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Purification and Characterization of Vitellin from the Freshwater Giant Prawn, *Macrobrachium rosenbergii*

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Ying-Nan Chen and Ching-Ming Kuo (1998) Purification and characterization of vitellin from the freshwater giant prawn, *Macrobrachium rosenbergii*. *Zoological Studies* **37**(2): 126-136. Vitellin of *Macrobrachium rosenbergii* was purified by the combined use of ultrafiltration, ion exchange high-performance liquid chromatography (HPLC), and PAGE. The vitellin fraction recovered from HPLC contained 4 proteins, B1-B4, each of which was composed of 2 major polypeptides (A and B), as well as other minor components (E and F polypeptides and others larger than 205 kDa in molecular weight) on SDS-PAGE. Polymorphism of vitellin is clearly demonstrated, and the E polypeptide was found to be the possible precursor of A and B polypeptides as revealed by peptide mapping followed by Western blotting. The molecular weights of A, B, and E polypeptides were estimated by SDS-PAGE to be 83.1, 88.8, and 147.8 kDa, respectively, and those of A and B polypeptides were further characterized by MALDI-TOF mass spectrometry to be 95.9 and 106.9 kDa. The vitellin components were associated with each other through non-disulfide bonds as revealed by non-reducing SDS-PAGE and MALDI-TOF mass spectrometry. Hemolymph vitellogenin (Vg) was composed of 3 polypeptides, i.e., vc1, vc2, and vc3. Purified vitellin is immunologically and electrophoretically identical to Vg. A and B polypeptides are immunochemically correlated with vc1 and vc3, and vc2 and vc3, respectively; vc3 could therefore be the possible precursor of vc1 and vc2. Partial amino acid sequences of A and B polypeptides are also presented, and the transformational processing of Vg into vitellin is further discussed.

Key words: MALDI-TOF mass spectrometry, Peptide map, Vitellin, Vitellogenin.

Vitellogenesis in crustaceans, an important physiological process associated with female reproduction, is characterized by vitellogenin (Vg) synthesis and its subsequent processing and accumulation within the developing oocytes. Vg, a female-specific protein circulating in the hemolymph, is the precursor of vitellin that is the nutritive material necessary for the development of an embryo. Various sites of Vg synthesis in crustaceans have been reported. Vg is synthesized by fat body (Picaud 1980, Suzuki et al. 1989), hemocytes in *Callinectes sapidus* (Kerr 1968), and ovary (Lui and O'Connor 1976, Eastman-Reks and Fingerman 1985, Yano and Chinzei 1987, Lee and Watson 1995) or hepatopancreas (Quackenbush 1989, Han et al. 1994). In spite of discrepancies in the principal site of Vg synthesis among crustacean species, the immunological identity of hemolymph Vg and the major yolk protein, vitellin, has repeatedly been demonstrated in several decapod crustaceans, *C. sapidus* (Kerr 1969), *Homarus americanus* (Byard and Aiken 1984), *Pandalus kessleri* (Quinitio et al. 1989), *Penaeus monodon* (Quinitio et al. 1990), and *Macrobrachium nipponense* (Han et al. 1994). It was also observed in the terrestrial isopods, *Armadillidium vulgare* (Suzuki 1987) and *Oniscus asellus* (Vafopoulou and Steel 1995). Information on the transformation process of crustacean Vg into vitellin is still lacking.

In the terrestrial isopod, *A. vulgare*, various forms of Vg (Vg1-4) and vitellin (Vn1-4) have been identified and partially characterized by electrophoretic and immunochemical methods (Suzuki et al. 1989). Changes in the appearance of Vg and

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vitellin were also observed during the molting cycle and oocyte growth (Derelle et al. 1986, Suzuki 1987, Shafir et al. 1992). Possible involvement of a Vg fragment with protease function in the fragmentation process of Vg into vitellin in sand crayfish, *Ibacus ciliatus*, was presented (Komatsu and Hayashi 1994). In *Macrobrachium rosenbergii*, 3 vitellin forms with identical composition were obtained and partially characterized (Chang et al. 1993b), and 3 subunits of Vg were further reported (Wilder et al. 1994). To date, the physiological relationship between Vg and vitellin in crustaceans and discrepancy in Vg and vitellin constituents still remain to be clarified. Moreover, the physiological function other than nutrition of Vg and vitellin during embryonic development is also worthy to be explored. The present study dealing with the purification and characterization of vitellin from ovaries of the freshwater giant prawn, *M. rosenbergii*, aims to verify the polymorphism of vitellin and Vg constituents, and to elucidate the possible mode of Vg transformation into vitellin.

MATERIALS AND METHODS

Sexually mature females of the freshwater giant prawn, *M. rosenbergii*, were obtained from prawn culture farms in Pingtung, Taiwan. Vitellogenic females with a gonadosomatic index larger than 5% were selected. Ovaries were removed and immediately frozen at –80 °C until use for vitellin extraction and fractionation. All procedures were performed at 4 °C.

Vitellin extraction

Frozen ovarian samples, first defrosted in homogenization buffer (0.5 M Tris-HCl buffer containing 0.5 M NaCl, pH 7.5) at 4 °C, were homogenized by use of a Kinematica AG polytron (PT1200C) at 2000 rpm, and then centrifuged at 2000 × g, 4 °C for 30 min (Hitachi CR20B2, Japan). The supernatant was retained for further fractionation and purification.

