

Hemostasis of Tiger Prawn *Penaeus monodon* Affected by *Vibrio harveyi*, Extracellular Products and a Toxic Cysteine Protease

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ABSTRACT: The effects of bacterial cells, extracellular products (ECP) and a purified cysteine protease of *Vibrio harveyi* on hemostasis and plasma components of tiger prawn (*Penaeus monodon*) were studied. The clotting ability of the hemolymph withdrawn from moribund prawns pre-injected with the bacteria, ECP, cysteine protease or PBS (control) was observed for 2 h at 25 C. Of these, only the control group was clottable while all the other groups were unclottable. A component of the plasma, previously identified as coagulogen-like protein, was further confirmed to be a coagulogen by the comparison of plasma with serum on non-reduced SDS-PAGE or using rabbit antiserum to the coagulogen-like protein (R α coagulogen) to neutralize the clotting ability of normal prawn hemolymph. The coagulogen was reduced in amount in plasma of moribund prawns after injection with the bacteria, ECP or cysteine protease while it apparently disappeared after pre-incubation with the ECP or cysteine protease for 2 h at 25 C compared with normal prawn plasma as observed in crossed immunoelectrophoresis (CIE) gels. The reduction of the amount of coagulogen in plasma of moribund prawns was also evident in CIE gels using R α coagulogen. In addition, the apparent disappearance of the coagulogen mentioned above was eventually proven to be due to the change of its migration rate in CIE gels after pre-incubation with ECP or cysteine protease, since the disappeared coagulogen arc (arc 2) (migrated into arc 1) could be visualized by using R α coagulogen or by reducing the time for pre-incubation from 2 h to 30 min. Thus, the effects of cysteine protease on plasma coagulogen observed *in vitro* and *in vivo* may markedly interfere with hemostasis leading to the occurrence of unclottable hemolymph. These complex events may significantly contribute to the pathogenicity of *V. harveyi* in the prawn.

Keywords: coagulogen, cysteine protease, extracellular products, hemostasis, *Penaeus monodon*, *Vibrio harveyi*

INTRODUCTION

Vibrio harveyi is commonly present in various marine and brackish habitats, i.e., warm marine waters, surfaces of marine animals, light organs of certain marine fish and cephalopods, and intestine of aquatic animals (1-8). Only in this decade has the virulence of this species been recognized in a small but expanding list of cultured marine animals particularly in penaeids in Asia and Australia (9-18). Mass mortalities of *Penaeus monodon* and larvae or juveniles associated with luminous vibrios have been observed in hatcheries or farms in Australia (9), China (10), India (11), Indonesia (12), Thailand (13), the Philippines (14, 15), and Taiwan (16, 17).

A variety of extracellular proteases produced by a few *Vibrio* species isolated from sea water,

fish and shellfish have been isolated and examined with respect to their enzymatic properties and/or virulence. The majority of these studies are focused on the species of *V. anguillarum* because of its foremost importance as a fish pathogen (19-24). Two proteases produced by *V. alginolyticus* NCMB 1339, one metalloprotease produced by *V. alginolyticus* S3y, and one serine protease produced by *V. alginolyticus* Swy have also been described to be toxic to larval *Ostrea edulis* (25, 26), juvenile *Epinephelus malabaricus* (27) and *Penaeus japonicus* (28), respectively. Three extracellular alkaline metal-chelator-sensitive proteases produced by *V. harveyi* isolated from sea water have also been reported (29, 30). However, no information is available concerning the pathological role of these proteases from *V. harveyi* played in aquatic animals.

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Recently, massive losses of *P. monodon* and *P. japonicus* attributed to this luminous *Vibrio* species have been observed frequently in farms in Taiwan (16, 17, 31). The pathogenicity of different strains of *V. harveyi* and their extracellular products (ECP) has been reported (16). In addition, a novel 38-kDa cysteine protease produced by this *Vibrio* species strain 820514 has also been purified (32) and characterized as a major exotoxin (33).

In the present communication, we investigate the effects of the bacteria, ECP and purified cysteine protease of strain 820514 on hemostasis and plasma components of *P. monodon* *in vitro* and *in vivo*. The reaction of the cysteine protease with the major affected plasma component, coagulogen, and its implication in the pathogenesis in *P. monodon* is discussed.

MATERIALS AND METHODS

Bacteria and Extracellular Products (ECP)

Luminous *V. harveyi* strain 820514, originally isolated from diseased tiger prawn *Penaeus monodon* in Taiwan in 1993, was used in this study (16). Stock cultures of strain 820514 were grown on tryptic soy agar (TSA; Oxoid, Basingstoke, England) supplemented with 1.5 % NaCl for 16 h at 25 C. Two swabs of these bacteria suspended in 5 ml phosphate buffered saline (PBS) pH 7.2 were either washed 3 times with PBS for adjustment of bacterial cells used in the experiments or spread onto TSA (+1.5 % NaCl) overlaid with sterile cellophane and grown for 24 h at 25 C. The ECP was harvested as previously described (34). Briefly, 15 ml of PBS was added to the surface of the cellophane overlay of TSA (+ 1.5 % NaCl) and spread completely. The harvested bacterial suspension was then centrifuged at 25,000 g for 60 min at 4 C; the pellet was discarded. The supernatant fluids were passed through a 0.22- μ m filter (Millipore Corp., Bedford, Mass.), and the ECP

was stored in 1-ml aliquots at -70 C. Total protein was measured by the method of Bradford (35) with bovine serum albumin (BSA) as the standard.

