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The hemolymph clottable proteins of tiger shrimp, *Penaeus monodon*, and related species

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Abstract

A clottable protein was purified from the hemolymph of tiger shrimp (*Penaeus monodon*) by sequential DEAE anion-exchange chromatography. The protein formed stable clots in the presence of Ca^{2+} and the transglutaminase in hemocyte lysate. It is thermostable at temperatures up to 66°C. The molecular mass of the clottable protein was determined to be 380 kDa by SDS–PAGE and MALDI–TOF mass spectrometry, and the protein exists as disulfide-linked homodimers and oligomers. The size and amino acid composition of the clottable protein are similar to those of several other shrimps, prawns, lobster and crayfish, and their N-terminal amino acid sequences are 60–80% identical. Monosaccharide analysis of the clottable protein revealed the presence of mannose, glucosamine or *N*-acetylglucosamine and possibly glucose in this glycoprotein of about 5% sugar content. Lipid in the protein upon electrophoresis was hardly detectable with the Oil Red O staining method. In immunodiffusion and immunoblotting analyses, the anti-clottable protein antibodies reacted with the clottable proteins from the penaeid shrimps but not with those from other crustaceans.

Keywords: Clottable protein; Hemolymph coagulation; Purification; Mass spectrometry; Amino acid sequence; Monosaccharide analysis; Immunodiffusion; Shrimp

1. Introduction

Coagulation of the hemolymph is an essential defense mechanism of crustaceans which possess an open circulatory system. It prevents both the loss of body fluid and the entry of opportunistic pathogens. Three types of hemolymph coagulation in crustaceans have been described [29]. Type A coagulation (where a dense hemocyte network is sufficient to seal the wound) is seen in the crab *Loxorhynchus grandis*; type B (hemocyte aggregation is followed by plasma coagulation) occurs in the Maine lobster (*Homarus americunus*); and type C (involving the explosive cells or hyaline cells) is present in the spiny lobster and shrimps [17]. These three types are likely variations of a basic mechanism involving both hemocyte aggregation and coagulation of hemolymph proteins [9,5]. The major hemolymph protein involved in coagulation is designated herein as the clottable protein [10,11,28]. The clottable proteins from the spiny lobster (*Panulirus interruptus*) [11,12] and the crayfish (*Pacifastacus leniusculus*) [21] have been identified to be homodimeric lipoglycoproteins of about 400 kDa. The N-terminal amino acid sequence of the lobster clottable protein is 30% identical to that of *Caenorhabditis elegans* vitellogenins but not similar to those of vertebrate fibrinogens [8]. A calcium-dependent

Abbreviations: HLS, hemocyte lysate supernatant; MALDI–TOF MS, matrix-associated laser desorption/ionization–time-of-flight mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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transglutaminase in the decapod hemocytes (hyaline cells) plays an essential role in the coagulation processes [12,23]. In addition, the Factor XIIIa-like enzyme is known to catalyze the covalent cross-linking between subunits of the clottable protein [13,23]. The tiger shrimp, *Penaeus monodon*, used in this study is an economically important species cultured in Taiwan and southeastern Asia. We wish to understand its basic coagulation system. The shrimp has a high hyaline cell count (54%) [6] and appears to perform type C coagulation. In this study we have purified and characterized the clottable protein from the shrimp and several related species. Biochemical and immunochemical comparisons between the clottable proteins of some decapod species in Taiwan are to be presented.

2. Materials and methods

2.1. Hemolymph and hemocyte lysate

The intermolt tiger shrimps of 20–25 g each were purchased from local markets and kept in aerated seawater. The hemolymph (1 ml) was withdrawn from the ventral sinus located at the base of the first abdominal segment, using a 23G needle syringe containing 0.1 ml anticoagulant (50 mM EGTA, 18 mM Tris–HCl, 0.35 M NaCl, 13 mM KCl, 1.67 mM D-glucose, pH 7.5) [4]. The hemocytes were immediately spun down at $300 \times g$ at 4°C for 10 min, and the supernatant was pooled for preparing the clottable protein. The pellets were washed once with the anticoagulant, then hypotonically lysed in 100 μl of 10 mM Tris–HCl (pH 7.5). The hemocyte lysate was centrifuged at $12000 \times g$ at 4°C for 30 min and the supernatant (HLS) was collected and used for experiments in the same day.

