unadsorbed proteins were eluted with PBS, and monitored by absorbance at 280 nm (A_{280}). As control, the reaction mixture without incubation was applied to the column. Activity was determined as the observed A_{280} minus the control value and expressed as ΔA_{280}.

Homology modeling of FHCE1 and FHCE2

The 3-D structure of MHCE was used as a template [Protein Data Bank (PDB) accession number: 3VTG] to build a homology model of FHCE1 and FHCE2 on the program Modeller (Sali et al., 1993). Ten models were obtained and the model with the minimum discrete optimized protein energy (DOPE) score was selected as the best model. The selected model was then assessed by the 'evaluate_model' script in the program to confirm that the obtained model was a reasonable one.

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 approximately 90%, and that of our *Fundulus* population was approximately 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 approximately half that in 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. The difference between the rate in SW and that in FW was statistically significant. Most (97.8%) embryos hatched immediately after the embryos cultured in SW were transferred to FW on 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 a constant rate in 1/4SW to SW. Thus salinity tolerance of medaka and *Fundulus* embryos is in accordance with the salinity of their 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,

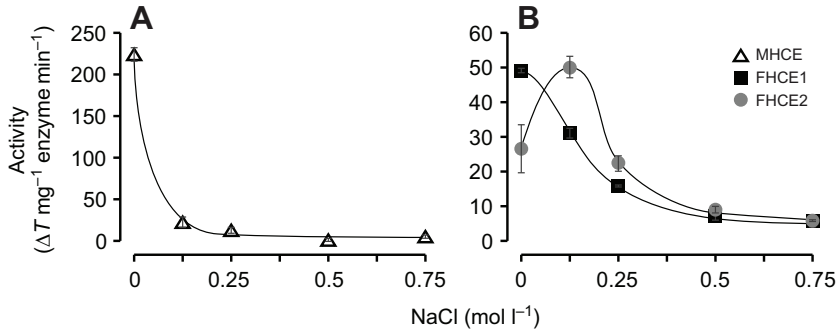

Fig. 2. Salt dependency of HCE activity in egg envelope digestion. Optimal salinities of purified MHCE, FHCE1 and FHCE2 were determined using medaka (A) and *Fundulus* (B) egg envelopes as substrate, respectively. Activities were determined by turbidimetry. Bars indicate standard error (*N*=3).

a 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., 1989b), 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) towards the medaka egg envelope was the highest in 0 mol l^{-1} NaCl (224 ΔT_{610} mg^{-1} enzyme min^{-1}), and was remarkably decreased with increase in salt concentration (Fig. 2A). Most of the activity was not observed in 0.25–0.75 mol l^{-1} NaCl.

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

Next, we determined salt dependency of medaka and *Fundulus* LCEs, which showed 63% similarity in amino acid sequence of the mature enzyme region (supplementary material Fig. S1). 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 0.125–0.5 mol l^{-1} NaCl, and decreased to one-third with 0 mol l^{-1} NaCl. However, the activity of medaka LCE was constant from 0 to 0.25 mol l^{-1} NaCl, and decreased to 63–64% in 0.5 mol l^{-1} NaCl. Although the activity of FLCE at 0 mol l^{-1} NaCl seems to be low, the swollen egg envelope was efficiently solubilized by FLCE under 0–0.5 mol l^{-1} 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.

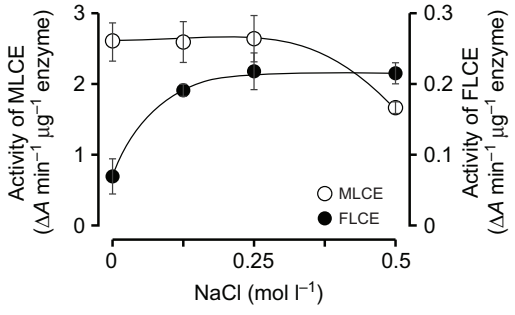

Fig. 3. Salt dependency of LCE activity in egg envelope digestion. Optimal salinities of purified MLCE and FLCE were determined using medaka and *Fundulus* egg envelopes as substrate, respectively. Bars indicate standard error (*N*=3).

