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ORIGINAL RESEARCH ARTICLE

Portunus trituberculatus cryptocyanin seizes swimming crab reovirus and improves crab survival

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Abstract

Crustaceans lack immunoglobulins, and their humoral immunity relies on immune factors present in the hemolymph. A native hemolymph protein with a molecular mass of approximately 120 kDa was found to bind the swimming crab (*Portunus trituberculatus)* reovirus (SCRV) via virus overlay protein binding assays. This protein was identified as cryptocyanin using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis and sequence alignment. The interaction between SCRV and cryptocyanin was confirmed by coimmunoprecipitation. In the antiviral assays *in vivo*, cryptocyanin enhanced the survival of *P. trituberculatus*. Our findings showed for the first time that cryptocyanin can seize viruses, suggesting that cryptocyanin may participate in anti-infective immunity. This study identifies cryptocyanin as a novel and essential component of the invertebrate innate immune system and provides a theoretical basis for elucidating its function in antiviral defense mechanisms.

Keywords: *Portunus trituberculatus*; Reovirus; Cryptocyanin; Virus overlay protein binding assay; Coimmunoprecipitation

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1. Introduction

Swimming crab, *Portunus trituberculatus*, is a globally important commercial crab species characterized by rapid growth and high economic value. It has been designated as a strategically important species for aquaculture in China since 1981.[1] However, with the rapid development of intensive and high-density farming, the aquaculture environment has become increasingly complex, and disease outbreaks are becoming increasingly frequent. Crab viral diseases with rapid transmission and high mortality rates now represent a primary constraint on the healthy and sustainable development of the *P. trituberculatus* aquaculture industry, inflicting substantial economic losses on the industry every year.

Swimming crab reovirus (SCRV) is an important pathogen discovered in recent years. It causes devastating outbreaks with high mortality in cultured *P. trituberculatus*, resulting in significant economic losses for the aquaculture industry.[2–5] To date, no effective

methods have been developed to prevent SCRV infection in crab culture, and traditional aquaculture management and water quality regulation measures cannot effectively block viral transmission and spread. Consequently, there is an urgent need to deeply investigate the intrinsic antiviral immune mechanisms of *P. trituberculatus* and identify key functional molecules involved in defending against SCRV infection.

Hemocyanin and cryptocyanin are members of the hemocyanin gene family. Cryptocyanins were initially misidentified as hemocyanin isoforms due to their high similarity in sequence, structure, and size.[6] Hemocyanin is a copper-containing oxygen-binding protein, while cryptocyanin is a copper-free protein that lacks oxygen-binding capacity.[7–9] Hemocyanin and its derived peptides have been widely found to possess immunological activity and are important members of innate immunity.[10,11] Some have been found to bind to viruses[12–15], and some derived peptides have been reported to improve the survival rates of crustaceans such as shrimp.[15–17] However, no literature has reported that cryptocyanin is involved in immunity. Yet, cryptocyanin may be related to immunity, as *Vibrio anguillarum* challenge induced higher expression of cryptocyanin mRNA in *Eriocheir sinensis*[18], and white spot syndrome virus infection induced higher cryptocyanin expression.[19] To date, studies on cryptocyanin have primarily focused on the involvement of cryptocyanin in cuticle synthesis, structural integrity, and the molt cycle.[20] There is only one publication reporting its association with viruses, in which Yuan *et al.*[21] discovered that the non-structural protein p35 of *Scylla serrata* reovirus interacts with cryptocyanin, but its immune relevance was not validated. Thus, the potential immune role of cryptocyanin remains completely uncharacterized.

In our preliminary study screening candidate viral receptor proteins, we extracted crab proteins and subjected them to virus overlay protein binding assays (VOPBA).[4] A specific protein band was identified as cryptocyanin. This finding prompted further investigation of cryptocyanin. In this study, we confirmed the direct interaction between cryptocyanin and SCRV and quantified its protective effect on crab survival using VOPBA, coimmunoprecipitation (Co-IP), and *in vivo* challenge assays. Collectively, these findings provide the first evidence that cryptocyanin can bind viruses and may function as part of the invertebrate immune system.

