Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 61 (2004) 93–99
Antiviral Research
www.elsevier.com/locate/antiviral

Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*

Xiaobo Zhang [a], Canhua Huang [b], Qiwei Qin [c,*]

[a] *Key Laboratory of Marine Biogenetic Resources, The Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, PR China*
[b] *Department of Biological Sciences, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*
[c] *Tropical Marine Science Institute, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

Received 8 May 2003; accepted 22 August 2003

Abstract

Penaeid shrimp aquaculture has suffered from many diseases, especially from viral origin such as white spot syndrome virus (WSSV). In an attempt to obtain antiviral-relevant proteins, two peptides with molecular masses at 73 and 75 kDa were isolated from shrimp *Penaeus monodon* using affinity chromatography coupled with the purified WSSV or a fish iridovirus (Singapore grouper iridovirus, SGIV), and identified as hemocyanin by mass spectrometry. The results, using fish viruses capable of cell culture, showed for the first time that the hemocyanin had non-specific antiviral properties and no cytotoxicity against host cells.

Keywords: Shrimp; *Penaeus monodon*; Hemocyanin; Antivirus

1. Introduction

Penaeid shrimp culture is a worldwide economic activity especially important for intertropical developed and developing countries. However, the intensification of shrimp farming has been accompanied by the development of many infectious diseases, especially from viral origin, which cause a decrease in growth in shrimp production resulting in vast economic losses (Destoumieux-Garzon et al., 2001). In cultured penaeid shrimp, about 20 shrimp viruses have been reported. Among them, white spot syndrome virus (WSSV), causing high mortality, has been the most economically important viral pathogen of farmed penaeid shrimp in the past decade (Zhang et al., 2002). In this context, control of disease is very important to insure the long-term survival of shrimp aquaculture.

Shrimp, like other invertebrates, lack a true adaptive immune response system (Hoffmann et al., 1999). However, living in an aquatic environment rich in microorganisms, shrimp have developed effective systems for detecting and eliminating noxious microorganisms, which depend entirely on non-specific innate immune response. The defense mechanisms, largely based on the activity of blood cells, include hemolymph coagulation, a rapid and powerful system that prevents blood loss upon wounding and participates in the engulfment of invading microorganisms (Destoumieux et al., 1997). Non-specific molecules including phenoloxidase, bactericidins and lectins, specifically antibacterial and anti-fungal peptides, have been documented and some genes have been characterized (Cuthbertson et al., 2002; Destoumieux et al., 2000; Destoumieux-Garzon et al., 2001; Gross et al., 2001; Rojtinnakorn et al., 2002; Roux et al., 2002). Surprisingly, in shrimp and other crustaceans, the antiviral peptide or protein has seldom been reported.

WSSV, a circular double-strand DNA virus, has become a major cause of shrimp mortality in aquaculture. Therefore, there is an urgent need to understand the immune response of shrimp against WSSV, which may be helpful in developing strategies for management of the disease and for long-term sustainability of penaeid shrimp farming worldwide. However, in contrast to extensive studies on the morphology and molecular biology of the virus, little work has been done on the shrimp defense against the WSSV infection. In this paper, we present the isolation, purification and identification of hemocyanin from shrimp *Penaeus monodon*. Antiviral properties of the hemocyanin were also investigated using fish virus–cell culture systems.

* Corresponding author. Tel.: +65-67749881; fax: +65-67749654.
E-mail address: tmsqinqw@nus.edu.sg (Q. Qin).

0166-3542/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2003.08.019

2. Materials and methods

2.1. Shrimp hemolymph and white spot syndrome virus (WSSV)

Shrimp *P. monodon* and crayfish *Cambarus clarkii* were obtained from a shrimp farm in Singapore. The healthy shrimp and crayfish were infected with WSSV by injection in the lateral area of the fourth abdominal segment as described by Zhang et al. (2002). At various time of post-infection, shrimp specimens were selected at random and their hemolymph were collected. The collected healthy and infected hemolymph were immediately frozen and stored at −70 °C until use.