2.2. Protein purification and clotting assay

The tiger shrimp plasma (containing 2.4 g proteins in 30 ml) was dialyzed against 50 mM Tris–HCl, 1 mM EDTA, pH 8.0 (TE buffer) for 14 h at 4°C. It was twice subjected to sequential chromatography at 4°C on a TSK DEAE-650 (S) column (2.5×9 cm) pre-equilibrated with TE buffer alone or with 0.1 M NaCl, and eluted with a step-wise gradient of NaCl. Protein concentrations were determined by the Bradford method [2], using bovine serum albumin as a standard. For clotting assay, each fraction was dialyzed against 50 mM Tris–HCl/0.1 M NaCl, pH 8.0, at 4°C overnight, and an aliquot (200 μg in 200 μl) of each fraction was added to 5 μl HLS and 10 mM $CaCl_2$ [19]. After incubation for 1 h at room temperature, the test tube was tilted to test whether the solution was transformed into a gel state.

2.3. Matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectrometry

The clottable protein was dialyzed against distilled water and adjusted to a concentration of 0.4 μg μl^{-1}. An equal volume of sinapinic acid was added to the sample and 1 μl of this solution was deposited onto the tip of the probes and crystallized under vacuum. The molecular mass of the protein was determined by MALDI–TOF mass spectrometry (Model G2025, Hewlett-Packard, USA).

2.4. Amino acid composition and sequence

Amino acid composition was analyzed after the vapor-phase hydrolysis of the sample at 158°C for 30 min using 7 M HCl/10% TFA/0.1% phenol [3], and the hydrolysate was derivatized to dimethylaminoazobenzenesulfonyl amino acids before separation by reversed-phase HPLC [20]. The hydrolysate of hen egg white lysozyme was used as a reference for the composition analyses. The N-terminal amino acid sequences of the clottable proteins were determined with a gas-phase sequencer coupled with an on-line HPLC system (Model 477A, Applied Biosystems, USA).

2.5. Monosaccharide analysis

The lyophilized clottable protein (50 μg) was dissolved in 100 μl of water. The sample was mixed separately with an equal volume of 4 M trifluoroacetic acid and with two volumes of 4 M HCl before being hydrolyzed at 100°C for 6 h. The hydrolysate was dried in a vacuum by centrifugation, and then analyzed by a anion-exchange chromatography system (Dionex, USA). The quantity of sugar peaks were analyzed with a pulsed amperametric detector and sugar standards were used for calibration of these peaks [15].

2.6. Antiserum

The clottable protein band (190 kDa) in 5% gel after conducting the SDS–PAGE was stained and cut-out for elution. The eluted and lyophilized protein was used for an antigen to immunize New Zealand white rabbits by multiple subcutaneous injections. The polyclonal antibodies were induced and harvested according to the method of Harlow and Lane [16].

2.7. Immunodiffusion

We have collected the hemolymph from the following decapods: *P. monodon*, *Penaeus japonicus*, *Metapenaeus ensis*, *Macrobrachium rosenbergii*, *Procambarus clarki*, *Scylla serrata*, *Charybdis feriatus*, and *Panulirus versi-*

color. Immunodiffusion was carried out on 1% agar gel (2 mm thickness) [26]. The antiserum and hemolymph were placed in different wells and the gel was incubated in a humid chamber for 24 h. The precipitation lines were observed after the gel was stained with Coomassie brilliant blue R-250.

