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Cloning and characterization of hemolymph clottable proteins of kuruma prawn (*Marsupenaeus japonicus*) and white shrimp (*Litopenaeus vannamei*)[☆]

Winton Cheng^a, Inn-Ho Tsai^{b,c,**}, Chang-Jen Huang^{b,c}, Pei-Chi Chiang^a, Chia-Hsiung Cheng^b, Maw-Sheng Yeh^{d,*}

^aDepartment of Aquaculture, National Pingtung University of Science and Technology, Pingtung, Taiwan

^bInstitute of Biological Chemistry, Academia Sinica, Taipei, Taiwan

^cInstitute of Biochemical Sciences, National Taiwan University, Taipei, Taiwan

^dDepartment of Food and Nutrition, Hungkuang University, Sha Lu, Taichung, Taiwan

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cDNA sequences;
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(*Marsupenaeus japonicus* and *Litopenaeus vannamei*)

Abstract

The hemolymph clottable protein (CP) of *Marsupenaeus japonica* (designated as Mj-CP) was purified by a DEAE anion-exchanger and a Sepharose CL-6B gel filtration column. In the presence of Ca²⁺, it formed stable clots in vitro upon the addition of the hemocytes lysate containing transglutaminase. Results of gel filtration chromatography and SDS-PAGE indicated that Mj-CP mainly existed as disulfide-linked homodimers of 390 kDa. Specific primers were designed; PCR as well as RACE help to clone and sequence Mj-CP cDNA of 5660 bp. The predicted CP-precursor contains a signal peptide followed by a subunit of 1671 amino acids (isoelectric point 5.6), including two RGD motifs and three potential N-glycosylation sites. Mj-CP is structurally 80% and 38% identical to the CPs of tiger shrimp and crayfish, respectively. Likewise, CP cDNA of white shrimp (*Litopenaeus vannamei*) was also cloned and sequenced; the predicted CP has 1666 amino acid residues and an isoelectric point of 5.2. Both CPs bear potential transglutaminase cross-linking sites, i.e. seven Ser–Lys–Thr repeats near the N-terminus, a Ser- and Gln-rich region in the middle, and polyGln ($n = 8–11$) near the C-terminus. Phylogenetic analyses of crustacean CPs and vitellogenins revealed divergent evolution of the two protein families. By RT-PCR, the subcuticular epidermis was identified as one of the major tissues that express CP in *M. japonica*. © 2007 Elsevier Ltd. All rights reserved.

Abbreviations: CP, clottable protein; Mj, *Marsupenaeus japonicus*; Lv, *Litopenaeus vannamei*; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; VTG, vitellogenin.

[☆]Note: The cDNA sequences of Mj-CP and Lv-CP were deposited in GenBank under accession Nos. [DQ915453](https://www.ncbi.nlm.nih.gov/nuccore/DQ915453) and [DQ984182](https://www.ncbi.nlm.nih.gov/nuccore/DQ984182), respectively.

^{**}Also corresponding author.

*Corresponding author. Fax: +886 42631 9176.

E-mail addresses: bc201@gate.sinica.edu.tw (I.-H. Tsai), yms1123@sunrise.hk.edu.tw (M.-S. Yeh).

1. Introduction

Efficient immune systems and blood coagulation are of vital importance for the survival of both vertebrates and invertebrates. Hemolymph coagulation is part of the crustacean innate immune response; it prevents leakage of hemolymph from sites of injury and dissemination of invaders such as bacteria throughout the body [1]. Coagulation systems of invertebrates are distinct from those of vertebrates. Previous studies showed that diverse coagulation pathways have evolved in marine arthropods, e.g. the coagulation system in horseshoe crab [2] is different from that in crustaceans [3,4]. It has been shown that polymerization of clottable proteins (CPs) by the hemocyte transglutaminases triggers hemolymph clotting in insects [5] and crustaceans [6,7].

Several crustacean CPs have been purified from the hemolymph and characterized [8–14]. They are homodimeric glycoproteins of about 400 kDa and bear no structural similarity to fibrinogen or the horseshoe crab coagulogen [2]. Stable clots of the CPs may form in the wound area by Ca^{2+} -dependent transglutaminase from the hemocytes [15–18]. Crustacean CPs might also be involved in lipid transport [6,13,19] in addition to the clotting role. Some features responsible for lipid binding have been identified in the low-resolution crystal structure of CP of lobster (*Panulirus interruptus*) [20].

Kuruma prawn (*Marsupenaeus japonicus*) and white shrimp (*Litopenaeus vannamei*) are economically important species cultured in Taiwan and southeastern Asia. Herein, we solved the complete nucleotide sequences of CP cDNAs of both shrimp species and characterized the purified CP of *M. japonicus*. Being the substrates for hemocyte transglutaminases, both CPs contain structural elements involved in the cross-linking reactions and these were examined and compared with those in other crustacean CP. CP expression levels in various *M. japonicus* tissues were also investigated by RT-PCR. Moreover, amino acid sequences for crustacean CPs and vitellogenins (VTGs) [21–26] were subjected to phylogenetic analysis, and their evolutionary relationships were discussed.

2. Materials and methods

2.1. Hemolymph and hemocyte lysate

Shrimps (20–25 g body weight) were purchased from local markets and kept in aerated seawater. The hemolymph (0.6–1 ml) was withdrawn using a 23 G 2.5 ml syringe containing 0.06–0.1 ml anticoagulant as described previously [27]. Hemocytes were immediately spun down at 300g, 4 °C for 10 min, and the supernatant was pooled for preparing the CP. The pellet was washed once and then hypotonically lysed in 1 ml of 10 mM Tris–HCl (pH 7.5). The hemocyte lysate was centrifuged at 12,000g at 4 °C for 30 min and the supernatant was collected and used in the same day.

2.2. Protein purification and clotting assay

Shrimp plasma was first dialyzed against 50 mM Tris–HCl, 1 mM EDTA, pH 8.0 (TE buffer) for 14 h at 4 °C. It was

subjected to ion exchange chromatography at 4 °C with a TSK DEAE-650 (s) column (2.5 × 9 cm) equilibrated with TE buffer at linear NaCl concentration ranging from 0 to 0.35 M NaCl [28]. The fractions from TSK DEAE-650 (s) column chromatography with clotting ability were further concentrated and subjected to gel filtration chromatography with a Sepharose CL-6B column. The elution buffer was 1 mM EDTA in 50 mM Tris–HCl (pH 8.0); 2.2 ml fractions were collected at a flow rate of 16 ml/h at 4 °C. Protein concentrations were determined by the Bradford method [29] using bovine serum albumin as standard. For clotting assay, the purified sample was dialyzed; then a 0.1 ml aliquot was transferred to a test tube and the hemocyte lysate was added [11]. After incubating for 1 h at room temperature, the test tube was tilted to check whether the sample was clotted.