Cloning of FHCE1 and FHCE2 cDNAs

In 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 these 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). Fig. 4B,C shows the salt dependency curves of rFHCE1 and rFHCE2 towards 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.

3-D structure of FHCE1 and FHCE2

We used a 3-D structure of HCE to argue how the different salt dependencies were derived. Recently, we have crystallized MHCE (Kudo et al., 2004), and determined the 3-D structure of MHCE (PDB accession number: 3VTG). The overall 3-D architecture of MHCE has a kidney-like shape (Fig. 5A), which is quite similar to that of astacin, a prototype in the astacin metalloprotease family (Bode et al., 1992), and zebrafish hatching enzyme (Okada et al., 2010). As shown in Fig. 4A, the molecule mainly consists of seven β-sheets (β1–7) and three α-helices (α1–3). On the basis of this X-ray crystallogram of MHCE, we constructed homology models for FHCE1 and FHCE2 (Fig. 5B,C). Comparison of the conformations of the three HCEs revealed that all of them possess a similar electric charge of the active site cleft. It is noteworthy that the position 36

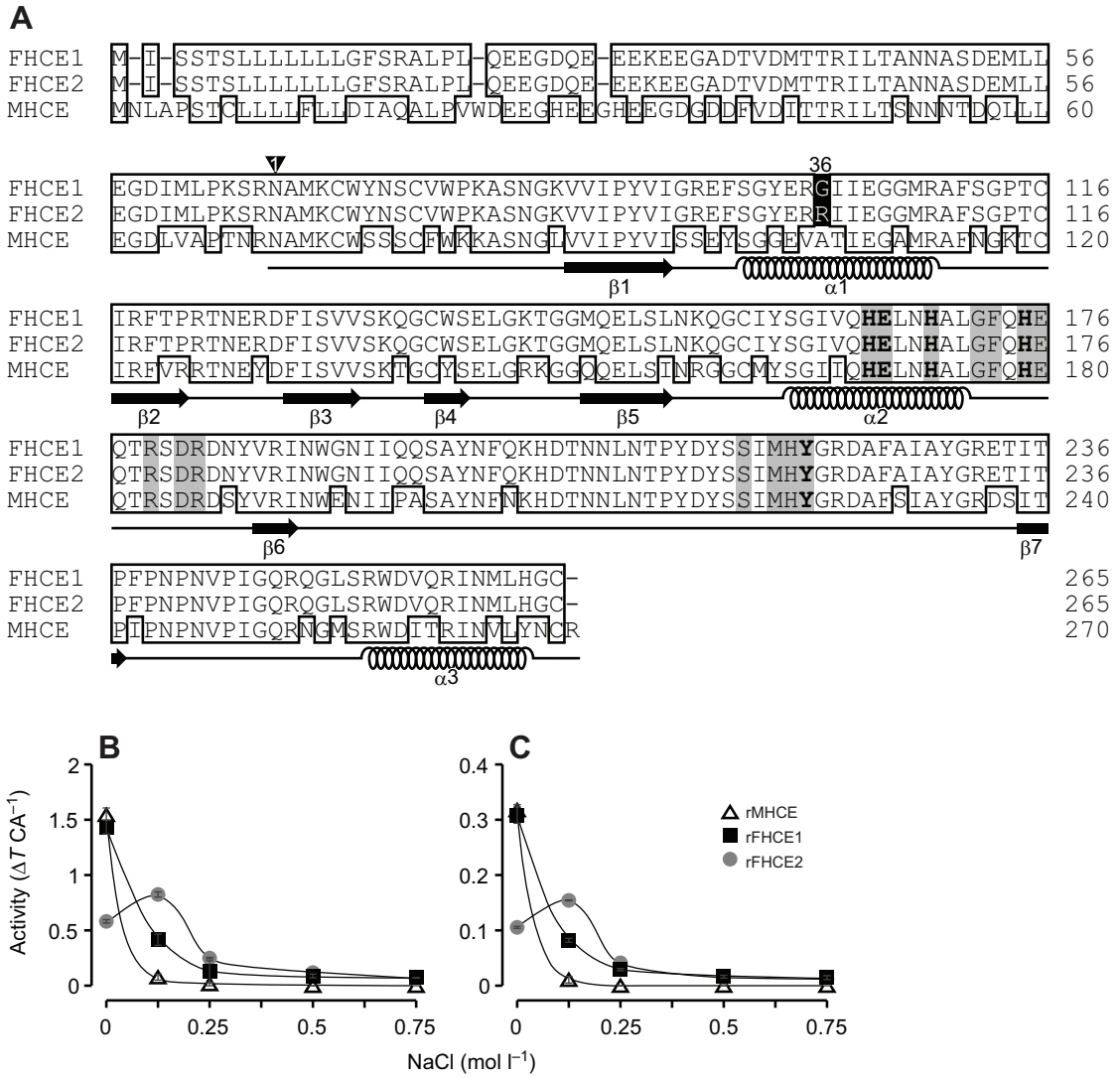

Fig. 4. Amino acid sequences of HCEs and their recombinant proteins. (A) Alignment of amino acid sequences, deduced from FHCE1, FHCE2 and MHCE cDNAs. Identical residues are boxed. Triangle indicates the N terminus of the mature enzyme. Position 36 is shaded in