2.8. Immunoblotting

The purified clottable protein, the hemolymph of *P. monodon* and *P. japonicus* were subjected to a reducing SDS–PAGE (5% gel) and transferred electrophoretically to a PVDF membrane [30]. The blotted membrane was allowed to react with rabbit anti-clottable protein antibodies, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG. A color reaction was generated by adding nitro blue tetrazolium and *5*-bromo-*4*-chloro-*3*-indolyl phosphate (Sigma).

3. Results

3.1. Purification of the clottable protein

Purification of the clottable protein from tiger shrimp was achieved by two sequential chromatography on a TSK DEAE-650 column (Fig. 1). The apparent molecular weight and purity of the eluted proteins were analyzed by SDS–PAGE with a 2.5–7.5% gel gradient [25] (Fig. 2). It appeared that fractions 26–34 of peak I contained mainly hemocyanin, fractions 35–69 of peak I contained hemocyanin and the dimeric clottable protein (Fig. 1A). Peaks II and III were found to contain aggregated form of the clottable proteins as revealed by SDS–PAGE (data not shown), and the yield of peaks II and III were 21.4 and 13.6 mg from 30 ml plasma, respectively (Fig. 1A). Fractions 35–69 of peak I (32 mg) were pooled and dialyzed with TE buffer containing 0.1 M NaCl, then re-chromatographed on a DEAE-650 column equilibrated with the same buffer. Hemocyanin was eluted by 0.21 M NaCl in TE, the clottable protein (13.9 mg) was eluted by 0.24 M NaCl in TE, and aggregated form of the clottable protein (2.4 mg) was eluted by 0.3 M NaCl in TE (Fig. 1B). Clottable proteins were also purified from *P. japonicus* [4] and *M. rosenbergii* (Fig. 3) by procedures similar to those described above.

3.2. Molecular weight determination

The purified clottable protein from *P. monodon* migrated as a single band of 190 kDa in SDS–PAGE gel under reducing conditions, and as a band of 380 kDa under non-reducing conditions (Fig. 2). Thus, the protein exists as a homodimer linked by disulfide bonds. A minor band (with a subunit of 180 kDa, presumably a degraded form of the clottable protein) was sometimes observed (Fig. 2). Moreover, the molecular mass of the clottable protein was confirmed to be 380750 ± 800 by MALDI–TOF mass spectrometry (Fig. 4).

3.3. Oligomerization of clottable protein by hemocyte lysate

To follow the time course of the clotting, 1 μl HLS was added to a microfuge tube containing 10 μg purified clottable protein in 30 μl of 50 mM Tris–HCl buffer containing 0.1 M NaCl (pH 8.0), reaction was started by the addition of 1 μl 0.3 M $CaCl_2$. The reaction was stopped at various time intervals by adding 8 μl sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) and heated immediately at 95°C for 5 min. The samples were subjected to SDS–PAGE under reducing condition on a 2.5–7.5% gradient gel. The higher oligomeric forms increased rapidly with incubation time, while the band of the 190 kDa subunit decreased concomitantly. For example, the 380-