2. Materials and methods

2.1. Crude extraction of hemolymph proteins

Hemolymph was collected from the hearts of healthy *P. trituberculatus* with hard exoskeletons and immediately mixed with anticoagulant at a ratio of 1:1 (v:v). The mixture was centrifuged at 8,000 rpm for 30 min at 4 °C. The collected supernatant fluid was further centrifuged at 10,000 rpm for 30 min at 4 °C. The final supernatant was stored at −20 °C for subsequent use.

2.2. Virus overlay protein binding assay

The SCRV was deposited at the China General Microbiological Culture Collection Center under accession number CGMCC No. 5379. SCRV virions were purified from the tissues of artificially infected *P. trituberculatus* as described.[4,5] Chicken-derived polyclonal antibody specific to SCRV was prepared according to Zhang *et al.*[5]

Virus overlay protein binding assay was performed according to a previously described method.[4] In brief, crude hemolymph proteins extracted from *P. trituberculatus* and prestained protein molecular weight markers (Cat. No. 26612, Thermo Scientific, USA) were resolved via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electrotransferred onto a 0.45 μm polyvinylidene fluoride (PVDF) membrane (Millipore, Germany). The membrane was blocked overnight at 4 °C with 5% low-fat milk in phosphate-buffered saline (PBS) supplemented with 0.5% Tween 20 (PBST), then rinsed three times with PBST. Next, the membrane was incubated with 12.9 μg purified SCRV suspended in PBS for 1 h at room temperature (RT). After three PBST washes, the membrane was probed with a chicken-derived polyclonal antibody specific to SCRV for 2 h at ambient temperature. After an additional triple rinse with PBST, the membrane was treated with horseradish peroxidase (HRP)-conjugated sheep anti-chicken IgG (CW6162, ComWin Biotech, China), diluted 1:5,000 in PBST containing 0.5% skim milk, for 1 h at RT. Finally, after three PBST washes, viral-binding bands on the membrane were developed with O-phenylenediamine (Thermo Scientific, USA) and documented by imaging.

2.3. Identification of the swimming crab reovirus-binding protein via mass spectrometry assay

To identify the composition of the screened SCRV-binding protein, the protein band matching the viral binding signal observed in VOPBA was carefully excised from the 8% SDS-PAGE gel and submitted to Guangzhou Fitgene Biotechnology Co., Ltd. (China) for Micromass matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) peptide mass fingerprinting analysis. The MassLynx software (Version 4.1, Waters Corporation, USA) was used to generate peak lists (.pkl files), which were then searched against the Brachyura crustacean protein database (containing 11,187 sequences) using the MASCOT search engine (Version 2.4,

Matrix Science Ltd., UK). MASCOT scoring was defined as $-10 \times \log(P)$, where P represents the probability of a random match, as described by Perkins *et al.*[22] Protein hits with scores above 53 were considered statistically significant ($p < 0.05$).

2.4. Preparation of the antibody against the swimming crab reovirus-binding protein

Polyclonal mouse antibody against the SCRV-binding hemolymph protein was prepared as described previously.[4] Briefly, crude hemolymph proteins extracted from *P. trituberculatus* and the protein molecular weight markers were resolved using 8% SDS-PAGE. The band matching the virus-binding protein in VOPBA was excised from the gel, homogenized, and resuspended in 0.9% sodium chloride (NaCl). The suspension was emulsified with an equal volume of complete Freund's adjuvant (Sigma, USA) for subcutaneous immunization of Institute of Cancer Research mice ($n = 5$). A first booster with incomplete Freund's adjuvant (Sigma, USA) was given two weeks later, followed by two more boosters at one-week intervals. Mouse sera were harvested seven days after the final injection and stored at −80 °C for later use; the non-immunized mouse serum was prepared as a control.

