Identification of a Female-Specific Hemocyanin in the Mud Crab, *Scylla olivacea* (Crustacea: Portunidae)

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Hong-Yu Chen, Shih-Hu Ho, Tzyy-Ing Chen, Keryea Soong, I-Ming Chen, and Jin-Hua Cheng (2007) Identification of a female-specific hemocyanin in the mud crab, *Scylla olivacea*. *Zoological Studies* **46**(2): 194-202. Copper-containing proteins, hemocyanins, are respiratory proteins of crustacean decapods. In this paper, a novel female-specific hemocyanin (FSH) in the mud crab, *Scylla olivacea* (Crustacea: Portunidae), is reported. FSH occurs in the hemolymph of adult females with maturing ovaries, but not in the hemolymph of juveniles of either sex or in adult males. FSH was purified from the hemolymph of females with maturing ovaries by liquid chromatography. Further analysis revealed that FSH is a carbohydrate-rich protein; no cross-immune reaction was found between FSH and vitellin; furthermore, it does not resemble vitelloprotein molecules. It has characteristics in common with other better-known hemocyanins, such as its copper content and oxygen-binding ability. The P_{50} of FSH is 28.3 mmHg O_2, and its oxygen saturation point is 152 mmHg. From evidence of its oxygen-binding ability, it is certain that FSH is a unique hemocyanin of female crabs with maturing ovaries, and its functions are supposedly correlated with reproduction in female crabs.
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Key words: Female-specific hemocyanin, Vitellin, Vitellogenin, Mud crab, *Scylla olivacea*.

Hemocyanins, copper-containing respiratory proteins, are found in arthropods including the Crustacea, Chelicerata, Myriapoda, and some species of Insecta (Markl 1986, van Holde and Miller 1995, Sánchez et al. 1998, Hagner-Holler et al. 2004). The basic structure of an arthropod hemocyanin is a hexamer of 6 similar or identical subunits; each subunit contains a binuclear dioxygen-binding site, and each site possesses 2 copper atoms, CuA and CuB, each of which is coordinated by 3 histidyl residues (Linzen et al. 1985, van Holde and Miller 1995). The molecular masses of each subunit of crustacean hemocyanins are estimated to range from 72,500 to 79,500. Hexamer or their multiples (2, 4, 6, and 8x) form functional native hemocyanin complexes in hemolymph (Markl and Decker 1992).

Non-respiratory hemocyanins have been reported in the Crustacea, such as cryptocyanin in the Dungeness crab, *Cancer magister* (Terwilliger et al. 1999), and pseudo-hemocyanin in the American lobster, *Homarus americanus* (Burmester 1999). These molecules do not contain copper, so have no respiratory function, but are supposed to be storage proteins related either to molting in *C. magister* or to reproduction in *H. americanus* (Burmester 1999, Terwilliger et al. 1999). Kusche et al. (2003) provided evidence that cryptocyanin and pseudo-hemocyanin belong to the same type of protein and originated from the decapod r-hemocyanins about 180 million yrs ago. In the Dungeness crab, hemocyanins of the mega-

lopa and juveniles differ from that of the adult in both structure and function. Subunit *C mag* 6 does not appear in the hemocyanin until an individual reaches the 6th juvenile instar. In the absence of *C mag* 6, juvenile hemocyanin has a lower oxygen affinity than that of the adult (Terwilliger 1998).

In this paper, a novel hemocyanin appearing only in females with maturing ovaries and named female-specific hemocyanin (FSH) was purified from the hemolymph of the mud crab, *Scylla olivacea*. The biochemical characteristics of FSH were compared with those of purified vitelloproteins and other hemocyanin-related proteins. The copper content and oxygen-carrying capability of FSH are also reported.

MATERIALS AND METHODS

Animal and hemolymph collection

Mud crabs, *S. olivacea*, were purchased from a local farm in Tungkang, Pingtung County, southwestern Taiwan or were cultured at the Tungkang Marine Laboratory. After being purchased, animals were kept in fiberglass reinforced plastic (FRP) tanks at ambient temperature and salinity and fed artificial shrimp pellets and Antarctic shrimp daily.

