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Purification and cDNA cloning of the ovigerous-hair stripping substance (OHSS) contained in the hatch water of an estuarine crab *Sesarma haematocheir*

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Summary

The egg attachment system of an estuarine crab *Sesarma haematocheir* is formed on the maternal ovigerous hairs just after egg laying, and slips off these hairs just after hatching. The stripping is caused by an active factor that we call OHSS (ovigerous-hair stripping substance), which is released by the embryo upon hatching. OHSS was purified, and its active form had a molecular mass of 25 kDa. The cDNA of OHSS cloned from an embryonic cDNA library was 1759 bp long, encoding 492 amino acids in a single open reading frame (ORF). The C-terminal part of the predicted protein was composed of a trypsin-like serine protease domain, with homology to counterparts in other animals of 33–38%. The predicted protein (54.7 kDa) secreted as a zymogen may be cleaved post-translationally, separating the C-terminal from the N-terminal region. The OHSS gene was expressed in the embryo at least 2 weeks before hatching. Expression was also detected in the zoea larva 1 day after hatching and in the brain of the female. However, it was not detected in the muscle, hepatopancreas or ovigerous seta of the female. Ultrastructural analysis indicated that the material investing maternal ovigerous hair, i.e. the outermost layer (E1) of the egg case, is attached at the special sites (attachment sites) arranged at intervals of 130–160 nm on the hair. It is suggested that OHSS acts specifically at these sites, lysing the bond with the coat, thus disposing of the embryo attachment system. This enables the female to prepare the next clutch of embryos without ecdysis.

Key words: crab, *Sesarma* (or *Chiromantes*) *haematocheir*, ovigerous hair, embryo attachment system, investment coat, stripping, ovigerous-hair stripping substance (OHSS), serine protease.

Introduction

Fertilized eggs in a number of decapod crustaceans (except Dendrobranchiata) attach to ovigerous hairs arranged on the abdomen of the female. The attachment is effected by an 'embryo attachment system' composed of an egg envelope, funiculus, and the coat that wraps around the hair, and the embryos are ventilated by the female (Herrick, 1895; Yonge, 1937, 1946; Cheung, 1966; Goudeau and Lachaise, 1983; Goudeau et al., 1987; Saigusa et al., 2002). On completion of embryonic development, the egg case is broken, and zoea larvae hatch.

Hatching in most crustaceans differs greatly from that of other animals. It is characterized not by dissolution of the egg case, but by its sudden rupture (Davis, 1981). Ultrastructural studies on the egg case of an estuarine crab *Sesarma haematocheir* indicated that no morphological changes occur in the thick outer layers (E1+E2; 1.5 μm in total) upon hatching. Only the innermost thin layer (E3; 0.2 μm) is markedly digested (Saigusa and Terajima, 2000). At least two kinds of active factors are contained in the hatch water (i.e. the filtered medium in which zoea larvae are released by the female), caseinolytic proteases and OHSS (ovigerous-hair stripping substance) (Saigusa, 1996). OHSS plays a role in the stripping of the embryo attachment system from the maternal ovigerous hairs just after hatching, in preparation for the next clutch of embryos (Saigusa, 1995). OHSS is clearly secreted by the embryo and not by the female (Saigusa, 1995). However, physiological mechanisms by which the stripping of ovigerous hairs is caused by OHSS are not known.

Embryos of a number of animals, including the sea urchin *Paracentrotus lividus* (Lepage and Gache, 1990), the ascidian *Ciona intestinalis* (D'Aniello et al., 1997), teleosteans *Oryzias latipes* (Yamagami, 1988) and *Hippoglossus hippoglossus*

(Helvik et al., 1991), release proteases upon hatching, and help to break down the fertilization envelope. They are called 'hatching enzymes'. The hatching enzyme of *Oryzias latipes* is in fact two distinct enzymes, each of which differs in its action against the egg case (Yasumasu et al., 1989, 1992), whereas the hatching enzyme of the sea urchin is a single protease (Lepage and Gache, 1990). The hatching enzyme would be contained in the water in which embryos have hatched. In *Sesarma haematocheir*, caseinolytic proteases might digest the innermost thin layer, but ultrastructural analysis did not reveal evidence that OHSS plays a role in digestion of the egg case (Saigusa et al., 2002).

To investigate its properties, OHSS has been partially purified by three steps of chromatography, and the molecular mass eluted on the molecular sieve chromatography was roughly estimated to be 30 and 32 kDa (Saigusa and Iwasaki, 1999). Furthermore, polyclonal antibodies raised against purified OHSS detected a 55 kDa protein. However, further investigation of the properties and functions of this substance require a more elaborate purification and cDNA cloning.

In the present study, we have purified OHSS from hatch water using a reverse phase high-performance liquid chromatography (RP-HPLC), and cloned the OHSS cDNA and its gene. The deduced amino acid sequence matched with partially determined N-terminus and internal amino acid sequences, and the cloned cDNA was identified as that of OHSS. The primary structure of OHSS indicates that it belongs to the family of trypsin-like serine proteases. We confirmed that OHSS is expressed in the embryos. Furthermore, this paper provides evidence of recycling of the maternal ovigerous hairs by the action of OHSS.

Materials and methods

Preparation of hatch water

Ovigerous females of *Sesarma haematocheir* De Haan the estuarine terrestrial crab used in this study, were collected at Kasaoka, Okayama Prefecture, Japan. The thicket inhabited by the crabs is separated from the shore of a small estuary by a small road (for the habitat, see Saigusa, 1982). Just after sunset from the beginning of July to the end of September, between 19.00 and 20.00 h, ovigerous females appear on this road on their way to the shore to release their zoea larvae. Thus exposed, they can be easily captured.

Ovigerous females captured on the road were first disinfected in ice-cold 30–70% ethanol for a few minutes, then washed with a large quantity of distilled water (DW), and finally placed individually into plastic containers (10 cm in diameter, 15 cm in height), but without water. These containers were transferred to the laboratory, where each crab was immediately placed in a small, covered plastic cup (5 cm in diameter, 6 or 8 cm in height) containing 10 ml of DW. As soon as zoea larvae were released, the zoeas were removed by filtration through nylon mesh, and the remaining water was then passed again through a filter paper. The resulting hatch water was pooled in a 50 ml plastic bottle and immediately stored at –40°C until used. Most females incubate their next clutch of embryos a few days after larval release (Saigusa et al., 2002). The females were therefore kept in the laboratory for about 2 months, and hatch water was obtained from their second larval release (for further details, see Saigusa 1995, 1996).

Purification of OHSS

OHSS was partially purified through three steps of chromatography (hydrophobic chromatography, ion-exchange chromatography and molecular sieve chromatography; Saigusa and Iwasaki, 1999). The procedures were all performed with a fast protein liquid chromatography system (FPLC; Amersham-Pharmacia, Piscataway, NJ, USA) in an experimental chamber with the temperature controlled at 4°C. The pooled active fractions (1 ml/fraction) eluted by gel filtration were collected, concentrated to about 50 µl by ultrafiltration (Centricon YM-10; Millipore, Bedford, USA), and fractionated by reverse phase high-performance liquid chromatography (RP-HPLC) (YMC-Pack ODS-A reverse-phase HPLC column; 150 mm× 6 mm; YMC Co., Ltd., Kyoto, Japan). The proteins were eluted using a linear gradient of 8–52% acetonitrile containing 0.1% HCl over 80 min. The flow rate was at 0.6 ml min^{-1}. The procedure was performed using a Waters 626 LC system (Millipore) equipped with a model 600E controller and a model 486 ultraviolet light (Millipore). The eluate was monitored at 278 nm. Each fraction was tested for biological activity of OHSS.

