J. Biochem. 2010;148(4):439–448 doi:10.1093/jb/mvq086

JB THE JOURNAL OF BIOCHEMISTRY

Mechanism of egg envelope digestion by hatching enzymes, HCE and LCE in medaka, *Oryzias latipes*

Received December 13, 2009; accepted July 5, 2010; published online August 2, 2010

Shigeki Yasumasu[1,*], Mari Kawaguchi[2,3], Satoshi Ouchi[4], Kaori Sano[4], Kenji Murata[5], Hitoshi Sugiyama[6], Tatsuo Akema[6] and Ichiro Iuchi[1]

[1]Department of Materials and Life Sciences, Faculty of Science and Technology, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo 102-8554; [2]Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564; [3]Research Fellow of the Japan Society for the Promotion of Science (JSPS); [4]Graduate Program of Biological Science, Graduate School of Science and Technology, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo 102-8554, Japan; [5]Department of Animal Science, University of California Davis, One Shields Avenue Davis, CA 95616, USA; and [6]Department of Physiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan

*Shigeki Yasumasu, Department of Materials and Life Sciences, Faculty of Science and Technology, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo 102-8554, Japan.
Tel: +81 3 3238 3393, Fax: +81 3 3238 3393,
email: s-yasuma@sophia.ac.jp

Hatching of medaka embryos from the fertilized egg envelope involves two enzymes, HCE and LCE. HCE swells the envelope and then LCE completely dissolves it. We determined HCE and LCE cleavage sites on the egg envelope that are primarily constructed of two groups of subunit proteins, ZI-1,2 and ZI-3. HCE and LCE cleaved different target sequences on the egg envelope proteins but shared one common cleavage site. HCE cleaved the N-terminal region of ZI-1,2 and ZI-3, mainly the Pro-Xaa-Yaa repeat sequence of ZI-1,2 into hexapeptides, but not the site within a zona pellucida (ZP) domain that is considered to be the core structure of the egg envelope. The cleavage of these N-terminal regions results in swelling and softening of the envelope. LCE cleaved the middle of the ZP domain of ZI-1,2, in addition to the upstream of the trefoil domain of ZI-1,2 and the ZP domain of ZI-3. This middle site is in the intervening sequence connecting two subdomains of the ZP domain. Cleaving this site would result in the solubilization of the swollen egg envelope by the disruption of the filamentous structure that is thought to be formed by the non-covalent polymerization of ZP domains.

Keywords: astacin metalloprotease family/egg envelope/hatching enzyme/medaka/ZP domain.

Abbreviations: FE, fertilized egg envelope; HCE, high choriolytic enzyme; LCE, low choriolytic enzyme; SDS, sodium dodecyl sulphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; UFE, unfertilized egg envelope; ZP, zona pellucida.

Most animal eggs are surrounded by an egg envelope. At hatching, embryos secrete one or more proteolytic enzymes that digest the egg envelope. These enzymes are called the hatching enzyme. The hatching enzymes of vertebrates have been identified from fish to birds (*1*–*4*), and known to be members of the astacin metalloprotease family (*5*). Their molecular structures have been conserved. Although mammalian hatching enzymes have not been identified at the protein level, the genes orthologous to other vertebrate hatching enzyme genes have been found in mammalian genomes (*6*). The envelopes of vertebrate embryos, substrates of the hatching enzymes, are constructed of several homologous glycoproteins. These glycoproteins, isolated from zona pellucida (ZP) of mouse eggs, are composed of three subunits: ZP1, ZP2 and ZP3 (*7*). They commonly possess a characteristic ZP domain consisting of ~260 amino acids. Comparative analyses of the other vertebrate homologues have revealed that the ZP domain is a common structure in all the egg envelope subunits (*8*). Recently, the egg envelope subunits of an invertebrate, abalone (archeogastropod) were reported to possess the ZP domain (*9*). Structural analysis of ZP domain-containing protein has suggested that the ZP domain consists of two independently-folding subdomains: an N-subdomain (ZP-N) and a C-subdomain (ZP-C), which are connected by an intervening protease-sensitive sequence (*10*, *11*). Recently, the 3D structural analysis of mouse ZP3 has revealed that its ZP-N is an independently-folding domain (*12*).

Hatching enzymes have been extensively studied in fish, especially in medaka *Oryzias latipes* (*13*–*17*). The hatching enzymes of medaka were found to consist of two proteases: HCE (high choriolytic enzyme, choriolysin H, EC 3.4.24.67) and LCE (low choriolytic enzyme, choriolysin L, EC 3.4.24.66). The two enzymes have been purified and well characterized (*15*, *16*). The fertilized egg envelope (FE) is comprised of a thick inner layer (15 μm) and a thin outer layer (0.3 μm) (*18*). HCE swells the inner layer up to ~100 μm in thickness by its proteolytic action, with concomitant release of water-soluble small peptides (*15*, *19*). LCE then efficiently solubilizes the swollen inner layer, but does not readily digest the inner layer without treatment of HCE. At hatching, the egg envelope is efficiently solubilized by the cooperative action of HCE and LCE, and the outer layer that is left undigested is mechanically broken by movement of the embryo.

The inner layer of a medaka egg envelope is comprised of two groups of subunits: ZI-1,2 and ZI-3 (*13*, *20*). They are synthesized in liver of the spawning female as precursors called choriogenin,

and transferred to the ovary via the blood stream (*21*). ZI-1,2 are believed to be heterogeneous glycoproteins derived from the precursors: choriogenin H (ChgH) (*22*) and choriogenin H minor (ChgHm) (*23*). ZI-3 is a homogeneous glycoprotein derived from its precursor: choriogenin L (ChgL) (*24*). These three choriogenins possess, in common, a signal peptide, an N-terminal region, a ZP domain and a consensus sequence of the C-terminal processing site (Arg-Lys-X-↓-Arg) that is processed prior to egg envelope assembly from the precursor choriogenins (*25*). In addition, ChgH and ChgHm have N-terminal regions that are longer than ChgL, and contain a characteristic repeat sequence (Pro-Xaa-Yaa repeat sequence), and a trefoil domain just upstream of the ZP domain. ChgH and ChgHm have similar characteristics (76% identity in amino acid sequence with the ZP domain), and are closely related to mammalian ZP1 and ZP2 (*22*, *23*). ZI-3 is homologous to mammalian ZP3 (*24*).

After fertilization, the egg envelope turns into a hard and tough structure to provide a physical barrier against the environment (*13*, *26*). This phenomenon is called 'egg envelope hardening'. During hardening, ZI-1,2 and ZI-3 are polymerized to form an insoluble, high molecular weight complex. Polymerization occurs via ε-(γ-glutamyl) lysine isopeptide bonds catalysed by transglutaminase (*26*, *27*). Due to these cross-links, the egg envelope increases both in mechanical strength and in resistance to environmental chemicals. For example, the inner layer of unfertilized egg envelope (UFE) is easily solubilized with 8 M Urea or 1% SDS (sodium dodecyl sulphate), while that of FE is not solubilized with either (*28*). This hardened FE is a substantial substrate for the hatching enzymes, HCE and LCE.