Before bleeding, the ovary was staged by observing ovarian development inside the carapace of female crabs using a spotlight. Hemolymph samples were withdrawn from the base of the swimming legs of crabs with a syringe; an equal volume of ice-cold anticoagulant (18 mM Tris-HCl, 50 mM EDTA, 32.4 g/l NaCl, 1.0 g/l KCl, and 0.3 g/l glucose; pH 7.5) was immediately added to the hemolymph samples. Then the hemolymph samples were centrifuged at 1000 x *g* for 5 min to remove cells and particulate matter, and the supernatant was collected and stored at -20°C. Ovarian tissues were taken from some crabs for vitellin purification, and oocyte diameters were measured under a microscope. Ovarian tissues were stored at -20°C.

Analysis of hemolymph samples by polyacrylamide gel electrophoresis (PAGE)

Hemolymph samples were added to the sample loading buffer (1.0 ml of 0.5 M Tris-HCl, 1.2 ml of glycerol, 0.2 ml of 0.1% bromophenol blue, and 5.6 ml of double deionized (dd)H_2O) for the nondenaturing gel electrophoresis. Electrophoresis was performed in mini-protein II dual slab cells (Bio-Rad). Separating gels were prepared with 5% acrylamide: bis-acrylamide (37.5: 1) dissolved in gel buffer (1.5 M Tris-HCl; pH 8.8). Sufficient ammonium persulfate and N,N,N',N'-Tetramethylethylenediamine (TEMED) were added as the catalyst and initiator, respectively. For the stacking gels, 3.9% acrylamide: bis-acrylamide and gel buffer (0.5 M Tris-HCl; pH 6.8) were used. Electrophoresis was run at 150 V in Tris-glycine buffer (3 g Tris base and 14.4 g glycine in 1 L ddH_2O; pH 8.3). Gels were stained with Coomassie blue after electrophoresis.

Protein purification

DEAE Sepharose Fast Flow column

The hemolymph of crabs with fully mature ovaries was collected and diluted with 0.01 M Tris-HCl (pH 8.0) at a ratio of 3: 40, after which samples were applied to a DEAE-Sepharose Fast Flow column (2.5 x 14 cm, Pharmacia). Elution was performed with a gradient of 0-0.33 M NaCl in 0.01 M Tris-HCl (pH 8.0). The gradient was created using a gradient former (Bio-Rad model 395). The column was flushed with 1 M NaCl. The flow rate was 0.8 ml/min. The effluent was collected every 10 min and detected at 280 nm UV light; the effluent was verified to contain FSH by 5% native-PAGE.

HTP hydroxylapatite column

FSH-containing fractions were further purified using a Bio-Gel HTP hydroxylapatite column (2.5 x 85 cm, Bio-Rad). Elution was performed with a gradient of 5-136 mM phosphate buffer (pH 7.5). The column was flushed with 0.4 M phosphate buffer. The flow rate was 0.75 ml/min. The effluent was collected every 10 min and detected at 280 nm UV light. The effluent was verified to contain FSH by 5% native-PAGE and stored for later use.

Vitellin, vitellogenin, and 2 other hemocyanin-related proteins (identified as 16S hemocyanin and a non-respiratory protein, NRP) were purified from the ovaries and hemolymph of mature females using the same methods described above with some modifications.

Biochemical characteristics of FSH

Distinguishing electrophoretic staining

Sudan black staining

Purified FSH, vitellin, and vitellogenin were premixed with 1/10 (v/v) Sudan black reagent (1% Sudan black (w/v) in 10% ethyl acetate and 90% propylene glycol). Pre-stained protein samples were allowed to sit at 4°C overnight, and then were run on native-PAGE. Protein bands containing lipids appeared as a dark-black color.

Carbohydrate staining – Periodic acid Schiff's reagent staining

After protein samples were run on native-PAGE, gels were soaked in 12.5% trichloroacetic acid for 30 min, rinsed several times in distilled water, transferred to 1% periodic acid and 3% acetic acid for 1 h, rinsed 5 times in distilled water (10 min each time), and stained overnight in Schiff's reagent (0.45 (w/v) basic fuchsin, 0.09 N HCl, and 0.45 (w/v) sodium bisulfite) at 4°C. Gels were then rinsed 3 times with 0.5% sodium bisulfite and 5 times with distilled water to remove the background color and were then transferred to 50% methanol for storage.