Purification of Extracellular Protease

Purification of the 38-kDa cysteine protease was described in a previous study (32). Briefly, the protease was purified from the extracellular products of strain 820514 to apparent homogeneity by a combination of ammonium sulfate precipitation, hydrophobic interaction chromatography and anion-exchange (Mono Q) Fast Protein Liquid Chromatography.

Experimental Prawns

Intermoult tiger prawns (*P. monodon*) weighing approximately 20 g were separately held in tanks (2,500 liter) supplied with air-lifted 3% salinity sea water at 25-28 C. Hemolymph was collected from a batch of 10 prawns by inserting a 25-gauge needle attached to a 1-ml syringe containing 0.25 ml of modified citrate/EDTA buffer (anticoagulant) (36) into the site between II and III pleopod and 0.75 ml of hemolymph was withdrawn from each prawn. The pooled hemolymph was then centrifuged at 850 g for 20 min, with the resultant supernatant being collected as prawn plasma. To obtain serum, hemolymph was collected as described above except that no anticoagulant was used in the collection syringe. Serum was collected by centrifuging (850 g, 20 min) hemolymph that had been allowed to clot.

Rabbit Antisera

Antiserum to normal tiger prawn plasma (R α plasma) was raised in a rabbit. The initial injection consisted of 0.2 ml of plasma (17 mg protein) mixed with 2.8 ml PBS which had then been emulsified in 3 ml complete Freund's adjuvant (Sigma). Two booster injections of the same

Freund's adjuvant (Sigma) was given at the 2 and 4 week post initial injection, separately. The antiserum was collected 2 weeks later and 1 ml aliquots stored at -70°C . Rabbit antiserum to the BSA ($\text{R}\alpha$ BSA) was obtained from our previous study (37). Antiserum to the plasma coagulogen-like protein was also raised in a rabbit using a protocol similar to that described above. The coagulogen-like protein antigen(s) was excised from a portion of the precipitation arc 2 in CIE gel as shown in Figure 1 (38) by using the Multiphor II System as previously described (39). In brief, 20 μl of prawn plasma was electrophoresed in the first dimension at 10 V/cm for 70 min. The second dimension gel contained 3% (v/v) $\text{R}\alpha$ plasma and electrophoresis was conducted at 2 V/cm for 16 h in 0.05 M tris-barbiturate buffer, pH 8.6. Gels were pressed, washed with 0.1 M NaCl three times, dried and stained with Coomassie brilliant blue R 250. The excised gels (from two similar CIE gels) were

mixed and homogenized prior to each immunization in the rabbit.

Clotting Ability of Hemolymph from Challenged Prawns

Batches of 6 prawns were injected intramuscularly (i.m.) with 0.1 ml of samples (4.63×10^6 colony forming unit *V. harveyi*/g prawn), 2.67 μg protein ECP/g prawn, or 0.61 μg protein cysteine protease/g prawn) at the site between 4th and 5th abdominal segments (40). Sterile PBS was injected i.m. into animals as parallel controls. The animals were observed for 24 h post injection. For the determination of clotting ability of the challenged prawns, 0.5 ml of hemolymph was withdrawn from moribund or control prawns and recorded for 2 h at 25°C . In addition, the hemolymph and plasma of the prawns were collected as described above and used for crossed immunoelectrophoresis (CIE).

SDS-PAGE

Phastgel gradient (4–15%) polyacrylamide (Pharmacia) was employed in sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and used to determine the differences of protein bands between prawn plasma and serum. Pharmacia high molecular-mass calibration kits were used as marker proteins. Electrophoresis was conducted under non-reducing condition on the PhastSystem (Pharmacia) according to the recommendations of the manufacturer. The SDS sample buffer (without adding 2-mercaptoethanol) was composed of 10 mM Tris-HCl, 1mM EDTA, 1% SDS, 0.5% bromophenol blue, 20% glycerol and the pH level was adjusted to pH 8. After electrophoresis, the gels were stained with Coomassie brilliant blue R 250.

Clotting Tests of Hemolymph from Normal Prawns

For clotting tests, 0.5 ml hemolymph was collected from each of eight normal prawns by the

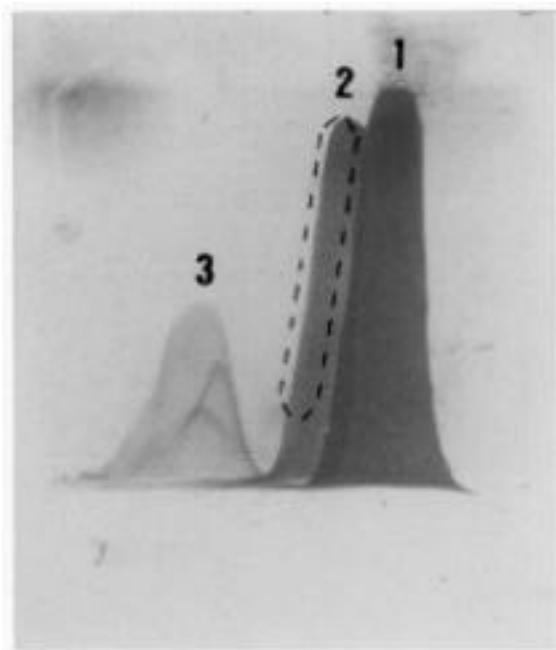


Figure 1. Profile of crossed immunoelectrophoresis (CIE) gel (Multiphor II system) of normal prawn plasma (pool of six tiger prawns, 84 mg protein/ml) against rabbit antiserum to normal plasma of tiger prawns ($\text{R}\alpha$ plasma), plasma + tris-barbital buffer (1:1, v/v).

method described above without adding the anticoagulant and then 0.1 ml of it was directly added to each Eppendorf tube, either empty or containing 0.1 ml of the anticoagulant, R α BSA, R α coagulogen and PBS, separately. The clotting ability of the treated samples was observed for 1 and 2 h at 25 C. The formation of a gel was scored as clottable otherwise scored as unclottable.