The native molecular weight of CP of *M. japonicus* (Mj-CP) was also estimated by its elution volume on a Sepharose CL-6B gel filtration column, based on a calibration plot of “ratios of the elution volume to the void volume” versus $\log(\text{molecular weight of the marker})$.

2.3. SDS-PAGE and amino acid sequencing

Purified Mj-CP was subjected to SDS-PAGE [30] with a 5% acrylamide gel, and stained with Coomassie brilliant blue R-250. Enzymatic oligomerization of Mj-CP was analyzed by 2.5% polyacrylamide/agarose gel. Markers used were myosin (212 kDa), β -galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), catalase (57 kDa), and aldolase (40 kDa) from Promega Co., USA.

The N-terminal sequence of purified Mj-CP (3 μg) was determined with a gas-phase sequencer coupled with an on-line HPLC system (Model 477A, Applied Biosystem, USA).

2.4. cDNA synthesis and cloning

Standard procedures in molecular biology were used for the preparation of plasmid DNA, restriction enzyme digestion, and DNA transformation to bacteria [30]. Total RNA was prepared from the gills of kuruma prawn and white shrimp separately, using the RNazol B kit (Biotechx, Friendswood, TX, USA) [31]. mRNA was purified using a QuickPrepR Micro mRNA purification kit (Amersham Pharmacia, Freiburg, Germany). The first-strand cDNA was synthesized using oligo(dT)-primer and random primers, and it was used as templates in subsequent polymerase chain reaction (PCR) [32].

To specifically amplify Mj-CP cDNAs, we used degenerate primers designed based on its highly conserved amino acid sequences: e.g. QPPQVMK (i.e. N-terminal sequence or Mj1), MEEDIQR (Mj2), FMEEDIQ (Mj3), TFTNQTLE (Mj9), EIDATKC (Mj10) and YDCTEWVY (Mj15) (Table 1) and Lv1–Lv11 as shown in Table 2. The PCR products were purified and ligated into pGEM-T (Promega). Each clone was sequenced using AmpliTaq DNA polymerase and fluorescent dideoxynucleotides on a DNA sequencer (Applied Biosystems Model 310) [33].

2.5. Rapid amplification of cDNA ends (RACE)

The 5' ends of Mj-CP and Lv-CP mRNAs were completed by a 5'-RACE system (Version 2.0, Invitrogene, Carlsbad, CA,

Table 1 Primers used in the cloning of Mj-CP gene

Primers	Sequence	Tm (°C)	Position
Mj1	5' CARCCNGGNCTNGARTAYCARTAY 3'	56.8	127
Mj2	5' TTYATGGARGARGAYATHCA 3'	48.7	637
Mj3	5' CGYTGDATRTCYTCYCCAT 3'	49.6	640
Mj4	5' CGACAGGTGACCTGGAGACGTCTGGCC 3'	62.8	751
Mj5	5' GTTCTCCTGACGGACCAGAGTGTGTCC 3'	64.3	794
Mj6	5' GTGTACTTGCTGCTGTGGATCTGCAGG 3'	65.8	855
Mj7	5' GAGCTCCGTGGCATCCTACCTCATCC 3'	64.3	900
Mj8	5' GGACACGCACCGAAGGATCGAACAGG 3'	64.3	931
Mj9	5' TCNAGNGTYTGRRTNGTRAANGT 3'	55.2	1016
Mj10	5' GARATHGAYGCNACNAARTG 3'	51.1	4081
Mj11	5' TCCTTGATGATGGACACGGCATAAGG 3'	59.5	4138
Mj12	5' GAGTGAAGAATGGATTCACCCACACC 3'	59.5	4176
Mj13	5' GCTACTACACGGACTCCAGCCTCTTCG 3'	64.3	4676
Mj14	5' ACATCTGCACTCACTTCACTTACGAGC 3'	59.7	4784
Mj15	5' TANACCCAYTCNGTRCARTCRTA 3'	55.4	4841

R = A/G, S = C/G, Y = C/T, H = A/T/C.

Table 2 Primers used in cloning of Lv-CP gene

Primers	Sequence	Tm (°C)	Position
Lv1	5' TTYATGGARGARGAYATHCA 3'	48.7	639
Lv2	5' TGGAAGACTTGCTAGTCTTCGATGTGG 3'	59.7	694
Lv3	5' GAACGTTCCGACAGTTGACCTGGAGACG 3'	64.3	766
Lv4	5' GAAGGATCGAGCAAGCCGTGGTGGAGG 3'	65.8	949
Lv5	5' TCNAGNGTYTGRRTNGTRAANGT 3'	55.2	1023
Lv6	5' GARATHGAYGCNACNAARTG 3'	51.1	4068
Lv7	5' GTTCCTTGATGATGGACACGGCATAGG 3'	59.5	4126
Lv8	5' GTGCAGGACACTGGCGTGCGACCGACG 3'	59.5	4226
Lv9	5' CATCCAACGACATGGTAGGACCTAAGG 3'	61.3	4633
Lv10	5' GATGTTGAACGCTACCAGGAGGACTGC 3'	62.8	4752
Lv11	5' TANACCCAYTCNGTRCARTCRTA 3'	55.4	4827

R = A/G, S = C/G, Y = C/T, H = A/T/C.

USA). Briefly, total RNA was isolated from both shrimps. The first-strand cDNA was synthesized by reverse transcriptase (SperScript II RT) using Mj4 primer or Lv4 and 5 µg of total RNA. The RNA template was removed by RNase and purified with S.N.A.P. column (Invitrogene, Carlsbad, CA, USA). The synthesized cDNA was further homopolymeric tailed by terminal deoxynucleotidyl transferase and dCTP, and then amplified with abridged anchor primer (AAP, 5' GGCCACGCGTCTGACTAGTACGGGII GGGIIGGGIIG 3') and Mj3 primer or Lv3 primer. An aliquot of the cDNA products was diluted 1:100 and subjected to nested PCR with abridged universal amplification primer (AUAP, 5' GGCCACGCGTCTGACTAGTAC 3') and Mj2 primer or Lv2 primer. Each of the 35 cycles run on the thermocycler was 94 °C for 30s, 55 °C for 30s, and 72 °C for 1.5 min, except the first and last cycles. The first cycle included an extended (2 min) denaturation period during which polymerase was added, while the last cycle had an extended (10 min) elongation period. PCR products were then cloned into pGEM-T vector (Promega) and sequenced.

2.6. Phylogenetic analysis and hydrophobic profiling

Sequences of crustacean CPs and VTGs were retrieved by program BlastP [34]. Their amino acid sequences were aligned by the program CLUSTALW [35]. A phylogenetic tree was built by the program PHYLIP (<http://www.evolution.genetics.washington.edu/phylip.html>) [36]. The degree of confidence of the lineage at each node was determined by the bootstrap analyses of 1000 replicates [37]. The hydrophobic profile of the Mj-CP sequence was plotted following the method of Kyte and Doolittle [38]. Windows of seven residues were used to calculate the average hydrophobicity for the central residue.