Determination of the molecular mass of the subunits

The subunit compositions of purified vitellin, vitellogenin, and FSH were determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were diluted with 4 volumes of sample loading buffer (1.0 ml of 0.5 M Tris-HCl (pH 6.8), 0.8 ml of glycerol, 0.2 ml of 0.1% bromophenol blue, 1.6 ml of 10% SDS, 10.4 ml of 2-mercaptoethanol, and 4.0 ml of ddH$_2$O) and heated to 95°C for 4 min before loading. Electrophoresis was performed in mini-protein II dual slab cells (Bio-Rad) according to the general method of Laemmli (1970) using 5% PAGE as the separating gel and 3.9% PAGE as the stacking gel. Electrophoresis was run at 150 V in Tris-glycine running buffer (3 g Tris base, 14.4 g glycine, and 5 g SDS in 1 L ddH$_2$O; pH 8.3). After electrophoresis, the gels were stained with Coomassie blue.

Antibody preparation and the immune assay

Preparation of polyclonal anti-FSH antibodies

Polyclonal antibodies against purified FSH were raised in rabbits. The same volume of FSH (0.5 mg/ml) was added to 0.5 ml Freund's adjuvant reagent, mixed thoroughly, and injected into the hypodermis of New Zealand rabbits. Complete Freund's adjuvant was used for the 1st injection and incomplete adjuvant for the other 3 injections, which were given every other week.

Blood samples were taken from the ear vein of rabbits, allowed to sit at room temperature for 1 h, and then centrifuged at 2500 x g for 35 min. The supernatant was collected, and the titers of anti-FSH serum were measured.

Double diffusion: the Ouchterlony technique

Antiserum and the substrates (FSH, vitellin, vitellogenin, NRP, and 16S hemocyanin) were loaded into individual wells made of a 1% agarose gel (1.5 mm thick). The gel was kept in a moist box until immunoprecipitation lines had formed between the wells containing the antiserum and antigen. The gel was then soaked in a 0.1 M NaCl solution for 12 h to fix the immunoprecipitate. After being rinsed with ddH$_2$O several times, the gel was stained with Coomassie blue.

Western blotting

Hemolymph and purified FSH, NRP, and 16S hemocyanin were analyzed on a 5% native-PAGE, and then the proteins bands of the gels were transferred to a PVDF membrane using a mini-transblot electrophoretic transfer cell (Bio-Rad). Anti-FSH serum (diluted 5000-fold in net buffer: 2.5 g gelatin, 8.775 g NaCl, 5 ml Tween-20, 6.055 g Tris, and 1.86 g ethylenediaminetetraacetic acid (EDTA) in 1 L) was used as the primary antibody, and goat-anti-rabbit antibody-conjugated horseradish peroxidase (Sigma, diluted 5000-fold in net buffer) was used as the secondary antibody. Finally, color formation was carried out by adding a diaminobenzidine solution (5 mg of 3,3'-diaminobenzidine in 100 ml of 50 mM Tris-HCl; pH 7.2) and 2 μl of 30% H$_2$O$_2$. PVDF membranes were rinsed with 0.05% Tween-20 containing phosphate-buffered saline (8.0 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$, and 0.24 g KH$_2$PO$_4$ in 1 L; pH 7.2) between each of the above-described steps.

Oxygen-binding ability assay

Copper content analysis

Copper contents of the protein samples were quantified using Polarized Zeeman atomic absorp-

tion spectroscopy (Hitachi). NRP and 16S hemocyanin were directly eluted from the native-PAGE gels. Protein samples were dried in a Savant dryer and then re-dissolved in 80% nitric acid before being analyzed.

Spectral analysis

The absorbance spectra of vitelloprotein and FSH were obtained by reading the absorbance on a spectrophotometer (Hitachi U-2000).

### Oxygen-binding curves of FSH and 16S hemocyanin

The optical absorbance changes of FSH and 16S hemocyanin due to oxygen binding were measured at 340 nm. Hemocyanin samples were loaded into photometer cuvettes and then hermetically sealed in a plastic bag. PO_2 in the bag was achieved by blending pure oxygen with pure nitrogen in a differential ratio. After the reaction reached equilibrium, the cuvettes were placed into a spectrophotometer (Hitachi U-2000) to measure the absorbance at 340 nm. The curve of PO_2 vs. absorbance was plotted, and P_{50} was calculated accordingly.