In this study, we observed the digestion manner of HCE and LCE onto the UFE and the FE, and determined the cleavage sites on the egg envelope subunits. We also proposed the model for digestion mechanism of egg envelope based on the information about the cleavage pattern of the digested products by these hatching enzymes, HCE and LCE, and the previous studies on the structure of egg envelope.

Materials and Methods

Materials

Embryos were obtained by natural mating of the orange-red variety of wild-type medaka, *O. latipes* and were cultured in tap water at 30°C in a shaking incubator. At the sixth day of post-fertilization, the embryos were transferred into a beaker containing a small amount of medium consisting of 10 mM NaCl and 2 mM $NaHCO_3$ (~pH 8.0), and allowed to hatch. When 80–90% of the embryos had hatched, the culture medium, now called hatching liquid, was filtered, frozen and stored at −20°C.

Purification of HCE and LCE from hatching liquid

Medaka hatching enzymes, HCE and LCE, were purified from the hatching liquid according to the procedures described earlier (*15*, *16*) with some modification. Ammonium sulphate powder (60% saturation) was added to the hatching liquid derived from 30,000 embryos, and the precipitate was collected by centrifugation and dissolved in a small volume (~10 ml) of 50 mM bicarbonate buffer (pH 10.2). We employed gel filtration as the second step for purification. In this step, HCE and LCE were roughly separated into two fractions. After dialysis against 50 mM bicarbonate buffer, the clear solution was applied onto a Toyopearl HW-50SF column (TOSOH Inc., Tokyo, Japan) equilibrated with 50 mM bicarbonate buffer (pH 10.2). The proteolytic activity was eluted as two peaks. The first peak mainly contained LCE, while the second peak contained HCE. For the purification of LCE, the former peak was concentrated again by ammonium sulphate precipitation, and re-chromatographed on the same column. The fractions having the activity were dialysed against 50 mM Tris–HCl buffer (pH 8.0), subjected to a Source 15S column (GE Healthcare UK Ltd.) on the HPLC system (Gilson, Middleton, WI, USA) and eluted with a linear gradient of 0–400 mM NaCl in 50 mM Tris–HCl buffer (pH 8.0). For purification of HCE, the latter peak in the first Toyopearl HW-50SF chromatography was dialysed against 50 mM Tris–HCl buffer (pH 8.0) and applied to an S-Sepharose column (GE Healthcare UK Ltd.) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The column was washed with the same buffer, and the adsorbed protein was eluted with the same buffer containing 400 mM NaCl. After being dialysed against 50 mM Tris–HCl buffer (pH 7.5), the samples were subjected to a Source 15S column on the HPLC system and eluted with a linear gradient of 0–400 mM NaCl in 50 mM Tris–HCl buffer (pH 7.5). The purity was checked by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). HCE and LCE thus purified were stored at 4°C.

Isolation of FE and UFE

FEs were manually isolated from pre-hatching embryos with sharp tweezers, and washed several times with Tris–buffered saline (TBS, 50 mM Tris–HCl pH 8.0 and 0.15 M NaCl). Unfertilized eggs were isolated from spawning female fish and crushed in TBS containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM iodoacetic acid. After centrifugation (5,000 r.p.m., 30 s), the supernatant was decanted. This procedure was repeated several times to completely remove yolk proteins and cell debris. The outer layer of the UFEs was observed to be intact in SDS sample buffer by microscopic observation. Thus, the purity of the isolated UFEs was confirmed, and only the bands derived from the inner layer were observed by SDS–PAGE. The isolated UFEs were used for digestion experiments.

Microscopic observation of egg envelope digestion by HCE and LCE

Observation by a low-power binocular microscope: Two or three isolated UFEs or FEs were incubated in 30 µl of 50 mM Tris–HCl (pH 8.0) containing 1 µg of purified HCE, 0.3 µg of purified LCE or their mixture for 30 min at 30°C.

Sectional observation by a light microscope. The isolated FEs were incubated in 50 µl of 50 mM Tris–HCl (pH 8.0) containing 5 µg of purified HCE for 2 or 5 min. After incubation for 5 min with HCE, some of the egg envelopes were briefly washed with TBS, and incubated in 50 µl of 50 mM Tris–HCl (pH 8.0) containing 1 µg of purified LCE for 10 min. Each of the envelope samples was fixed with 4% paraformaldehyde in phosphate-buffered saline [PBS, 20 mM phosphate (pH 7.2) and 150 mM NaCl], dehydrated by alcohol series and embedded in Epoxy resin. Sectioned samples (0.5 µm-thick) were stained with toluidine blue.

Polyacrylamide gel electrophoretic analysis

SDS–PAGE was performed by the method of Laemmli using a 12.5% gel (*29*). The gel was stained with Coomassie Brilliant Blue G (CBB).

Digestion of UFE by HCE and/or LCE

Fifty UFEs were incubated in 150 µl of 50 mM Tris–HCl (pH 8.0) containing 2.3 µg of purified HCE or 1.2 µg of purified LCE at 30°C for 1 h. After aliquots were analysed by SDS–PAGE, the resulting mixture was fractionated by a Superdex 75 10/300 GL column equilibrated with PBS. Fractions containing lower molecular weight digests were applied onto a C18 reverse phase column (YMC Co., Ltd. Tokyo, Japan) equilibrated with 0.1% trifluoroacetic acid (TFA) on the HPLC system, and eluted with a liner gradient of 0–45% acetonitrile in 0.1% TFA.

Determination of N-terminal amino acid sequence

The digests of UFEs by HCE or LCE were separated by SDS–PAGE, electrically blotted onto a PVDF membrane (Hybond-P, GE Healthcare UK Ltd), and stained with CBB. The band was cut out and subjected to a protein sequencer (Procise 491HT,

Applied Biosystems, Foster City, CA, USA). Low molecular weight peptides isolated from the digests were directly blotted on to the PVDF membrane and subjected to the sequencer.

Analysis of hatching enzyme-cleavage sites using synthetic peptides

Synthetic peptides consisting of 11 or 12 amino acid residues were used in the analysis. The synthetic peptides were custom synthesized by Hayashi-Kasei Co. Ltd (Osaka, Japan). The sequences were designed from HCE- and LCE-cleavage sites that were determined after egg envelope digestion. A 100 µl reaction mixture consisting of 100 µM peptide and an appropriate amount of enzyme in 50 mM Tris–HCl (pH 8.0) was incubated at 30°C for 30 min. After incubation, the reaction was stopped by the addition of 10 µl of 100 mM EDTA. The final mixture was applied onto a C18 column of the HPLC system equilibrated with 0.1% TFA, and eluted with a linear gradient of 0–36% acetonitrile in 0.1% TFA. The activity was calculated from the ratio of peak area of digested peptide to that of undigested peptide. The cleavage site on the peptide was determined by amino acid sequencing, or determined by the estimation of molecular weight by mass spectrometry. For determination of the Michaelis constant (K_m), synthetic peptides were used as substrates in a concentration range up to 1 mM. Initial rates were determined by single time points of reactions in which the consumption of substrate was <30% of the starting concentration. The K_m values were determined by using Lineweaver–Burk plot.