The ovarian extract was first cut-off by ultrafiltration using molecular weight-cut off concentrator (Centreprep-30, Amicon Inc., USA), followed by buffer exchange with 50 mM Tris-HCl buffer (pH 7.5). The extract was further concentrated with the same concentrator and its absorbance measured at 474 nm to confirm the presence of vitellin, in which a carotenoid constitutes a component (Zagalsky et al. 1967, Chang et al. 1993b).

High-performance liquid chromatography

The concentrated vitellin extract was then fractionated by high-performance liquid chromatography (HPLC, L-6000 pump, L-4500 diode array detector, Hitachi, Japan) on an ion exchange column (POROS Q/M, 100mm × 4.6 mm i.d., PerSeptive Biosystem, USA) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) for further purification. A linear gradient was established by the elution buffer (50 mM Tris-HCl buffer containing 1 N NaCl, pH 7.5) from 5 min. The flow rate was maintained at 3 ml/min. The eluates were monitored at 280 nm, and each 3- ml fraction was recovered by a fraction collector (Gilson FC203, USA). Vitellin-containing fractions were identified by absorbance at 474 nm (Zagalsky et al. 1967), and further concentrated by ultrafiltration.

Electrophoresis

The purity of vitellin fractions was determined by use of a discontinuous polyacrylamide gel electrophoresis (disc-PAGE), which was carried out on 10% polyacrylamide separating gel with 5% polyacrylamide stacking gel. The electrophoretic separation of vitellin fractions was performed with an electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3) in a vertical slab gel unit (Mighty Small II, SE250, Hoefer Scientific Instruments, USA). The gels were stained with Coomassie blue G-250 (9% acetic acid, 45% methanol, 0.1% Coomassie blue G-250). The stained protein portions of the gels were sliced, and then incubated in reducing incubation buffer (125 mM Tris-HCl buffer containing 10% glycerol, 1 mM EDTA, 0.3% 2-mercaptoethanol, a minute amount of bromophenol blue, pH 6.8) twice for 30 min each time, and finally electrophoresed on 8% separating and 5% stacking polyacrylamide gel in discontinuous sodium dodecyl sulfate PAGE (disc-SDS-PAGE) with electrophoretic buffer (25 mM Tris, 0.1% SDS, 192 mM glycine, pH 8.3) (Laemmli 1970). Silver staining was used to identify protein-bands on the gels from which molecular weights were estimated following the method described by Weber and Osborn (1969). Six markers of known molecular weight (MW-SDS-200, Sigma, USA) were carbonic anhydrase (29 kDa), albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa), and rabbit muscle myosin (205 kDa).

The vitellin fraction was pre-stained with Sudan Black B and run on 10% PAGE, or run first

on 8% SDS-PAGE, followed by staining by periodic acid-Schiff's reagent or methyl green to determine whether it contained lipids, carbohydrates or phosphorus (Cutting and Roth 1973, Covens et al. 1988). Presence of disulfide bonds in vitellin was verified by the difference in 8% SDS-PAGE profiles obtained from vitellin with or without 2-mercaptoethanol pretreatment.

MALDI-TOF mass spectrometry

The molecular weights of major components of vitellin were further determined by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF mass spectrometry) (G2025A MALDI-TOF Mass Spectrometer System, Hewlett-Packard Co., USA) following a method modified from Karas et al. (1995). The positive ion mode was followed, and equine cardiac cytochrome c (12359.2 Da), equine cardiac myoglobin (16951.5 Da), and bovine serum albumin (66430.2 Da) were used for calibration. Vitellin was first quantified using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA), and bovine serum albumin was used as the standard. A 0.5-μg sample was mixed with 1 μl 100 mM sinapinic acid in acetonitrile:methanol:water (60:36:8, v/v/v) and co-crystallized under vacuum. The crystalline product was then ionized using a 5-6 μjoule/cm^2 N_2 laser (337 nm). The molecular weight was estimated from the mass spectrum in which the masses of positive-ions were calculated based on the time elapsed from ionization untill collision of the molecules onto the detector.

Preparation of antisera

Purified vitellin (0.4 mg) was dissolved and emulsified with an equal volume of 0.9% saline and complete Freund's adjuvant (Life Technologies, Inc., Grand Island, NY, USA), which was injected into the spleen of a New Zealand white rabbit once. Blood collected by ear puncture after 35 d of immunization at intervals of 15 d was kept at room temperature for 1 h, and then under 4 °C overnight. The blood was centrifuged at 1000 × g, 4 °C for 20 min, and then the titers of antisera were determined following the method described by Hong et al. (1989).

Antisera against the major components of vitellin were further prepared. Vitellin (0.6 mg) was first electrophoresed on preparative SDS-PAGE. Each gel fraction of a major vitellin component was sliced out and then emulsified with an equal volume of 0.9% saline and complete Freund's adjuvant. Multiple subcutaneous injections into New Zealand white rabbit were performed at intervals of 7 d, and titers of antisera were also monitored. Subsequent boosters used incomplete Freund's adjuvent (Life Technologies, Inc., Grand Island, NY, USA). The prepared antisera were frozen at –80 °C until use.

Immunoprecipitation

The presence of antigens precipitatable with vitellin antiserum in various tissues and hemolymph was examined by immunoprecipitation as described by Ouchterlony (1968) on 2% agar gels overlaid on gelBond film (FMC BioProducts, USA). Diffusion was allowed to proceed for up to 48 h at room temperature, and the precipitin lines were further stained with Coomassie blue G-250. Tissues of prawn were dissected and fragmented in ice-cold prawn saline (Nagamine et al. 1980), and subsequently washed with several changes of saline. The fragments of tissues were then homogenized and centrifuged, and the supernatants were separately collected for immunoprecipitation analysis.