2.7. RNA isolation from various tissues and RT-PCR

Total RNA was isolated from the central nervous system (CNS), gill, heart, hemocyte, hepatopancreas, intestine,

lymphoid organ, eyestalk, sub-cuticular epidermis, and muscle of *M. japonicus* using the RNazol reagent (Biotecx, Friendswood, TX, USA). First-strand cDNA was amplified from 2 μ g total RNA using 10 pmol oligo-dT primer in a 25 μ L reaction containing 30 U RNasin (Promega), 1 mM dNTP, 10 mM dithiothreitol, and 300 U Superscript II (Life Technologies). After incubating at 42 °C for 1 h, 2 μ L of the solution was added to a 48 μ L solution of the primers designed from Mj-CP cDNA sequence (forward, 5' ACTACACGGACTC-CAGCCTCTTCG 3'; reverse, 5' GTAAGTCAGGGTGTAGGAGT-GAGG 3') in 1.5 mM MgCl₂, 0.25 mM dNTP, and 0.5 U ExTaq (Takara Shuzo Co., Japan). After amplifying by PCR at the annealing temperature of 55 °C for 30s (25–40 cycles), the 376 bp PCR products were examined on a 1.2% agarose gel. The primers β -actin-F (5' ACGACATGGAGAAGATCTGG 3') and β -actin-R (5' GAAGCTCGTACGACTTCTCC 3') were used to amplify a 489 bp β -actin fragment, which was used as an internal control [39]. The PCR reaction was the same as that described above.

3. Results

3.1. Purification and characterization

Purification of Mj-CP was achieved by two chromatographic steps (Fig. 1). The apparent molecular weight and purity of eluted proteins were analyzed by SDS-PAGE using 5% gel (Fig. 2A). Fractions 22–50 (peak II) contained mainly hemocyanin, and fractions 63–78 (peak IV) contained Mj-CP. About 15 mg protein from peak IV was dialyzed against TE buffer, and then loaded on a Sepharose CL-6B column equilibrated with the same buffer. A major peak followed by a small shoulder was eluted from the column; it appeared to contain higher molecular weight forms of Mj-CP since the mass of the major peak of Mj-CP was estimated to be about 0.8–1.2MDa based on elution volume from the column (Fig. 1B and inset). The N-terminal sequence of the cross-linked bands excised from the gel could also be identified as Mj-CP. Some native Mj-CP dimers appeared to be cross-linked into tetramers and hexamers during collection and handling of the hemolymph.

3.2. Oligomerization of Mj-CP by hemocyte lysate

To test whether it was the transglutaminase substrate capable of clotting, 1 μ L hemocyte lysate was added to 10 μ g purified Mj-CP in 30 μ L of 50 mM Tris-HCl (pH 8.0) with 0.1 M NaCl, and reaction was started by the addition of 1 μ L 0.3 M CaCl₂. After incubation for 1 and 5 min, the reaction was stopped and analyzed by SDS-PAGE under reducing condition (Fig. 2B). It was found that Mj-CP oligomerized in 1 min while most of the protein transformed into insoluble clots in 5 min.

3.3. Full sequence of Mj-CP

The N-terminal 1–15 amino acid sequence of purified Mj-CP was determined as LQPGLEYQYDYDAXV by automated sequencing. It shares up to 80% similarities to those of CPs of other shrimps [10–14,26]. It was found that the antiserum

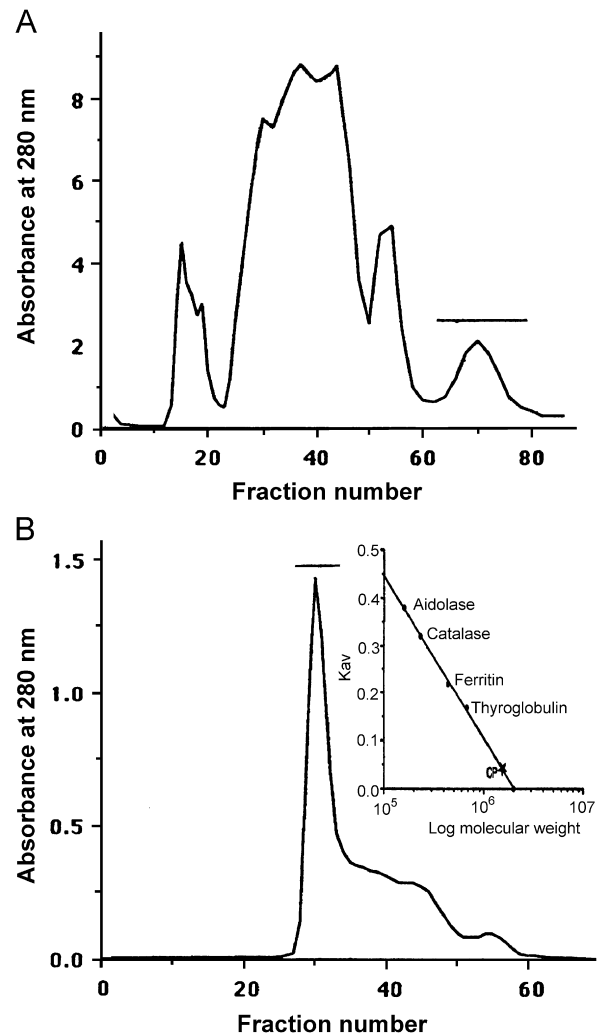


Fig. 1 Purification of Mj-CP at 4 °C. (A) DEAE ion exchange chromatography of *M. japonicus* plasma. Elution was carried out with 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA followed by a gradient of 0–0.4 M NaCl; fractions of 4 ml were collected. (B) Further gel filtration of Mj-CP on Sepharose CL-6B equilibrated with 1 mM EDTA in 50 mM Tris-HCl (pH 8.0). Fractions of 2.2 ml were collected. Bars denote fractions capable of clotting. Inset: the native molecular weight of Mj-CP was estimated by its elution volume from this column, as calibrated with elution volumes of four marker proteins.

against *P. monodon* CP could cross-react with Mj-CP in immuno-diffusion and Western blot analyses [7]. Degenerate PCR primers (Table 1) were useful for cloning Mj-CP. Three clones encoding the N-terminal and the C-terminal sequences of Mj-CP were isolated (CP1, CP2, and CP5, Fig. 3). An internal cDNA fragment of 3208 bp was also amplified by PCR (CP4, Fig. 3). The full length of the CP cDNA was completed by 5' RACE and 3' RACE (CP3 and CP6, Fig. 3). Six clones were isolated and sequenced to complete the entire 5660 bp cDNA sequence of Mj-CP (accession no. DQ915453). According to Kozak's rule [40], the putative initiating codon ATG is at nucleotide 82. The open reading frame contains a 14-residue signal peptide followed by Mj-CP of 1671 amino acids, which has a predicted mass of 188,420 Da and an