WSSV was purified using the hemolymph from infected crayfish as described previously (Zhang et al., 2002). Briefly, the infected hemolymph was layered on the top of a 10–40% (w/v) continuous sodium bromide gradient and centrifuged at 110,000 × *g* for 2 h at 4 °C. Virus bands were collected by side puncture, diluted in 1:10 in TNE buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) and pelleted at 119,000 × *g* for 1 h at 4 °C. The resulting pellets were resuspended in TNE buffer. Virus samples were examined under transmission electron microscope (JEOL 100 cxII, Japan) for purity.

2.2. Fish viruses and cell cultures

The following six fish viruses were used in this study, Singapore grouper iridovirus (SGIV), frog virus 3 (FV3) and lymphocyctis virus (LDV) belong to Iridoviridae family with a circular double-stranded (ds) DNA genome; threadfin reovirus (ThRV), Reoviridae family, dsRNA virus; angelfish birnavirus (ABV) and infectious pancreatic necrosis virus (IPNV) belong to family of Birnaviridae, dsRNA viruses (Ahne, 1994). They were propagated in grouper (GP), fathead minnow (FHM) and bluegill fry (BF2) cell lines, respectively. Grouper embryo cells (GP) from brown-spotted grouper, *Epinephelus tauvina* was cultured in Eagles' minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS), 0.116 M sodium chloride, 100 IU ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin (Qin et al., 2001). Bluegill fry (BF2) cells (Wolf and Quimby, 1966) and fathead minnow (FHM) cells (Gravell and Malsberger, 1965) were cultured in EMEM containing 10% FBS, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and 16 mM Tris–HCl (pH 7.5). All cell cultures were cultured and maintained at 25 °C.

2.3. Purification of SGIV

GP cells were infected with SGIV, originally isolated from diseased groupers *Epinephelus malabaricus* and *E. tauvina* in Singapore (Qin et al., 2003), at a multiplicity of infection (MOI) of approximately 0.1. After completed cytopathic effect (CPE), viruses were purified as described by Qin et al. (2001). Briefly, the virus-infected cultures were harvested and centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant (SN1) was collected and stored at 4 °C while the pellet of virus–cell debris was resuspended with SN1, followed by three cycles of rapid freezing/thawing. The virus–cell debris suspension was then centrifuged at 4000 × *g* for 20 min at 4 °C. The supernatant (SN2) was collected and finally pooled with SN1 overnight at 4 °C. Virus particles were pelleted from the pooled SN1 and SN2 by centrifugation at 10,000 × *g* for 8 h at 4 °C. The pellet was resuspended in 2 ml TN buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.5) and layered onto gradients of 15–60% (w/v) sucrose. After centrifugation at 150,000 × *g* for 1 h at 4 °C, the resulting virus bands were collected and resuspended in TN buffer. Virus samples were examined under transmission electron microscope.

2.4. Affinity chromatography of shrimp hemolymph

A suitable amount of CNBr-activated Sepharose 4B (Pharmacia Biotech) was suspended in 1 mM HCl and washed for 15 min. The ligand (purified WSSV or purified SGIV) to be coupled was dissolved in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). The Sepharose 4B gel was mixed with the coupling solution containing the ligand and rotated end-over-end for 1 h at room temperature. After washing with 5 gel volumes of coupling buffer, the gel was transferred to 0.1 M Tris–HCl (pH 8.0) and incubated for 2 h at room temperature. Subsequently, the gel was washed with 3 cycles of pH-alternated buffer A (0.1 M acetate buffer, 0.5 M NaCl, pH 4.0) and buffer B (0.1 M Tris–HCl, 0.5 M NaCl, pH 8.0) at 5 gel volumes of each buffer.

The column filled with ligand-coupled gel was equilibrated using 0.1 M Tris–HCl (pH 8.0). The collected shrimp hemolymph were filtered (0.22 μm) and loaded into the column. After washing with 10 bed volumes of 0.1 M Tris–HCl, the column was eluted with elution buffer (0.1 M Tris–HCl, 1 M NaCl, pH 8.0).