RESULTS

Distinctive protein found in the hemolymph of adult females with maturing ovaries

There were two major protein bands in the hemolymph of males and immature females on the native PAGE analysis (Fig. 1). Since hemocyanins are abundant proteins in the hemolymph, they were first assumed to be 16S (the lower one) and 24S (the upper one) hemocyanin. Nevertheless, the upper major protein band does not contain copper (see below); it is not a respiratory protein and was named the NRP instead. While the levels of 16S hemocyanin were about the same in all individuals, those of NRP showed high variations among individuals (Fig. 1). There was an additional major protein band, which was located between the above 2 hemocyanins and was only found in the hemolymph of adult females with maturing ovaries (Fig. 1). It was thought to be a hemocyanin as well, and was provisionally named FSH. Levels of FSH were more abundant than those of the other 2 hemocyanins in some female individuals (Fig. 1). Vitellin and vitellogenin were also present in the hemolymph of female crabs with maturing ovaries with much lower Rf values (ratio of the distance travelled by the center of the spot to the distance simultaneously travelled by the mobile phase) than the NRP, but their concentrations were too low to show definitely visible bands in the gel (Fig. 1).
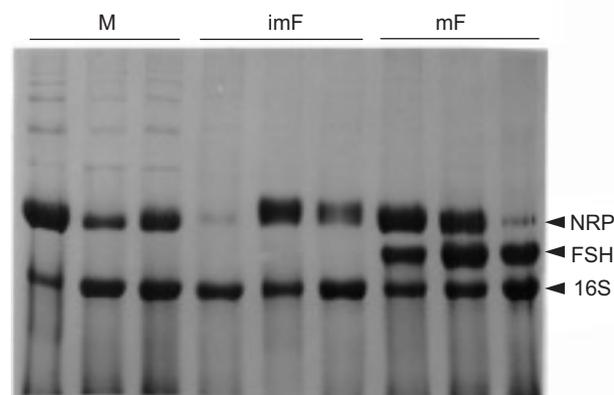
Fig. 1. Native-PAGE (5% acrylamide) of adult hemolymph. M, male; imF, immature female; mF, mature female; NRP, non-respiratory protein; FSH, female-specific hemocyanin; 16S, 16S hemocyanin.
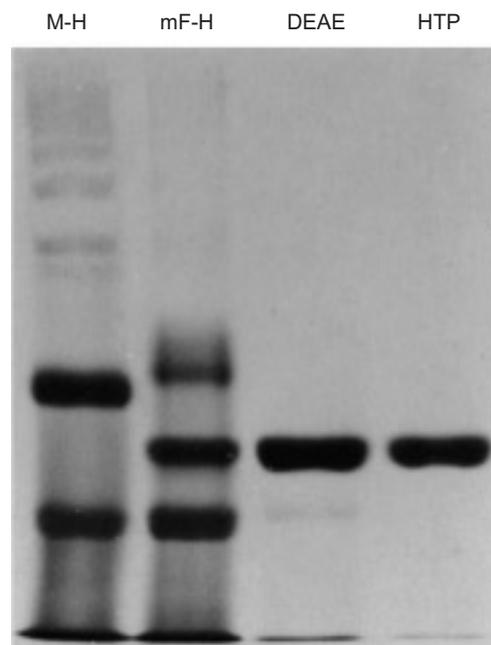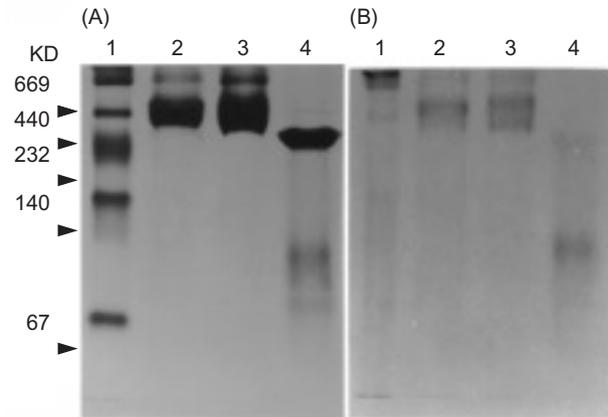

Fig. 2. Female-specific hemocyanin (FSH) purified on 5% native-PAGE. M-H, male hemolymph; mF-H, mature female hemolymph; DEAE, FSH purified using a DEAE Sepharose Fast Flow column; HTP, FSH further purified using a Bio-Gel HTP hydroxylapatite column.