Results

Microscopic observation of egg envelope digestion by hatching enzymes

The UFE is soft and fragile (Fig. 1A). After fertilization, the envelope transforms into a tough, hard structure called the FE (Fig. 1D). The digestion manner of the hatching enzymes was observed by light microscopy using UFE as the substrate. The inner layer of UFE was found to be completely solubilized either by HCE alone, or by LCE alone (Fig. 1B and C). For comparison, we also show the result of FE as reported previously (*15*, *16*). The inner layer of FE was swelled by the action of HCE (Fig. 1E), and completely solubilized by the actions of both HCE and LCE (Fig. 1F). No morphological change was observed by treatment with LCE alone (data not shown) (*16*). To examine the digestion manner more precisely, we performed the sectional observation. Figure 2 shows the time-course of FE swelling by HCE. HCE predominantly acted on the inside of FE during the process (Fig. 2A and B). The swollen and intact portions of the inner layer were clearly distinguishable. This demonstrates that HCE gradually digests their inner layer of envelope, and the envelope expands towards the inside, probably due to the presence of an outer layer that is scarcely digested by the hatching enzymes (*18*). The partially-swollen envelope in (B) was washed with buffer and incubated with LCE (Fig. 2C). LCE solubilized only the swollen portion of the HCE-treated envelope, but did not affect the intact portion of HCE-treated envelope. The results suggest that the solubilization of the inner layer at hatching is performed by the two-step action of two enzymes: HCE swells it and then LCE solubilizes its swollen portion. In natural hatching, however, any swelling of the inner layer was not observed. It is probably synchronous action of HCE and LCE to the egg envelope. Thus, as soon as HCE swelled the inner layer, its swollen portion was solubilized by LCE.

Determination of HCE and LCE cleavage sites in UFE

Since the inner layer of UFE was completely solubilized both by HCE alone and by LCE alone, isolated UFEs were employed as the substrate to determine the cleavage sites. As shown in lane 1 of Fig. 3, SDS–PAGE of intact UFE exhibited two bands, at 64–74 kDa and 48 kDa. According to the previous reports, the broad, upper band contains several bands called ZI-1,2 (64–74 kDa), and the lower band, ZI-3 (48 kDa) (*13*, *20*). We determined the cleavage sites of HCE and LCE by comparing the N-terminal sequences of their digests with the sequences deduced from cDNAs encoding the precursors of ZI-1,2 (ChgH, Accession number: D89609 and ChgHm, AB025967) and the precursor of ZI-3 (ChgL, D38630).

On incubation with HCE alone, the inner layer of UFE was completely digested. Two major bands (46 and 35 kDa) and one minor band (37 kDa) were observed (Fig. 3A, lane 2). The N-terminal sequence determined for the 46-kDa product (DPTQQ) matched that from D40 of ChgL, demonstrating that the 46-kDa product is derived from ZI-3, and that the HCE cleavage site is Q39/D40 of ChgL (Figs 3 and 4). The molecular mass of this band determined by SDS–PAGE was larger than that calculated from the ChgL cDNA (38,880; from D40 to T393 of the C-terminal processing site). This difference is postulated to be due to the *N*-glycosylation of ZI-3 (*21*). The sequence (TPPIGPPPP) identified for the 35-kDa band was found in the sequence from T221 of ChgH, and its molecular mass accorded with the predicted molecular weight of 36,539 from T221 to G557 of the C-terminal processing site. These results revealed that the 35-kDa product contains the trefoil domain and ZP domain of ZI-1,2 (Figs 3 and 4). The sequence (YPVPA) identified for the minor band of 37 kDa matched the predicted sequence from Y74 in ChgL cDNA, indicating that this band is the digest derived from ZI-3.

SDS–PAGE of the LCE-digests of UFE exhibited three protein bands at 37 kDa, 17 kDa and 16 kDa (Fig. 3A, lane 3). The N-terminal sequence of the 37-kDa band (YPVPA) matched the predicted sequence from Y74 located in the ChgL cDNA, and also that of the 37-kDa band of HCE-digests. The intensity of this band was much denser in the LCE-digests (Fig. 3A, lane 3) than that in the HCE-digests (Fig. 3A, lane 2). Therefore, this site in ZI-3 (ChgL) is considered to be one of the major cleavage sites for LCE. The N-terminal sequence (TPPIG) of the 17-kDa product matched that of the 35-kDa product in HCE-digests. Thus, the site N220/T221 in ChgH is suggested to be the common cleavage site for HCE and LCE. The sequence in the 16-kDa product (SPLSIAE) matched that from S388 in ChgH. Therefore, the 16-kDa product is also derived from ZI-1,2 and D387/S388 in ChgH cDNA was determined as the LCE cleavage site (Figs 3 and 4). This site was found at the middle of the ZP domain in ZI-1,2, or ChgH cDNA. The molecular masses of 17-kDa and 16-kDa product approximately agreed with their molecular sizes predicted from the ChgH cDNA (17,852 from T221 to D387 and 18,704