2.5. Mass spectrometry

The isolated proteins from affinity chromatography were separated by a 12% SDS–PAGE gel and stained with Coomassie Blue R 250. Protein bands were excised and subjected to in-gel digestion and mass spectral analyses (matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS/MS)) as previously described (Huang et al., 2002). Briefly the excised bands were dehydrated several times with acetonitrile. After drying in a Speedivac, in-gel protein digestions were performed using trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate at 37 °C for 15 h. The digests and the matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile) (1:1, v/v) were

loaded on the target plate. MALDI-TOF spectra of the peptides were obtained as before (Huang et al., 2002) with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA, USA). Data mining against GenBank was performed using MS-Fit software. For nano-ESI-MS/MS, the digest samples were analyzed in a quadrupole time-of-light (Q-TOF) mass spectrometer-Q-TOF2 mass spectrometer (Micromass, Manchester, UK) following the previous procedure (Huang et al., 2002). Data search against the GenBank database was performed using Globule Server (Micromass).

2.6. Separation of hemocyanin subunits

After separation of the affinity-isolated proteins on SDS–PAGE, a section of the gel was excised and then stained in 0.3 M $CuSO_4$ to determine the positions of the hemocyanin subunits (Zhang et al., 2002). The protein bands in the remaining non-stained gel were excised according to their positions in the stained gel and transferred into dialysis tubing, respectively. After electrophoresis in the SDS–PAGE electrode buffer for 1 h, the eluate in the tubing was loaded into Econo-Pac 10 DG disposable chromatography column (Bio-Rad, CA, USA) and eluted with the renaturation buffer (50 mM Tris–HCl, pH 8.0, 1 mM DTT, 1 M NaCl). Subsequently, the eluate was stirred gently at 4 °C overnight to renature the protein (Lin and Cheng, 1991).

2.7. Antiviral assays

2.7.1. Inhibition against virus

Inhibitory effect of the test hemocyanin on viruses was monitored by the inhibition of virus-induced cytopathic effect (CPE) in GP cells (for SGIV) or FHM (for FV3) or BF2 cells (for LDV, ThRV, ABV and IPNV). Cells were seeded in 96-well plates (10^5 cells per well) and incubated at 25 °C. After serial dilution, 10 μl of various concentrations of hemocyanin were mixed with equal volumes of viruses (SGIV, FV3, LDV, ThRV, ABV and IPNV, respectively) at a MOI of 0.02. Then the mixtures of viruses and hemocyanin were incubated for 1 h at room temperature. When the cells were confluent, cell monolayers were infected with the mixtures of hemocyanin and viruses, viruses mixed with the elution buffer (0.1 M Tris–HCl, 1 M NaCl, pH 8.0) and viruses only as controls, respectively. After a 0.5–1 h adsorption period, culture media were added, and the plates were incubated at 25 °C. Each treatment was repeated four times. When the control cultures (inoculated with viruses but not exposed to hemocyanin) showed complete destruction (usually 3 days after virus inoculation), the number of viable cells was determined by staining of the cells with 1% crystal violet in ethanol (Villalon et al., 1993). Following the 2–3 min staining period, excess dye was removed by washing the cell monolayer three times with phosphate buffered saline (PBS). The plates were then let to dry upside down and the amount of dye bound was determined directly by inspection of the cells against a white background. The concentration of the hemocyanin required to inhibit virus-induced CPE to 50% of the virus-infected control (without the hemocyanin) was estimated as the 50% effective concentration (EC_{50}) (Villalon et al., 1993; Matsuda et al., 1999).

2.7.2. Cytotoxicity

Cells were seeded in 96-well plates (10^5 cells per well). When confluent, culture media were replaced by the media containing different concentrations of the hemocyanin. Seven days later, number of viable cells was determined by staining of the cells with 1% crystal violet in ethanol. Cytotoxicity of hemocyanin was evaluated as CC_{50}, which corresponds to the concentration required to reduce the viable cells to 50% of control (without the test hemocyanin) (Villalon et al., 1993; Matsuda et al., 1999).

3. Results

3.1. Isolation of antiviral protein from shrimp *Penaeus monodon*

In an attempt to identify and characterize the proteins involved in shrimp immune response against WSSV, the purified virus was coupled to Sepharose for the affinity isolation of shrimp proteins bound to WSSV. After affinity chromatography with the healthy and WSSV-infected shrimp hemolymph, two proteins were obtained with molecular masses of 73 and 75 kDa as determined by SDS–PAGE (Fig. 1, lanes 1–3). This indicated that the two proteins