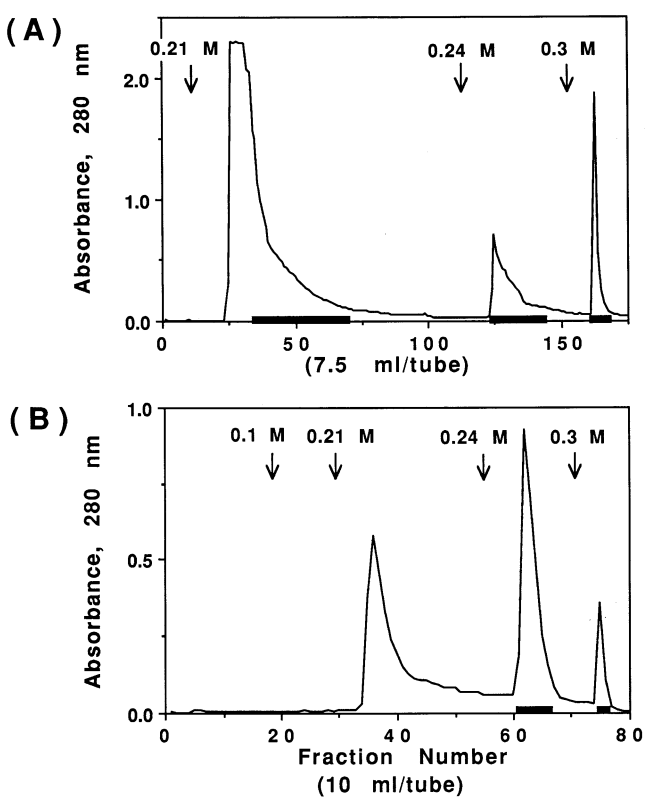

Fig. 1. Purification of the clottable protein from *P. monodon*. (A) First DEAE anion-exchange chromatography. The plasma (30 ml) was loaded onto a column equilibrated with Tris–EDTA buffer, and subsequently eluted with 0.21, 0.24 and 0.3 M NaCl–TE buffer. (B) Second chromatography of fractions 35–69 from (A) using the DEAE column equilibrated with the buffer plus 0.1 M NaCl. The bars denote fractions capable of clotting.

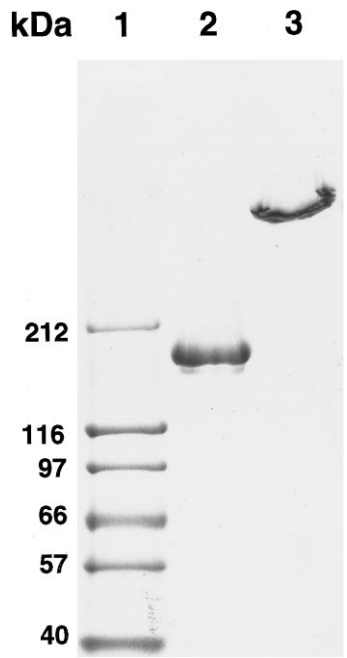

Fig. 2. SDS–PAGE of the purified clottable protein. Lane 1, molecular mass marker; lane 2, reduced clottable protein; lane 3, non-reduced clottable protein.

kDa dimers were detectable within 30 s after addition of the HLS, larger polymers were detected in 5 min, and the clot became too large to enter the gel after 15 min (Fig. 5). After 15 min the minor band of the 360-kDa protein was also shifted to larger forms which could not enter the gel (Fig. 5, lane 5). In control experiments where EDTA instead of Ca^{2+} was added, there was no polymerization of the clottable protein (data not shown).

The cross-reactivities between the tiger shrimp HLS and the clottable proteins from other species including several penaeid shrimps and *M. rosenbergii* were confirmed by conducting gel-shifting test similar to that of Fig. 5 (data not shown).

3.4. Amino acid composition and sequence

Amino acid composition of the purified tiger shrimp clottable protein and those of the homologous proteins from lobster [11] and crayfish [21] are listed in Table 1. These proteins have similar amino acid compositions. The N-terminal amino acid sequence of the shrimp protein up to the 30th residue was determined by automated sequencing. It is shown in Table 2 that the *P. monodon* sequence is about 60–80% identical to those of the clottable proteins of the shrimp *P. japonicus*, the lobster *P. interruptus* [8], the crayfish (*P. leniusculus*) [14], and the freshwater prawn (*M. rosenbergii*).

3.5. Glycan analysis

Positive results of the PAS staining [22] of the *P. monodon* clottable protein on SDS–PAGE suggests that it contains sugar. The monosaccharide content of the protein was determined after acid hydrolysis by anion-exchange chromatography [15]. The clottable protein contains 0–1.4% glucose, 2.6% mannose and 1.2% glucosamine (presumably from *N*-acetylglucosamine). The protein probably contains *N*-glycan of high mannose type, as commonly found in invertebrate glycoproteins.