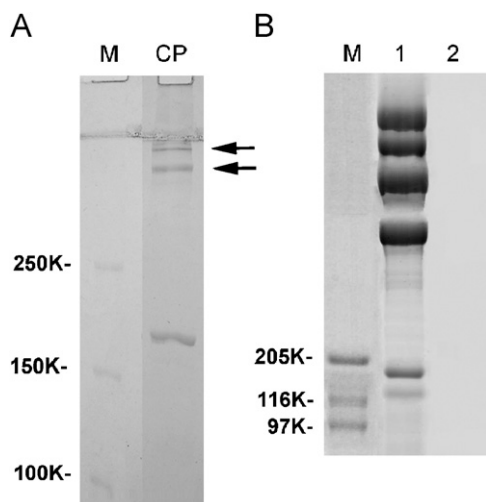


Fig. 2 SDS-PAGE analyses. (A) Purified Mj-CP on 5% polyacrylamide gel; (B) polymerization of Mj-CP by hemocyte lysate and Ca^{2+} . A 2.5% polyacrylamide/agarose gel was used; lane 1, purified Mj-CP was clotted by hemocyte lysate; lane 2, supernatant after centrifugation of the clotted sample. Arrows indicate cross-linked form of CP.

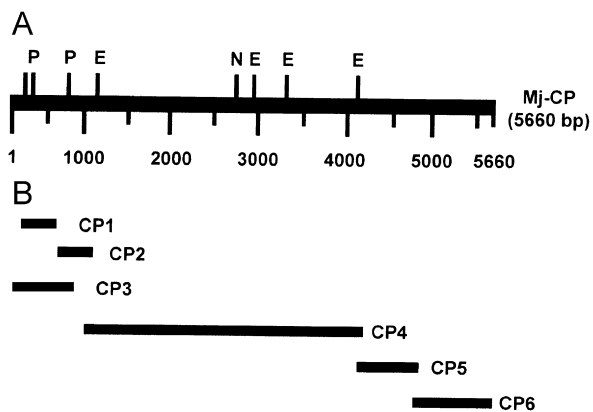


Fig. 3 Restriction enzyme map (A) and cloning strategy (B) of Mj-CP cDNA. Oligonucleotides CP1, CP2, CP4, and CP5, were obtained by PCR. CP3 and CP6 were obtained by 5' RACE and 3' RACE, respectively. Abbreviations of restriction enzyme sites are denoted as E, *EcoRI*; N, *NcoI*; P, *PstI*.

isoelectric point of 5.6 (Fig. 4). However, the N-terminal sequence of Mj-CP was deduced as LQPGLEYQYRYSARV based on its cDNA, while it was determined as LQPGLEYQYDYDAXV by protein sequencing. The discrepancies at residues 10 (D/R) and 12 (D/S) may be due to errors in the identification of PTH-amino acid released in the two steps since residues 10 and 12 are conserved as R and S in the other three shrimp CP, or possible geographic variations of the shrimps due to different batches of *M. japonicus* specimens used in cloning and hemolymph collection.

3.4. cDNA cloning of Lv-CP

Similar cloning strategy and primers (Table 2) were successfully used to clone the Lv-CP gene of white shrimp,

L. vannamei. The cDNA has a total length of 5541 bp, including an open reading frame of 4998 bp (DQ984182). According to Kozak's rule [40], the putative initiating codon ATG is at nucleotide 83. The open reading frame is predicted to encode a protein of 1666 amino acids (188,064 Da) and an isoelectric point of 5.2 (Fig. 4). The amino acid sequences of several fragments previously published for Lv-CP [41] also match the sequence predicted from Lv-CP cDNA.

3.5. Sequence comparison and phylogenetic analysis

Amino acid sequences of four crustacean CPs so far solved were aligned in Fig. 4. The predicted Mj-CP sequence is 80.6%, 80.3%, and 37.5% identical to those of CPs of white shrimp, tiger shrimp, and crayfish, respectively. These CPs appear to be homologous to insect VTGs and share low sequence similarities with other lipoproteins (e.g. mammalian apolipoprotein B and microsomal triglyceride transfer protein) [10,25]. Like insect VTGs, the crayfish CP contains a conserved but possibly non-functional proteolytic processing site RXRR [25]; however, this site is absent in the CPs of penaeid shrimps.

A phylogenetic tree was constructed based on amino acid sequences of four CPs and the VTGs of various species including representative vertebrates, insects, and shrimps (Fig. 5). High bootstrap values on all nodes ($\geq 78\%$) suggest robustness of the tree. All CPs are in a distinct cluster and penaeid CPs are more closely related to each other than to crayfish CP in this tree, suggesting that crustacean CP genes and VTG genes have evolved divergently.

3.6. RT-PCR and tissue expression profile

The expression patterns of Mj-CP mRNA in various tissues of *M. japonicus* were analyzed by RT-PCR. As shown in Fig. 6, a 376 bp DNA fragment could be amplified, in contrast to the negative control. The expression levels were highest in subcuticular epidermis, moderate in gill, CNS, eyestalk, heart, muscle, and lymphoid organ, and lowest in hepatopancreas, hemocyte, and intestine (Fig. 6).

4. Discussion

In shrimps, coagulation is initiated by the release of hemocyte transglutaminase, which effectively polymerizes the hemolymph CP to form stabilized gel [17]. Mj-CP was purified (Fig. 1), and it formed stable clots in the presence of Ca^{2+} and hemocyte transglutaminases, as shown by SDS-PAGE analysis of Mj-CP (Fig. 2B). By RT-PCR analysis, Mj-CP was found to be expressed in most shrimp tissues, especially in the sub-cuticular epidermis, gill, CNS, and heart (Fig. 6). The finding was similar to the tissue expression profiles of tiger shrimp CP [43]; the present study further shows that shrimp epidermis is an important expression site of CP.