Egg attachment system and bioassay of OHSS

Embryos of crabs attached to ovigerous hairs arranged on the four pairs of the ovigerous seta of the female (Fig. 1A,B). The egg attachment system consists of an outermost envelope (E1) originating from the vitelline membrane (envelope of the ovum) (Saigusa et al., 2002). The adhesion and plasticity of this envelope changes just after egg-laying, and kneading of the eggs by the ovigerous setae forms the investment coat on the ovigerous hair (Fig. 1C).

After hatching, the larvae are released into the water by a vigorous fanning movement of the abdomen (Saigusa, 1982), but the egg attachment system (broken egg envelope, funiculus, and investment coat) remains on the hairs (Fig. 1D). The egg attachment system is finally removed from the hairs by the actions of OHSS (Fig. 1E). If the embryos attached to the female are gently pulled with forceps, the ovigerous hairs are broken (Fig. 1F,G), whereas the embryo clusters treated with an OHSS solution easily slip off the hairs without damage (Fig. 1H).

The biological assay of OHSS is based on the ability of living or chemically fixed ovigerous setae to respond to the OHSS solution. In brief, an ovigerous seta with its attached embryos, all in the early stages of development, was excised from a female, fixed in 70% ethanol, and then stored at 4°C until used. Shortly before the bioassay, the fixed ovigerous setae were suspended in DW to wash out the ethanol, and then placed in a glass dish with DW. The ovigerous seta was subdivided into four segments under a stereomicroscope (for further details of biological assay, see Saigusa, 1995).

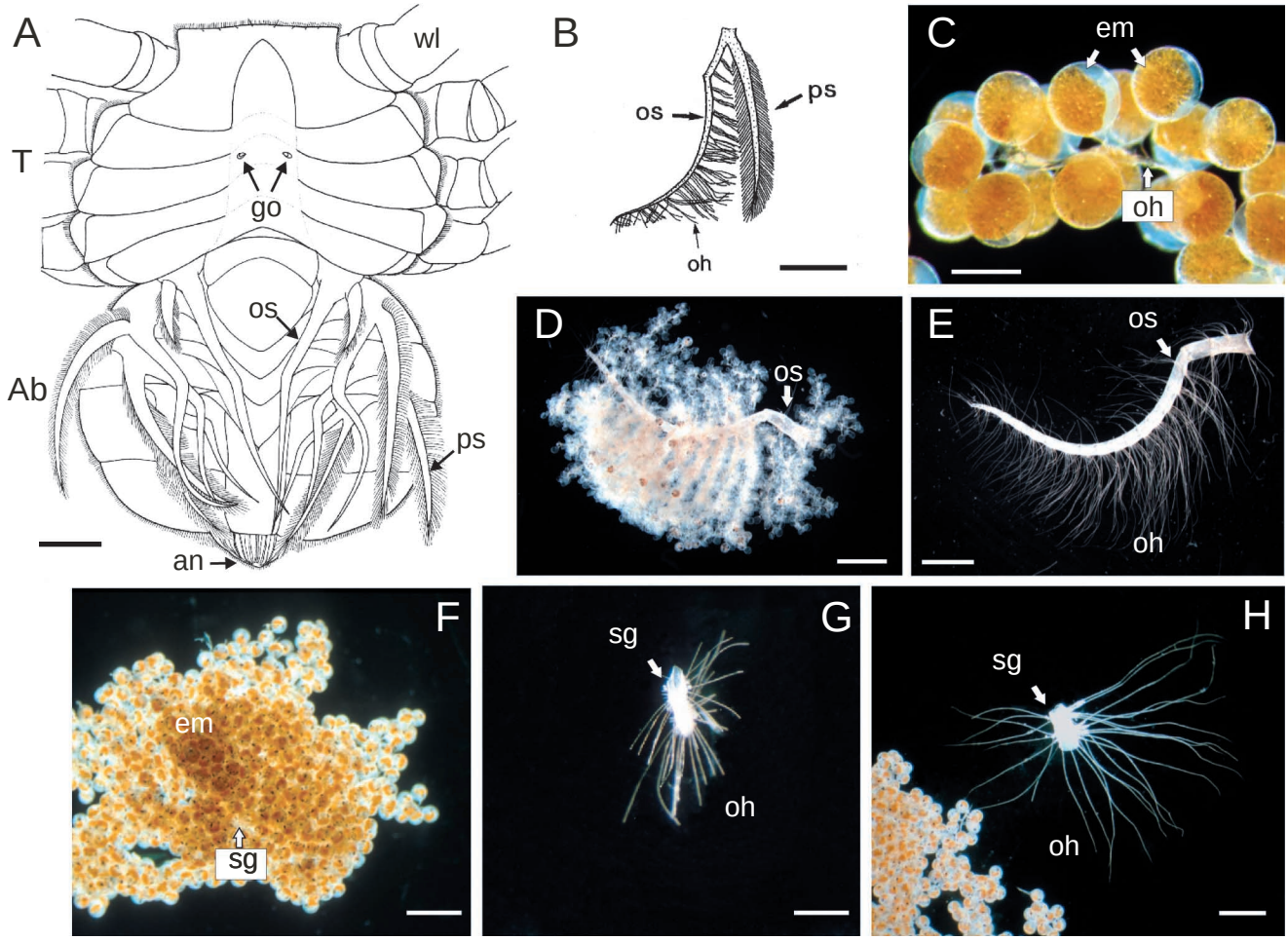

Fig. 1. Embryo attachment system and its stripping from the ovigerous hairs after hatching, in the estuarine crab *Sesarma haematocheir*. (A) Diagram of the incubation chamber of the female (space between the thorax T and the abdomen Ab). (B) The abdominal appendage of the female. Ovigerous hairs (oh) are arranged in whorls on the ovigerous seta (os). Embryos are attached to the ovigerous hairs by the stalk (funiculus), but not to the fine hairs arranged on the plumose seta (ps). (C) Embryos attached to an ovigerous hair (living specimen). (D) The egg attachment system remained on the ovigerous hairs just after hatching. (E) Ovigerous hairs stripped several hours after hatching. (F) The ovigerous seta with its attached embryos was divided into five segments, one of which is shown here. (G) A segment of the ovigerous seta from which the embryos were gently pulled with a forceps after detaching from the female and subdividing. (H) A segment of the ovigerous seta from which the embryos were gently pulled with a forceps after immersion in the hatch water for 1.5 h. wl, walking leg; go, gonopore; an, anus; em, embryo cluster; sg, segment of the ovigerous seta. Scale bars: A, 5 mm; B,D,E, 2 mm; C, 0.5 mm; F–H, 1 mm.