black where the numbering system is started 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 in bold. Spirals and arrows represent α-helix and β-sheet, respectively, on the basis of the 3-D 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* (C) egg envelopes as substrate. Bars indicate standard error (*N*=3).

residue is located in the middle of an α-helix (α1) 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

The above findings seem to support the idea that the mutation does not affect the recognition of the cleavage site of substrate by the catalytic site of the enzyme. Therefore, to determine salt dependency towards small peptides, we first examined substrate specificity of HCEs using 18 kinds of MCA peptides. As shown in supplementary material Fig. S2, 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 0–0.5 mol l^{-1} 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 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

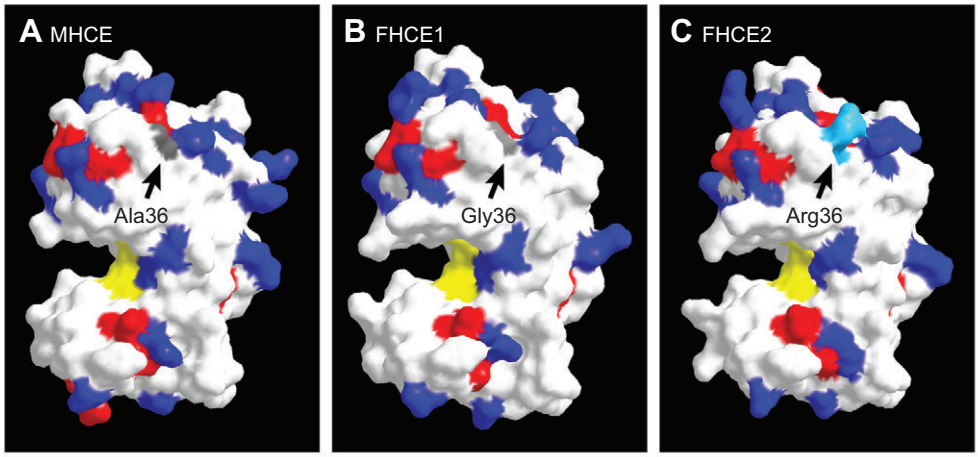

Fig. 5. 3-D structures of MHCE, FHCE1 and FHCE2. Crystal structure of MHCE (A), and homology models of FHCE1 (B) and FHCE2 (C) constructed from the 3-D structure of MHCE. Amino acid residue at position 36 is highlighted with light blue for basic amino acid residue (Arg for FHCE2) or gray for non-charged 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 in bold in Fig. 4A.

difference is due to recognition by HCE of higher-ordered structure of the envelope.