Protein purification

FSH was purified from the hemolymph of female crabs with maturing ovaries using a DEAE Sepharose Fast Flow column; it presented a subsequent and unresolved peak ahead of native 16S hemocyanin. FSH was further purified using a Bio-Gel HTP hydroxylapatite column. After either chromatographic procedure, purified FSH was confirmed by 5% native-PAGE (Fig. 2, lanes 3 and 4).

Biochemical features of FSH, vitellin, and vitellogenin

Fig. 3. Native-PAGE (7.5%) of the standard (lane 1), vitellin (lane 2), vitellogenin (lane 3), and female-specific hemocyanin (FSH) (lane 4). (A) Stained with Coomassie blue; (B) pre-stained with Sudan black.
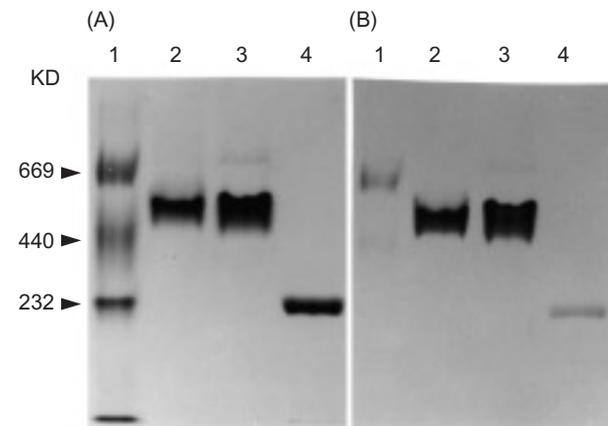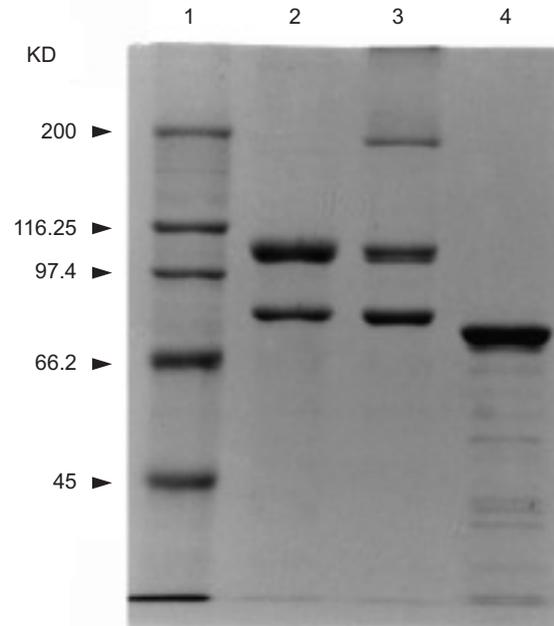

Fig. 4. Native-PAGE (5%) of the standard (lane 1), vitellin (lane 2), vitellogenin (lane 3), and female-specific hemocyanin (FSH) (lane 4). (A) Stained with Coomassie blue; (B) stained with periodic acid-Schiff's reagent.

Sudan black staining

After staining with the Sudan black reagent, vitellin and vitellogenin turned black but FSH did not (Fig. 3), indicating that both vitellin and vitellogenin are lipoproteins, but FSH is not.

Schiff's reagent staining

After staining with Schiff's reagent, vitellin, vitellogenin, and FSH all turned red (Fig. 4) indicat-
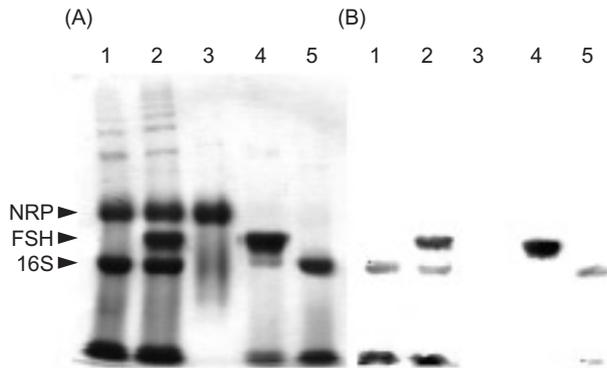