The antibody titer was quantified using enzyme-linked immunosorbent assay according to a previously established method.[23] The specificity of the mouse antibody was evaluated using a standard Western blotting assay. Briefly, crude hemolymph proteins extracted from *P. trituberculatus* and the protein molecular weight markers were resolved with 8% SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Germany). The membranes were blocked overnight at 4 °C using 5% low-fat milk prepared in PBST, washed thrice with PBST, incubated with the mouse antibody for 1 h at RT, and washed thrice with PBST. The mouse anti-SCRV-binding protein antibody specifically adsorbed on the membrane was detected using HRP-conjugated goat anti-mouse antibody (CW0102S, ComWin Biotech, China).

2.5. Coimmunoprecipitation assays

We further assessed the interaction between the SCRV-binding protein identified in VOPBA and SCRV using Co-IP assays. Co-IP was performed as previously described with minor modifications.[4] Freshly prepared crude hemolymph protein (800 μL) extracted from swimming crab was incubated with 30 μL purified SCRV under gentle rocking at 4 °C for 8 h. The titer of the prepared mouse antibody against the SCRV-binding protein was 1:10,000. To immunoprecipitate cryptocyanin, 10 μL of mouse antibody against the SCRV-binding protein (or irrelevant immunoglobulin Y against soft-shelled turtle systemic septicemia spherical virus as a negative control) was added, followed by overnight incubation at 4 °C. Protein A/G-conjugated agarose beads (60 μL, Santa Cruz, USA) were added to the mixture, followed by incubation at 4 °C for 3 h with gentle rocking. The beads were collected by centrifugation, washed four times with high-salt NETN buffer (20 mM Tris-HCl, 0.5% NP-40, 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 25 μg/mL phenylmethylsulfonyl fluoride [PMSF], pH 7.6) and once with regular NETN buffer (20 mM Tris-HCl, 0.5% NP-40, 100 mM NaCl, 1 mM EDTA, 25 μg/mL PMSF, pH 7.6). Bound proteins were eluted by boiling in loading buffer for 4 min, separated by 12% SDS-PAGE, and SCRV was detected via Western blotting using the anti-SCRV polyclonal antibody.

2.6. Antiviral assay of *P. trituberculatus* cryptocyanin *in vivo*

P. trituberculatus, averaging 20 g in weight, were purchased from a crab farm in Ningbo, Zhejiang Province, China, and cultured in our laboratory in aerated water for seven days at 28 °C. The crabs with hard exoskeletons were randomly divided into three groups (20 crabs/group) in triplicate. The cryptocyanin was purified as described.[8] A 50 μl aliquot of cryptocyanin was incubated with 100 μl purified SCRV for 1 h at 4 °C, and injected into each swimming crab of the test groups via the second pereiopod. A 50 μl aliquot of PBS was incubated with 100 μl purified SCRV for 1 h at 4 °C and injected into each crab of the control groups. Crabs injected with PBS (150 μL/ crab) served as a blank control. All the crabs were maintained with daily water changes and continuous aeration. The number of deaths was recorded daily to calculate the cumulative mortality rate. Since no additional deaths were observed four days post-injection, the statistical analysis was limited to the 4 days. The cumulative mortality rate was calculated using the following formula: Cumulative mortality rate (%) = (Number of deaths/total number) × 100%. The results were analyzed using Statistical Package for Social Sciences (SPSS 20.0, IBM, United States) with one-way analysis of variance, followed by Tukey's post-hoc test to assess significant differences among experimental groups, where $p < 0.05$ was considered statistically significant. Data were represented by three independent experiments and shown as the mean ± standard deviation.