Western blotting

The cross-reaction of hemolymph proteins with anti-vitellin sera was further verified by Western blotting. The hemolymphs from male and vitellogenic female prawns, and purified vitellin were all run on 8% SDS-PAGE. The fractionated proteins were then electrophoretically transferred from the gel onto a PVDF membrane (polyvinylidene difluoride, Immobilon-P, 0.45 μm, Millipore, USA) in a transferring buffer (192 mM glycine, 25 mM Tris, 1.3 mM SDS, 10% methanol) using a semi-dry blotting system (EBU-4000, CBS Scientific Co. Inc., USA). The processed PVDF was immersed first in Tris buffer saline (TBS, 20 mM Tris, 50 mM NaCl, pH 7.4) containing 3% bovine serum albumin for 30 min, and then rinsed thrice with TBS containing 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate) (TBST). Following subsequent incubation for 1 h in anti-vitellin or anti-vitellin-components sera in TBS containing 1% BSA, the PVDF membrane was rinsed with TBST and then reacted with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS containing 1% BSA for 1 h. Unreacted 2nd antibody was removed, and the PVDF membrane was then immersed in BCIP/NBT (5-bromo-4-chloro-3-

indolyl-phosphate/nitro blue tetrazolium) substrate solution (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, containing 0.3 mg/ml NBT, 0.15 mg/ml BCIP, pH 9.5) (Promega, USA) for color development under darkness. Finally, the PVDF membrane was immersed in color stop solution (1 mM EDTA in 20 mM Tris-HCl, pH 2.9) and then rinsed sequentially with TBS and double-distilled water.

Peptide maps of vitellin components

To clarify the homogeneity among vitellin components, peptide maps of vitellin components were developed by electrophoresis following a method modified from Cleveland et al. (1977) and Cleveland (1983) and visualized by Western blotting. The vitellin fraction eluted from HPLC was run first on 10% PAGE, and then stained with Coomassie blue G-250 to identify major protein bands. The gels containing B3 protein were incubated in reducing incubation buffer and then run on 8% SDS-PAGE to separate the vitellin components. Gel portions containing the vitellin component were sliced and soaked in 10 ml equilibration buffer (450 mM Tris-HCl buffer containing 12% glycerol, 4% SDS, 0.008% Coomassie blue G-250, 0.003% phenol red, 0.15% 2-mercaptoethanol, pH 8.45) twice for 10 min each, then intruded into sample wells of 10%-20% tricine gradient gel (Novex pre-cast gel, Novel Experimental Technology, USA) and overlaid with equilibration buffer containing 20% glycerol. Endoproteinase Glu-C (protease V8) (Promega, USA) was dissolved in the equilibration buffer and electrophoresed in the electrophoretic buffer (100 mM Tris buffer, 100 mM tricine, 0.1% SDS, pH 8.25) (Schagger and Jagow 1987) after introduction of protease solution into the sample well. Power interruption was performed every half hour, twice for 30 min each time. Electrophoresis was terminated when the tracking dye arrived at the position 1 cm above the bottom of the gel. Western blotting then followed, and each anti-vitellin component serum was separately employed for immunochemical detection of the polypeptide fragments derived from protease digestion.

Amino acid sequences of vitellin components

The amino acid sequences of N-terminal and several internal peptides of vitellin components were determined with a protein sequencer (476A, Applied Biosystems, Inc., USA) to elucidate their partial primary structure. Vitellin components and their fragments derived from digestion by protease V8 in polyacrylamide gel were all electrophoretically transferred onto a PVDF membrane, and portions of the membrane with desired blotted proteins were cut out for sequencing as described by Liu et al. (1993).

RESULTS

Purification of vitellin

Vitellin extract was fractionated by ion exchange HPLC and monitored at wavelengths of 280 nm and 474 nm. Vitellin was eluted in the 1st fraction based on peak absorbances at both 280 and 474 nm (Fig. 1). The vitellin fraction recovered from HPLC fractionation was electrophoresed on 10% PAGE, and the electropherogram revealed 4 stained bands, namely B1, B2, B3, and B4. B3 was shown to be the major band. It constituted more than 90% total protein on the basis of staining density and width of the band (Fig. 2a). Each protein, subsequently run on 8% disc-SDS-PAGE, revealed that B1, B2, and B3 were similar in electrophoretic profiles and consisted of 2 major components, A and B polypeptides, with the presence of