CIE Using the PhastSystem

In studies of the effects of bacterial cells, ECP and the 38-kDa cysteine protease of *V. harveyi* on the plasma components of tiger prawns, CIE was performed using the PhastSystem (Pharmacia) as described by the manufacturer's note (PhastSystem Technical Note No. 3, Pharmacia LKB Biotechnology, Sweden). Briefly, 2 μ l of each sample was electrophoresed in the first dimension at 70 V for 30 min. The second dimension gel contained 10% (v/v) rabbit antiserum and was electrophoresed at 50 V for 75 min in 0.07 M tris-barbital buffer (buffer strip, 0.35 M tris-barbital buffer), pH 8.6. CIE gels were pressed, washed with 0.1 M NaCl three times, dried and stained with Coomassie brilliant blue R 250. Bacterial cells (10^9 colony forming unit/ml), ECP (648 μ g protein/ml), the cysteine protease (50.4 μ g protein/ml) or 0.07 M tris-barbital buffer were incubated with an equal volume of normal prawn plasma or serum for 2 h or 30 min at 25 C prior to the CIE.

RESULTS

ECP and Purified Cysteine Protease

The ECP of *V. harveyi* strain 820514 were harvested after 24 h of incubation of the culture at 25 C. The total protein of the ECP and purified 38-kDa cysteine protease was 648 and 50.4 μ g protein/ml, respectively.

Clotting Ability of Hemolymph from Challenged Prawns

All the prawns pre-challenged with bacteria, ECP or cysteine protease were moribund while none of the prawns pre-injected with PBS (controls) died within 24 h. The hemolymph withdrawn from the moribund prawns and the controls was unclottable within 2 h and clottable within 1 h, respectively.

SDS-PAGE

One 380 kDa protein band and one high molecular-mass polymer were present in the plasma but both of them were absent from the serum (Figure 2).

Clotting Tests of Hemolymph from Normal Prawns

Table 1 shows that R α coagulogen and the anticoagulant (modified EDTA/citrate buffer) could cause the normal prawn hemolymph to become unclottable. In the other treatments, all the hemolymph was clottable within 1 h except hemolymph of the No. 7 prawn pre-injected with PBS. However, it was clottable within 2 h.

CIE

As component 2 (arc 2) was present in the plasma (Figures 1 and 3a) but was absent from the serum (Figure 3b), it appeared to be a coagulogen (also see reference 38). Components 1 and 3 have been previously suggested to be another type of hemocyanin or a moult-related protein and hemocyanin, respectively (38). Using R α coagulogen against plasma in CIE gels, only one major arc (component 2) and one smaller arc (component u) were visualized in the plasma (Figure 3c). This component u was an unknown factor (contaminant) present in both of the plasma and serum (Figures 3c and 3d).

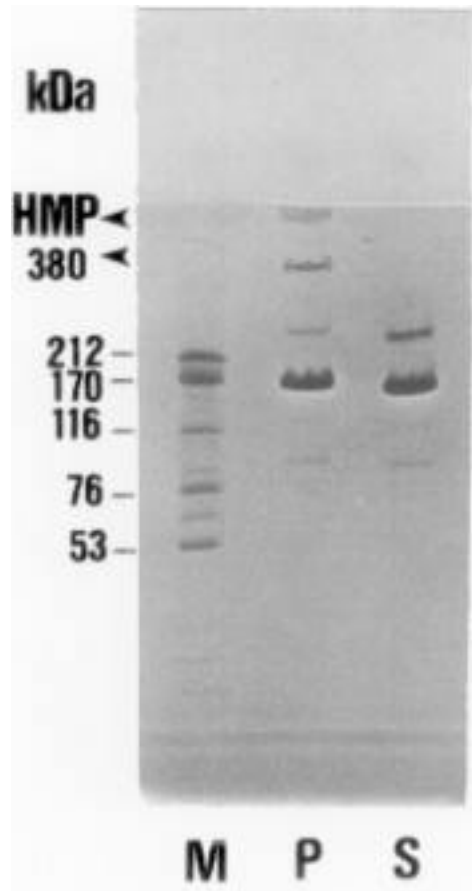


Figure 2. Unreduced SDS-PAGE of normal plasma and serum of tiger prawns stained with Coomassie brilliant blue R250. M: marker proteins (2 µg protein) (myosin, 212 kDa; α 2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa). P: normal prawn plasma (4.2 µg protein). S: normal prawn serum (5.0 µg protein). HMP: high molecular-mass polymer.