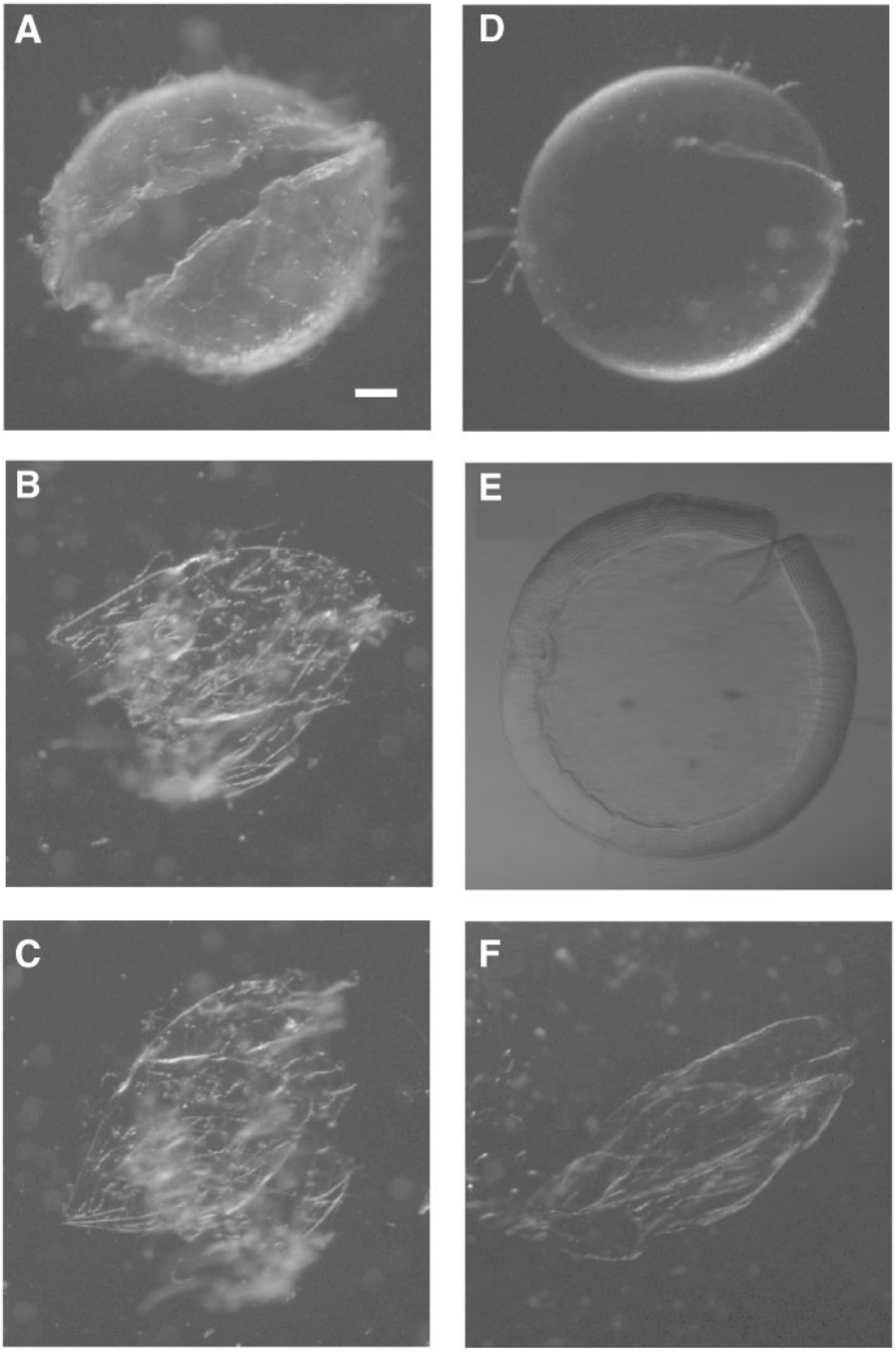

Fig. 1 Solubilization of UFE and FE by hatching enzyme(s). Isolated UFE (A) was incubated with purified HCE (B) or LCE (C) for 30 min at 30°C. Isolated FE (D) was incubated with purified HCE (E) or the mixture of HCE and LCE (F) for 30 min at 30°C. The undigested thin outer layer was observed in B, C and F. Scale bar, 100 μm.

from S388 to G557). These results showed that LCE cleaved at the following three sites: one just upstream of a trefoil domain of ZI-1,2, a second one in the middle of the ZP domain of ZI-1,2 and a third one near the N-terminus of the ZP domain of ZI-3 (Figs 3B and 4).

The SDS–PAGE pattern of UFE digested with a mixture of HCE and LCE was quite similar to that of the LCE-digests (Fig. 3A, lane 3 and 4). Their N-terminal sequences were the same as those in the LCE-digests.

To determine the cleavage sites from the digests having molecular masses smaller than those described in above paragraphs, we analysed the HCE- and LCE-digests of UFE by gel filtration column chromatography. Thus, the HCE-digests were fractionated on a Superdex 75 10/300 GL column into two peaks: a large broad peak eluted at the void volume of the column and an irregular peak near the bed volume (Fig. 5A). SDS–PAGE of the former peak exhibited two bands at 47 kDa and 35 kDa, corresponding to the major bands detected by the earlier SDS–PAGE

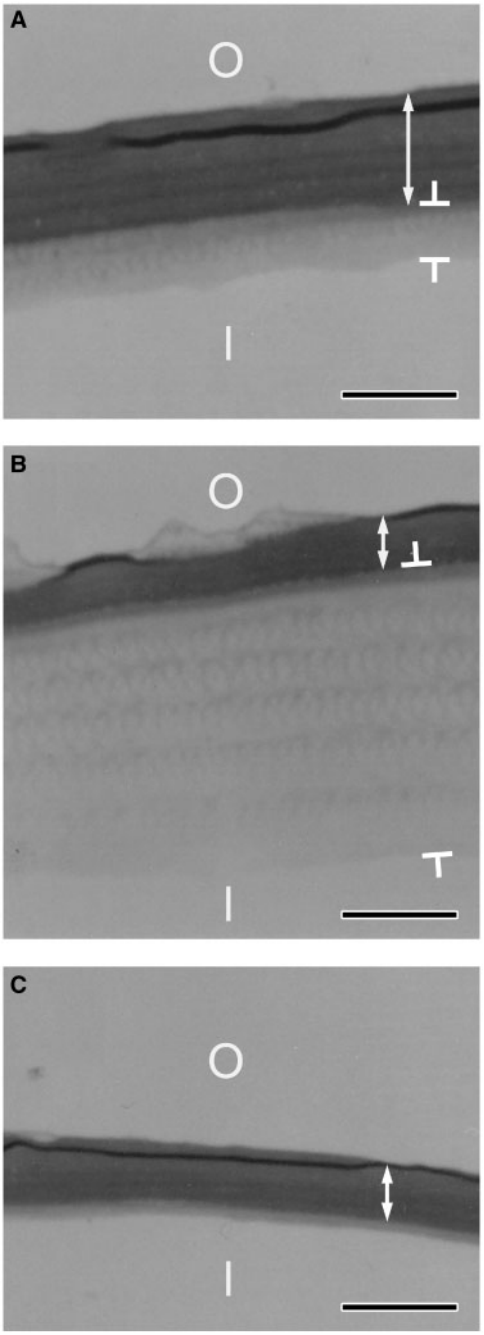

Fig. 2 Light microscopic observation of digestion of FE by HCE and LCE. Isolated FE was incubated with purified HCE for 2 min (A) and 5 min (B) at 30°C. Partially-swollen envelope in (B) was further incubated for 10 min with purified LCE (C). The sections were stained with toluidine blue. Scale bar, 20 μm. Two bars and left right arrow represent thickness of the swollen part and intact part of inner layer, respectively. O, outside of the envelope; I, inside of egg envelope.

analysis. The latter peak was further fractionated on a reversed phase column into three peaks (Pa to Pc, Fig. 5C) with several minor peaks. Each of the three peaks contained hexapeptides. The sequences of Pa and Pc were identical, showing YPSKPQ in the Pro-Xaa-Yaa repeat sequences of ChgH and ChgHm. Analysis of Pb exhibited (Y/N)P(S/Q)(Y/V)PQ, a mixture of several peptides. Referring to the sequence deduced from ChgH cDNA, Pb was predicted to be a mixture of three peptides, YPSYPQ, NPQVPQ and NPSYPQ (Fig. 4B). Thus, HCE cleaved the Pro-Xaa-Yaa repeat sequence into short peptide fragments, and its major products were hexapeptides.

When the LCE-digests were fractionated on the same column, two major peaks appeared slightly after the void volume and two minor peaks (peak A and B) at the later positions (Fig. 5B). The two major peaks contained three products (37 kDa, 17 kDa and 16 kDa), which correspond to the bands detected by the earlier SDS–PAGE analysis of the LCE digests. No band was detected for the two minor peaks by SDS–PAGE, but each of them gave a sharp single peak by reversed phase column chromatography (data not shown). The sequence contained in peak A was DGKPSNPQQ, and this sequence was found from D137 in Pro-Xaa-Yaa repeat sequence of the predicted amino acid sequence of ChgH. However, sequencing of peak B generated no sequence, suggesting that its N-terminus is blocked. It is possible that this fragment contains N-termini of ZI-1,2 or ZI-3, as it is known that they are blocked (*22*, *24*). Thus, it was found that LCE was able to cleave a limited number of site(s) in the N-terminal region of ZI-1,2 but could not easily cleave the Pro-Xaa-Yaa repeat sequence as did HCE.