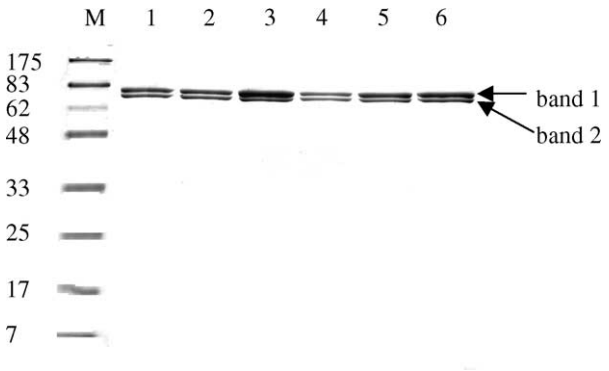

Fig. 1. SDS–PAGE of affinity-purified proteins from shrimp *Penaeus monodon* hemolymph using purified WSSV and purified SGIV ligands. Lane M: protein marker (kDa); lane 1: healthy shrimp hemolymph, purified WSSV ligand; lane 2: WSSV-infected shrimp hemolymph (2 h post-infection), purified WSSV ligand; lane 3: WSSV-infected shrimp hemolymph (24 h post-infection), purified WSSV ligand; lane 4: healthy shrimp hemolymph, purified SGIV ligand; lane 5: WSSV-infected shrimp hemolymph (2 h post-infection), purified SGIV ligand; lane 6: WSSV-infected shrimp hemolymph (24 h post-infection), purified SGIV ligand. Bands 1 and 2 were excised for mass spectral analyses.