DISCUSSION

Medaka embryos hatch in freshwater, and their hatching rate was higher in freshwater than in brackish water and seawater. The egg envelope digestion activity of MHCE was highest in freshwater, and decreased with increasing salt concentrations. The changes of the activity in 0–0.5 mol l^{-1} NaCl 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.

However, *Fundulus* embryos that 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 mol l^{-1} NaCl, and had broad salt dependency in 0–0.25 mol l^{-1} 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 regions would tend to fluctuate under natural conditions, the broad salt dependencies of FHCEs are 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 reported that even a single non-synonymous substitution affects a protein function

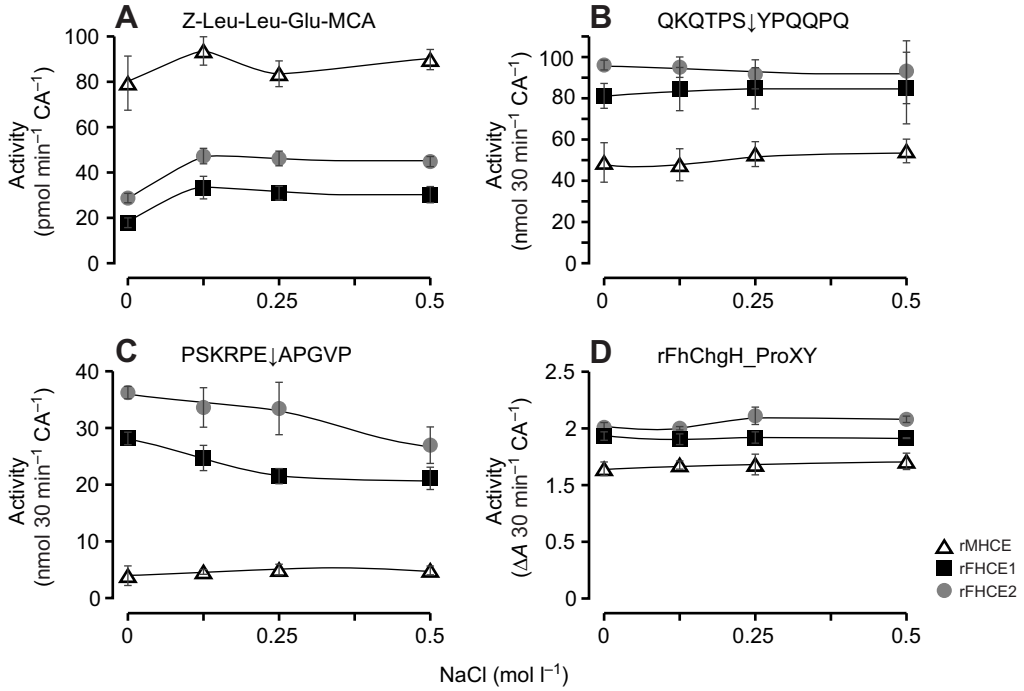

Fig. 6. Salt dependency of HCE towards peptidyl and soluble proteinous substrates. Salt dependency of rMHCE, rFHCE1 and rFHCE2 activity was examined using Z-Leu-Leu-Glu-MCA (A), synthetic peptides (B,C) and recombinant protein, rFhChgH_ProXY (D). The peptide sequences are shown at the top of each panel, and arrows in the sequences indicate cleavage sites. Bars indicate standard error (*N*=3).