2.7. Denaturing gel electrophoresis

In VOPBA, the undenatured cryptocyanin of *P. trituberculatus* was subjected to gel electrophoresis without the addition of dithiothreitol (DTT), β-mercaptoethanol (β-ME), or boiling. To estimate the apparent molecular mass of *P. trituberculatus* cryptocyanin in the presence of β-ME and after boiling pretreatment under denaturing

conditions, the protein was mixed with SDS-PAGE sample buffer containing β-ME and boiled for 5 minutes. Electrophoresis was performed on self-cast polyacrylamide mini-gels (1 mm thick) using a discontinuous buffer system. The separation gel (pH 8.8) contained 8% polyacrylamide. The stacking gel (pH 6.8) contained 5% polyacrylamide. The running buffer contained 25 mM Tris (pH 8.6), 192 mM glycine, and 0.1% SDS (w/v) in water. All solutions were freshly prepared prior to use. SDS-PAGE was performed on a vertical polyacrylamide gel system at a current of 80 V until the protein bands reached the interface of the separating gel. Separation was performed at 120 V for two h. After electrophoresis, the gel was immersed in Coomassie blue R250 solution. The Coomassie stain was removed by aspiration after staining. The gel was then immersed in the destaining solution composed of 50% (v/v) methanol, 10% (v/v) acetic acid, and 40% (v/v) water, allowing the gel to destain with gentle agitation. The destaining step was repeated several times, with removal of destaining solution at each change by aspiration. Destaining was continued until the protein bands were seen clearly without any background staining of the gel. In addition, Western blot assays were performed to detect the protein bands using the antibody against the SCRV-binding protein prepared in Section 2.4.

3. Results

3.1. Screening of swimming crab reovirus-binding protein in swimming crab hemolymph via virus overlay protein binding assay

Swimming crab crude hemolymph proteins were prepared, separated by SDS-PAGE, and transferred to PVDF membranes. After incubation with SCRV, the membranes were treated with anti-SCRV polyclonal antibodies followed by HRP-conjugated secondary antibodies for band detection. VOPBA analysis revealed a specific interaction between SCRV and an approximately 120 kDa hemolymph protein of the swimming crab. No other significant bands were observed (Figure 1).

3.2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis

The mass spectrographic results are shown in Figure 2. As summarized in the official Mascot search report, the SCRV-binding protein generated a high-confidence match to *Portunus pelagicus* cryptocyanin (National Center for Biotechnology Information accession gi|121484232), with a Mascot score of 76. Therefore, the putative SCRV-binding protein was identified as cryptocyanin.

3.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of pre-boiling *P. trituberculatus* cryptocyanin in the presence of β-mercaptoethanol

Boiled *P. trituberculatus* cryptocyanin samples in the presence of β-ME had an apparent molecular mass of approximately 80 kDa (Figure 3), which is similar to that of the other reported crustacean cryptocyanin.[8,24]

3.4. Interaction between swimming crab cryptocyanin and swimming crab reovirus identified using coimmunoprecipitation

Western blot analysis of the crude hemolymph extract with the anti-cryptocyanin antibody revealed a single specific band, indicating the antibody's specificity. The binding of cryptocyanin to SCRV, discovered in VOPBA, was further validated using Co-IP. The results of the Co-IP showed that SCRV (approximately 60 kDa) was specifically precipitated from the mixture of SCRV and crude hemolymph proteins of swimming crab with the antibody against cryptocyanin, while SCRV could not be precipitated with an unrelated viral antibody (immunoglobulin Y to soft-shelled turtle systemic septicemia spherical virus) (Figure 4).

3.5. Antiviral efficacy of cryptocyanin in *P. trituberculatus*

To elucidate whether cryptocyanin has anti-SCRV properties, the antiviral experiments were carried out *in vivo*. The average cumulative mortality rate in groups injected with PBS alone was only 5%, suggesting that

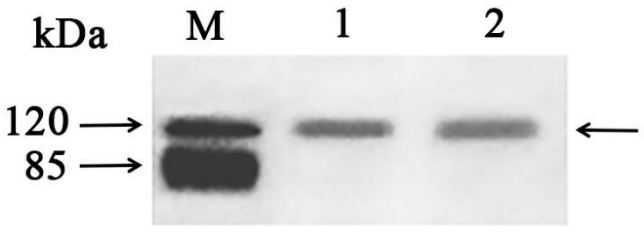


Figure 1. Virus overlay protein binding assay analysis of swimming crab reovirus binding to *P. trituberculatus* crude hemolymph proteins
Notes: M: Prestained protein molecular weight marker; Lane 1–2: The prominent binding bands with molecular weights of approximately 120 kDa are indicated with black arrows on the right.