The proteins derived from ChgHm were not detected in any digests. This is consistent with a previous report that the content of the egg envelope proteins derived from ChgHm is much lower than that from ChgH (*26*).

Determination of HCE and LCE cleavage sites in FE

The real substrate of hatching enzyme is FE. The main constituents of the hatching liquid are the hatching enzyme (HCE/LCE)-digest of the FE at natural hatching. We then analysed the digests in the hatching liquid, and compared them with those of the HCE/LCE-digests of UFE (Fig. 3A, lane 4, 5). The N-terminal sequence of the 37-kDa band in the hatching liquid matched that of the 37-kDa band in the UFE digests. However, we found two differences; the 16-kDa band in the UFE digests was not present in the digests from the hatching liquid, and a 25-kDa band was only observed in the digests from the hatching liquid. The sequence of the 17-kDa product in the hatching liquid was predicted to be a mixture of two sequences (TPPIGPPPPK, lined from T221 and SPLSIAELGP, lined from S388 in the predicted amino acid sequence in ChgH cDNA) matched those of the 16-kDa product and 17-kDa product of UFE. It is puzzling that the 17-kDa product in the hatching liquid contains a mixture of two peptides whose N-terminal sequences coincide with those of 16-kDa and 17-kDa product of UFE. This may be due to structural changes caused by the hardening of the egg envelope following fertilization. The sequence of the 25-kDa product was identified on the inside of the ZP domain in the predicted amino acid sequence of ChgL cDNA from T168 (TSQAVVI) (Figs 3 and 4A). The molecular mass of the product matched the predicted molecular weight of 24,820 (from T168 to T393 of ChgL), suggesting that some part of the

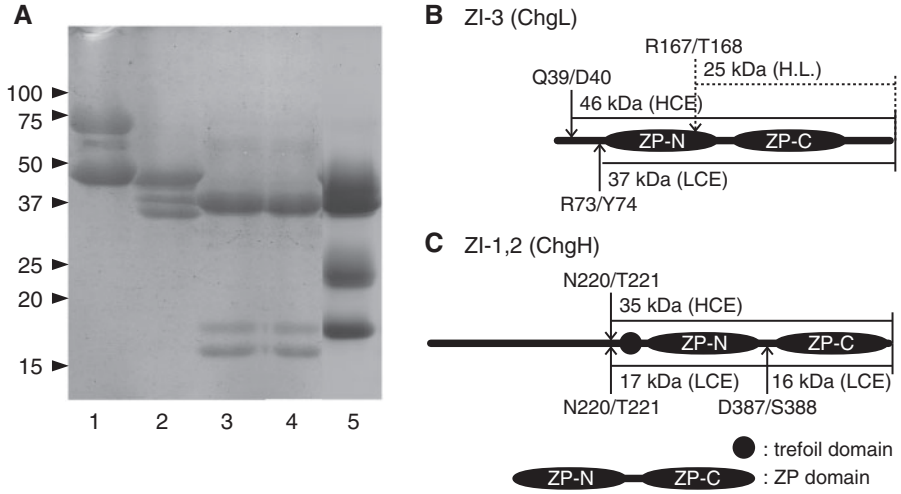

Fig. 3 SDS–PAGE patterns of hatching enzyme-digests and schematic representation of hatching enzyme cleavage sites. (A) Isolated UFE (lane 1), HCE-digests (lane 2), LCE-digests (lane 3), HCE/LCE-digests (lane 4) or hatching liquid (lane 5) was electrophoresed on 12.5% gel. Numbers on the left indicate the sizes of molecular markers (kDa). An amount of 10 μg (lane 1–4) and 20 μg (lane 5) of protein were loaded in each lane. (B) and (C) show the structure of ZI-3 and ZI1,2, respectively. The regions of the products produced by HCE and LCE cleavage, and their cleavage sites are indicated. ZP-N and ZP-C indicate the two subdomains in the ZP domain.

37-kDa product was further cleaved either by HCE or by LCE. The N-terminal sequences of digests in the hatching liquid correspond well to those of the HCE/LCE-digests of UFE except for the 25-kDa product in the hatching liquid which is not present in the HCE/LCE-digests of UFE. The results suggest that the HCE and LCE cleavage sites determined using UFE well reflect the FE digestion that really occurs at embryo hatching.

Substrate specificity of HCE and LCE with synthetic peptides

The cleaving efficiency of HCE and LCE was quantitatively estimated using synthetic peptide substrates that were designed from the cleavage sites determined by the egg envelope digestion experiments. HCE and LCE are highly specific toward these synthetic peptides. The efficient substrates for HCE were two peptides designed from Pro-Xaa-Yaa repeat sequences (Table I), suggesting that HCE prefers to cleave the Pro-Xaa-Yaa repeat sequence. The K_m value toward NPQVPQ↓YPSKPQ was 480 mM. The most efficient substrate for LCE was the peptide designed from the sequence located in the middle of the ZP domain in ZI-1,2, EVQPPD↓SPLSI; the K_m value was 48 mM. The specific activity of LCE toward the LCE cleavage site in the Pro-Xaa-Yaa repeat sequence, PKLFQ↓DGKPSN, was low, and the activities toward two other peptides designed from the Pro-Xaa-Yaa sequence were also low. These results support the finding of the present egg envelope digestion experiment that LCE inefficiently cleaves the Pro-Xaa-Yaa repeat region. Both the specific activities of HCE and LCE toward the peptides designed from the 25-kDa product in hatching liquid, SAPVVR↓TSQAV, were low, suggesting that the site located at the inside of the ZP domain of ZI-3 is an inefficient cleavage site.

Discussion

In the present study, we analysed the digests of egg envelopes by HCE and/or LCE, and determined HCE and LCE cleavage sites on the proteins constituting medaka egg envelope.

We postulated the locations of ε-(γ-glutamyl) lysine isopeptide cross-links on egg envelope proteins. If the digests by the hatching enzymes were cross-linked by covalent bonds, their bands should exhibit lower electrophoretic mobility than the uncross-linked digests on SDS–PAGE analysis. In SDS–PAGE of hatching liquid, the three digests corresponding to the ZP domain of ZI-3 and the N- and C-terminal halves of the ZP domain of ZI-1,2 were detected, and no band at molecular weight positions higher than those was observed (Fig. 3A, lane 5). The result suggests that ε-(γ-glutamyl) lysine isopeptide cross-links are very rare within these ZP domains. This result is consistent with the previous report that most of ε-(γ-glutamyl) lysine isopeptide cross-links are located in the N-terminal Pro-Xaa-Yaa region of ZI-1,2 (*19*).

Next, based on the digestion profile of HCE or LCE in the present experiment, we discuss an egg envelope digestion mechanism of hatching enzymes, in addition to the information accumulated in similar studies of ZP domain proteins of other animals.