Mj-CP was eluted from the anion exchanger at higher concentration than most other plasma proteins of *M. japonicus* (Fig. 1A), consistent with its predicted *pI* of 5.6. It was further purified by a Sepharose CL-6B gel filtration column (Fig. 1B). The molecular mass of Mj-CP is estimated to be 390 kDa (Fig. 2), which is slightly smaller

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Mj-CP : MKALILL-LLGACQALQPGLEYQYRYSARVATGIPSLNRQFAAAGIQADVTVQVADFTTAVQFSNVEVGDNLNELLDCDL : 79
Lv-CP : MKVILL-LLGACQALQPGLEYQYRYSARVATGIPSLNRQFAAAGIQADVTVQMAADYSTAVQFSNVEVGDNLNVLDCDL : 79
Pm-CP : MKALILL-LLGACQALQPGLEYQYRYSARVASGIPSLNRQFAAAGIQADVTVQMAADYSVAVQFSNVEVGDNLNVLDCDL : 79
Pl-CP : MKGVILL-LLGACGLHNSNLEYQYRYSGRVASGIPFLKSYSGAGIQADVFLGITSTKVPFFKI-SNTMVGVEYHDEL-EDT : 80

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Mj-CP : RAPLPIEYQPIQEHADLLERPFVRVIMNESGIPFHMGGPPEFVWISNIRKAFVNI FRVPLFVNTESHAI---KLNYSKAVK : 155
Lv-CP : RAPLPIEYHPLEDYVDLLEKPFVRVIMNESGIPFHMGGPPEFVWISNMRKAFVNI FRVPLLVNTERHST---KLNYSKLE : 155
Pm-CP : RAPLPIEYHPLEDYVDLLEKPFVRVIMNESHVFSYMEGPEEPINISNVRRCFVNI FRVPLFVSTEDHST---KLNYSKAVE : 155
Pl-CP : RAPLPIEYHPVEGKELLEKPFQALPGEORGEELVGGPAPAVITNKKAIWRASISIPIMANAGRQVDAFKRRTVSHNG : 160

                                     -> -> -> -> -> ->
Mj-CP : ESLVGRCONWYSSLRFFANQAKINQREHFMEEDIQR-----DAYSSSYTKTKSKTS--KTSKTS--KSSRSTKSGT : 224
Lv-CP : ETVVGRCONWYSSLRFFENLAEEINQREHFMEEDIQR-----DAYSSSYTKTKSKTS--KSSKTSKSTPSKTSKSGT : 226
Pm-CP : ETLVGRCONWYSSLRFLFNLAEEVNVQREHFMEEDIQR-----DAYSSSYTKTKSKTSKTSKTSKTSKTSKTSKSGT : 228
Pl-CP : TFIHEDCMCYSMIHLNKEEARENROREAFMEEDIQREEQDESDFRFTSSKVPSTKSTKTSKTKTKTGTKTKTKTKTGT : 240

Mj-CP : -----KTSFGHLSNVPCIEDTLWVRRRTDFDMCENLVSLQIHSSKYTEEVIQRSSVASYLIRG-DTHRRIEQAVLEGSI : 298
Lv-CP : -----KTSFGQANVPHIEDTLWVRRRTDFDMCENLVSLQIHSSKYTEEDIQRSSVASYLIRG-DTHRRIEQAVVEGTI : 300
Pm-CP : -----KTSFGQANVPHIEDTLWVRRRTDFDMCENLVSLQIHSSKYTEEDIQRSSVASYLIRG-DTHRRIEQAVVEGTI : 302
Pl-CP : KNTGKGAFAKLTTPERIDNTLWMSRITVDFDSCELDLVKWRTHGNKIKDKIKRQTSIGTYLIRGNARDTRVEYAVIEGSI : 320

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Mj-CP : SVITNKEHEHVDFTFNQTLLEKAVREVGGPIITVYDVEPHYDNDLIER-----POGDFEIVPLEQVIFGRPIITQIV : 371
Lv-CP : SVITNKEHEHVDFTFNQTLLEKAVREVGGPIITVYVHNHYNHHYNIER-----POGDFEIVPLEQVIFGRPIITQIV : 373
Pm-CP : TVITNKEHEHVDFTFNQTLLEKAVREVGGPIITVYDVEPHYDNDLIER-----POGDFEIVPLEQVIFGRVITQIV : 375
Pl-CP : TVITGTSQSEHISTFTFNQTLLEKRVFRIRIQNLFSFDVETVTHQSFFHEVDKFDVSEQEGHIIHPSLEGLVTCGRITQGV : 400

Mj-CP : NAFVYIKKTVDDLSHRMERPAKPENLAYIIGRLVEAVANLDYQHIOQTLNHFESQSTLHR--YIFQVLFVAGTEPSV : 449
Lv-CP : NCIIMDYIKKSIDDLSHRMEHYPAKPENLAYIIGRLVEAVANLDYPHIQTLYSHFFGKGNLQK--YIFQVLFVAGTEPSV : 451
Pm-CP : NAFVYIKKTVDDLSHRMEHYPAKPENLAYIIGRLVEAVANLDYPHIQTLYTHFDGKGTMR--YIFVQVLFVAGTEPAV : 453
Pl-CP : TDKTLIRLRLKMLVKRTTTHYSTDFLVKDMSTLVDPASISRPDIEALCSQIQHEELHAKKLEIYELVLTAGTEPAI : 480

Mj-CP : TFVAVNIREPQKTA-----FYSIDVNVVRFAPIPKILN---LILSEBEGLPSTG-LMNFANLANKACLSEDRKHF : 518
Lv-CP : TFALGHLNNIPYKT-----IYDSIGVNLRSAPILKIMT---SLIDGEBGVVHSTG-VMNFPATLAHDAACLEDKHF : 520
Pm-CP : NFAVNLSDIIFYVT-----FYESLGVSLRPPAPIPKIMH---SLINGEBGLHSTG-LMNFATLAHDAACLEDKHF : 522
Pl-CP : RYLLPKIKDPBERKINVASVKTIENFHTSVKNEELLFQIDLEQTLTWDEBNQOFKMTLLNLASLAKQLCLSSDKWES : 560

Mj-CP : YFDYSCGPKSSCDPELIISTFIPYLVRGLGNQETDIWQRLTYLQALSNLGTPQTINVLKPIILGVETN--VFMRNTNAIW : 596
Lv-CP : YFDYSCGPKSSCDPELIINTFIPYLVRNGLNHEKDVNQRILYQALSNLGTPQTINVLKPIILGVETN--IFMRNTNAIW : 598
Pm-CP : YFDYSCGPKSSCDPELIITNFIPYLVRGLGNQGVNQRILYQALSNLGTPQTINVLKPIILGVETN--IFMRNTNAIW : 600
Pl-CP : YGNTCDGQHTCTEDFLIENSLEFLIQGVQDDSSLIWRLVITQALANLGIPTIDVLKHFALGDKPSTNHIIVRTNAIL : 640

Mj-CP : SLSAYNMKALSQIYDILMPIIENKGEQFEIRNIAFLTLATWCPGSSWQQLAVSTWHDPSQFANFVTTIYSIARSH : 676
Lv-CP : SLSAYNMKALSQIYDILMPIIENKGEQFEIRNIAFLTLATWCPGHAWQQLAVSTWHDPSQFANFVTTIYSIADSH : 678
Pm-CP : SLSAYNMKALSQIYDILMPIIENKGEQFEIRNIAFLTLATWCPGHAWQQLAVSTWHDPSQFANFVTTIYSISDSH : 680
Pl-CP : GLSGYVLPEPACSSVFGILMPEVFENTGEHNVAVAFLAMMFKESLAWWERIATSTRDPSSQVAVVSTTIFSLANAN : 720