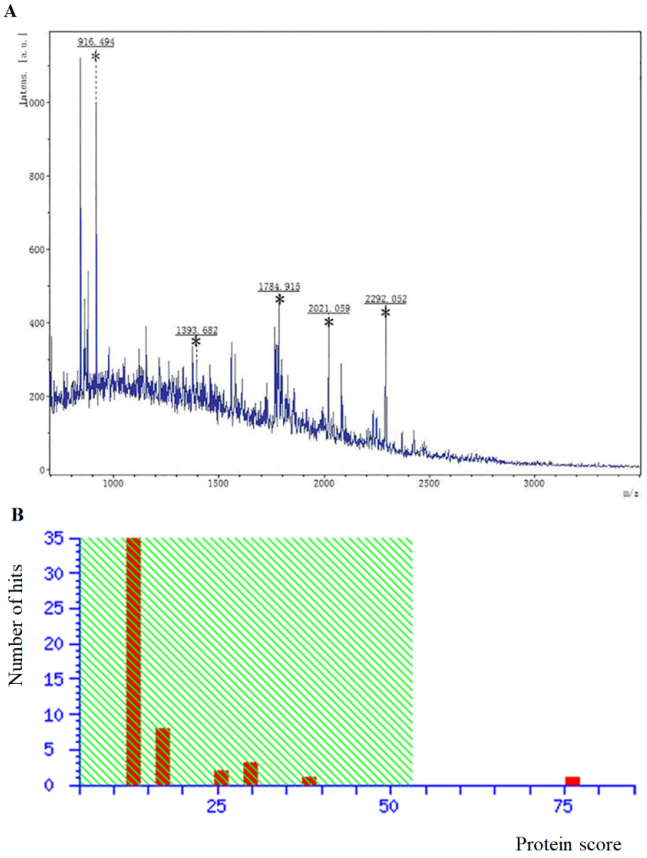


Figure 2. Mass spectrographic analysis of the SCRV-binding protein. (A) Fingerprint of the protein; matched peaks are marked by asterisks. (B) Mass spectrographic analysis of the protein fingerprint using Mascot. Accession information is as follows: National Center for Biotechnology Information accession no.: gi|121484232, score:76, description: cryptocyanin (*P. pelagicus*).

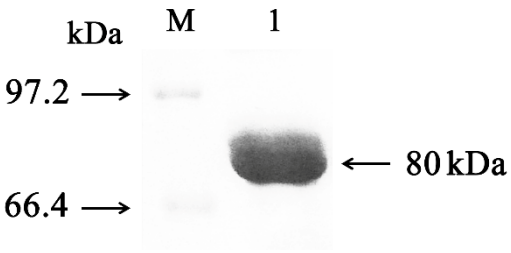


Figure 3. *P. trituberculatus* cryptocyanin was separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The cryptocyanin samples in the presence of β-mercaptoethanol were prepared by boiling. Notes: M: Broad protein molecular weight marker (Cat. NO. 3452Q, TaKaRa, Japan); Lane 1: The cryptocyanin was indicated with black arrows on the right.

injection and cultivation had no significant negative effect on the crabs. The average cumulative mortality rate of the groups artificially infected with SCRV reached 100% within four days, while that of the groups artificially infected with SCRV pre-incubated with cryptocyanin was 73.33% (Figure 5). Afterward, no further deaths occurred. This result showed that cryptocyanin substantially improved the survival rate of swimming crabs infected with the virus, with approximately a 25% increase in survival after four days.