3.6. Lipid staining

We used the staining method of Oil Red O [24] after SDS–PAGE of the clottable protein to check whether it contains lipid or not. The results were negative even after up to 80 μg of the purified protein were loaded on the gel (data not shown).

3.7. UV spectra and thermal stability

The clottable protein was dialyzed against 50 mM Tris, pH 8.0, and adjusted to 0.3 mg ml^{-1}. UV spectra of the protein were obtained by scanning on a UV-Vis spectrophotometer (Hitachi, Model U 3200, Japan) at least twice. The thermostability of the protein was studied by incubating the sample for 5 min at temperatures between 25 and 55°C with an increment of 5°C and between 58 and 76°C with an increment of 2°C, then cooled down to 25°C for UV absorption spectrophotometry. The protein had an absorbance maximum at 279.5 nm at 25°C as a typical tryptophan-containing protein. The protein appears to be stable at temperatures below 66°C, and the UV spectra was almost identical to that at 25°C. However, the protein precipitated out at 76°C and above.

3.8. Immunodiffusion and immunoblotting

The hemolymph was collected from eight local common crustacean species (see Section 2). The antibodies against the clottable protein were shown to react with the hemolymph of *P. monodon* and *P. japonicus*, but not those decapods of different genera (Fig. 6A). Both the hemolymphs of *P. monodon* and *P. japonicus* were subjected to Western blot analysis. A single band of the *P. japonicus* protein on the membrane reacted with the antibodies (Fig. 6B, lane 3), as did the cross-linked form of the clottable protein (Fig. 6B, lane 2).

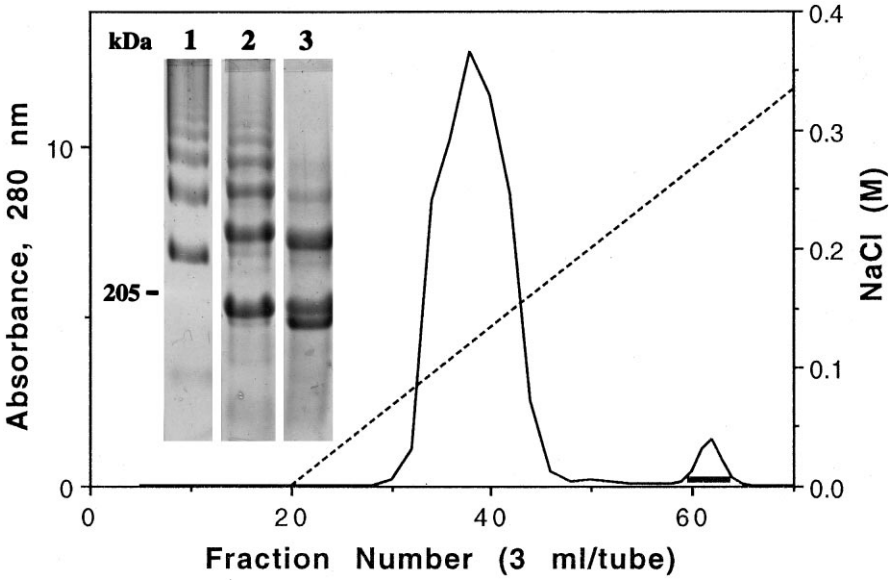

Fig. 3. Purification of the clottable protein from *M. rosenbergii* on a TSK DEAE-650(S) column. From 2 ml of the hemolymph loaded, 12 mg of the clottable protein were purified (second peak). The first peak contained hemocyanin. Inset: SDS–agarose gel (2.5%) showing the molecular mass of the clottable protein of: lane 1, *M. rosenbergii* (non-reduced); lane 2, *M. rosenbergii* (reduced); lane 3, *P. monodon* (reduced). Molecular masses of the markers are shown on the left.