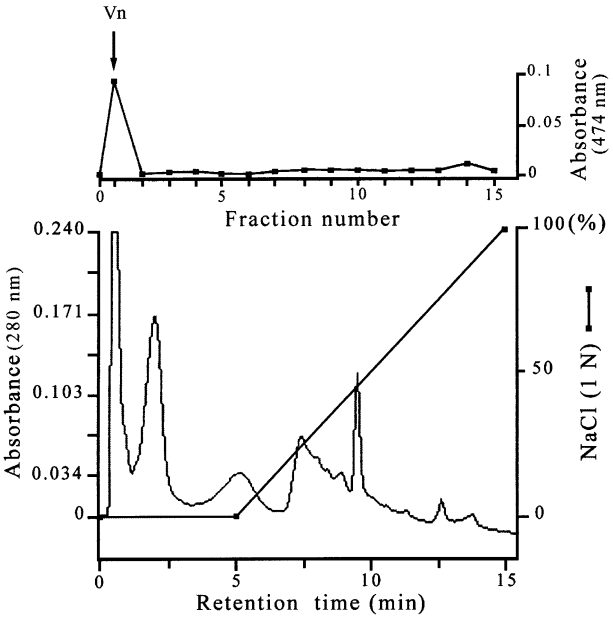

Fig. 1. Chromatogram of vitellin (Vn) extract of *Macrobrachium rosenbergii* fractionated by ion exchange HPLC on a POROS Q/M column, which was equilibrated with 50 mM Tris buffer and then eluted with a linear gradient of 0-1 N NaCl in 50 mM Tris buffer. Flow rate: 3 ml/min; fraction volume: 3 ml; and absorbance monitored at wavelengths of 280 nm and 474 nm, respectively.

other minor components such as E polypeptide. B4 was composed mainly of C and D polypeptides, with a small portion of E polypeptide (Fig. 2b). Polymorphic vitellins of *M. rosenbergii* evidently accumulated in the oocytes, and could be purified through the procedure including ultrafiltration, ion exchange HPLC, and polyacrylamide gel electrophoresis. The mobilities of C and D polypeptides were notably different from those of A and B polypeptides; i.e., D moved more slowly than B, while C was closer to A. B1, B2, B3, and B4 were all composed of E polypeptide, though B3 was less stained. In addition, B1-B4 all contained other minor components, such as F polypeptide, which was more heavily stained in B3 than in the other ovarian proteins (B1, B2, and B4) by silver staining, and those polypeptides larger than 205 kDa in molecular weight.

Characterization of vitellin

The molecular weights of 2 vitellin major polypeptide components were estimated from SDS-PAGE to be 83.1 kDa and 95.9 kDa for A and B polypeptides, respectively (Fig. 2b). A and B polypeptides were the major components of vitellin in *M. rosenbergii*, with only 2 major peaks of purified vitellin exhibited on the MALDI-TOF mass spectrum, of which the m/z values were 88 760 ± 118 and 106 865 ± 204, equivalent to 88.8 kDa and 106.9 kDa in molecular weight, respectively (Fig. 3).

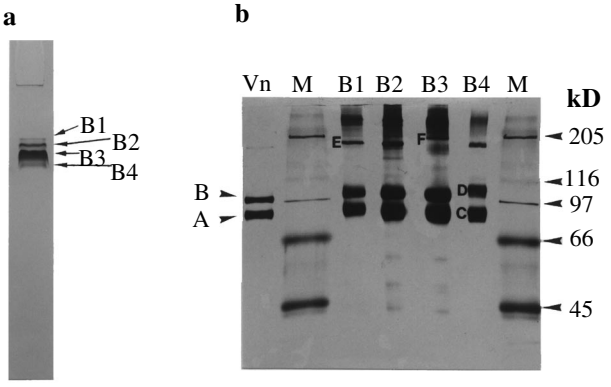

Fig. 2. Electropherograms of vitellin fraction recovered from ion exchange HPLC. A 20-μg protein sample was electrophoresed on 10% PAGE (a), and further run on 8% SDS-PAGE followed by silver staining (b). Samples of 1 μg protein of vitellin fraction (Vn), B1-B4 proteins, and a mixture of standard proteins (M) were electrophoresed simultaneously. Varying quantities of recovered B1-B4 proteins were introduced to SDS-PAGE for better discrimination of each band. B1, B2, and B3 were similar in electrophoretic profiles consisting of 2 major components, A and B polypeptides, while B4 was composed mainly of C and D polypeptides. Other minor constituents, including E and F polypeptides and those larger than 205 kDa in molecular weight, were also observed.

Vitellin separating into its components, shown in non-reducing SDS-PAGE (Fig. 4) and MALDI-TOF spectrum which exhibited 2 peaks, confirmed that A and B polypeptides are associated with each other through non-disulfide bonding. Besides, vitellin is composed of lipids, carbohydrates and phosphorus as revealed by the respective stainability of vitellin to Sudan Black B, periodic acid-Schiff's reagent, and methyl green (Fig. 5), so vitellin is therefore considered a lipoglycophosphocarotenoprotein compound. The results agree with the characteristics of vitellin first purified from 6 crustacean species (Wallace et al. 1967). The partial amino acid sequences of vitellin components are shown as follows:

partial sequence of N-terminal of A polypeptide:
NH_2-APEPQ (G/K) (V/T) NLTV (K/E) LD;
partial sequence of N-terminal of B polypeptide:
NH_2-SIDLSQ (L/I) THLFDKLY;
internal partial sequence of A polypeptide:
NH_2-IARP(I/W)(L/W)Q; and
internal partial sequence of B polypeptide:
NH_2-KI(D/L)L(K/S)QI.