4. Discussion

Given the urgent need to identify key antiviral molecules against SCRV, this study aimed to screen SCRV-binding proteins from the hemolymph of *P. trituberculatus*. Through VOPBA screening, a hemolymph protein with an approximate molecular mass of 120 kDa was found that specifically binds to SCRV. Subsequent MALDI-TOF-MS analysis revealed this protein as cryptocyanin. The interaction between cryptocyanin and SCRV was further validated using Co-IP assays. An *in vivo* antiviral assay demonstrated that cryptocyanin significantly increased the survival rate of *P. trituberculatus* challenged with SCRV, indicating that cryptocyanin plays a crucial role in crab antiviral defense.

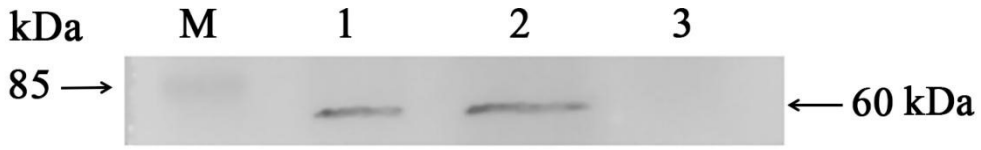


Figure 4. The immunoprecipitates of swimming crab reovirus (SCRV) and cryptocyanin precipitated by the antibody against cryptocyanin, and detected via Western blot analysis using the antibody against SCRV
Notes: M: Prestained protein molecular weight marker; Lane 1: Swimming crab crude hemolymph proteins + SCRV+ anti-cryptocyanin Ab; Lane 2: SCRV; Lane 3: Swimming crab crude hemolymph proteins + SCRV + immunoglobulin Y to soft-shelled turtle systemic septicemia spherical virus.

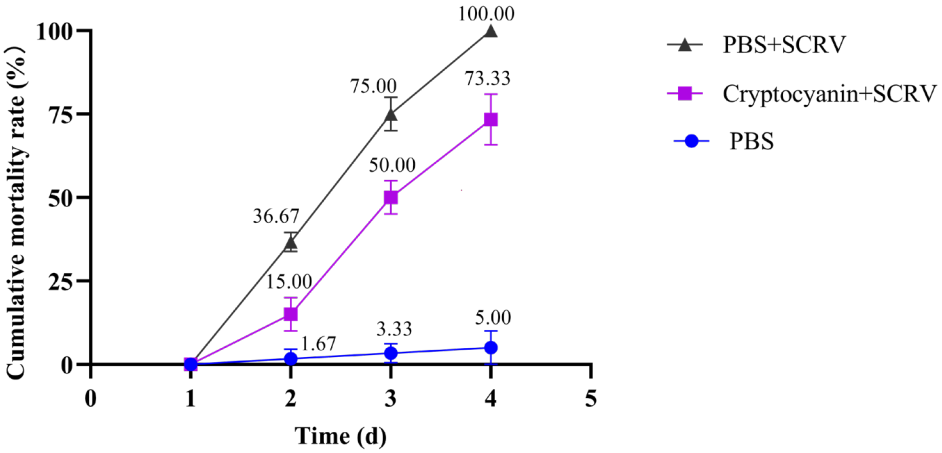


Figure 5. Antiviral efficacy of *P. trituberculatus* cryptocyanin *in vivo*

Invertebrates lack adaptive immunity but possess a potent innate immune system. Unlike vertebrates that deploy antibody- and lymphocyte-mediated adaptive immunity, invertebrates entirely depend on germline-encoded innate immune receptors and humoral effectors for pathogen resistance, and crustaceans particularly utilize hemolymph-derived immune molecules to clear viral invaders. Various humoral immune factors, including lectins, hemocyanins, hemocyanin-derived peptides, lysozymes, antimicrobial proteins, C-reactive proteins, phenoloxidases, and thioester-containing proteins, have been shown to recognize and bind to pathogens. These molecules mediate agglutination, opsonize pathogens for phagocytosis, directly kill microorganisms, and activate downstream immune cascades such as the prophenoloxidase system and complement-like pathways.[25,26]