Mj-CP : TKLABTVSRVERLCKPAAPASVLSHTNFFLHEYLFSEEHGSRVNFASFVHGAIPBEVFLRLCTEFFYGYTKVKRIDAQ : 756
Lv-CP : TKLABTVSRVKHLCKPAAPASVLSHTNFFLHEYLFSEEHGSRVNFASFVHGLPBEVFLRLCTEFFYGYTKVAREDAQ : 758
Pm-CP : TKLABTVSRIKHLCKPAAPASVLSHTNFFLHEYLFSEEHGSRVNFASFVHGAIPBEVFLRLCTEFFYGYTKVKRIDAQ : 760
Pl-CP : YPMKSIAGRNVHLAKPPTHVSLTHSSMIFLNDVLYNCKSQNYENEGWASNOGLFESKILYQLQLRTHLGLTKFRVVFVG : 800

Mj-CP : QRLGFHLVQIMRQAMAK-PSKPKPOSSAQEIVAGMWEELKEKIFGSAPETDSQLQLWKLDRILYLTIESDKGFPYTPHL : 835
Lv-CP : QRLGLYLMQFLRQKMTK-PLKRPPOSSAQEIVAGMWEELKEKLEFESPESESLLWLKLDRLIYVAVNDRMEGNLIP-- : 834
Pm-CP : QRLGYNLMQMKRMTK-PKRPPOSSAQEIVAGMWEELKEKIFGSAPESASDATLWLKLDRLIYVALQYHMEGAPTF-- : 837
Pl-CP : QAEINKIKKAYSSVFQNKILRTKSKDAETVQEMFDEIVDQLGVIHTKREADMSFYIKITDFISLTPVLSSPE-NILAIL : 879

Mj-CP : LDQVTTGLFHSLEQFDRDLNTEALPTEIGIPFSVQYIPEADAWNINNGPEYSITSGKIRGNVGFIFDSSQVQYVDART : 915
Lv-CP : -----TTVLAQLFVYDRDLNTEALPTEIGIPFSVQYIPEADAWNINNGPEYSITSGKIRGNVGFIFDSSQVQYVDARA : 909
Pm-CP : -----YGMYSQKPFYDRDLNTEALPTEIGIPFSVQYIPEADAWNINNGPEYSITSGKIRGNVGFIFDSSQVQYVDARA : 912
Pl-CP : SEEDRDNMENS-PHIERSVDYTEVLPDLELPEMIHYTBENAYLVDWKVTKSQSIPT-KRRDFALTLDFSCDNTLETKT : 957

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Fig. 4 Alignment of crustacean CP sequences. The sequences of Mj-CP (DQ915453) and Lv-CP (DQ984182) and those from tiger shrimp (Pm-CP, AAF19002) and crayfish (Pl-CP, AAD16454) were compared. Gaps (-) are added to improve alignment. Residues that are identical in three sequences are shown in black. The Lys-rich and Gln-rich regions are boxed. Arrowheads indicate the Ser-Lys-Thr repeats. Asterisks and bars above the residues denote the conserved N-glycosylation sites and conserved RGD motifs, respectively.

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Mj-CP : RLPSNRPVAVASGLYADTQLVLPNVHASIDSYSQEIQLSLEPTNTKRIQVVKFEFVPTVLENNYPSDVLVENEYKPI : 995
Lv-CP : RVPWSNRPVAVASGLYADTQLVLPNVHASFDIYSQEIQLSLEPTNTKRMQVVKFEFVPTVLENNYPSDVLVENEYKPI : 989
Pm-CP : RVPWSKRPVAVASGLYADTQLVLPNVHASFDIYSQEIQLSLEPTNTKRIQVVKFEFVPTVLENNYPSDVLVENEYKPI : 992
P1-CP : LLPWSKRFKFGALHNVVSLVLFVSHVHTNLDNEHSELKVTLEEQDSSKRVQLFNHNYEFTVWTGPFETALHTQQSEYKPI : 1037

Mj-CP : LHGEVPLFNDKHQAFFPSDVLGWVEVATDGDVHHTPSIYEIITQLFSAHT-TSHIWEQNI VFDPTLSTTRVVSFTFNVST : 1074
Lv-CP : LHGEVPLFNDKHQAFFPSDVLGWVQVATEGDIHHTPSIYEIITQLFSAHT-TSHIWEQNI VFDPTLSTTRVVSFTFNVST : 1068
Pm-CP : LHGEVPLFNDKHQAFFPSDVLGWVQVATEGDIHHTPSIYEIITQLFSAHT-TSHIWEQNI VFDPTLSTTRVVSFTFNVST : 1071
P1-CP : SAFFLETQSVQRIEYVPSVVGSLVWEASWARDFPSQFDPLHFSSDDEDFPFMPAYKSMDYSLVLDHAASSTRSITFTBAYVTL : 1117

Mj-CP : GSSGSLDRGDYDD-GGVSCNS--EYELGQFSQVGGVDDDL--LEVDSFG----TQSQTRERIVRLQOALIVS-GGHVRT : 1145
Lv-CP : GGSGLVDRGDYDD-GGVSCNS--EYELGQFSQVGGVDDDL--LEVDSFG----TQSQTRERIMRLQOALIAS-GGHVRT : 1140
Pm-CP : GSSGSLDRGDYDD-GGVSCNS--EYELGQFSQVGGVDDDL--LEVDSFG----TQSQTRERIMRLQOALIAS-GGHVRT : 1143
P1-CP : EKAEDIQQENEFQEGGETQCAADYVDFSQTHVGALEFQFGQGETBSFGQEQQQTETSNORIVQLQDFMPITGIVRT : 1197

Mj-CP : VSVVEVLEKGTPTRN--YEASVTWAISSASARSSKQVQVTLMKHPAVGVLEEAHTICLNAQIVKPHFNPFETEDVLTANYH : 1224
Lv-CP : VSVVEVLEKGTPTRN--YEASVTWAISSASARSSKQVQVTLMKHPAVGVLEEAHTICLNAQIVKPHFNPFETEDVLTANYH : 1219
Pm-CP : VSVVEVLEKGTPTRN--YEASVTWAISSASARSSKQVQVTLMKHPAVGVLEEAHTICLNAQIVKPHFNPFETEDVLTANYH : 1222
P1-CP : VSMGVEVLEKATKKKSYESVVTWAISSASARSSKQVQVTLMKHPAVGVLEEAHTICLNAQIVKPHFNPFETEDVLTANYH : 1277