such as enzyme activity and protein–protein interaction (Tracewell and Arnold, 2009). For example, (i) GAG (Val) to GTG (Glu) substitution in the sixth 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); and (iii) a 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 a good example, 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 mol l^{-1} NaCl, had a non-charged small residue at position 36, while FHCE2, showing the highest activity in 0.125 mol l^{-1} 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 the active site affects its enzyme activity due to conformational changes in the active site cleft (Somero, 2004; Fields et al., 2006). In this study, the enzyme activities of both FHCE1 and FHCE2 towards 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 (supplementary material Fig. S2). These biochemical data do not suggest that the mutation at position 36 alters the structure of the active site cleft and influences the recognition of primary structure of the cleavage site. However, the different salt dependency of the two FHCEs was observed only when the egg envelope was used as a substrate. HCE has been reported to bind tightly to the egg envelope when acting on the envelope (Yasumasu et al., 1989a). In order to digest such a solid substrate, HCE would be necessary to recognize the 3-D conformation of the substrate in addition to the primary structure around the 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.

AUTHOR CONTRIBUTIONS

M.K., S.Y., I.I. and M.N. designed the research; M.K., S.Y., A.S., N.K., K.S., I.I. and M.N. performed the research; M.K., S.Y., I.I. and M.N. analyzed the data; M.K., S.Y., I.I. and M.N. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

FUNDING

The present study was supported in part by a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) Fellows to M.K., and by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology to M.N.

REFERENCES

Bode, W., Gomis-Rüth, F. X., Huber, R., Zwilling, R. and Stöcker, W. (1992). Structure of astacin and implications for activation of astacins and zinc-ligation of collagenases. *Nature* **358**, 164-167.

DiMichele, L. and Taylor, M. (1980). The environmental control of hatching in *Fundulus heteroclitus*. *J. Exp. Zool.* **214**, 181-187.

Fields, P. A., Rudomin, E. L. and Somero, G. N. (2006). Temperature sensitivities of cytosolic malate dehydrogenases from native and invasive species of marine mussels (genus *Mytilus*): sequence–function linkages and correlations with biogeographic distribution. *J. Exp. Biol.* **209**, 656-667.

Griffith, R. W. (1974). Environmental and salinity tolerance in the genus *Fundulus*. *Copeia* **1974**, 319-331.

Ingram, V. M. (1956). A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. *Nature* **178**, 792-794.

Ingram, V. M. (1957). Gene mutations in human haemoglobin: the chemical difference between normal and sickle cell haemoglobin. *Nature* **180**, 326-328.

Inoue, K. and Takei, Y. (2002). Diverse adaptability in *Oryzias* species to high environmental salinity. *Zoolog. Sci.* **19**, 727-734.

Kawaguchi, M., Yasumasu, S., Shimizu, A., Hiroi, J., Yoshizaki, N., Nagata, K., Tanokura, M. and Iuchi, I. (2005). 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*. *FEBS J.* **272**, 4315-4326.

Kawaguchi, M., Nakagawa, M., Noda, T., Yoshizaki, N., Hiroi, J., Nishida, M., Iuchi, I. and Yasumasu, S. (2008). Hatching enzyme of the ovoviviparous black rockfish *Sebastes schlegelii* – environmental adaptation of the hatching enzyme and evolutionary aspects of formation of the pseudogene. *FEBS J.* **275**, 2884-2898.

Kawaguchi, M., Yasumasu, S., Shimizu, A., Sano, K., Iuchi, I. and Nishida, M. (2010). Conservation of the egg envelope digestion mechanism of hatching enzyme in euteleostean fishes. *FEBS J.* **277**, 4973-4987.

Kudo, N., Yasumasu, S., Iuchi, I. and Tanokura, M. (2004). Crystallization and preliminary X-ray analysis of HCE-1, a hatching enzyme of medaka fish, *Oryzias latipes*. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 725-726.