4. Discussion

We were able to purify the hemolymph clottable protein from *P. monodon* successfully by repeated sequential chromatography on a TSK DEAE-650 column. The results of a linear gradient of NaCl were not as good as the step-wise elution for purification of the clottable protein. With their lysine ϵ-amino groups modified in the cross-linking reaction, polymerized clottable proteins became more acidic than the dimeric protein and were eluted from the anion exchanger with a higher concentration of salt (Fig. 1A). Previously, lobster clottable protein was purified by isoelectric precipitation at pH 5.0 followed by gel filtration [11]. In analogy, the clottable protein of crayfish was isolated by repeated precipitation in 10 mM phosphate buffer, pH 6.0 [21]. We also have tried to isolate tiger shrimp clottable protein by precipitation in phosphate buffer (pH 5.0 or 6.0), but the yield and purity were not satisfactory.

The molecular mass of the clottable protein of *P. monodon* is estimated to be 380 kDa (Fig. 4), which is slightly smaller than those reported for the proteins from spiny lobster [10] and crayfish [21]. However, their amino acid composition and N-terminal sequences are similar (Tables 1 and 2), suggesting they are homologous proteins. The shrimp clottable protein is able to form stable clots in the presence of Ca^{2+} and the HLS which contains Ca^{2+}-dependent transglutaminase activities (Fig. 5). Similar coagulation systems and hemolymph clottable proteins appear to be present in lobster [12], fresh water prawn (Fig. 3) and crayfish [21]. In contrast, the coagulogen and amebocyte of horseshoe crab [18] bear no structural and mechanistic similarities to the crustacean coagulation system.

The shrimp clottable protein was thermostable up to 66°C. However, proteolysis at the C-terminal region of the protein probably occurred during its collection and purification since we found that the 360-kDa minor form has an identical N-terminal sequence to that of the 380-kDa form. Similar type of degradation was previously reported for the purification of lobster fibrinogen [7] and crayfish clotting protein [21]. By immunoblotting of the crude hemolymph after reduced gel electrophoresis, we detected both forms of the 190- and 180-kDa subunits from the stored, but only the 190-kDa subunits from freshly prepared hemolymph. Moreover, there were relatively higher contents of the degraded form when the clottable protein was purified from the hemolymph stored at -18°C for 2 years (data not shown). Since both forms are capable of clotting (Fig. 5), proteolytic activation appears not to play such an important role in the shrimp clotting system as in the horseshoe crab system [18].

The clottable proteins from the spiny lobster [11,12] and the crayfish [21] have been reported to be lipoglycoproteins and suggested to be involved in lipid transport [14]. However, negative results were obtained for the shrimp clottable protein even when large amounts were loaded on the gel and subjected to staining with Oil Red O. Thus, the lipid content of the shrimp protein may be under the detection limit or the lipid moiety was dissociated from the protein in the presence of SDS during the electrophoresis.