Antisera against purified vitellin and its components specifically reacted only with female hemolymph, ovarian extract, and electrophoretically fractionated ovarian vitellin, but not with male hemolymph of *M. rosenbergii* (Figs. 6, 7). The vitellin purified in this study was consequently assumed to be a female-specific protein and immunologically identical to Vg as revealed in immunoprecipitation analysis. In addition to vitellogenic female hemolymph and ovarian extract, precipitin lines were also observed between wells loaded with extract of female hepatopancreas or anti-vitellin serum, respectively (Fig. 6). Furthermore, female-specific Vg, a precursor of yolk protein in female hemolymph, was separated into its components on SDS-PAGE and 3 bands, namely vc1, vc2, and vc3, were then immunochemically detected by Western blotting with anti-vitellin sera (Fig. 7). These 3 components of Vg were electrophoretically identical to the vitellin components, A, B, and E polypeptides, and the molecular weights were estimated to be 83.1, 95.9, and 147.8 kDa for vc1, vc2, and vc3, respectively. Only 2 components of Vg were recognized when an anti-vitellin component, A or B polypeptide serum, was used;

i.e., vc1 and vc3 were cross-reacted with anti-A polypeptide serum, and vc2 and vc3 with anti-B polypeptide serum. Anti-vitellin A polypeptide serum would not cross-react with B polypeptide, and vice versa. It is therefore evident that vc1 and vc2 are structually correlated with vc3.

Since E polypeptide could be immunochemically recognized in Western blotting with anti-vitellin

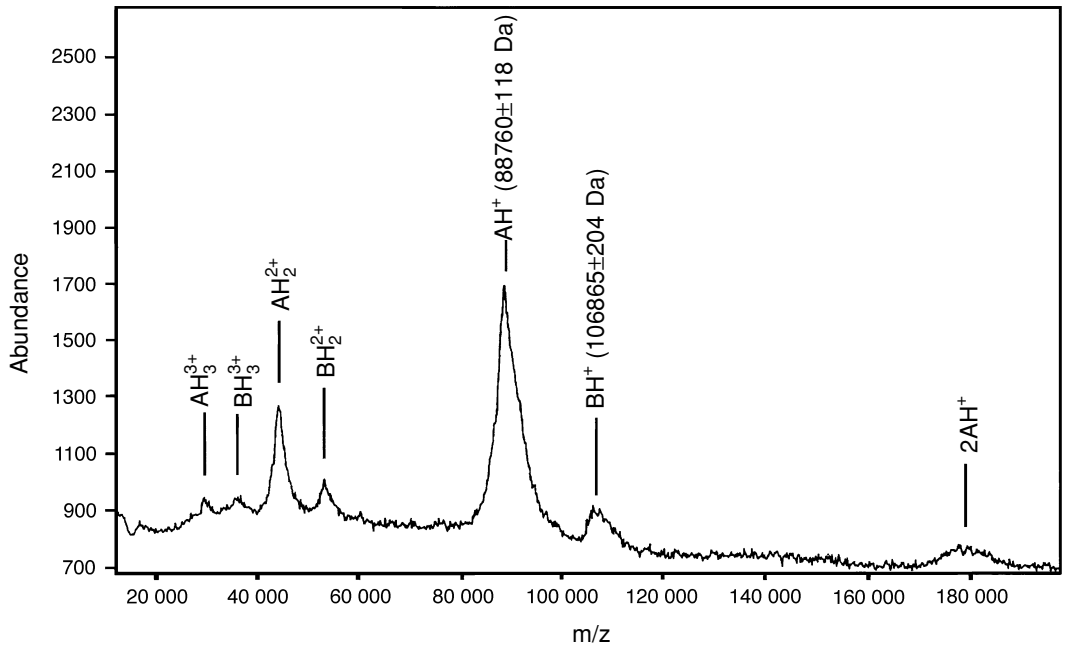

Fig. 3. MALDI-TOF mass spectrum of vitellin of *Macrobrachium rosenbergii*. Signals from variously protonated molecules of vitellin components were detected. Each line indicates the central position of the peaks in the spectrum. The molecular weights of A and B polypeptides estimated from the mean m/z values of singly protonated vitellin components are indicated in the figure. Laser power density was 5-6 μ joule cm^{-2} at a wavelength of 337 nm, and positive-ion mode was employed. Purified vitellin at 0.5 μg was co-crystalized with sinapinic acid under vacuum prior to the ionization by N_2 laser.

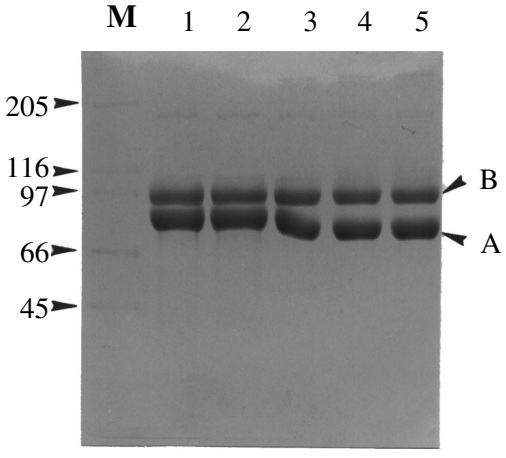

Fig. 4. Electropherograms of vitellin and a mixture of standard proteins (M) on 8% SDS-PAGE. Vitellin was either pretreated with 2-mercaptoethanol prior to electrophoresis (lanes 1-2) or given no reducing agent pretreatment (lanes 3-5). The side diffusion effect due to the diffusion of 2-mercaptoethanol is observable in lane 3.