Mj-CP : STVKAELYDEVTCEEASVLSLEASFDVSNVATERVKEAIDKSCD--YSTYAPGIDIITSPLYDHSHTATWTPDFPDYLNK : 1303
Lv-CP : STVKAELYDEVTCEEAPVLLLEASFDVSNDAKERVKEAIDKSCD--YSTYAPGIDIITSPLYDHSHTATWTPDFPDYLNK : 1298
Pm-CP : STVKAELYDEVTCEEAPVLLLEASFDVSNVATERVKEAIDKSCD--YSTYAPGIDIITSPLYDHSHTATWTPDFPDYLNK : 1301
P1-CP : TTVTAELYDEVTCEEASVLSLEASFDVSNVATERVKEAIDKSCD--YSTYAPGIDIITSPLYDHSHTATWTPDFPDYLNK : 1357

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Mj-CP : LTYVVDLIRASLSKKEFDHTTAHYDARVEIDATKCIETGLWTVRTEKPYAVSIRKELIIPAWANPIFTCPPLATTLTY : 1383
Lv-CP : LTYVVDLIRASLSKKEFDHTTAHYDARVEIDATKCIETGLWTVRTEKPYAVSIRKELIIPAWANPIFTCPPLATTLTY : 1378
Pm-CP : LTYVVDLIRASLSKKEFDHTTAHYDARVEIDATKCIETGLWTVRTEKPYAVSIRKELIIPAWANPIFTCPPLATTLTY : 1381
P1-CP : LSYVHDDIIQHELYSVTTYDHTAINPEHTIDIDATKCKCNKWTENREREMVAVATGSPVWILKDFLGPDEIRKVFYD : 1437

Mj-CP : DLAVGRTPVSCVTEETKVRTFDAKEFAYEPSECWNAMSIDMSPNERG-----MLVRRHTGQWEVRIIWHMREGLVDFDISP : 1457
Lv-CP : DLAVGRTPVSCVTEETKVRTFDAKEFAYEPSECWNAMSIDMSPNERG-----MLVRRHTGQWEVRIIWHMREGLVDFDISP : 1452
Pm-CP : GLTVGRTPVSCVTEETKVRTFDAKEFAYEPSECWNAMSIDMSPNERG-----MLVRRHTGQWEVRIIWHMREGLVDFDISP : 1455
P1-CP : NFVGRTPVSCVTEETKVRTFDAKEFAYEPSECWNAMSIDMSPNERG-----MLVRRHTGQWEVRIIWHMREGLVDFDISP : 1517

Mj-CP : TNFLVNGEPEAKGTDNRYTVLHHEDSHTIVFESGTSIKVSKDVEFLMAMYHRRHTAGLCCNFDGEPNSNDMVGPKGCYTD : 1536
Lv-CP : TNFLVNGEPEAKGTDNRYTVLHHEDSHTIVFESGTSIKVSKDVEFLMAMYHRRHTAGLCCNFDGEPNSNDMVGPKGCYTD : 1531
Pm-CP : TNFLVNGEPEAKGTDNRYTVLHHEDSHTIVFESGTSIKVSKDVEFLMAMYHRRHTAGLCCNFDGEPNSNDMVGPKGCYTD : 1534
P1-CP : SQLLVNGEPEAKGTDNRYTVLHHEDSHTIVFESGTSIKVSKDVEFLMAMYHRRHTAGLCCNFDGEPNSNDMVGPKGCYTD : 1597

Mj-CP : SSLFALSWSAPGEGCASFI FSGKKRVECYDICTHFTYEPTGVTHSDALYDCTEWVYHERTEGHHYHCKALSPMPVCRPE : 1616
Lv-CP : GHLFALSWSAPGEGCAAFNFKSLKRDVERVQEDCPHFAHEPTGVAHSDALYDCTEWVYHERTEGHHYHCKALSPMPVCRPE : 1611
Pm-CP : GPLFALSWSAPGEGCASFMYKLNLRDVERVQEDCPHFAHEPTGVAHSDALYDCTEWVYHERTEGHHYHCKALSPMPVCRPE : 1614
P1-CP : PALFALWMTTKGEGCAQSLRSKRAVENFQVCPREPHIPTGVSHPNVLYDCTEWVYHERTEGHHYHCKALSPMPVCRPE : 1677

Mj-CP : CVANSPITTSVEYEKRETEGHQQAQQQQQ--VEGTQNDCECFPHSYTLTYPSSCVPH : 1671 identity
Lv-CP : CVANSPITTSVEYEKRETEGHQQAQQQQQ--VEGTQNDCECFPHSYTLTYPSSCVPH : 1666 80.6%
Pm-CP : CVANSPITTSVEYEKRETEGHQQAQQQQQ--VEGTQNDCECFPHSYTLTYPSSCVPH : 1670 80.3%
P1-CP : SKAARTFAKPLDYDQVFCQRRNRT-----LECHKHLLTSEFAACVPH : 1721 37.5%

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Fig. 4 (Continued)

than those reported for CPs of lobster [8], white shrimp [41], and crayfish [4], but slightly higher than that of tiger shrimp [6]. In analogy to the tiger shrimp CP [17], purified Mj-CP was a glycoprotein, high-mannose type of N-glycans could explain for the mass increase of 13 kDa for the predicted CP dimer protein of 377 kDa. A minor 180 kDa degradation form of Mj-CP, bearing identical N-terminal sequences as the native 195 kDa subunit, was detected in purified Mj-CP. Similar degraded forms were also found during purification of crayfish CP [14] and tiger shrimp CP [7].

Apparently, the CP genes of marine penaeid shrimps were highly conserved. Similarity between sequences of tiger

shrimp CP and Mj-CP is about 80% (Fig. 4). Their protein hydrophobicity profiles were also similar [10], and no extensive hydrophobic region or membrane-spanning domain can be identified in both molecules (Fig. 7). By immunodiffusion, we showed that the hemolymph of *M. japonicus* could cross-react with the antibodies against tiger shrimp CP [6]. As a substrate for hemocyte transglutaminase, shrimp CPs contain lysine and glutamine side chains to be cross-linked into isopeptide bonds. According to the substrate specificity of mammalian transglutaminase [44–46], we predict that special regions of the shrimp CPs probably are involved in the clotting reactions: (a) a Lys-rich