Following *in vivo* bacterial, ECP or cysteine protease challenge, the amount of plasma coagulogen all appeared to be reduced with the greatest reduction occurring in the bacteria treated group using Rα plasma against each plasma in CIE gels (*cf.* Figures 4A and 3a). Similar observations were also made using Rα coagulogen against each plasma in the CIE gels (Figure 4B). Component 3 apparently migrated faster in plasma of ECP challenged prawns (*cf.* Figures 4Ab and 3a). After *in vitro* bacterial, ECP or cysteine protease exposure for 2 h, a fast-migrating component 2 could only be visualized in the bacteria treated group (Figure 5Aa) while

component 2 seemed to have disappeared in the other two groups treated with ECP or cysteine protease (Figures 5Ab and 5Ac) using Rα plasma against each plasma in CIE gels. Component 3 also apparently migrated faster in plasma treated with ECP (Figure 5Ab).

After further *in vitro* ECP or cysteine protease exposure for 30 min, a shoulder in component 2 area was visualized in ECP treated group (Figure 6Aa) but a fast migrating component 2 was visualized in cysteine protease treated group (Figure 6Ab), using Rα plasma against the respective plasma in CIE gels. An apparently fast-migrated component 3 could also be visualized in ECP treated group (Figure 6Aa).

Component 2 which seemed to have disappeared (see Figures 5Ab and 5Ac) could be visualized using Rα coagulogen against the respective plasma in CIE gels (Figures 5Bb and 5Bc). A shoulder in component 2 area in ECP treated group (see Figure 6Aa) or the fast migrating component 2 in protease treated group (see Figure 6Ab) could also be visualized using Rα coagulogen against the respective plasma in CIE gels (Figures 6Ba and 6Bb). The results

Table 1. Clotting tests of hemolymph withdrawn from eight normal tiger prawns *Penaeus monodon* in different treatments

Treatment	Normal <i>P. monodon</i>								
	1	2	3	4	5	6	7	8	
Blank	1 h	+	+	+	+	+	+	+	+
	2 h	+	+	+	+	+	+	+	+
+ anticoagulant**	1 h	-	-	-	-	-	-	-	-*
	2 h	-	-	-	-	-	-	-	-
+ Rα coagulogen***	1 h	-	-	-	-	-	-	-	-
	2 h	-	-	-	-	-	-	-	-
+ Rα BSA***	1 h	+	+	+	+	+	+	+	+
	2 h	+	+	+	+	+	+	+	+
+ PBS	1 h	+	+	+	+	+	+	-	+
	2 h	+	+	+	+	+	+	+	+

* +, clottable; -, unclottable.

** Modified EDTA/citrate buffer.

*** Rα coagulogen, rabbit antiserum to coagulogen; Rα BSA, rabbit antiserum to bovine serum albumin.

revealed that the *in vitro* exposure of plasma to the bacteria, ECP or cysteine protease all caused component 2 (coagulogen) to migrate faster in the CIE gels.

DISCUSSION

In the present study, we attempted to investigate the effects of the pathogenic *V. harveyi* 820514 strain, its ECP and purified 38-