The ZP domain in the ZP3 homologues of egg envelopes is commonly known to contain eight conserved Cys residues, while that of ZP1/ZP2-like protein contains two additional Cys residues (Ca, Cb) in its C-terminal region, i.e. 10 Cys residues in total (C1–C8, Ca and Cb) (*10*). All of these Cys residues are also conserved in the ZP domains of medaka ZI-3 (ZP3 homologue) and ZI-1,2 (ZP1/ZP2 homologues) (Fig. 4). The disulphide linkages in the ZP domains of native mouse ZP (*30*) and rainbow trout VE (vitelline envelope) (*31*) have been determined by mass spectrometry. These analyses revealed that the egg envelope proteins homologous between mouse and

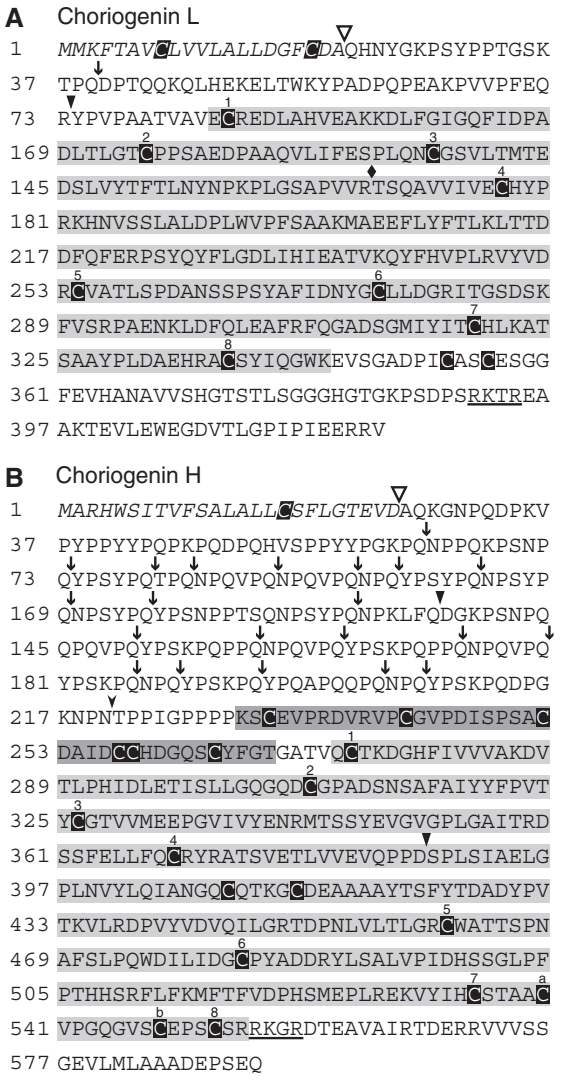

Fig. 4 HCE- and LCE-cleavage sites mapped on the deduced amino acid sequences of Choriogenin L cDNA and Choriogenin H cDNA. The HCE- and LCE-cleavage sites are indicated by vertical arrows and inverted black triangles, respectively. A vertical arrowhead indicates the common cleavage site for HCE and LCE. The diamond is the site found in the 25 kDa-digests from the hatching liquid. The cleavage sites except for HCE-cleavage sites in Pro-Xaa-Yaa repeat sequence were determined by amino acid sequencing of egg envelope digests. HCE-cleavage sites in Pro-Xaa-Yaa repeat sequence were predicted to be PQ↓(Y/N)P from the sequences of low molecular weight HCE-digests. The trefoil domain and ZP domains are indicated in dark and light grey boxes, respectively. The cysteine residues are shown by black boxes with white letters. The numbers above the black boxes show the conserved cysteine residues in ZP domains of ChgL (1–8) and in ChgH (1–8 and a, b). Signal sequences are italicized and the cleaving sites of signal peptidase are indicated by inverted open triangles. Underlines show the consensus sequence of the C-terminal processing site.

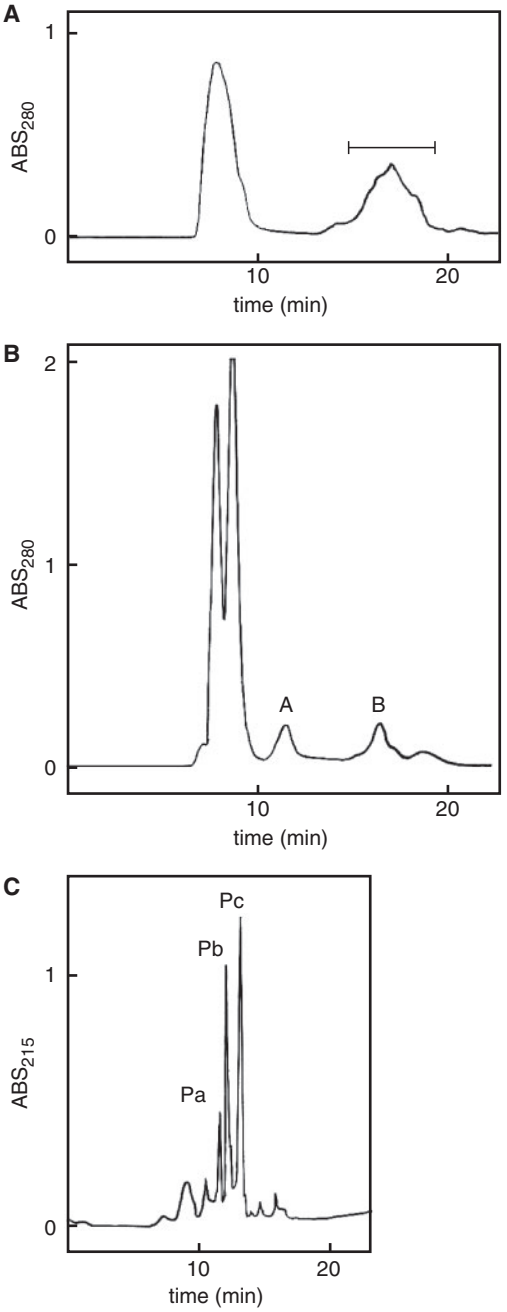

Fig. 5 Chromatograms of HCE- and LCE-digests. (A) HCE-digests of UFE were fractionated on a Superdex 75 10/300 GL column equilibrated with PBS (pH 7.2). (B) LCE-digests of UFE were fractionated on the same column. (C) The low molecular weight fractions of the HCE-digests, indicated by a bar in (A), were applied on a reversed phase C18 column and eluted with a linear gradient of 0–45% acetonitrile in 0.1% TFA.

rainbow trout, ZP/VE proteins, share the same disulfide-bonding patterns within the ZP domain. The results in turn suggest that the 3D structure of the ZP domain is highly conserved both in mammals and fish.

It was reported that the purified egg envelope proteins of mouse and rainbow trout can be polymerized into long filaments through their non-covalent interactions by ultrastructural observations (*32*, *33*). It was also suggested that the ZP domain itself is responsible for polymerization into filaments (*34*). The results obtained from these experiments suggested that the characters of the ZP domain, such as its self polymerization or assembly, and the resultant filamentous structure should contribute to forming the macromolecular structure of egg envelope.

The electron microscopic observations of the filament of mouse ZP protein (*7*, *35*) and the 3D model

Table I. Specific activity of HCE and LCE examined with synthetic peptides.