The subdivided segments with their attached embryos were placed in the well of a plastic culture dish, with medium (300 µl) in which 50 µl of each fraction eluted by RP-HPLC was diluted with 250 µl of PBS (phosphate-buffered saline; pH 7.4). The culture dish was shaken on a mechanical shaker at constant temperature (25±1°C). After incubation for 1 and 1.5 h, each segment with its attached embryos was again placed in a glass dish with DW. The embryos were gently pulled away from the ovigerous hairs using fine forceps. The percentage of ovigerous hairs that were stripped clean but were still undamaged was calculated under the stereomicroscope (for further details, see Saigusa, 1995).

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) in a 15% polyacrylamide gel. Prior to SDS-PAGE, the aqueous phase of each fraction obtained by RP-HPLC was evaporated on a centrifugal evaporator (CVE-1000; EYELA, Tokyo, Japan) equipped with a cold trap (EYELA, UT-2000). The amount of protein in each fraction was calculated using a Protein Assay Kit (BioRad, Hercules, USA). 200 ng of precipitated protein was dissolved in lysis buffer (0.0625 mol l^{-1} Tris, 2.5% SDS, 2.5% β-mercaptoethanol, 4 mol l^{-1} urea, 0.025 mol l^{-1} EDTA, 2.5% sucrose and 0.0025% Bromophenol Blue) and then denaturated at 95°C for 3 min. Electrophoresis was performed for 3 h at 30 mA in Tray buffer (0.025 mol l^{-1} Tris, pH 8.3, 9.6 mol l^{-1} glycine and 0.1% SDS), according to the method of Ikeuchi and Inoue (1988). The molecular mass marker employed was a Rainbow colored protein molecular mass

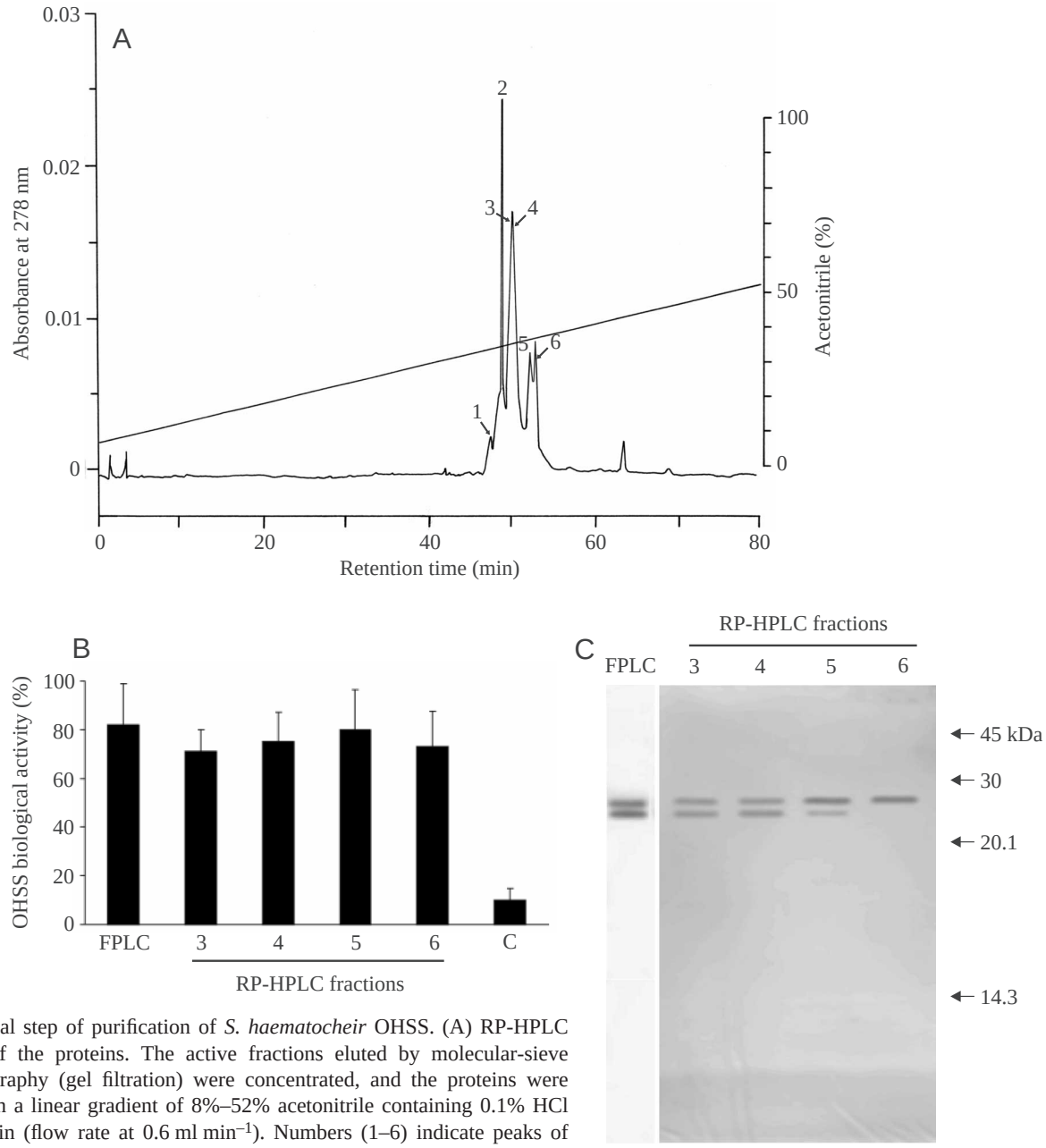

Fig. 2. Final step of purification of *S. haematocheir* OHSS. (A) RP-HPLC analysis of the proteins. The active fractions eluted by molecular-sieve chromatography (gel filtration) were concentrated, and the proteins were eluted with a linear gradient of 8%–52% acetonitrile containing 0.1% HCl over 80 min (flow rate at 0.6 ml min^{-1}). Numbers (1–6) indicate peaks of protein. (B) OHSS biological assay of fractions eluted by FPLC (gel filtration) and RP-HPLC. Bioassay carried out for 1.5 h with two ovigerous setal segments per fraction (see Saigusa and Iwasaki, 1999). C, control assay in distilled water. Values are means ± S.D. (C) Protein analysis by SDS-PAGE. The polyacrylamide gel was stained with Coomassie Brilliant Blue. The marker proteins were ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa; Amersham). Two bands (25 and 22 kDa) appeared in fractions 3–5, while a single band (25 kDa) appeared in fraction 6.

marker (Amersham-Pharmacia). The gels were stained with Coomassie Brilliant Blue (CBB) R-250.

Amino acid sequencing

The N terminus of OHSS eluted on RP-HPLC was determined with the amino acid sequencer (Applied Biosystems, Foster, USA). The N terminus of OHSS was determined using fraction no. 6. Furthermore, the OHSS containined in fraction no. 6 (Fig. 2C) was digested by lysylendopeptidase, eluted by RP-HPLC, and amino acid sequences of the peptides were determined by the amino acid sequencer.