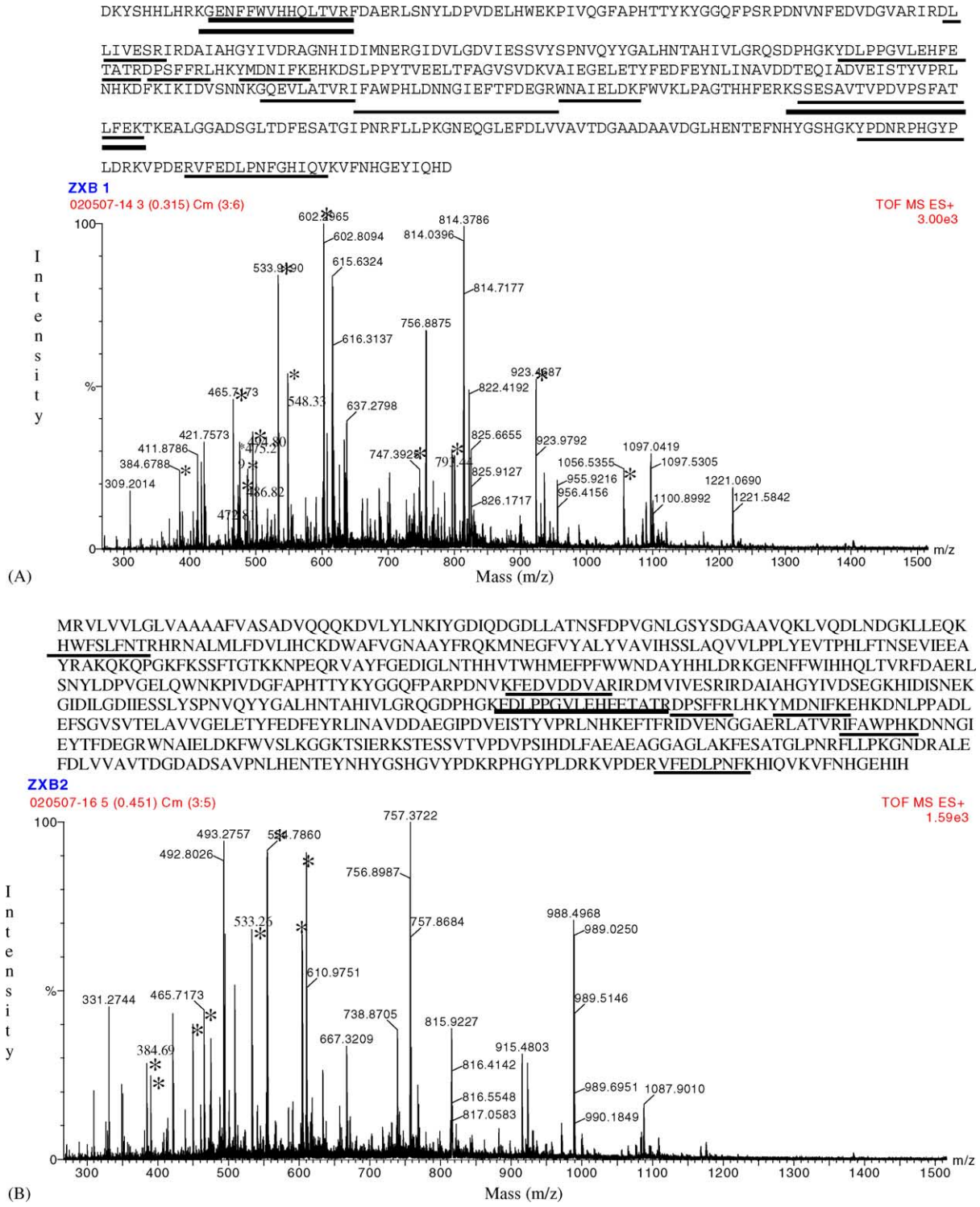

Fig. 2. Identification of two affinity-isolated proteins by mass spectral analyses. Peptides were produced by in-gel tryptic digestion. The tryptic peptides that match to hemocyanin sequences within a mass accuracy of 100 ppm were indicated by solid underlines. The matched peaks were marked by stars. (A) nano-ESI-MS spectrum of band 1 (B) nano-ESI-MS spectrum of band 2.

might be relevant to antiviral activity because of their binding capacities to WSSV. When the purified SGIV, a fish virus, was coupled to the Sepharose, two same proteins were obtained from the shrimp hemolymph (Fig. 1, lanes 4–6). The result showed that the two proteins were able to bind different viruses. However, no protein from the shrimp hemolymph was found to bind the glutathione S-transferase (GST) or anti-mouse IgG coupled to the Sepharose column. This indicated that the two proteins were specially bound to virus.

Table 1
Antiviral properties of the hemocyanin isolated from *Penaeus monodon*

	SGIV	FV3	LDV	ThRV	ABV	IPNV
EC_{50} ($\mu g\,ml^{-1}$)	6.64 ± 0.02	4.61 ± 0.01	5.53 ± 0.04	4.88 ± 0.02	4.66 ± 0.03	4.56 ± 0.01
CC_{50} ($\mu g\,ml^{-1}$)	>200	>200	>200	>200	>200	>200

Effects of the hemocyanin on virus replication were assayed with six fish viruses in cell cultures in vitro. EC_{50} was estimated as effective concentration of hemocyanin required to inhibit virus-induced CPE to 50% of the virus-infected control (without hemocyanin addition) as assayed by the dye uptake method. CC_{50} was evaluated as cytotoxic concentration required to reduce the viable cells to 50% of the control (without the test hemocyanin). Each treatment was repeated four times.

3.2. Primary structure determination of the affinity-isolated proteins

The two affinity-isolated protein bands in SDS–PAGE were excised and subjected to MS analyses to identify their primary structures. After in-gel tryptic digestion, they were first analyzed by MALDI-TOF-MS. Based on searching against GenBank with the peptide mass fingerprints, only band 2 (Fig. 1) was identified to be shrimp hemocyanin. To identify the two proteins, therefore, tryptic peptides of the two proteins were further subjected to nano-ESI-MS/MS. Major peaks from nano-ESI-MS spectra of the two proteins were selected for manual sequencing respectively. Subsequently, peptide sequences derived from the MS/MS data were searched against GenBank database using a global server. The two protein (Fig. 1) were all revealed to be shrimp hemocyanin with 31.2% (band 1) and 10.8% (band 2) coverage of amino acids sequences respectively (Fig. 2A and B).