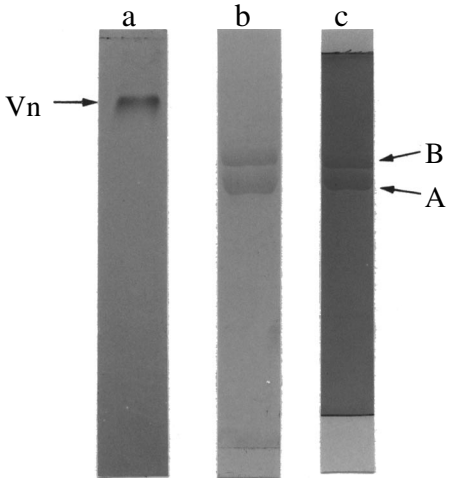

Fig. 5. Characterization of vitellin of *Macrobrachium rosenbergii*. Vitellin fraction was prestained with Sudan Black B and run on 10% PAGE; major protein component, B3, clearly displayed (a); major vitellin components, A and B polypeptides, were stained with periodic acid-Schiff's reagent (b) and methyl green (c) on 8% SDS-PAGE

serum, the primary structural relationship among vitellin components was further verified by peptide mapping followed by Western blotting. The peptide maps of A, B, and E polypeptides derived from digestion by protease V8 were developed and recognized respectively with anti-A or -B polypeptide serum. We found that not only the map of A polypeptide was identical to that of E polypeptide when anti-A polypeptide serum was employed as the primary antibody in Western blotting, but also the map of B polypeptide was identical to that of E polypeptide when anti-B polypeptide serum was employed (Fig. 8). We therefore concluded that A and B polypeptides are likely derived from the fragmentation of E polypeptide, i.e., E polypeptide is the possible precursor of A and B polypeptides.

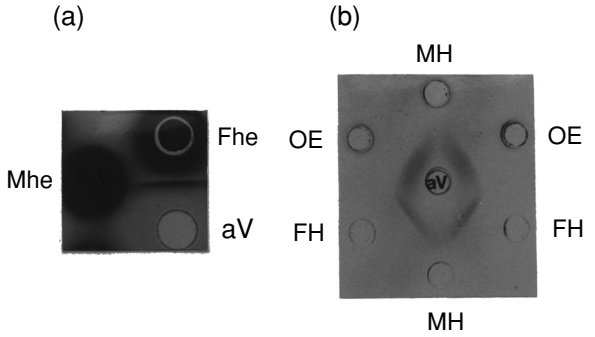

Fig. 6. Immunoprecipitation reaction: (a) aV (antisera against vitellin), Fhe (extract of female hepatopancreas), Mhe (extract of male hepatopancreas); (b) aV (antisera against vitellin), FH (vitellogenic female hemolymph), OE (ovarian extract), MH (male hemolymph).

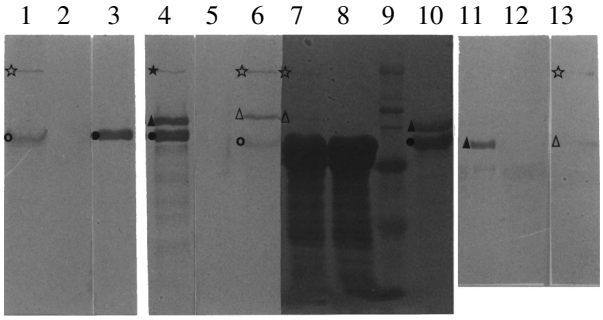

Fig. 7. Relationships between ovarian vitellin and hemolymph protein of *Macrobrachium rosenbergii*. Total proteins at 24 μg in the hemolymph from males (lanes 2, 5, 8, 12) and females (lanes 1, 6, 7, 13), 2 μg purified vitellin (lanes 3, 4, 10, 11), and a mixture of standard proteins (lane 9) were reduced with 2-mercaptoethanol and then separated on 8% SDS-PAGE. Immunostaining with anti-vitellin sera (lanes 4-6), anti-A polypeptide serum (lanes 1-3), anti-B polypeptide serum (lanes 11-13), and staining with Coomassie blue G-250 (lanes 7-10) were employed. The symbols marked at the left side of each stained band indicate the components of vitellin (●A polypeptide, ▲B polypeptide, ★E polypeptide) and vitellogenin (○ vc1, △ vc2, ☆vc3). Fragments coming from the degradation of A and/or B polypeptides can be observed in lane 4. E polypeptide being barely observable in lanes 3 and 11 is due to the short duration for color development in immunostaining.

DISCUSSION

Vitellin, a major portion of ovarian proteins in *M. rosenbergii*, was efficiently fractionated from other ultrafiltered ovarian proteins by ion exchange HPLC with a POROS Q/M column, and was found to be only eluted in the non-adsorbed fraction. By using a DEAE-Sephacel column eluted with 10 mM Tris buffer (pH 8.0) along with a linear gradient of 0-0.5 N NaCl, vitellin was detected in both non-adsorbed and adsorbed fractions in the same species, and 3 forms of vitellin varying in molecular weight between 240 and 3800 kDa were eluted out

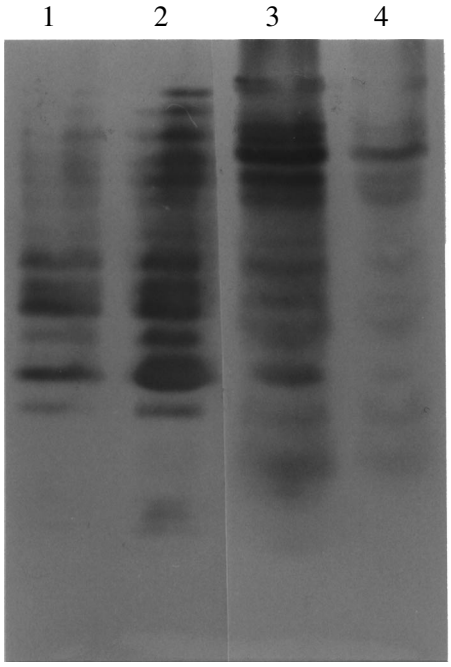

Fig. 8. Peptide maps of vitellin components after digestion by protease V8 developed with tricine SDS-PAGE in 10%-20% gradient gel, followed by Western blotting. Vitellin components were first separated from each other on 8% SDS-PAGE and then electrophoresed in 10%-20% tricine gradient gel, during which the digestion by protease V8 was processed. The derived fragments of each component of vitellin, A (lane 3), B (lane 2), and E polypeptides (lanes 1, 4) were electrophoretically transferred onto a PVDF membrane followed by immunostaining with anti-B polypeptide serum (lanes 1, 2) or anti-A polypeptide serum (lanes 3, 4).