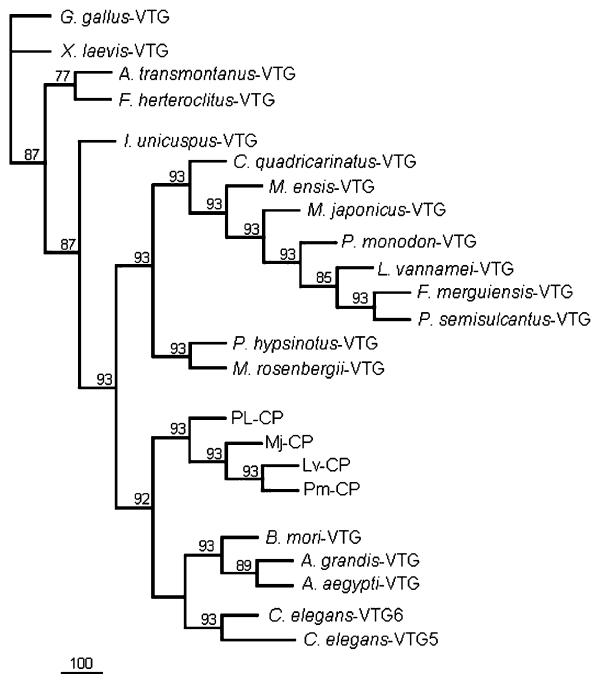


Fig. 5 Phylogenetic tree based on the amino acid sequences of representative VTGs and CPs. Species and GenBank accession nos. for the VTGs are white sturgeon, *Acipenser transmontanus* (AAA87392); yellow fever mosquito, *Aedes aegypti* (AAA18221; S46404); boll weevil, *Anthonomus grandis* (Q05808); silkworm moth, *Bombyx mori* (BAA06397); nematode, *Caenorhabditis elegans* (NP_508589 and AAC04423); mummichog, *Fundulus heteroclitus* (AAA93123); chicken, *Gallus gallus* (CAA31942); lamprey, *Ichthyomyzon unicuspis* (AAA49327); nine shrimp species including *Fenneropenaeus merguensis* (AAR88442), *Litopenaeus vannamei* (AAP76571), *Marsupenaeus japonicus* (BAD98732), *Macrobrachium rosenbergii* (BAB69831), *Metapenaeus ensis* (AAT01139), *Penaeus monodon* (ABB89953), *Penaeus semisulcatus* (AAL12620), *Pandalus hypsinotus* (BAD11098), and *Cherax quadricarinatus* (AAG17936); frog, *Xenopus laevis* (CAA68433). The numbers at the nodes are bootstrap values.

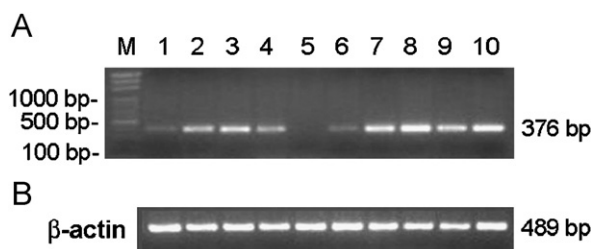


Fig. 6 Transcription of Mj-CP in various tissues. RT-PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide. (A) CP-mRNA levels in Mj-tissues as analyzed by PCR amplification of a 376 bp fragment of the mRNA. Lane 1, hemocyte; lane 2, muscle; lane 3, central nervous system; lane 4, lymphoid organ; lane 5, hepatopancreas; lane 6, intestine; lane 7, gill; lane 8, sub-cuticular epidermis; lane 9, eyestalk; lane 10, heart. (B) As an internal control, β -actin mRNA in each tissue was amplified.

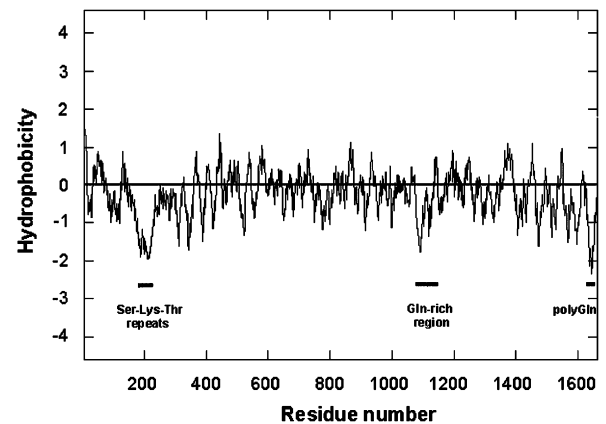


Fig. 7 Hydropathic profile of the Mj-CP. The average hydrophobicities over seven residues were calculated [36] along the entire amino acid sequence of Mj-CP. Potential sites of cross-linking by transglutaminase were marked.

stretch at residue positions 200–250, which contain about seven Ser–Lys–Thr (SKT or TKT) repeats; (b) a Ser- and Gln-rich region at positions 1100–1200; and (c) a polyGln stretch containing 8–11 Gln residues (e.g. XQQAQQQQQVQGTQ) near the C-terminus (Fig. 4). These stretches reside in the major hydrophilic regions of Mj-CP, i.e. residues 175–238, 368–376, 523–573, 1175–1248, 1467–1487, and 1619–1658 (Fig. 7). Thus, they presumably are exposed on the surface of CP molecules and accessible to the transglutaminase, allowing multiple linkages between the CP molecules or their subunits to form.

Remarkably, 10 cysteine residues and a GI/LCG motif (at position 1512–1771 of Mj-CP) are conserved in the C-terminal regions of CPs (Fig. 4). A similar motif (TCGLCG) was conserved in invertebrate and vertebrate VTGs, and in domains D1 and D2 of human von Willebrand factor (vWF) and domain D3 of human mucin 2 [47,48]. These D-domains play essential roles in the polymerization of mucin 2 and vWF, and are involved in the disulfide bonding between vWF subunits [49,50]. Possibly, the C-terminal regions of shrimp CPs are also involved in their dimerization or subunit interactions. There are two RGD motifs conserved in penaeid CPs (Fig. 4), but their functional significance is not clear. The binding of tiger shrimp CP to the hemocytes was not detected [10], although functional RGD-recognizing receptors on the granular hemocytes of crayfish have been reported [51].

Being the major constituent of the egg yolk of almost all oviparous animals, VTGs are huge (200–700 kDa) phosphoglycolipoproteins [22,24,52]. They display sequence similarity to crustacean CP [26] and other lipoproteins such as apolipoprotein B [23,24], and microsomal triglyceride transfer protein [53]. The cladogram in Fig. 7 reveals the following: (i) All VTGs form a cluster and are related to each other according to the known biosystematics of vertebrate and invertebrate species [42]. (ii) All vertebrate VTGs are rather different from the invertebrate VTGs and CPs, and loop-out in the tree. (iii) VTG genes of terrestrial arthropods and nematode are closer to the crustacean CPs than to the crustacean VTGs. VTG and CP genes of marine or freshwater crustaceans probably are paralogous genes, which

have been diverged before the merging of terrestrial arthropods or insects. Thus, CPs possibly were derived from ancient animal VTG-like molecules as suggested previously [10,25], like VTGs of today's terrestrial invertebrates. Apparently, both CP and VTG are indispensable in crustacean species, and the CP and the VTG genes have been separately evolved before insect and crustacean split. In addition, we found that the CP mRNAs are expressed in most *M. japonica* tissues, while the sub-cuticular epidermis appears to be the tissue with the highest level of Mj-CP expression. This finding is in accord with the important role of CP in the defense, healing, and growth of the shrimp.

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