3.3. Inhibitory activity of affinity-isolated hemocyanin against viruses

The results from affinity chromatography showed that the shrimp hemocyanin was able to bind different viruses (WSSV and a fish virus). Moreover, up to date, there is no suitable cell line to culture shrimp virus. In an attempt to reveal anti-viral properties of the hemocyanin, therefore, the fish viruses capable of cell culture were applied to facilitate CPE analysis. After serial dilutions, the affinity-isolated hemocyanin from *P. monodon* was mixed with six fish viruses of SGIV, FV3, LDV, ThRV, ABV and IPNV, respectively. Cells were seeded in 96-well plates, and then infected with six fish viruses only, viruses mixed with the elution buffer as controls and the mixtures of virus and hemocyanin, respectively. Three days after virus inoculation, the control cultures (inoculated with viruses but not exposed to hemocyanin) showed complete destruction, and the cells inoculated with mixtures of virus and hemocyanin were very lightly destructed. Subsequently, the numbers of viable cells were determined. The effects of hemocyanin on viruses are shown in Table 1. For all the six fish viruses (DNA or RNA viruses), the EC_{50} ranged between 4.56 and 6.64 $\mu g\,ml^{-1}$. This showed that the hemocyanin was a potent inhibitor of virus at low concentration. To investigate cytotoxicity of the hemocyanin to host cells, serial dilutions of hemocyanin alone were added to the cells. The results showed that CC_{50} of the hemocyanin to host cells (GP, BF2 and FHM) were all more than 200 $\mu g\,ml^{-1}$. Based on the high concentration of CC_{50}, the hemocyanin had no cytotoxicity at low antiviral concentration. However, the hemocyanin was not able to inhibit virus replication completely. When the cells (inoculated with virus–hemocyanin mixtures) were cultured continuously, the complete CPEs were observed eventually. Hemocyanin in the culture supernatant was analyzed by SDS–PAGE at various culturing time, and no degradation of hemocyanin was observed during cell cultures.

To investigate the antiviral activities of the two subunits (bands 1 and 2 with molecular masses of 73 and 75 kDa, respectively) of hemocyanin, they were purified from SDS–PAGE gel and then renatured (Fig. 3). Each of the purified two subunits and mixture of the subunits were assayed for their activities against virus. After serial dilutions, the subunit 1 (band 1), subunit 2 (band 2) and mixture

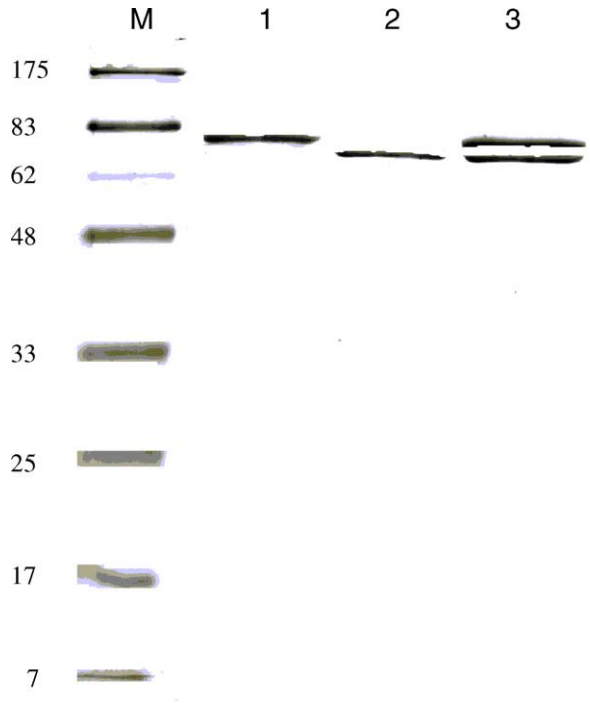

Fig. 3. Purification of two subunits of hemocyanin. The two bands of hemocyanin, separated by SDS–PAGE, were excised and electrically eluted. Lane M: protein marker (kDa); lane 1: the upper subunit (band 1); lane 2: the lower subunit (band 2); lane 3: unpurified hemocyanin.

Table 2
Effects of two separated subunits of the hemocyanin on SGIV replication in cell cultures in vitro

	Subunit 1 (73 kDa)	Subunit 2 (75 kDa)	Mixture of the two subunits
EC_{50} ($\mu g\,ml^{-1}$)	6.25 ± 0.04	6.23 ± 0.07	5.57 ± 0.05
CC_{50} ($\mu g\,ml^{-1}$)	>200	>200	>200

After serial dilutions, purified hemocyanin subunit 1 (73 kDa), subunit 2 (75 kDa) and mixture of the two subunits were mixed with SGIV, and then infected to GP cells. When the control cultures showed complete CPE, EC_{50} of each protein treatment was determined as assayed by the dye uptake method, meanwhile the CC_{50} for each treatment was also evaluated. Each treatment was repeated four times.

of subunits were mixed with SGIV, and then infected to GP cells. When the control cultures showed complete CPE, numbers of viable cells were determined. The results revealed that they all presented antiviral property. On the other hand, no cytotoxicity against host cell was observed (Table 2).