Extraction of total RNA

Total RNA was extracted from the embryos, zoea larvae, and tissues of adult females. Female crabs including ovigerous individuals were maintained under 15 h:9 h light:dark cycle (LD15:9), similar to that in the field in summer (lights-on at 05.00 h and lights-off at 20.00 h), and at constant temperature

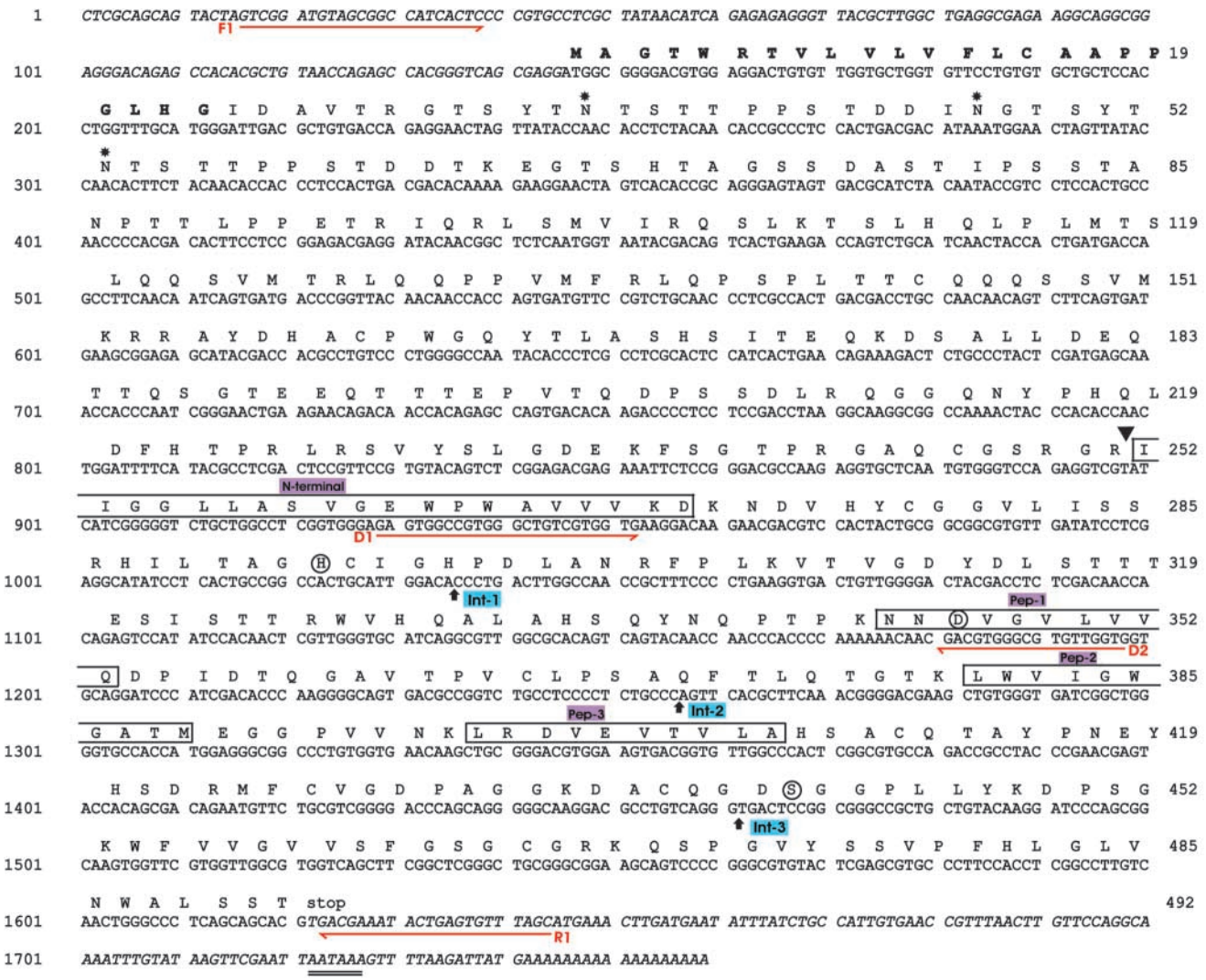

Fig. 3. Nucleotide sequence and deduced amino acid sequence of *S. haematocheir* OHSS. Nucleotides are numbered to the left from the first base at the 5′ end, and amino acids are numbered to the right from the initiating methionine. The putative signal peptide sequence is shown in bold. The boxed amino acids indicate determined sequences. The putative cleavage site accompanying activation is shown by a solid triangle. The catalytic triad of the serine protease domain is enclosed by a circle. A putative polyadenylation signal is doubly underlined. Putative N-glycosylation sites are indicated by asterisks. Locations of three introns (Int-1, 240 bp; Int-2, 316 bp; and Int-3, 842 bp) are marked by upward-pointing arrows. Positions of nucleotide primers used for cloning and expression analysis of OHSS gene are indicated by red arrows (see text for details).

(25±1°C). Muscles, ovigerous hair, brains and hepatopancreas were excised from the adult female. Embryos at different stages of development were removed from ovigerous females. Just after the larval release, zoeas were transferred to an aquarium containing clean seawater with very weak aeration. Zoeas were collected on the day of hatching and 3 days after hatching. All these samples were individually frozen in liquid nitrogen and stored at –80°C until used.

Total RNA was extracted and purified with an RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To prevent possible DNA contamination in the RNA, samples were subjected to DNase treatment using DNA-free Kit (Ambion, Austin, TX, USA). The obtained total RNA was dissolved in nuclease-free DW and stored at –80°C until used.

Construction of the cDNA library of embryos

The poly(A)$^+$RNA (9 μg) was purified from total RNA (400 μg) extracted from the embryos using the QuickPrep Micro mRNA purification kit (Amersham-Pharmacia). The embryonic cDNA library was constructed using a Marathon cDNA amplification kit (BD Biosciences, Palo Alto, CA, USA). In brief, first strand cDNA synthesis was carried out using 2 μg of poly(A)$^+$RNA, the modified lock-docking oligo(dT) primer provided with the Marathon cDNA amplification kit (BD Biosciences), and the Superscript II reverse transcriptase (BD Biosciences). Second strand synthesis was achieved using the Marathon cDNA amplification kit following the manufacturer's instructions.

cDNA cloning and DNA sequencing of OHSS

The following two degenerate primers were used: 5′-GA-(A/G)TGGCCATGGGC(C/T)GT(C/T)GT(C/T)GT(C/T)-3′ (D1 in Fig. 3) and 5′-(C/T)ACCAA(A/G)AC(G/T)CC(C/T)AC-(G/T)TC-3′ (D2 in Fig. 3) corresponding to the amino acid sequences EWPWAVVV and DVGVLV, respectively. The reactions were carried out in a total volume of 20 μl of solution containing 1× PCR reaction buffer, 150 μmol l^{-1} dNTP mix, 0.5 U of Taq DNA polymerase, 2 mmol l^{-1} $MgCl_2$, each of the primers at 0.6–0.8 μmol l^{-1}, and 50 ng of cDNA. The amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) programmed for 35 cycles of 94°C (1 min), 57°C (1 min) and 72°C (1 min).

The PCR products were separated on a 2% agarose gel, and the DNA fragment (about 270 bp) was cut out from the gel and purified using a QIAEX II Gel Extraction kit (Qiagen). The yields were cloned into pCR2.1-TOPO cloning vector using a TOPO TA Cloning kit (Invitrogen, Carlsbad, USA) according to the supplier's protocol.