Miyamoto, T., Machida, T. and Kawashima, S. (1986). Influence of environmental salinity on the development of chloride cells of freshwater and brackish-water medaka, *Oryzias latipes*. *Zool. Sci.* **3**, 859-865.

Naruse, K. (1996). Classification and phylogeny of fishes of the genus *Oryzias* and its relatives. *Fish Biol. J. Medaka* **8**, 1-9.

Naruse, K., Shima, A., Matsuda, M., Sakaizumi, M., Iwamatsu, T., Soeroto, B. and Uwa, H. (1993). Distribution and phylogeny of rice fish and their relatives belonging to the suborder Adrianichthyoidei in Sulawesi, Indonesia. *Fish Biol. J. Medaka* **5**, 11-15.

Nelson, J. S. (2006). *Fishes of The World*, 4th edn. Hoboken, NJ: John Wiley & Sons.

Okada, A., Sano, K., Nagata, K., Yasumasu, S., Ohtsuka, J., Yamamura, A., Kubota, K., Iuchi, I. and Tanokura, M. (2010). Crystal structure of zebrafish hatching enzyme 1 from the zebrafish *Danio rerio*. *J. Mol. Biol.* **402**, 865-878.

Sali, A. and Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **234**, 779-815.

Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic 'strategies'. *Comp. Biochem. Physiol. B* **139**, 321-333.

Tracewell, C. A. and Arnold, F. H. (2009). Directed enzyme evolution: climbing fitness peaks one amino acid at a time. *Curr. Opin. Chem. Biol.* **13**, 3-9.

Umeno, D., Tobias, A. V. and Arnold, F. H. (2005). Diversifying carotenoid biosynthetic pathways by directed evolution. *Microbiol. Mol. Biol. Rev.* **69**, 51-78.

Yamagami, K. (1973). Some enzymological properties of a hatching enzyme (chorionase) isolated from the fresh-water teleost, *Oryzias latipes*. *Comp. Biochem. Physiol.* **46B**, 603-616.

Yasumasu, S., Katow, S., Umino, Y., Iuchi, I. and Yamagami, K. (1989a). A unique proteolytic action of HCE, a constituent protease of a fish hatching enzyme: tight binding to its natural substrate, egg envelope. *Biochem. Biophys. Res. Commun.* **162**, 58-63.

Yasumasu, S., Iuchi, I. and Yamagami, K. (1989b). Purification and partial characterization of high choriolytic enzyme (HCE), a component of the hatching enzyme of the teleost, *Oryzias latipes*. *J. Biochem.* **105**, 204-211.

Yasumasu, S., Iuchi, I. and Yamagami, K. (1989c). Isolation and some properties of low choriolytic enzyme (LCE), a component of the hatching enzyme of the teleost, *Oryzias latipes*. *J. Biochem.* **105**, 212-218.

Yasumasu, S., Yamada, K., Akasaka, K., Mitsunaga, K., Iuchi, I., Shimada, H. and Yamagami, K. (1992). Isolation of cDNAs for LCE and HCE, two constituent proteases of the hatching enzyme of *Oryzias latipes*, and concurrent expression of their mRNAs during development. *Dev. Biol.* **153**, 250-258.

Yasumasu, S., Iuchi, I. and Yamagami, K. (1994). cDNAs and the genes of HCE and LCE, two constituents of the medaka hatching enzyme. *Dev. Growth Differ.* **36**, 241-250.

Yasumasu, S., Kawaguchi, M., Ouchi, S., Sano, K., Murata, K., Sugiyama, H., Akema, T. and Iuchi, I. (2010). Mechanism of egg envelope digestion by hatching enzymes, HCE and LCE in medaka, *Oryzias latipes*. *J. Biochem.* **148**, 439-448.

Yokoyama, S., Radlwimmer, F. B. and Blow, N. S. (2000). Ultraviolet pigments in birds evolved from violet pigments by a single amino acid change. *Proc. Natl. Acad. Sci. USA* **97**, 7366-7371.