4. Discussion

Penaeid shrimp culture is an important economic industry worldwide. However, this industry has suffered from many diseases especially from viral origin. Among the shrimp viruses, WSSV is currently one of the most important pathogens. Up to date, however, there is no effective method to control virus. A better understanding of shrimp immune response, therefore, will be very helpful for disease control. Shrimp, like other invertebrates, lack a true adaptive immune system and rely instead on various innate immune responses against invading pathogens. While the intricacies of the shrimp immune system have been explored with respect to a number of bacterial and fungal pathogens, little is known about the shrimp response to viral infections. In this study, a protein fraction was isolated from shrimp haemolymph employing affinity chromatography and was identified to be hemocyanin by mass spectrometry. Results showed, for the first time in crustaceans, that the hemocyanin had an anti-viral property. This indicates that the hemocyanin plays an important role in shrimp innate immune response against viruses.

Hemocyanin occurs in several classes: Crustacea, Myriapoda, Merostomata and Arachnida. As the main protein component of hemolymph, hemocyanin typically represents up to 95% of the total amount of protein (Sellos et al., 1997). Hepatopancreas has been shown to be the site of hemocyanin synthesis (Spindler et al., 1992). The hemocyanin, isolated from shrimp hemolymph, is composed of three 75–76 kDa structural and functional subunits (Stoeva et al., 2001). The polypeptide monomer of hemocyanin contains 600–660 amino acid residues depending on species. Hexamer, with a molecular mass of 4.5×10^5 Da, is the predominant form in the most primitive crustacean Decapoda such as *Penaeus setiferus* or *P. monodon* (Sellos et al., 1997). In addition to their primary function as an oxygen carrier for many arthropods, it has been suggested that hemocyanins would be multifunctional proteins involved in physiological processes such as osmoregulation, protein storage or enzymatic activities. In the case of tarantula *Eurypelmeta californicum*, partial in vitro hydrolysis of hemocyanin and cleavage of N-terminal sequences of the protein was shown to activate a phenoloxidase activity (Decker and Rimke, 1998). In some reports, the isolated antibacterial and antifungal peptides from shrimp were found to be generated from the C terminus of hemocyanin (Destoumieux-Garzon et al., 2001; Destoumieux et al., 1997). Actually the hemocyanin itself had the inhibitory property against bacteria in our study (data not shown). Moreover, for the first time, our data revealed that the shrimp hemocyanin itself had antiviral property. However, as shown in fish virus assay, viruses could not be killed by the hemocyanin. It only inhibited virus replication. The virus-infected shrimp maybe die at late infectious stage, even with large amount of hemocyanin in shrimp. Therefore, the hemocyanin, dioxygen-transporting protein, may function as an innate host defense in shrimp that lacks specific antibody immunity and merit further study.

Till today, there is no suitable cell line for shrimp virus culture. In this study, fish viruses capable of cell culture were used to facilitate the antiviral assay. Our data showed that the hemocyanin isolated from shrimp was broadly antiviral against a variety of viruses including DNA and RNA viruses. However, its antiviral mechanism is still unknown. We have mixed the hemocyanin with purified WSSV and SGIV, respectively, and then incubated the mixtures for 2–24 h. No morphological change for the viruses was observed under electron microscope (data not shown). To further investigate inhibitory properties of the hemocyanin against shrimp viruses, studies using shrimp itself exposed to shrimp viruses are under way.

Acknowledgements

This work is supported in part by Hi-Tech Research and Development Program of PR China (863 Program of China), and by A*STAR (Agency for Science, Technology and Research) of Singapore (Grant No.: R347-000-027-305).

References

Ahne, W., 1994. Viral infections of aquatic animals with special reference to Asian aquaculture. Ann. Rev. Fish Dis. 4, 375–388.

Cuthbertson, B.J., Shepard, E.F., Chapman, R.W., Gross, P.S., 2002. Diversity of the penaeidin antimicrobial peptides in two shrimp species. Immunogenetics 54, 442–445.