Location	Enzyme[a]	Peptide sequence	Activity (nmol/30 min/μg enzyme)	
			HCE	LCE
Q39/D40 in ChgL (ZI-3)	HCE	GSKTPQ↓DPTQQ	28.5	n.d.
R73/Y74 in ChgL (ZI-3)	LCE	VPFEQR↓YPVPA	0.5	76.3
N220/T221 in ChgH (ZI-1,2)	HCE and LCE	PGKNPN↓TPPIG	11.3	23.9
D387/S388 in ChgH (ZI-1,2)	LCE	EVQPPD↓SPLSI	1.3	136
R167/T168 in ChgL (ZI-3)	Hat. Liq.	SAPVVR↓TSQAV	n.d.	1.9
Pro-Xaa-Yaa repeat sequence in ZI-1,2	HCE	NPSYPQ↓NPSYPQ	81.7	0.3
Pro-Xaa-Yaa repeat sequence in ZI-1,2	HCE	NPQVPQ↓YPSKPQ	43.7	1.7
Q136/D137 in ChgH (ZI-1,2)	LCE	PKLFQ↓DGKPSN	0.1	0.2

HCE- or LCE-cleavage sites on each peptide are indicated by arrows. [a]Name of enzyme(s) that cleaves each site. Hat. Liq., Cleavage site detected from egg envelope digests in hatching liquid. n.d., Not detected.

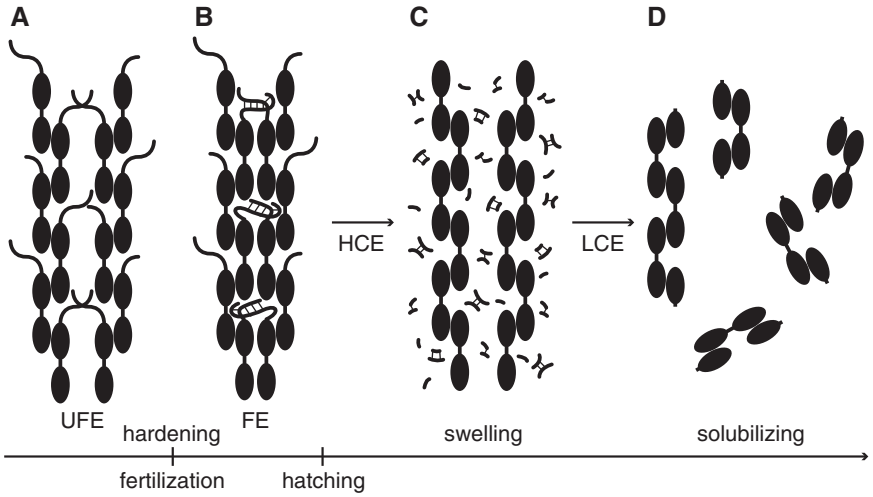

Fig. 6 The predicted structural change of the inner layer of medaka egg envelope at fertilization, and at hatching. After fertilization, ε-(γ-glutamyl) lysine cross-links are formed between the N-terminal regions of the subunit proteins, making the egg envelope compact and hard. (A) and (B) show the predicted molecular structure of the unfertilized soft egg envelope and the fertilized hardened egg envelope, respectively. At hatching, HCE swells and loosens the egg envelope by cleaving the N-terminal regions of egg envelope subunits into fine fragments (C). LCE cleaves the part between ZP-N and ZP-C, and solubilizes the swollen envelope by disruption of the filamentous structure formed by assembly of ZP-domains (D). Relative ZP domain orientation and arrangements are hypothetical.

of the N-terminal fragment of mouse ZP2 (*12*) suggest that the N-terminal region protrudes from ZP filaments. Given the structural similarity of the ZP domain between mouse and fish, we postulate that the N-terminal regions of ZI-1,2 and ZI-3 may loop out from the ZP domain filaments in UFE (Fig. 6A). If so, the molecular mechanisms underlying both the hardening process and the digestion of FE by HCE and LCE can be explained as follows. At fertilization, ε-(γ-glutamyl) lysine isopeptide cross-links are formed between the N-terminal regions, making the inter-filament interaction tight, and change the structure of the egg envelope into a hard, tough structure (Fig. 6B). At hatching, HCE cleaves the N-terminal region of ZI-3 and ZI-1,2, mainly the Pro-Xaa-Yaa sequence of ZI-1,2, into short peptide fragments, releasing them from the FE. As a result, most of the cross-links located in the Pro-Xaa-Yaa sequences are eliminated by proteolysis of HCE. This HCE-cleaving would render the architecture of the egg envelope loose, resulting in swelling of the envelope (Fig. 6C). The result that LCE can digest UFE, but cannot easily digest FE, also supports the following model, i.e. the interaction between the ZP domain filaments of UFE is loosened enough for LCE to gain access to them, but the filaments of FE are tightly bunched, preventing the access of LCE. At hatching, the digestion of FE by HCE makes LCE accessible to its cleaving site.

After HCE-digestion, the swollen egg envelope is considered to be composed of a ZP domain-dependent filamentous structure exclusively constructed from the polymerization or assembly of ZI-1,2 and ZI-3. LCE cleaves only the three sites on egg envelope proteins, one of which locates in the intervening protease-sensitive sequence between ZP-N and ZP-C of ZI-1,2. Therefore, it is conceivable that, in the process of LCE-cleaving at this site, disruption of the filamentous structures would occur, resulting in the solubilization of the swollen FE (Fig. 6D).

According to the phylogenetic analysis of fish hatching enzyme genes, fishes in the early phase of teleost evolution possessed a single hatching enzyme. Since

then, two enzymes, HCE and LCE, have been produced by duplication and diversification of the gene (*36*). The study of the egg envelope-digestion mechanism suggests that a single enzyme-dependent swelling of egg envelope is an ancestral form of the digestion (*37*). Therefore, HCE inherits the role of the ancestral enzyme, i.e. swelling of the egg envelope. LCE was produced by changing the substrate specificity of an ancestral HCE-like enzyme thereby acquiring a new function, solubilization of the swollen egg envelope. Such an evolutionary pathway is understandable when one considers the structure-dependent digestion by HCE and LCE which developed over the long history of evolution.

Acknowledgements

We express our cordial thanks to Dr Fred S. Conte in the Department of Animal Science, University of California, Davis, and Dr Jean Michalec, retired lecturer of Scientific and Technical English, Environmental Issues and General English courses at Sophia University for their kind reading of this article. The present study was supported in part by Grants-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science, Japan (19570221 to S.Y.).

Conflict of interest
No conflict of interest.