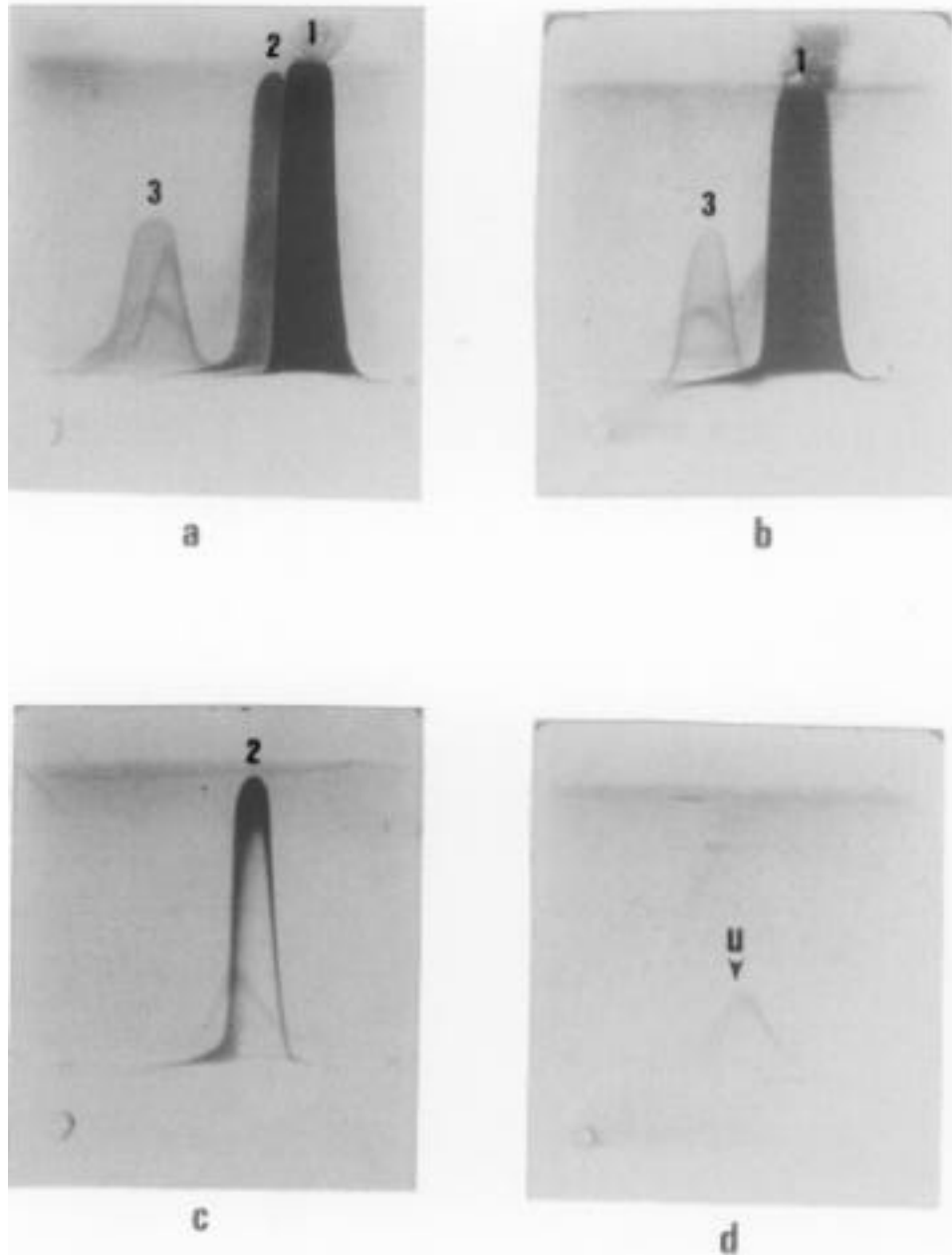


Figure 3. Profiles of CIE gels of normal prawn plasma (84 mg protein/ml) or serum (101 mg protein/ml) against rabbit antiserum to normal prawn plasma (R α plasma) or rabbit antiserum to coagulogen (R α coagulogen). a: normal plasma + tris-barbital buffer, (1:1, v/v) against R α plasma; b: normal serum + tris-barbital buffer, (1:1, v/v) against R α plasma; c: normal plasma + tris-barbital buffer, (1:1, v/v) against R α coagulogen; d: normal serum + tris-barbital buffer, (1:1, v/v) against R α coagulogen.

kDa cysteine protease on hemostasis of tiger prawn. Since prawn/shrimp were more sensitive to vibriosis when infected in premoult than

intermoult (41), intermoult tiger prawns were used in all of the experiments reported here to avoid possible errors. Although coagulation in