The cDNA clones were cycle-sequenced using a Thermo Sequenase Cycle Sequencing kit (Amersham-Pharmacia) with M13 forward (–20) and M13 reverse primers specific to the flanking regions of a multi-cloning site in the pCR2.1-TOPO cloning vector according to the manufacturer's direction, and analyzed with an automatic DNA sequencer, the DSQ-2000L (Shimadzu, Kyoto, Japan).

Rapid amplification of cDNA ends (RACE)-PCR

To clone full-length cDNA encoding the entire open reading frame (ORF) of OHSS, RACE-PCR was performed with embryonic cDNA library as template, using a Marathon cDNA Amplification kit (BD Biosciences). The OHSS-sequence-specific primer: 5′-AGGACAAGAACGACGTCCAC-3′ (corresponding to nucleotides 954-973 in Fig. 3) and the AP1 primer provided in the kit (BD Biosciences) were used for 3′RACE. For 5′RACE, we used the OHSS-sequence-specific primer 5′-GTCGTTGTTTTTTGGGGTGG-3′ (complementary to nucleotides 1164–1183 in Fig. 3) and the AP1 (BD Biosciences). The RACE-PCR conditions were: 94°C for 30 s followed by 25 cycles of 94°C for 5 s and 70°C for 2 min. The PCR products were separated on 1% agarose gel and DNA fragments were cut out from the gels, purified, cloned into pCR2.1-TOPO vector (Invitrogen), and sequenced.

Isolation of genomic DNAs

Genomic DNAs were prepared from individual frozen embryo clusters using the method described by Blin and Stafford (1976). The purified genomic DNA was suspended in TE (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 8.0) and stored at –20°C until used.

Reverse transcription PCR (RT-PCR)

2 μg each of total RNA obtained from embryos, zoeal larvae, hepatopancreas, muscles and brains of adult female *S. haematocheir* were subjected to reverse transcription using a First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland). The PCRs were carried out in a total volume of 20 μl of solution containing 1×PCR reaction buffer, 150 μmol l^{-1} dNTP mix, 0.5 U of Taq DNA polymerase, 2 mmol l^{-1} $MgCl_2$, each of the primers at 0.3–0.4 μmol l^{-1} and 50 ng of cDNA. For a negative control, a PCR using genomic DNA was performed with the same reaction mixture using 150 ng of genomic DNA as template. The amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) programmed for 30 cycles of 94°C (1 min), 57°C (1 min) and 68°C (3 min). Samples were removed from each reaction during the PCR every four cycles starting from 15th cycle (i.e. 15th, 19th, 23rd, 27th) and PCR products were separated on 2% agarose gel.

The primers 5′-GTCGGATGTAGCGGCCATCACTC-3′ (F1 in Fig. 3, corresponding to nucleotides 16–38) and 5′-GCTAAACACTCAGTATTTCGTC-3′ (R1 in Fig. 3, complementary to nucleotides 1623–1644) were used.

PCR on genomic DNA

To isolate the OHSS gene, approximately 150 ng of genomic DNA were used in 20 μl PCR reactions that consisted of 1× PCR reaction buffer, 150 μmol l^{-1} of each nucleotide, 0.5 units of Taq DNA polymerase (Takara, Otsu, Japan), 2 mmol l^{-1} $MgCl_2$, and each of the primers at 0.3–0.4 μmol l^{-1}. The primers 5′-GTCGGATGTAGCGGCCATCACTC-3′ (F1 in Fig. 3, corresponding to nucleotides 16–38) and 5′-GCTAAACACTCAGTATTTCGTC-3′ (R1 in Fig. 3, complementary to nucleotides 1623–1644) were used.

The amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) programmed for 35 cycles of 94°C (1 min), 57°C (1 min) and 68°C (4 min), followed by elongation for 10 min at 72°C. The PCR products were separated on 1% agarose gel and DNA fragments were cut out from the gels, purified, cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced.

Sequence analyses

Multiple sequence alignment and comparisons were made using GeneDoc Multiple Sequences Alignment Editor 2.6 computer software (Nicholas et al., 1997).

A homology search in a protein database was carried out using BLAST 2.0. Protein features were analyzed using ProtoScale software *via* the Internet (http://us.expasy.org). A putative signal peptide sequence was predicted with SignalP V1.1 software (http://www.cbs.dtu.dk/services/SignalP/). For the calculation of molecular mass and primer design and analysis, we used Vector NTI Suite 8 (InforMax) computer software.

Results

Purification of OHSS

The biological activity of OHSS elutes as a single peak on molecular sieve chromatography, and the molecular mass of OHSS was roughly estimated to be 30–32 kDa (Saigusa and Iwasaki, 1999). The pooled active fractions from molecular

sieve chromatography (1 ml per fraction) were combined, concentrated to 50 μl, and subjected to reverse phase chromatography (Fig. 2A). Strong OHSS biological activity was observed in four consecutive fractions number 3–6 (Fig. 2B). Each peak was analyzed by SDS-PAGE (Fig. 2C). Two common protein bands (22 and 25 kDa) appeared in fractions 3–5, but only one protein band (25 kDa) appeared in fraction 6. Comparison of Fig. 2B with C clearly indicates that the 25 kDa protein at least has OHSS activity.

The biological activity of OHSS was detected in fractions 3–6, if the bioassay was carried out immediately after the chromatography (Fig. 2B). But when active fractions were stored in a refrigerator (4°C, overnight) or in a deep freezer (–30°C, overnight), the biological activity was lost.

Amino acid sequence of OHSS

Twenty residues of the N-terminal amino acid sequence of purified OHSS were determined: IIGGLLASCGEWPWAV-VVKD. The N-terminal amino acid sequences of three polypeptides obtained by digestion of purified OHSS (fraction 6) with lysylendopeptidase were: NNDVGVLVVQ (Pep-1), LWVIGWGATM (Pep-2) and LRDVEVTVLA (Pep-3). The polypeptides are boxed in Fig. 3.

cDNA cloning and nucleotide sequencing

A PCR-based approach was employed for cloning a complete sequence of OHSS cDNA. The degenerate primers designed to the sequences corresponding to the determined sequence of the N terminus (D1 in Fig. 3) and Pep-1(D2 in Fig. 3) generated a 270 bp PCR (data, not shown). The full-length cDNA amplified by 5′ RACE and 3′ RACE PCR was 1759 bp long, containing a single open reading frame (ORF) encoding 492 amino acids (sequence submitted to GenBank, accession number AY306010), a putative polyadenylation signal (AATAAA), and a poly(A)$^+$ tail (Fig. 3). The calculated molecular mass of the deduced amino acids was 54.7 kDa.

N-terminal sequences of OHSS (IIGGLLASVGEWPWAV-VVKD) corresponded to the residues 252–271. The partially determined amino acid sequences of the digest of purified OHSS were in agreement with the deduced amino acid sequence: Pep-1, NNDVGVLVVQ corresponding to residues 344–353; Pep-2, LWVIGVGATM corresponding to residues 380–389; and Pep-3, LRAVEVTVLA corresponding to residues 398–407 (Fig. 3). Only two residues were different: the sixth residue of Pep-2 was V (Valine) instead of W (Tryptophan); and the third residue of Pep-3 was A (Alanine) instead of D (Aspartic acid).