Fig. 5. SDS-PAGE (7.5%) of the standard (lane 1), vitellin (lane 2), vitellogenin (lane 3), and female-specific hemocyanin (FSH) (lane 4).
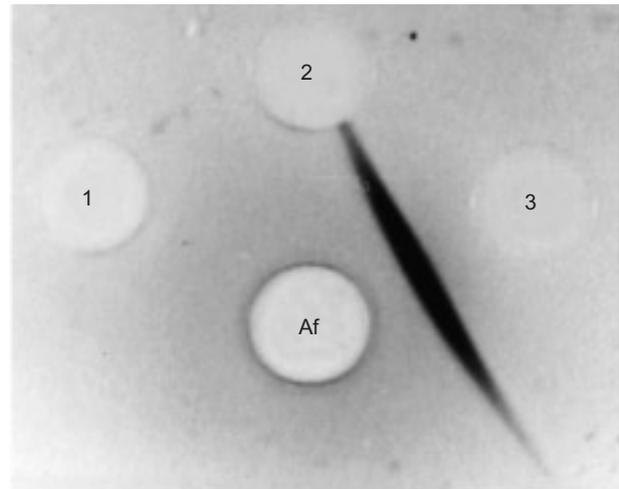

Fig. 6. Immunoprecipitation reactions of vitellin (1), vitellogenin (2), and female-specific hemocyanin (FSH) (3) with the anti-FSH (Af) antibody.

ing that all three of these female-specific proteins contain carbohydrates.

Fig. 7. Immunological analysis of *Scylla olivacea* hemolymph proteins. (A) 5% Native-PAGE with Coomassie blue staining; (B) Western blot, anti-female-specific hemocyanin (FSH) antibody. 1, Hemolymph of a male crab; 2, hemolymph of a mature female crab; 3, non-respiratory protein (NRP); 4, FSH; 5, 16S hemocyanin (16S).
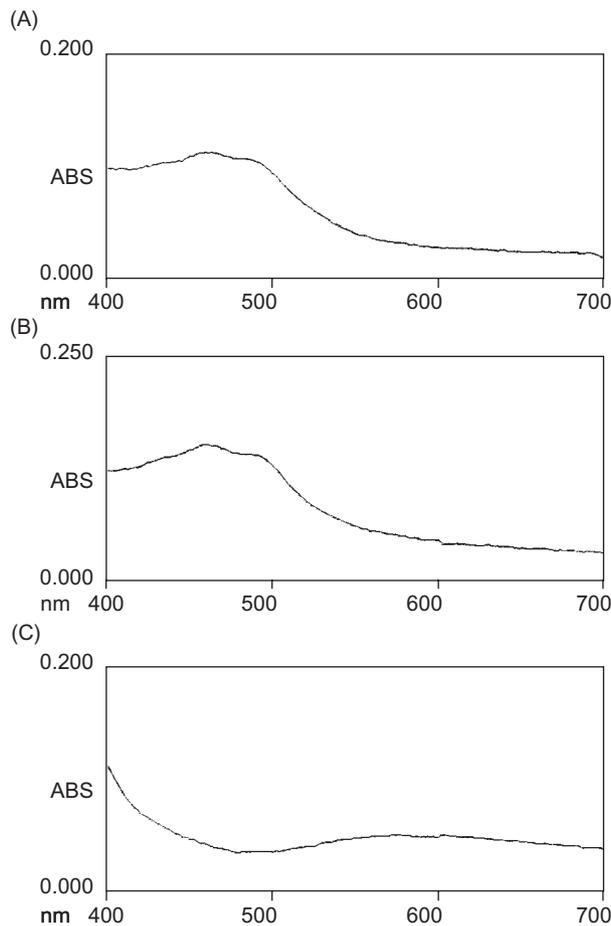
Fig. 8. Absorption spectra (400-700 nm) of vitellin (A), vitellogenin (B), and female-specific hemocyanin (FSH) (C).

Subunit analysis

From the result of 7.5% SDS-PAGE, FSH presented a major 78-kDa subunit band (Fig. 5), indicating that FSH is a hemocyanin-like protein. In contrast, vitellin had 2 subunits (of 85 and 111 kDa), and vitellogenin had 4 subunits (of 85, 108, 113, and 177 kDa); the sizes of these subunits conformed to those of crabs (Chen et al. 2004, Mak et al. 2005).