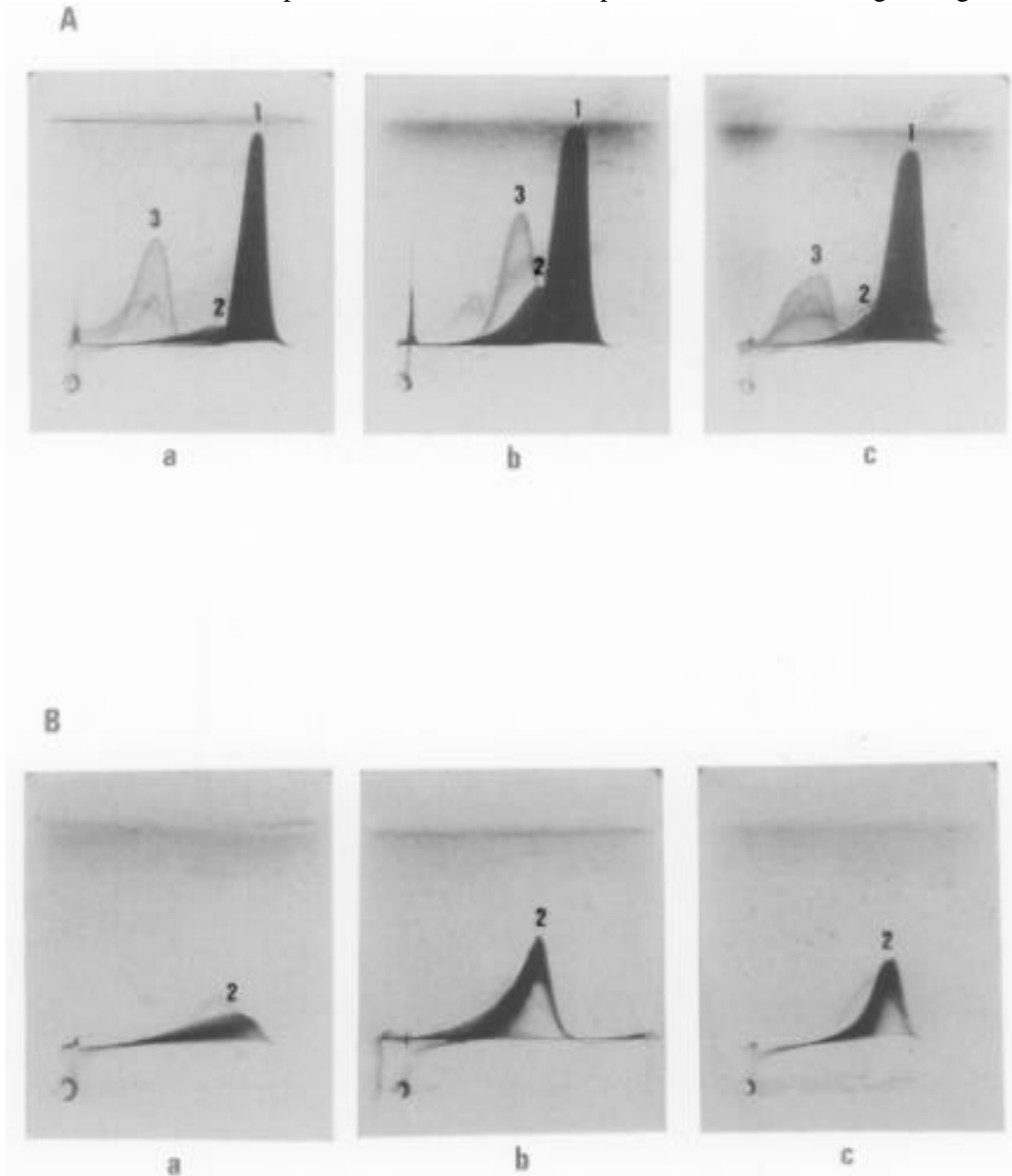


Figure 4. Profiles of CIE gels of moribund prawn plasma pre-challenged with *V. harveyi*, ECP or cysteine protease against Rα plasma or Rα coagulogen. A: First dimension: (a) pre-challenged with *V. harveyi* (4.63×10^6 CFU/g prawn); (b) pre-challenged with ECP (2.67 μg protein/g prawn); (c) pre-challenged with cysteine protease (0.61 μg protein/g prawn). Second dimension: Rα plasma. B: First dimension: (a) pre-challenged with *V. harveyi* (4.63×10^6 CFU/g prawn); (b) pre-challenged with ECP (2.67 μg protein/g prawn); (c) pre-challenged with cysteine protease (0.61 μg protein/g prawn). Second dimension: Rα coagulogen.

prawn is generally very strong and rapid (42), the present results showed that intramuscular injection of the bacteria, ECP or cysteine protease

into tiger prawns resulted in the occurrence of moribund prawns with unclottable hemolymph. This interesting phenomenon stimulated us to

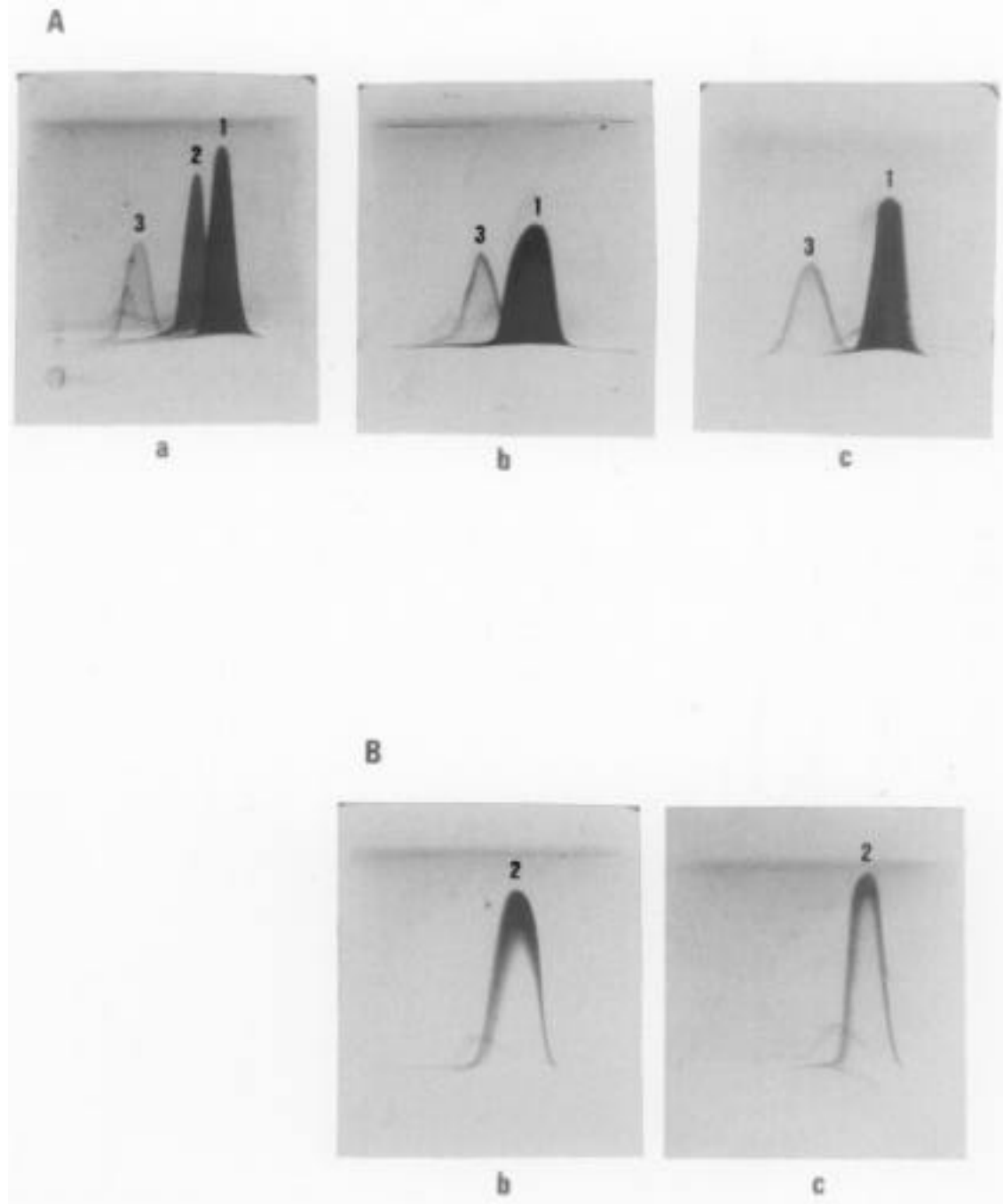


Figure 5. Profiles of CIE gels of normal prawn plasma (84 mg protein/ml) pre-incubated with *V. harveyi*, ECP or cysteine protease for 2 h against R α plasma or R α coagulogen. A: First dimension: (a) pre-incubated with *V. harveyi* (10^9 CFU/ml) (1:1, v/v); (b) pre-incubated with ECP (648 μ g protein/ml) (1:1, v/v); (c) pre-incubated with cysteine protease (50.4 μ g protein/ml) (1:1, v/v). Second dimension: R α plasma. B: First dimension: (b) pre-incubated with ECP (648 μ g protein/ml) (1:1, v/v); (c) pre-incubated with cysteine protease (50.4 μ g protein/ml) (1:1, v/v). Second dimension: R α coagulogen.