References

1. Ishida, J. (1944) Hatching enzyme in the freshwater fish, *Oryzias latipes*. *Annot. Zool. Jpn.* **22**, 137–164
2. Ishida, J. (1985) Hatching enzyme: past, present and future. *Zool. Sci.* **2**, 1–10
3. Katagiri, C., Maeda, R., Yamashika, C., Mita, K., Sargent, T.D., and Yasumasu, S. (1997) Molecular cloning of *Xenopus* hatching enzyme and its specific expression in hatching gland cells. *Int. J. Dev. Biol.* **41**, 19–25
4. Yasumasu, S., Mao, K.M., Sultana, F., Sakaguchi, H., and Yoshizaki, N. (2005) Cloning of a quail homologue of hatching enzyme: its conserved function and additional function in egg envelope digestion. *Dev. Genes Evol.* **215**, 489–498
5. Bond, J.S. and Beynon, R.J. (1995) The astacin family of metalloendopeptidases. *Protein Sci.* **4**, 1247–1261
6. Quesada, V., Sanchez, L.M., Alvarez, J., and Lopez-Otin, C. (2004) Identification and characterization of human and mouse ovastacin: a novel metalloproteinase similar to hatching enzymes from arthropods, birds, amphibians, and fish. *J. Biol. Chem.* **279**, 26627–26634
7. Wassarman, P.M. and Mortillo, S. (1991) Structure of the mouse egg extracellular coat, the zona pellucida. *Int. Rev. Cytol.* **130**, 85–110
8. Spargo, S.C. and Hope, R.M. (2003) Evolution and nomenclature of the zona pellucida gene family. *Biol. Reprod.* **68**, 358–362
9. Aagaard, J.E., Yi, X., MacCoss, M.J., and Swanson, W.J. (2006) Rapidly evolving zona pellucida domain proteins are a major component of the vitelline envelope of abalone eggs. *Proc. Natl Acad. Sci. USA* **103**, 17302–17307
10. Jovine, L., Darie, C.C., Litscher, E.S., and Wassarman, P.M. (2005) Zona pellucida domain proteins. *Annu. Rev. Biochem.* **74**, 83–114
11. Llorca, O., Trujillo, A., Blanco, F.J., and Bernabeu, C. (2007) Structural model of human endoglin, a transmembrane receptor responsible for hereditary hemorrhagic telangiectasia. *J. Mol. Biol.* **365**, 694–705
12. Monné, M., Han, L., Schwend, T., Burendahl, S., and Jovine, L. (2008) Crystal structure of the ZP-N domain of ZP3 reveals the core fold of animal egg coats. *Nature*. **456**, 653–657
13. Yamagami, K., Hamazaki, T.S., Yasumasu, S., Masuda, K., and Iuchi, I. (1992) Molecular and cellular basis of formation, hardening, and breakdown of the egg envelope in fish. *Int. Rev. Cytol.* **136**, 51–92
14. Yasumasu, S., Iuchi, I., and Yamagami, K. (1988) Medaka hatching enzyme consists of two kinds of proteases which act cooperatively. *Zool. Sci.* **5**, 191–195
15. Yasumasu, S., Iuchi, I., and Yamagami, K. (1989) 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
16. Yasumasu, S., Iuchi, I., and Yamagami, K. (1989) 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
17. 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
18. Yamamoto, T. and Yamagami, K. (1975) Electron microscopic studies on choriolysis by the hatching enzyme (chorionase) of the teleost, *Oryzias latipes*. *Dev. Biol.* **43**, 313–321
19. Lee, K.S., Yasumasu, S., Nomura, K., and Iuchi, I. (1994) HCE, a constituent of the hatching enzymes of *Oryzias latipes* embryos, releases unique proline-rich polypeptides from its natural substrate, the hardened chorion. *FEBS Lett.* **339**, 281–284
20. Hamazaki, T.S., Iuchi, I., and Yamagami, K. (1987) Isolation and partial characterization of a spawning female-specific substance in the teleost, *Oryzias latipes*. *J. Exp. Zool.* **242**, 343–349
21. Hamazaki, T.S., Nagahama, Y., Iuchi, I., and Yamagami, K. (1989) A glycoprotein from the liver constitutes the inner layer of the egg envelope (zona pullucida interna) of fish, *Oryzias latipes*. *Dev. Biol.* **133**, 101–110
22. Murata, K., Sugiyama, H., Yasumasu, S., Iuchi, I., Yasumasu, I., and Yamagami, K. (1997) Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion). *Proc. Natl Acad. Sci. USA* **94**, 2050–2055
23. Sugiyama, H., Yasumasu, S., Murata, K., Iuchi, I., and Yamagami, K. (1998) The third egg envelope subunit in fish: cDNA cloning and analysis, and gene expression. *Dev. Growth Differ.* **40**, 35–45
24. Murata, K., Sasaki, T., Yasumasu, S., Iuchi, I., Enami, J., Yasumasu, I., and Yamagami, K. (1995) Cloning of cDNAs for the precursor protein of a low-molecular-weight subunit of the inner layer of the egg envelope (chorion) of the fish *Oryzias latipes*. *Dev. Biol.* **167**, 9–17
25. Sugiyama, H., Murata, K., Iuchi, I., Nomura, K., and Yamagami, K. (1999) Formation of mature egg envelope subunit proteins from their precursors (choriogenins) in the fish, *Oryzias latipes*: loss of partial C-terminal sequences of the choriogenins. *J. Biochem.* **125**, 469–475

26. Sugiyama, H. and Iuchi, I. (2000) Molecular structure and hardening of egg envelope in fish. *Recent Res. Dev. Comp. Biochem. Physiol.* **1**, 139–161
27. Ha, C.R. and Iuchi, I. (1988) Enzyme responsible for egg envelope (chorion) hardening in fish: purification and partial characterization of two transglutaminases associated with their substrate, unfertilized egg chorion, of the rainbow trout, *Oncorhynchus mykiss*. *J. Biochem.* **124**, 917–926
28. Masuda, K., Iuchi, I., and Yamagami, K. (1991) Analysis of hardening of the egg envelope (chorion) of the fish, *Oryzias latipes*. *Dev. Growth Differ.* **33**, 75–83
29. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
30. Boja, E.S., Hoodbhoy, T., Fales, H.M., and Dean, J. (2003) Structural characterization of native mouse zona pellucida proteins using mass spectrometry. *J. Biol. Chem.* **278**, 34189–34202
31. Darie, C.C., Biniossek, M.L., Jovine, L., Litscher, E.S., and Wassarman, P.M. (2004) Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. *Biochemistry* **43**, 7459–7478
32. Darie, C.C., Janssen, W.G., Litscher, E.S., and Wassarman, P.M. (2008) Purified trout egg vitelline envelope proteins VEβ and VEγ polymerize into homomeric fibrils from dimers in vitro. *Biochim. Biophys. Acta* **1784**, 385–392
33. Litscher, E.S., Janssen, W.G., Darie, C.C., and Wassarman, P.M. (2008) Purified mouse egg zona pellucida glycoproteins polymerize into homomeric fibrils under non-denaturing conditions. *J. Cell. Physiol.* **214**, 153–157
34. Qi, H., Williams, Z., and Wassarman, P.M. (2002) Secretion and assembly of zona pellucida glycoproteins by growing mouse oocytes microinjected with epitope-tagged cDNAs for mZP2 and mZP3. *Mol. Biol. Cell* **13**, 530–541
35. Jovine, L., Qi, H., Williams, Z., Litscher, E., and Wassarman, P.M. (2002) The ZP domain is a conserved module for polymerization of extracellular proteins. *Nature Cell. Biol.* **4**, 457–461
36. Kawaguchi, M., Yasumasu, S., Hiroi, J., Naruse, K., Suzuki, T., and Iuchi, I. (2007) Analysis of the exon-intron structures of fish, amphibian, bird and mammalian hatching enzyme genes, with special reference to the intron loss evolution of hatching enzyme genes in Teleostei. *Gene*. **392**, 77–88
37. Sano, K., Inohaya, K., Kawaguchi, K., Yoshizaki, N., Iuchi, N., and Yasumasu, S. (2008) Purification and characterization of zebrafish hatching enzyme – an evolutionary aspect of the mechanism of egg envelope digestion. *FEBS J.* **275**, 5934–5946