Immune assay

Double diffusion: the Ouchterlony technique

Respective polyclonal antibodies against vitellin and FSH were raised in rabbits. The anti-FSH serum specifically recognized FSH but did not recognize the other 2 female-specific proteins (vitellin and vitellogenin) purified from adult females of the same species (Fig. 6). These
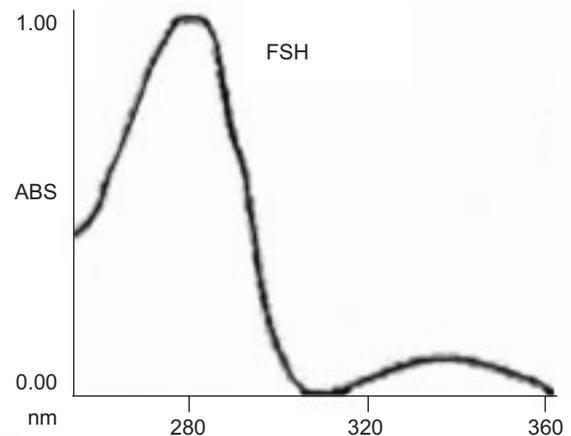
Fig. 9. Absorbance spectrum (265-365 nm) of female-specific hemocyanin (FSH).
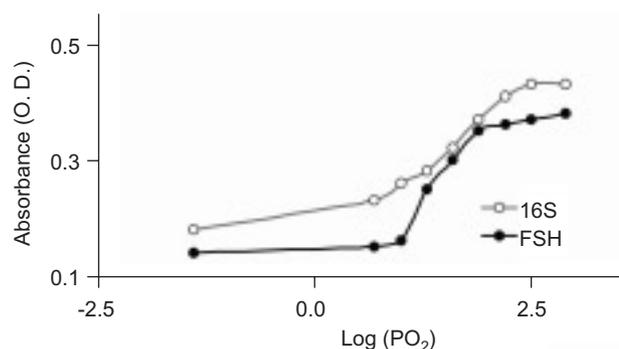
Fig. 10. Oxygen-binding curves of female-specific hemocyanin (FSH) and 16S hemocyanin. ●, FSH; ○, 16S hemocyanin.

results indicated that FSH and vitelloprotein are not related to each other.

Western blotting of hemocyanin by the anti-FSH antibody

Western blot analysis indicated that polyclonal antibodies against FSH recognized FSH, 16S hemocyanin, and a monomer (5S) hemocyanin (Fig. 7). There were no immune reactions other than these 3 protein bands in the gels. Anti-FSH antibodies had a strong reaction to FSH, but a weak one to 16S hemocyanin. Results of Western blotting indicated that FSH and 16S hemocyanin have similar structures. There was no immune reaction between the anti-FSP antibodies and the NRP (the other hemocyanin-related protein), suggesting that the NRP is not as closely related to FSH as is 16S hemocyanin.

Copper content

The copper contents of FSH, the NRP, 16S hemocyanin, and vitellin were analyzed using atomic absorption spectroscopy. FSH and 16S hemocyanin respectively contained 0.19% and 0.14% copper, which are close to published data (of around 0.17%) (Redfield et al. 1928, Allison and Cole 1940). The NRP and vitellin contained no copper at all. The above results indicated that FSH and 16S hemocyanin, but not the NRP, are respiratory proteins.

Oxygen-binding ability

Absorption spectrum

The absorption spectra of vitellin and vitellogenin were similar to each other with the highest absorption peak at 462 nm (Fig. 8). In contrast to vitellin and vitellogenin, FSH showed 2 absorption peaks at 280 and 337 nm that corresponded to the characteristic features of typical hemocyanin (Fig. 9).

Oxygen-binding ability analysis

The oxygen-binding curves of FSH and 16S hemocyanin were studied. As calculated from the curves (Fig. 10), the P_{50} values of FSH and 16S hemocyanin were 28.3 and 22.9 mmHg, and their oxygen saturation points were 152 and 300 mmHg O_2, respectively. The above results of both absorption spectra and oxygen-binding abilities

provide strong evidence that in addition to 16S hemocyanin, FSH is another functional hemocyanin in the hemolymph of *S. olivacea* females with maturing ovaries.