Decker, H., Rimke, T., 1998. Tarantula hemocyanin shows phenoloxidase activity. J. Biol. Chem. 273, 25889–25892.

Destoumieux, D., Bulet, P., Loew, D., Dorsselaer, A.V., Rodriguez, J., Bachere, E., 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. 272, 28398–28406.

Destoumieux, D., Munoz, M., Bulet, P., Bachere, E., 2000. Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). Cell. Mol. Life Sci. 57, 1260–1271.

Destoumieux-Garzon, D., Saulnier, D., Garnier, J., Jouffrey, C., Bulet, P., Bachere, E., 2001. Crustacean immunity: antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge. J. Biol. Chem. 276, 47070–47077.

Gravell, M., Malsberger, R.G., 1965. A permanent cell line from the fathead minnow (*Pimephales promelas*). Ann. N.Y. Acad. Sci. 126, 555–565.

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapman, R.W., Warr, G.W., 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes a hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. Dev. Comp Immunol. 25, 565–577.

Hoffmann, J.A., Kafatos, F.C., Janeway Jr., C.A., Ezekowitz, R.A.B., 1999. Phylogenetic perspectives in innate immunity. Science 284, 1313–1318.

Huang, C., Zhang, X., Lin, Q., Xu, X., Hu, Z., Hew, C.L., 2002. Proteomic analysis of shrimp white spot syndrome viral proteins and characterization of a novel envelope protein VP466. Mol. Cell. Proteomic. 1, 223–231.

Lin, K.H., Cheng, S.Y., 1991. An efficient method to purify active eukaryotic proteins from the inclusion bodies in *Escherichia coli*. Biotechniques 11, 748–752.

Matsuda, M., Shigeta, S., Okutani, K., 1999. Antiviral activity of marine polysaccharides and their oversulfated derives. Mar. Biotechnol. 1, 68–73.

Qin, Q.W., Lam, T.J., Sin, Y.M., Shen, H., Chang, S.F., Ngoh, G.H.C.L., Chen, C.L., 2001. Electron microscopic observation of a marine fish iridovirus isolated from brown-spotted grouper, *Epinephelus tauvina*. J. Virol. Meth. 98, 17–24.

Qin, Q.W., Chang, S.F., Ngoh-Lim, G.H., Gibson-Kueh, S., Shi, C., Lam, T.J., 2003. Characterization of a novel ranavirus isolated from grouper *Epinephelus tauvina*. Dis. Aquat. Org. 53, 1–9.

Rojtinnakorn, J., Hirono, I., Itami, T., Takahashi, Y., Aoki, T., 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. Fish Shellfish Immunol. 13, 69–83.

Roux, M.M., Pain, A., Klimpel, K.R., Dhar, A.K., 2002. The lipopolysaccharide and β-1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp(*Penaeus stylirostris*). J. Virol. 76, 7140–7149.

Sellos, D., Lemoine, S., Wormhoudt, A.V., 1997. Molecular cloning of hemocyanin cDNA from *Penaeus vannamei* (Crustacea, Decapoda): structure, evolution and physiological aspects. FEBS Lett. 407, 153–158.

Spindler, K.D., Hennecke, R.G., Gellissen, G., 1992. Protein production and the molting cycle in the crayfish *Astacus leptodactylus* (Nordmann, 1842). II. Hemocyanin and protein synthesis in the midgut gland. Gen. Comp. Endocrinol. 85, 248–253.

Stoeva, S., Idakieva, K., Georgieva, D.N., Voelter, W., Genov, N., 2001. *Penaeus monodon* (tiger shrimp) hemocyanin: subunit composition and thermostability. Z. Naturforsch. 56, 416–422.

Villalon, M.D.G., Gil-Fernandez, C., Clercq, E.D., 1993. Activity of several S-adenosylhomocysteine hydrolase inhibitors against African swine fever virus replication in Vero cells. Antiviral Res. 20, 131–144.

Wolf, K.M., Quimby, M., 1966. Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. Science 151, 1004–1005.

Zhang, X., Huang, C., Xu, X., Hew, C.L., 2002. Identification and localization of a prawn white spot syndrome virus gene that encodes an envelope protein. J. Gen. Virol. 83, 1069–1074.