The first 23 N-terminal residues of the deduced amino acid sequence were highly hydrophobic, and were predicted to be a signal peptide. Three potential N-glycosylation sites were found at the 35th, 47th and 53th residues after the putative signal peptide (Fig. 3).

Serine protease domain

The amino acid sequence deduced from the cDNA (Fig. 3) was compared with other proteins using a BLAST homology search. The search showed that the residues in the C-terminal region of the OHSS extending from positions 243–492 had high similarities to trypsin-like serine protease domain. An alignment of the homologous sequence of this domain of OHSS and other serine proteases is shown in Fig. 4. Homologies with these proteases ranged from 33% to 38%. Homology with prawn *Penaeus vanameii* chymotrypsin (Sellos and Van Wormhoudt, 1992) was 35%, that with crab *Paralithodes camtschaticus* trypsin (Rudenskaya et al., 1998) was 38%, that with a proclotting enzyme of the horseshoe crab *Tachypleus tridentatus* (Muta et al., 1990) was 34%, that with prophenoloxidase activating enzyme (defensin) of the freshwater crayfish *Pacifastacus leniusculus* (Wang et al., 2001) was 37%, that with human hepsin (Leytus et al., 1988) was 33%, and that with matriptase (Lin et al., 1999) was 33%.

The OHSS serine protease domain contained the invariant catalytic triad His-293, Asp-346 and Ser-441. The substrate specificity pocket (S1) of OHSS is likely to be composed of Asp-435, positioned at its bottom, with Gly-463 and Gly-473 at its neck, indicating that OHSS is a typical trypsin-like serine protease.

Genomic analysis

The primary structure of the OHSS gene was examined. A series of PCRs were conducted using genomic DNA as a template with a set of two gene-specific primers (F1 and R1 in Fig. 3). The primers were designed to correspond to the 5′ and 3′ ends of the OHSS cDNA. The PCR product was a single DNA fragment of 3.4 kb (lane G in Fig. 5). Further cloning and sequencing of the fragment revealed that it was the OHSS gene. Three introns of 240 bp (Int-1), 316 bp (Int-2) and 842 bp (Int-3) were present within the coding region of the serine protease domain of OHSS (Fig. 3). All introns displayed canonical GT-AG boundaries and were flanked by consensus matching exonic acceptor and donor sequences (Table 1). So far we have not observed any PCR signals indicating the existence of alternatively spliced transcripts of the OHSS gene.

Table 1. *Position, length and flanking region of introns found in the OHSS gene*

Intron	Position on cDNA	Sequence			Length (bp)
		Exon	Intron	Exon	
Int-1	1035	TGGACA	GTaagtt…tcccgtAG	CCCTGA	240
Int-2	1256	CTGCCC	GTgggtc…tgttgcAG	AGTTCA	316
Int-3	1461	TCAGGG	GTaatgc…gcattcAG	TGACTC	842

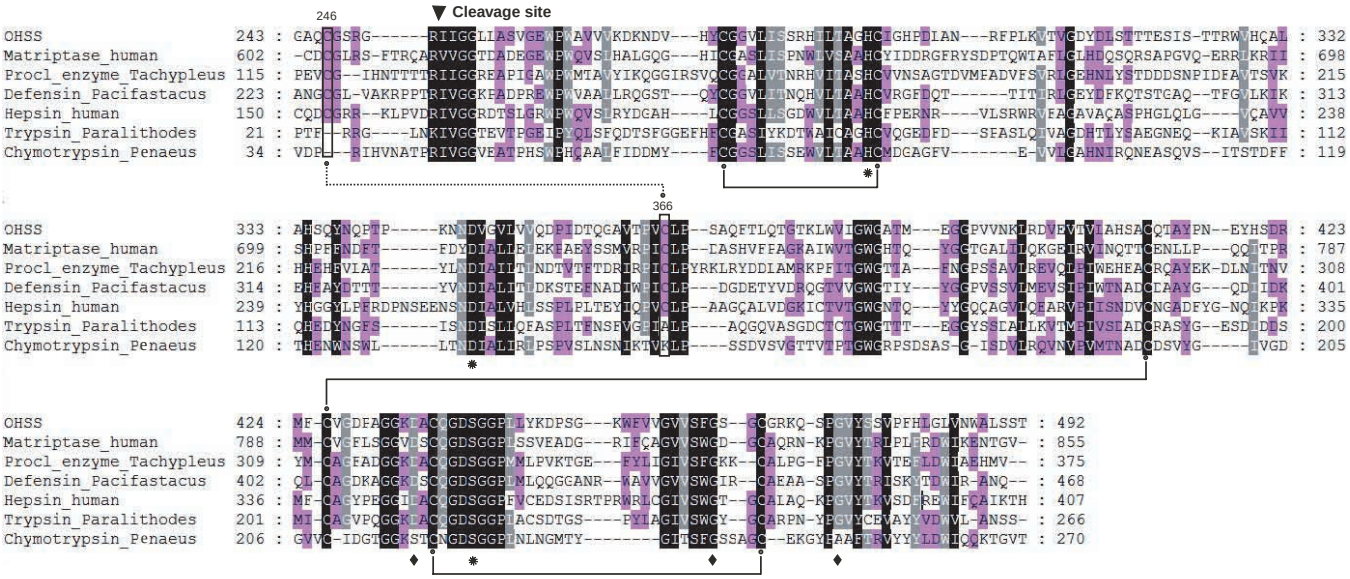

Fig. 4. Multiple alignment of the deduced amino acid sequence of the serine protease domain of *S. haematocheir* OHSS with those of known serine proteases of six species. Regions of high homology across all species are highlighted in black (100–90%); regions with less homology are highlighted in gray (>80%) and purple (>60%). The numbering of amino acids corresponds to the original sequences in each animal. Information for comparison: chymotrypsin of *Penaeus vanameii* (database accession number S29239); trypsin of *Paralithodes camtschaticus* (AF461036); proclotting enzyme (procl.) of *Tachypleus tridentatus* (P21902); defensin of *Pacifastacus leniusculus* (AJ007668.1); human hepsin (P05981); human matriptase (Q9Y5Y6). Residues in the catalytic triad (His-293, Asp-346 and Ser-441) are indicated by an asterisk. Residues in the substrate pocket (Asp-435, Gly-463 and Gly-473) are indicated by diamonds. Six conservative cysteines needed to form three intramolecular disulfide bonds are likely pairings as follows: Cys278–Cys294, Cys411–Cys426, and Cys437–Cys466. The disulfide bond Cys246–Cys366 (the cysteines are boxed) is observed in two-chain serine proteases, but not in trypsin and chymotrypsin.

Expression of the OHSS gene

The expression patterns of the OHSS gene were examined by RT-PCR (Fig. 5). 2 μg of total RNA extracted from embryos in different stages of development, zoeas (larval stage) and tissues of the adult female were reverse-transcribed and used as template for RT-PCR. After 15 cycles, very weak visible products were amplified only from embryos 1 day before hatching and zoeas just after hatching (data not shown). After 27 cycles of PCR, amplified DNA fragments that reflect the OHSS gene expression were found at all stages examined, excluding zoeas 3 days after hatching, in the brain, but not in either muscle, ovigerous setae or hepatopancreas of the female (Fig. 5).