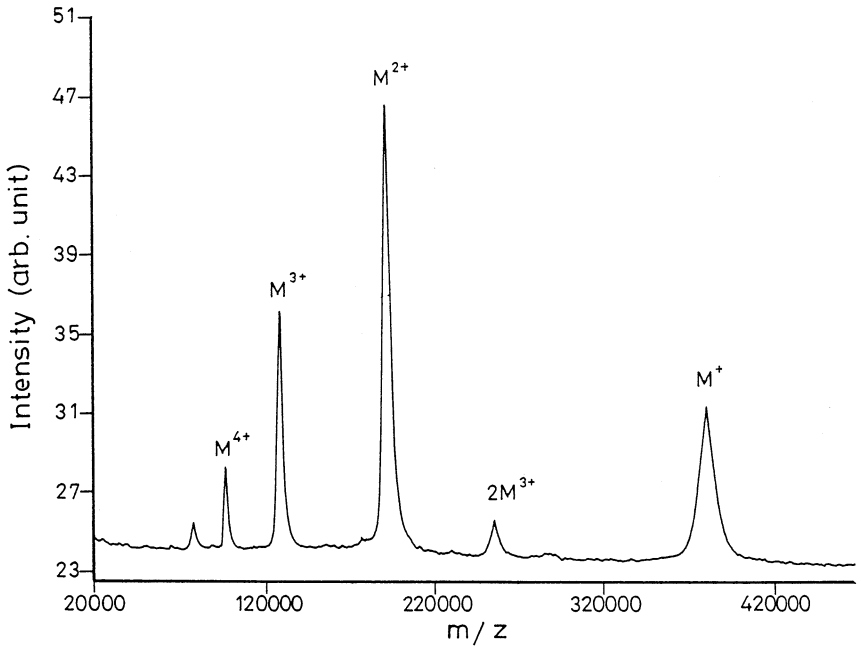

Fig. 4. MALDI–TOF MS analysis. Positive-ion MALDI-TOF mass spectrum of the clottable protein is shown. The relative molecular mass of M^+ is 380750 ± 800.

Notably, the hemolymph of *P. japonicus* and *P. monodon* reacted with the anti-clottable protein antibodies (Fig. 6A). The precipitation line with a spur indicates that the *P. japonicus* clottable protein is structurally very similar to that of *P. monodon* but contain epitopes not identical with that of the *P. monodon* protein. The subunit molecular mass of the *P. japonicus* protein is slightly larger than that of the *P. monodon* protein (Fig. 6B). Apparently, the clottable proteins of lobsters, crabs, and freshwater prawns are immunochemically distinct from the penaeid clottable protein. Similar high-molecular weight clottable proteins appear to be present in the hemolymphs of most of the decapods and may be responsible for coagulation and haemostasis of the animals [4,8,21] (Fig. 3). The structural similarities between the clottable proteins of *P. monodon* and those of lobster and crayfish are probably not very high (Table 2), therefore there were no im-

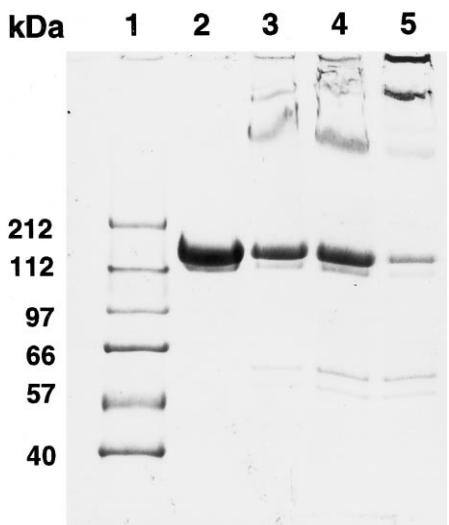

Fig. 5. Polymerization of *P. monodon* clottable proteins after adding the HLS and Ca^{2+}. Lane 1, molecular mass marker; lane 2, the clottable protein only; lanes 3–5, the protein incubated with HLS and Ca^{2+} for 30 s, and 5 or 15 min, respectively.

Table 1
Amino acid composition of clottable proteins isolated from tiger shrimp (this study), lobster (*P. interruptus*) [11] and crayfish (*P. leniusculus*) [21]

Amino acid	% Composition (m/m)		
	Tiger shrimp	Lobster	Crayfish
Asx	9.2	9.9	9.1
Thr	6.3	7.1	8.7
Ser	7.7	8.2	7.7
Glx	11.8	10.9	12.3
Pro	5.8	5.3	5.0
Gly	5.6	6.7	5.8
Ala	7.5	5.5	5.7
Cys	1.2	1.3	1.5
Val	6.9	6.9	6.5
Met	1.9	1.5	1.7
Ile	5.9	5.1	6.3
Leu	7.9	9.6	8.3
Tyr	4.2	3.2	3.1
Phe	5.1	4.1	5.2
His	4.2	4.2	3.2
Lys	4.8	4.2	6.3
Arg	4.2	4.8	3.8