examine the fate of the plasma clotting related protein, coagulogen, *in vitro* and *in vivo*. Component 1 and 3 in the plasma or the serum are possibly another type of hemocyanin or a moult-related protein and hemocyanin, respectively, comparable to those reported in

other studies (38, 43, 44). As shown in Figures 1 and 3a, component 2 was present in the plasma but was absent from the serum (Figure 3b), suggesting that it may be the coagulogen similar to previous studies (28, 42-44). In addition, one

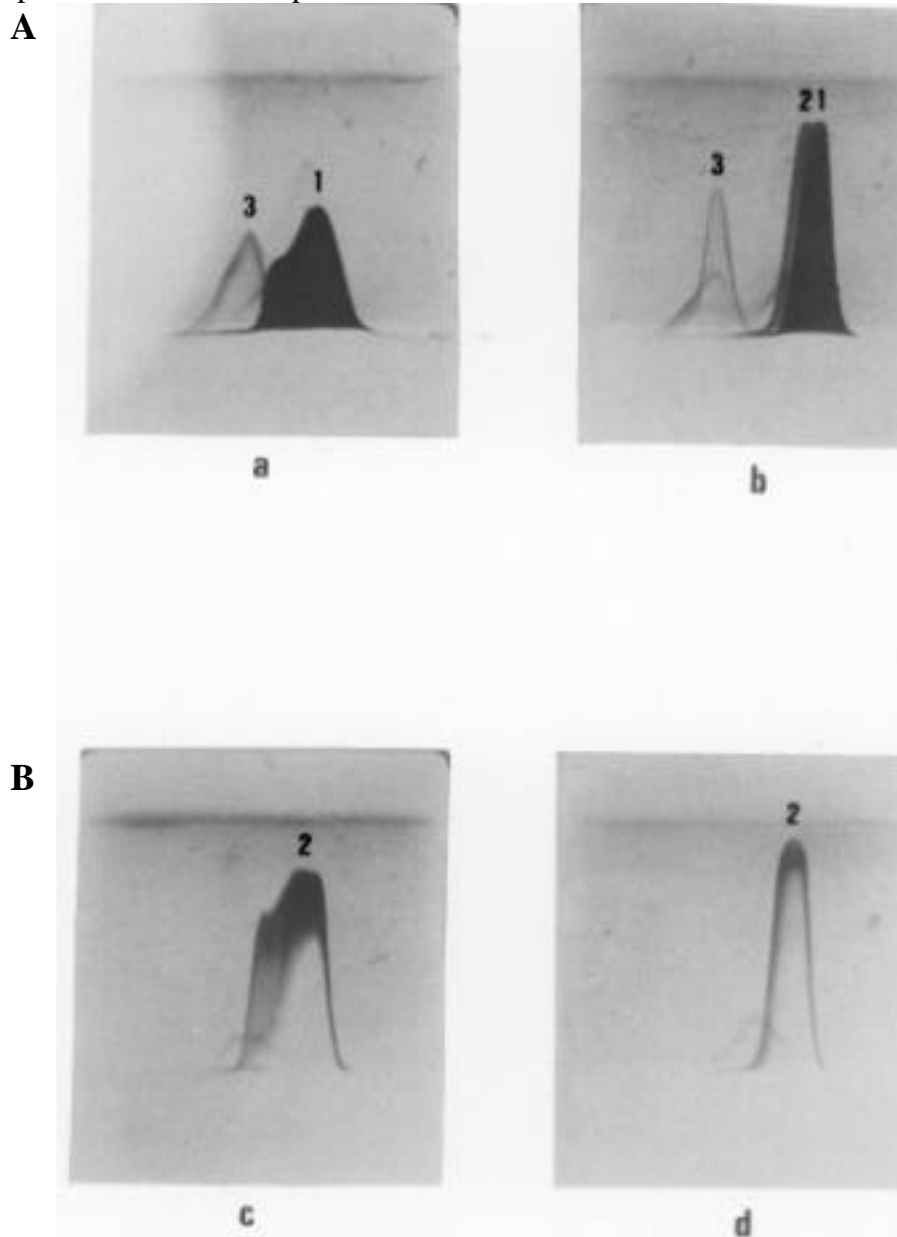


Figure 6. Profiles of CIE gels of normal prawn plasma (84 mg protein/ml) pre-incubated with ECP or cysteine protease for 30 min against R α plasma or R α coagulogen. A: First dimension: (a) pre-incubated with ECP (648 μ g protein/ml) (1:1, v/v); (b) pre-incubated with cysteine protease (50.4 μ g protein/ml) (1:1, v/v). Second dimension: R α plasma. B: First dimension: (a) pre-incubated with ECP (648 μ g protein/ml) (1:1, v/v); (b) pre-incubated with cysteine protease (50.4 μ g protein/ml) (1:1, v/v). Second dimension: R α coagulogen.