Discussion

OHSS (ovigerous-hair stripping substance) contained in the hatch water of an estuarine terrestrial crab *Sesarma haematocheir* was purified. OHSS had a molecular mass of 25 kDa in its active form. A cDNA clone of 1759 bp encoding 492 amino acids in a single ORF was obtained. The C-terminal part of the sequence (residues 252–492) was composed of a trypsin-like serine protease domain, which was well aligned with those from other animals. These results raise the following two major issues: (1) a putative process of conversion from the 54.7 kDa form to an active 25 kDa form, and (2) the action of the active OHSS on the egg attachment system, causing stripping of ovigerous hairs.

Putative process of conversion from the 54.7 kDa form to an active 25 kDa form

The OHSS cDNA clone of 1759 bp was found to encode a protein of 492 amino acids whose molecular mass was estimated to be 54.7 kDa (Fig. 3). A homology search indicated that the C-terminal part of the deduced amino acid sequence was composed of a trypsin-like serine protease domain (Fig. 4). Serine proteases are involved in many biological process including digestion, blood clotting, proenzyme activation and complement activation (e.g. Neurath, 1984; Lanz et al., 1993; Rawlings and Barrett, 1994; Klein et al., 1996; Levine et al., 2001). Numerous serine proteases are synthesized as inactive zymogens. Zymogens prevent premature physiological functioning of the active portion of the protease, thus protecting host cells from enzymatic damage (Neurath and Walsh, 1976; Rawlings and Barrett, 1994).

The protein encoded in the OHSS cDNA would be a zymogen of OHSS, which is likely to be proteolytically activated. The Arg-Ile-Ile-Gly-Gly motif (Fig. 3) clearly corresponds to the typical Arg-Ile-(Ile or Val)-Gly-Gly motif in other serine proteases (Fig. 4), indicating that Arg251-Ile252 is a putative proteolytic activation site of OHSS.

The six conserved cysteines required to form three intramolecular disulfide bonds that stabilize the catalytic pocket were demonstrated in trypsin-like serine proteases (e.g. Lin et al., 1999). The most likely cysteine pairings for OHSS

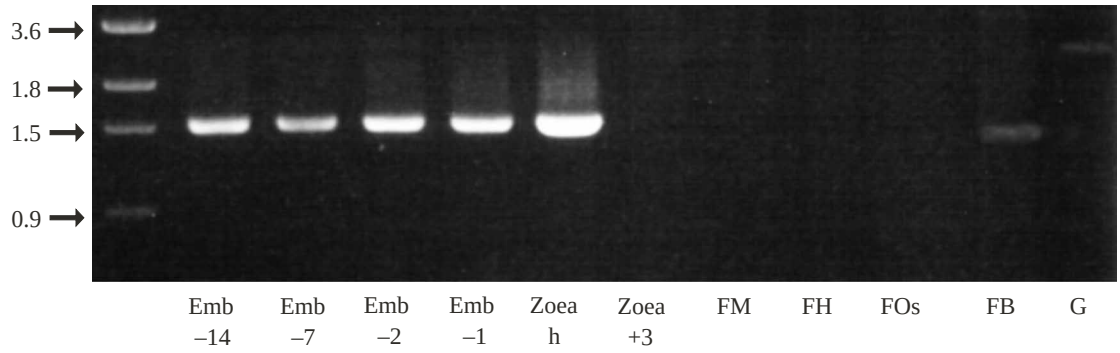

Fig. 5. RT-PCR analysis of the expression of the OHSS gene. PCR products of 27 cycles of RT-PCR amplification using OHSS cDNA were loaded in 0.8% agarose gel. RNAs were extracted from embryos detached from the female (Emb), the zoea larvae (Zoea), and muscles (FM), hepatopancreas (FH), ovigerous setae (FOs) and brain (FB) of the adult female. Numbers below Emb and Zoea indicate days before (–) and after (+) hatching, and the day of hatching (h). Lane G represents the products of PCR using genomic DNA from embryos as template. Size standards in kb are shown on the left.

are: Cys278-Cys294, Cys411-Cys426, and Cys437-Cys466. Furthermore, an additional cysteine (Cys366) is also contained in the OHSS serine protease domain. While this cysteine is not present in a single chain protease such as trypsin (Rudenskaya et al., 1998) and chymotrypsin (Sellos and Van Wormhoudt, 1992), it is found in two-chain proteases, e.g. hepsin (Leytus et al., 1988), prophenoloxidase activating enzyme (defensin) (Wang et al., 2001) and matriptase (Lin et al., 1999). The active form of the two-chain protease, representing the majority of plasma serine proteases, consists of two polypeptides held together by a disulfide bond, a highly conserved catalytic chain derived from the C-terminal region of the precursor polypeptide, and a unique noncatalytic chain derived from the N-terminal region of the polypeptide chain. The presence of noncatalytic chain(s) distinguishes the plasma serine proteases from digestive proteases (Neurath and Walsh, 1976). Noncatalytic chain(s) mediate interaction with other proteins, affecting the action of proteases on their selected substrates (Leytus et al., 1988).

Comparative sequence analysis (Fig. 4) suggests that OHSS is originally synthesized as a single-chain zymogen, and then proteolytically activated to take the two-chain form. The conserved intramolecular disulfide bond in OHSS is likely to be formed at Cys246 and Cys366 (Fig. 4). If this is the case, the majority of OHSS molecules in developing embryos would be present in the zymogen form. This suggestion is consistent with immunoblotting data obtained before. In our previous study, a polyclonal antiserum was raised against the active fractions (corresponding to the mixture of 25 kDa and 22 kDa proteins in Fig. 2C) eluted by molecular-sieve chromatography (Saigusa and Iwasaki, 1999). Antibodies purified from this antiserum (anti-OHSS antibody) recognized not only both proteins but also a band at about 55 kDa. It is highly probable that the 55 kDa protein detected by the anti-OHSS antibody is a zymogen form of OHSS. The estimated molecular mass of the polypeptides encoded in the OHSS cDNA was 54.7 kDa, and agreed well with the results of immunoblotting. While the 55 kDa protein was clearly detected from 2 weeks to 2 days before hatching, the 25 kDa proteins (active form) appeared from 4 days before hatching to the day of hatching. OHSS biological activity appeared only 1 day before hatching (Saigusa and Iwasaki, 1999). It is plausible that OHSS activity would be suppressed until 1 day before hatching, and that OHSS is activated by some (unidentified) factor(s) before hatching.

Action of OHSS on the egg attachment system

Just after laying her eggs, the female kneads them by moving the ovigerous setae. By this action the layer investing the embryo (E1) is stretched, and wraps around the ovigerous hair (Saigusa et al., 2002). The wrapping of and adherence to the ovigerous hair (enclosed by open rectangle in Fig. 6A) has been speculated to occur without any adhesive substance (Cheung, 1966; Goudeau and Lachaise, 1980, 1983; Goudeau et al., 1987). However, we found a electron-dense, slender structure arranged at intervals of 130–160 nm around the hair (Fig. 6B). The stretched embryonic envelope (E1) would attach to the ovigerous hair on this structure, finally forming the investment coat (Fig. 6A). An adhesive substance, which is possibly secreted from the maternal ovigerous seta, would appear at this structure (i.e., egg attachment site) upon egg-laying, making the bond with the investment coat (Fig. 6C).