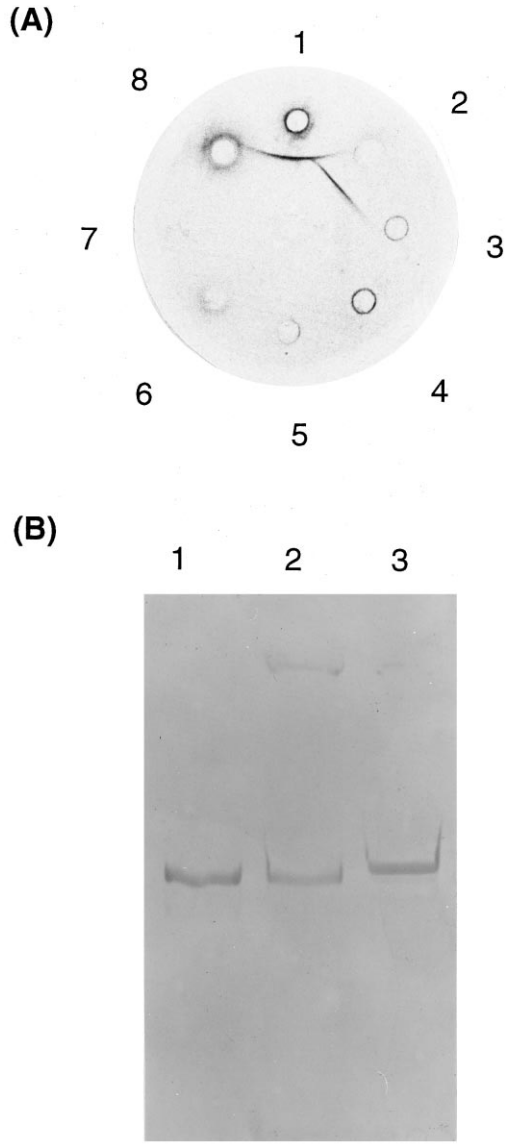

Fig. 6. (A) Immunodiffusion. Center well, anticlottable protein antibodies. Wells 1–8 contained fresh hemolymph of: (1) *P. monodon*; (2) *P. japonicus*; (3) *M. ensis*; (4) *M. rosenbergii*; (5) *P. clarki*; (6) *S. serrata*; (7) *Portuna sanguinolentus*; and (8) *P. versicolor*. (B) Western blot analyses. Lane 1, purified clottable protein; lane 2, hemolymph of *P. monodon*; and lane 3, hemolymph of *P. japonicus*.

munochemical cross-reactivities between their clottable proteins (Fig. 6).

We found previously that the concentration of penaeid shrimp clottable protein was higher in summer than in winter, and was varied by the molting cycle: its plasma levels increased by 2-fold before molting and decreased after molting to the normal level [4]. However, sex difference in the clottable protein level was not apparent. The concentration level and extent of oligomerization of the protein in the hemolymph also varied between different species (Figs. 1–3). For example, *P. japonicus* clottable proteins tend to form more oligomers or polymers and clot faster than the *P. monodon* protein during hemolymph withdrawal.

Table 2
Comparison of the N-terminal amino acid sequences of the clottable proteins from five decapods

	Sequence
P. monodon	LQPGLEYQYRYSARVASGIPSINRQ-FALXD
P. japonicus	LQPGLEYQYDYDAXV
P. interruptus	LQPKLEYQYKYHGIVALGIPSYKTQFY-DAH
P. leniusculus	LHSNLEYQYRYSGRVASGIP
M. rosenbergii	LQPGLQXHYRYXXXXXTGIM

One-letter codes of amono acids were used; X denotes those not being confirmed. References for the sequence are: *P. interruptus* [8]; *P. leniusculus* [14]; the other sequences, this study.

The N-terminal amino acid sequences of the crustacean clottable proteins show slight similarity to that of the *C. elegans* vitellogenin [8] which also bears sequence similarity to the vertebrate von Willebrand factor. These plasma proteins are all homodimers of about 400 kDa, and may exist as oligomers in plasma [1,27]. More sequence information on the crustacean clotting protein is needed to confirm possible evolutionary relationship between these plasma proteins.

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