380 kDa protein and one polymer were present in the plasma but absent from the serum on non-reduced SDS-PAGE (Figure 2). This was exactly the same as that of the purified 380-kDa clottable protein of the tiger prawn (45), and was also similar to the purified coagulogen of kuruma prawn which shows one 360-kDa protein and one polymer band using similar protocol (46). A portion of component 2 (as indicated in Figure 1) was accordingly excised and used to raise antiserum in a rabbit (R α coagulogen). The clotting ability of hemolymph withdrawn from normal prawns could be inhibited/neutralized by the addition of R α coagulogen similar to the use of anticoagulant (Table 1). The use of R α BSA did not hinder the clotting ability of hemolymph indicating the neutralization of hemolymph clotting ability we have demonstrated is not associated with the presence of rabbit serum components including high amount of non-specific immunoglobulins. Since component 2 was only present in the plasma using R α plasma or R α coagulogen in CIE, and together with the above results of non-reduced SDS-PAGE and the neutralization of hemolymph clotting ability, we confirm it to be the plasma coagulogen of the tiger prawn.

As the bacterium and its ECP is virulent to the prawn (16), and cysteine protease is the major exotoxin of the bacterium (33), the effects we have reported may be a direct consequence and are crucial in determining the pathogenic mechanism. Presumably the coagulogen was degraded in the treatments and is somewhat analogous to fibrinolysis in the well-known human system. A further study on the change of molecular weight of the coagulogen in *in vitro* and *in vivo* treated plasmas to resolve this possibility is now under investigation. To our knowledge, the present work is the first which demonstrates the *in vivo* effects of bacteria, ECP and protease on prawn hemostasis though an *in vitro* study has been performed on penaeids using ECP and a partially purified serine protease of *V. alginolyticus* (38). In the latter study, exposure to

the toxic serine protease of *V. alginolyticus* alters the migrating rate of the putative plasma coagulogen. This phenomenon was also found in the present *in vitro* study using the purified cysteine protease of *V. harveyi* which is also toxic to tiger prawn (33). However, we still have no explanation for such a physical alteration caused by toxic *Vibrio* proteases. In addition, we also have no explanation for the appearance of the fast-migrating coagulogen after *in vivo* or *in vitro* exposure of prawns or plasma to the ECP (Figures 4Ab, 5Ab and 6Aa). As the apparent disappearance of plasma coagulogen after *in vitro* exposure to the ECP or protease is simply a time-course effect found in the present study, such a finding may be useful in explaining why *in vitro* exposure to the ECP results in a similar phenomenon and exposure to the partially purified protease only alters the migration rate, a phenomenon which has not been resolved in a previous report (38).

In the *in vivo* study, the apparent reduction of plasma coagulogen found in all the three challenged groups may be the cause of the unclottable hemolymph. This result is not the same as that found in the *in vitro* exposure, which apparently caused formation of fast-migrating coagulogen. However, coagulogen is the shared plasma component affected by these treatments. From the results presented here, it is clear that the cysteine protease plays an important role in interfering with prawn hemostasis and coagulogen is the evident target. As coagulation of the crustacean hemolymph plays an essential defense role concerning the prevention of both the loss of blood (hemolymph) through breaks in the exoskeleton and the dissemination of bacteria throughout the body (44), the present observable phenomenon of unclottable hemolymph may well facilitate the dissemination and propagation of *V. harveyi* in its natural host (tiger prawn) though the definite mechanism responsible for the *in vivo* inactivation of clotting ability by the bacteria remains unproven. Presumably the secreted cysteine protease is the factor that provides the

bacterium with this ability, leading to bacteremia in the prawn. A similar appearance of unclottable hemolymph, as that found in our present study, has also been demonstrated in a previous study injecting a partially purified serine protease of *V. alginolyticus* into kuruma prawns (28). However, this unique aspect resulting in the inactivation of hemolymph clotting ability by *Vibrio* proteases *in vivo* is in contrast to the activation of blood clotting factors by microbial proteases in vertebrates leading to the generation of thrombin *in vitro* (47).

The present interesting finding may possibly reflect the existence of different hemostatic response between vertebrates and invertebrates (penaeids) toward microbial infection. As well-reviewed by Muta and Iwanaga (48), there are two types of clotting mechanisms in invertebrate animals. One of these is found in crustacean and insects where a gel is formed through the polymerization of clottable protein(s), catalyzed by a Ca^{2+} -dependent transglutaminase that is released from the hemocytes or muscle cells through an unknown mechanism. The other type of coagulation (in horseshoe crab or limulus) proceeds as a cascade-type of reaction composed of serine protease zymogens as is mammalian blood coagulation system. Therefore, another possibility may exist that the lack of clotting ability in prawn hemolymph is an indirect effect caused by bacteria and toxins by interfering with the polymerization of clottable protein or Ca^{2+} -dependent transglutaminase, but these complex events are still unclear.

Currently, no bacterial protease has been shown to contribute to the inactivation of blood clotting. Our present results describe the *in vivo* and *in vitro* effects of bacterial, ECP and cysteine protease on prawn coagulogen/hemostasis. As the crustacean clotting mechanism is much simpler than that of vertebrates so far known (49, 50), the present results have allowed development of *in vitro* and *in vivo* models for investigating pathophysiological changes of penaeids caused by *V. harveyi* infection, with a particular emphasis on

the relationship among hemolymph coagulation, disease and bacteremia/sepsis.

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