Although cryptocyanin belongs to the hemocyanin gene family, previous studies have documented its functions in cuticle synthesis, structural integrity, and the molt cycle.[20] Terwilliger *et al.* found that a marked decline in cryptocyanin synthesis occurs during intermoult in the crab *Cancer magister*, whereas divergent expression patterns have been documented in other crab species.[8] Cryptocyanin expression in *P. pelagicus* increases during intermoult, remains stable during the premoult period, and markedly decreases in the post-molt stage.[20] In the study of Wang *et al.*,[18] the significant up-regulation of cryptocyanin expression was observed in both premoult and intermoult stages in *E. sinensis* hepatopancreas; high levels of cryptocyanin expression in the intermoult stage were also present in the hemolymph. Collectively, cryptocyanin exhibits a molt stage-dependent expression pattern, characterized by relatively low abundance in the postmoult stage and increased expression during premoult, although considerable variation exists among species and developmental stages. Although the exact molt stage of *P. trituberculatus* was not determined in this study, it is unlikely that they were in the postmoult stage, as their exoskeletons were no longer pliable.

Given that hemocyanin family members are also involved in immune-related processes, whether cryptocyanin contributes to host defense remains unclear. To date, no evidence has been reported regarding its involvement in anti-infective immunity. Most studies focused on its structural role in exoskeleton formation and molting

regulation,[20] ignoring its potential non-classical functions. In contrast, our discovery of its antiviral activity represents a breakthrough in understanding the functional diversity of the hemocyanin gene family. To the best of our knowledge, this study is the first to show that cryptocyanin can bind SCRV. Furthermore, we provide the first evidence that this binding enhances the survival rate of animals challenged with the virus. Given these findings, cryptocyanin may represent a novel component of the invertebrate immune system, and its potential to confer cross-protection against viral or bacterial pathogens in other invertebrates warrants further investigation. Although cryptocyanin has not been previously reported as an invertebrate immune factor, its expression level has been found to be upregulated in invertebrate species undergoing stress exposure or pathogen infection.[18,19,27,28] It has been proposed that cryptocyanin has antifungal, antimicrobial, and antiviral activity. Its high expression levels during molting, when the outer shells become soft, may help protect against infections.[8,24,29] Qi *et al.*[30] found that the relative mRNA levels of crustin, lysozyme and cryptocyanin genes were significantly upregulated by arginine supplementation in the diet. Among these, crustin and lysozyme have all been identified as important immune factors.[31–34] Bao *et al.*[35] reported that crustin may participate in the innate immunity of *Sinopotamon henanense* by binding to bacteria and promoting hemolymph phagocytosis to eliminate invading pathogens. The lysozyme identified from the scallop *Argopecten purpuratus* was involved in the immune response and microbial homeostasis of *A. purpuratus* hemolymph.[36] In this study, we found that *P. trituberculatus* cryptocyanin can seize SCRV and increase the survival rate of crabs challenged with the virus.

Previous studies have reported that denatured cryptocyanin in some crustaceans treated with DTT or boiled for 3 min exhibits a monomeric apparent molecular mass between 68 kDa and 88 kDa.[8,9,24] Proteins may show different molecular weights on electrophoresis under different conditions. For instance, cryptocyanin of pre-molt *C. magister* had an apparent molecular mass of 430–450 kDa on the nondissociating, nondenaturing PAGE at pH 7.4. Yet, in the presence of DTT, *C. magister* cryptocyanin electrophoresed one major and two minor polypeptide chains with apparent molecular masses of approximately 88, 85, and 74 kDa.[8] Similarly, in this study, we observed that the undenatured cryptocyanin of *P. trituberculatus* migrated at approximately 120 kDa in VOPBA assays. After treatment with β-ME and boiling, the apparent molecular weight of *P. trituberculatus* cryptocyanin in denaturing gel electrophoresis was around 80 kDa (Figure 3). The precise mechanism of the differing electrophoretic mobility of cryptocyanins warrants further in-depth study. It may be related to glycosylation or incomplete dissociation of stable oligomeric complexes during sample preparation.