Several hours after hatching, the egg attachment system of *S. haematocheir* slips off the ovigerous hairs due to actions of OHSS (Saigusa, 1995, 1996; Saigusa et al., 2002). Ultrastructural analysis indicated that the stripping is due to separation of the attachment sites from the ovigerous hair (Fig. 6D, left). OHSS might act specifically at the attachment sites of the investment coat, lysing the bond with the coat (Fig. 6C,D, right), thus disposing of the embryo attachment system in preparation for the next clutch of embryos.

The embryo has a special developmental program for hatching (hatching program) for 2 nights (48–49.5 h), during which ecdysis occurs twice (Saigusa and Terajima, 2000). OHSS biological activity begins to appear 1 day before hatching (Saigusa and Iwasaki, 1999). OHSS may cause

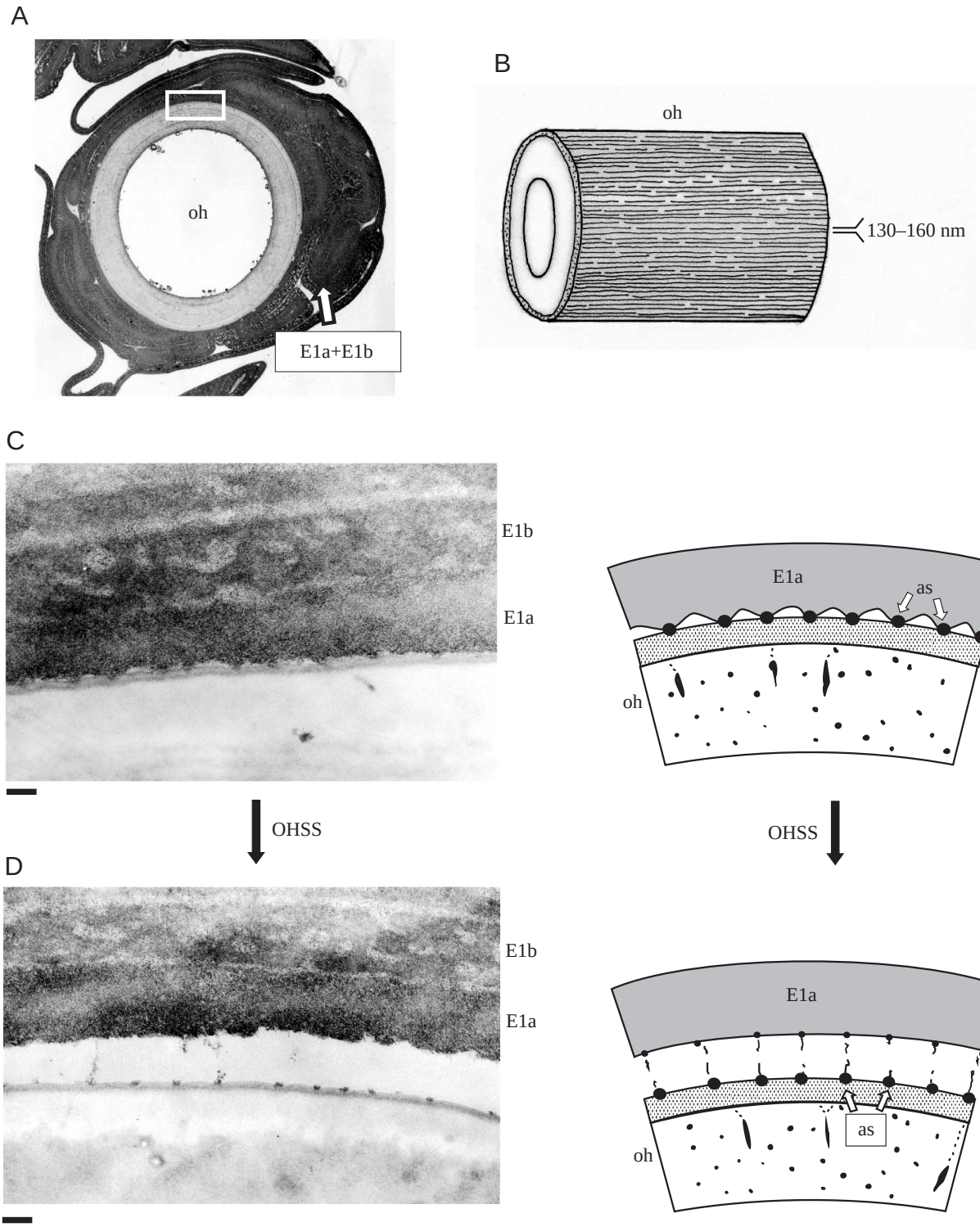

Fig. 6. Egg attachment system formed on the maternal ovigerous hair and its stripping after hatching. (A) The egg attachment system formed on an ovigerous hair. This system consists of a single layer with two sublayers (E1a, E1b). The fine structure of the attachment sites (enclosed by the open rectangle) is shown in C. (B) The egg attachment sites arranged at intervals of 130–160 nm on the maternal ovigerous hair. (C) Fine structure of the attachment of layer E1 to the ovigerous hair (left), and schematic drawing of this structure (right). A portion of the attachment site is shown by two small arrows (as). (D) Separation of layer E1 after the embryos have been immersed in hatch water (left), and its schematic drawing (right). Two small arrows (as) show a portion of the attachment sites separated from the investment coat (E1a). oh, maternal ovigerous hair. Scale bars, 100 nm.

separation of the embryonic exuviae from the zoeal cuticle before hatching. Furthermore, a PCR based analysis of mRNA expression showed that OHSS is highly expressed in the embryos just before hatching. However, less intensive expression of the gene also can be detected in the earlier stage embryos. The OHSS gene was also expressed not only in the brain of the female (Fig. 5), but also the eyestalk ganglia of the female (O. Gusev, H. Ikeda and M. Saigusa, unpublished data), and further studies are needed to elucidate the effects of OHSS gene expression in the brain of females in addition to the expression in the earlier embryonic stages.

Yasumasu et al. (1989) reported that the hatching enzyme of the fish *Oryzias latipes* consists of two kinds of proteases that act together on the egg envelope; one of them (HCE: high choriolytic enzyme) has two isomers (HCE-1 and HCE-2), as demonstrated by cation-exchange chromatography. Two distinct cDNAs were obtained and the nucleotide sequences had 92.8% similarity (Yasumasu et al., 1992). At present, we do not have any evidence that OHSS takes part directly in hatching, having obtained only one sequence of OHSS cDNA. However, we found a slight discrepancy in the deduced amino acid sequence with that of the purified protein and some minor variation in nucleotide sequences of the PCR products (O. Gusev, H. Ikeda and M. Saigusa, unpublished data). Thus, OHSS might consist of multiple isomers as well as one of the medaka hatching enzymes (HCE). This possibility remains to be explored.

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