as a single peak at 0.175 N NaCl gradient in the adsorbed fraction (Chang et al. 1993b). The vitellin form in the non-adsorbed fraction, 240 kDa in molecular weight, was also found to be identical to the one in the adsorbed fraction. They were both composed of 2 subunits with molecular weights of 90 and 104 kDa, respectively. The possibility of vitellin becoming insoluble or even aggregated in a low ionic strength solution has been postulated in several decapod species (Wallace et al. 1967). Discrepancies in vitellin fractionation observed in *M. rosenbergii* to date, could possibly have resulted from the aggregation of vitellins during the processes of fractionation and purification, or from the difference in the prawn's ovarian development phase from which the vitellin extracts were prepared.

The vitellin fraction was composed of 4 proteins (B1-B4) identified in PAGE; B3 was found to be the major one. B1, B2, and B3 showed similar patterns in SDS-PAGE with respect to mobilities of 2 major constituents, A and B polypeptides, and the electrophoretic profiles of these major constituents after cleavage by protease V8 were found to be identical among B1, B2, and B3 (data not shown). On the contrary, B4 was different from the others and was mainly composed of C and D polypeptides. Polymorphism of ovarian vitellin in *M. rosenbergii* was clearly demonstrated. B1-B4 proteins in the vitellin fraction all contained a component of similar mobility, E polypeptide, and a few other protein constituents larger than 205 kDa. Identities of peptide maps within E and A polypeptides, and E and B polypeptides, respectively, suggest that A and B polypeptides are possibly derived from the fragmentation of E polypeptide. A similar result was reported in *P. monodon* (Chen and Chen 1993). The relationship between the constituents larger than 205 kDa with Vn still remain to be clarified. Similarity of E polypeptide in the B4 protein to those found in B1, B2, and B3 proteins is still unclear, and the correlation between C and D polypeptides with Vg also remains to be elucidated.

Diversity in vitellin components, revealed by SDS-PAGE, has been reported in several crustacean species. These vitellins were composed of 2-8 subunits with molecular weights ranging from 35 to 190 kDa (de Chaffoy de Courcelles and Kondo 1980, Eastman-Reks and Fingerman 1985, Zagalsky 1985, Suzuki 1987, Tom et al. 1987, Lee and Puppione 1988, Quinitio et al. 1989, Quinitio et al. 1990, Tom et al. 1992, Chang et al. 1993a, Chen and Chen 1993, Vafopoulou and Steel 1995). Two vitellin forms composed of 2 and 6 subunits, respectively, and with molecular weights estimated from 109 to 105 kDa and 65 to 42 kDa, respectively, in *Emerita asiatica* were reported (Tirumalai and Subramoniam 1992). Only 2 subunits, 84-92 kDa and 92.2-105 kDa estimated from SDS-PAGE, in *M. rosenbergii* were proposed in the literature (Derelle et al. 1986, Chang et al. 1993b, Wilder et al. 1994).

The combined molecular weight of the main vitellin components, A and B polypeptides, was 179 kDa and 195.7 kDa, as estimated from SDS-PAGE or MALDI-TOF mass spectrometry, respectively. Vitellin contains carbohydrate and lipid components, which affect to some extent the mobility of vitellin on SDS-PAGE, and resulted in variation of molecular weight estimations. The estimate obtained from MALDI-TOF mass spectrometry seemed more accurate than that from SDS-PAGE. The difference in molecular weight estimates from MALDI-TOF mass spectrum and SDS-PAGE was calculated to be 6.4% for A and 10.3% for B polypeptide, respectively. The molecular weight of vitellin was mostly estimated by gel-filtration, gradient centrifugation, or pore-limited electrophoresis (PAGGE) in other crustaceans. Variability in the molecular weight estimates of vitellin in crustaceans reported to date obviously exists, but generally these estimates fall in the range of 280 to 700 kDa (Wallace et al. 1967, Croisille et al. 1974, Fyffe and O'Connor 1974, de Chaffoy de Courcelles and Kondo 1980, Zagalsky 1985, Suzuki 1987, Quinitio et al. 1989, Quinitio et al. 1990, Tom et al. 1992, Han et al. 1994, Vafopoulou and Steel 1995).

The low-energy laser applied in MALDI-TOF mass spectrometry is known to be unable to destroy disulfide bonding, while solvents such as methanol and acetonitrile can possibly segregate components which are associated by non-disulfide bonding in the molecule. Vitellin was shown to have 2 peaks in the MALDI-TOF spectrum, and it was separated into its components in non-reducing SDS-PAGE. Such findings suggest that the vitellin components are associated with each other by non-disulfide bonds. Similar results were reported elsewhere on the basis of use of non-reducing SDS-PAGE (Quinitio et al. 1989, Quinitio et al. 1990, Chang et al. 1994, Chang and Jeng 1995), and also in *M. rosenbergii* as deduced from the non-detectable level of cysteine in the amino acid composition (Chang et al. 1993b).