DISCUSSION

A novel hemocyanin was found in the hemolymph of female crabs with maturing ovaries. This protein appeared only in mature females, not in male or juvenile crabs; therefore, it was named female-specific hemocyanin (FSH). Its electrophoretic mobility was slightly lower than that of 16S hemocyanin, which is present in all adult crabs. In this study, the lower protein band on the native PAGE gel in figure 1 was the only copper-containing protein (besides the monomer hemocyanin located in the gel bottom) found in all crab hemolymph samples, and its oxygen-binding ability was demonstrated (Fig. 10); therefore it is a hemocyanin of *S. olivacea*. Only a hexamer but no dodecamer being seen in the native PAGE system may have been due to a lack of Ca^{2+} ions in the gel system (Decker et al. 1986) and confirms that the above hemocyanin is a hexamer (16S) not a dodecamer (24S).

The molecular mass of FSH was roughly estimated by native PAGE (Figs. 3, 4), and was underestimated, as the native PAGE is not a good way to predict precise molecular masses. Factors which affect the speed at which proteins move include not only the molecular mass, but also the shape and the charge they carry. For example, phycocyanin (232 kDa), a blue accessory pigment in cyanobacteria, migrates faster than human hemoglobin A (64.5 kDa) in the native PAGE system (Fujieda et al. 2005). The molecular mass of FSH was estimated to be about 495-543 kDa according to that of 16S hemocyanin and the ratio of migration distances of FSH and 16S hemocyanin on native PAGE.

FSH was demonstrated to be a hemocyanin of *S. olivacea*, due to its copper content, its oxygen-binding ability (with a P_{50} of 28.3 mmHg and an oxygen saturation point of 152 mmHg O_2), and the molecular mass of its subunits which ranged from 73 to 80 kDa, which correspond to characteristics of all crustacean hemocyanins.

From the evidence of the immunoassay, FSH is an independent hemocyanin and partially resembles 16S hemocyanin. It is the 1st respiratory hemocyanin accompanying ovarian development discovered in the Crustacea.

In *C. magister*, *C mag* 6, a subunit of hemocyanin does not appear until the 6th juvenile instar. In the absence of *C mag* 6, juvenile hemocyanin has a lower oxygen affinity than that of the adult (Terwilliger 1998). Although *C mag* 6 and FSH only appear in certain developmental stages of individuals, *C mag* 6 is a subunit of hemocyanin while FSH is a completely different hemocyanin.

The P_{50} values of *S. olivacea* FSH and 16S hemocyanin are 28.3 and 22.9 mmHg, respectively. The P_{50} range of the adult *C. magister* hemocyanin is from 11.1 to 48.5 mmHg (Terwilliger and Brown 1993); thus the detected P_{50} values of FSH and 16S hemocyanin are in a reasonable range.

The function of FSH has yet to be clearly delineated. Since it is only present in females with maturing ovaries, its functions may be related to the reproductive behavior of females. After copulation, female *S. olivacea* crabs swim to the open sea to lay their eggs, and they incubate the embryos under their abdomen (Arriola 1940, Hill 1975). At this stage, females are consuming more oxygen due to their swimming and laying behaviors, and water in the open sea has less oxygen than in the mud where extra oxygen from the air is directly exchanged through the gills. FSH possibly serves as an additional hemocyanin which provides for the extra needs of oxygen transport for brooders who are taking care of their embryos.

Non-respiratory hemocyanins have been found in the hemolymph of some decapod Crustacea, such as cryptocyanin in the Dungeness crab, *C. magister* (Terwilliger et al. 1999), and pseudo-hemocyanin in the American lobster, *Homarus americanus* (Burmester 1999). A non-respiratory hemocyanin-like protein (NRP, upper major band in Fig. 1) also appeared in most hemolymph samples from *S. olivacea*. This protein does not contain copper, so it is definitely not a respiratory protein. Moreover, this NRP was not recognized by the anti-FSH antibody, which recognized both FSH and 16S hemocyanin. While non-respiratory hemocyanins are thought to be storage proteins related to molting or reproduction (Burmester 1999, Terwilliger et al. 1999), the function of this NRP still needs to be studied. The result that the NRP showed high variation among individuals suggests that it may be a storage protein as well. However, the fact that the NRP appears in juveniles, adult males, and females with maturing ovaries, suggests that it might not be related to reproduction.

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