The fact that vitellin originates from Vg is widely accepted. The molecular weights of the 3 Vg components estimated in this study were closer

to those reported in the same species, of 90, 102, and 199 kDa (Wilder et al. 1994). That vitellin is immunologically identical to Vg in crustaceans has been reported elsewhere (Kerr 1969, Byard and Aiken 1984, Suzuki 1987, Quinitio et al. 1989 1990, Han et al. 1994, Vafopoulou and Steel 1995), while Vg in some crustaceans is electrophoretically identical to vitellin (Fyffe and O'Connor 1974, Suzuki 1987, Lee and Puppione 1988, Chen and Chen 1993). The electrophoretic and immunological identities of Vg and vitellin components were observed in *M. rosenbergii*, and we suggest that these 3 Vg components in the hemolymph are possibly all taken in by pinocytosis into oocytes and then processed into vitellin. Anti-A and -B polypeptides sera in this study were cross-reacted to vc1 and vc3, and to vc2 and vc3, respectively, and this suggests that vc3 is closely related to vc1 and vc2 in its molecular structure. Discrepancies in the findings in the literature can possibly be attributed to the specificities of antisera prepared (Derelle et al. 1986). It is also possible that the enriched Vg recovered from PAGE is consisted mainly of 2 smaller polypeptide components.

The way that Vg is processed into vitellin has been a controversial subject. In chicken, frogs, and many insects, Vg is first synthesized and then fragmented into vitellin components (Gerber-Huber et al. 1987, Nardelli et al. 1987, Wahli 1988). A 50-kDa fragment of Vg possessing protease function was observed in the Pacific eel, *Anguilla japonica* (Komatsu et al. 1996). A low-density lipoprotein (LDL) in oocytes and one of the Vg fragments both possessed protease function and were involved in the fragmentation process of Vg into vitellin in sand crayfish, *Ibacus ciliatus* (Komatsu and Ando 1992, Komatsu and Hayashi 1994).

Transformation of larger molecular vitellin fragments to small molecular forms is also indicated as ovarian maturation proceeds in a terrestrial isopod, *Armadillidium vulgare* and in *Penaeus vannamei* (Suzuki 1987, Tom et al. 1992). In *M. rosenbergii*, the Vg component at a molecular weight of 199 kDa was first detected after eyestalk ablation in female subadults, followed by the appearance of 2 smaller molecules of 102 and 90 kDa in molecular weight. Vg in mature female hemolymph is composed of these 3 polypeptides (Wilder et al. 1994). The present study confirms that vc1 and vc2 are not only structurally correlated with A and B polypeptides, respectively, but also with vc3. E polypeptide was further confirmed to be the possible precursor of A and B polypeptides and is immunologically and electrophoretically identical to vc3. These findings suggest that vc1 and vc2 are possibly derived from the fragmentation of vc3. Such fragmentation process presumably takes place outside of oocytes during vitellogenesis. The Vg components after pinocytosis into oocytes are transformed to polymorphic vitellins not only by association with each other, but also with other intraovarian proteins.

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淡水長臂大蝦(*Macrobrachium rosenbergii*)卵黃素之分離及特性研究

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經由超微過濾(ultrafiltration)、離子交換高效液相層析(ion exchange high-performance liquid chromatography)及聚丙烯銨膠體電泳(PAGE)可將淡水長臂大蝦(*Macrobrachium rosenbergii*)主要卵黃蛋白－卵黃素(vitellin)純化。由離子交換高效液相層析回收的卵黃蛋白含有 B1 、 B2 、 B3 及 B4 四種蛋白質， B1~B3 含有兩個主要組成份(component)，即 A 和 B 胜 胨(polypeptide)，以及 E 、 F 及分子量大於 205 kDa 的次要胜 胨組成，因此卵黃素的組成具有多樣性(polymorphism)。 E 胜 胨由胜 胨圖析及西方點墨試驗解析為是 A 和 B 胜胨 的可能前驅物(precursor)。由 SDS-PAGE 電泳圖譜估得 A 、 B 及 E 等三種胜胨的分子量分別為 83.1 、 88.8 及 147.8 kDa ，前二者的分子量更進一步以飛行時間質譜儀析(MALDI-TOF mass spectrometry)測定為 95.9 及 106.9 kDa 。卵黃素組成份間非以雙硫鍵(disulfide bond)結合。卵黃素在免疫學及電泳上與卵黃前質相似，雌蝦的肝胰臟被解析出為其卵黃前質生成的可能部位，而其卵黃前質則由 vc1 、 vc2 和 vc3 三種胜胨所組成，分子量分別與 A 、 B 及 E 者相當。 A 及 B 分別與 vc1 、 vc3 ，和 vc2 、 vc3 呈現免疫相關性， vc3 可能即為 vc1 和 vc2 的前驅物，在分子結構上可能與卵細胞內的 E 胜胨相同或相近。卵黃素主要組成份的部份胺基酸序列經蛋白質定序儀分別予以定序，而卵黃前質與卵黃素間轉進(processing)關係則在文內有所探討。

關鍵詞：基質輔助雷射去吸附及離子化－飛行時間質譜儀析，胜 胨圖譜，卵黃素，卵黃前質。

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