Glycosylation modifications, pretreatment conditions, and SDS-PAGE conditions can all affect protein apparent molecular weight. Boiling the protein sample for several minutes prior to electrophoresis may cause deglycosylation.[37–39] Accumulating evidence has demonstrated that arthropod hemocyanin and its homolog cryptocyanin undergo extensive post-translational modifications, mainly glycosylation and phosphorylation, which are critical for regulating protein structure, electrophoretic mobility, and immune-related biological functions.[38,39] Cryptocyanin harbors highly conserved glycosylation motifs modifying the apparent molecular weight, which were verified in both molluscan and crustacean hemocyanin superfamily proteins.[8,40,41] For instance, cryptocyanin of pre-molt *C. magister* was identified as a glycoprotein via periodic acid Schiff staining.[8] In crustaceans including shrimp and crabs, glycosylation not only maintains the structural stability of hemocyanin oligomers but also mediates immune recognition and effector activity, while reversible phosphorylation serves as a pivotal switch for the functional conversion of hemocyanin from a respiratory protein to an active immune effector.[39,42]

To date, two SCRV-binding proteins have been identified via VOPBA. One was derived from the gill cell membrane proteins and identified as tubulin mediating SCRV infection.[4] The other is cryptocyanin in this study. There is also an earlier report on the interaction between crabs and reoviruses: an interaction between mud crab reovirus VP12 and voltage-dependent anion-selective channel protein was found via yeast two-hybrid cDNA library screening and determined by Co-IP.[43]

Our findings could translate into several applications, including but not limited to the following. Recombinant cryptocyanin, or its virus-binding domain, may be developed as a passive immunotherapeutic agent delivered via immersion or oral feeding to neutralize SCRV during outbreaks. The cryptocyanin-SCRV binding interface represents a novel drug target; structural studies will allow virtual screening of small-molecule mimetics that block viral attachment. In a vaccine-like approach, cryptocyanin may serve as a molecular adjuvant or an antigen-delivery vehicle that potentiates innate immune memory when complexed with inactivated virus. Moreover, cryptocyanin levels could be used as a biomarker in marker-assisted selection for disease-resistant broodstock, while dietary immunostimulants that upregulate endogenous

cryptocyanin could be formulated into functional feeds. These strategies hold promise for delivering novel disease control measures for sustainable crustacean aquaculture.

Collectively, our study found that cryptocyanin can bind to SCRV, and *in vivo* assays revealed that cryptocyanin enhances the survival rate of virus-infected crabs. Nevertheless, this study has several limitations. The precise viral-binding domain of cryptocyanin, the specific signaling pathways triggered by cryptocyanin-mediated viral recognition, and the potential of RNA interference targeting the cryptocyanin gene to attenuate its antiviral effect in crabs remain to be elucidated.

5. Conclusion

Our study reveals that cryptocyanin can seize viruses and cryptocyanin may be involved in anti-infective immunity. The interaction of cryptocyanin from the swimming crab (*P. trituberculatus*) with SCRV was identified using VOPBA, mass spectrometry, and Co-IP. Cryptocyanin increased the survival rate of crabs challenged with the virus. These findings lay a foundation for future studies to elucidate the comprehensive antiviral mechanism of cryptocyanin in crustaceans and develop antiviral agents.

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Conflict of interest

Dengfeng Li is an Editorial Board Member of this journal, but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. The authors declare no conflicts of interest.

Author contribution

Conceptualization: Dengfeng Li
Investigation: Jing Fang, Dengfeng Li
Methodology: Jing Fang
Writing–original draft: Jing Fang
Writing–review & editing: All authors

Ethics approval and consent to participate

The animal study was reviewed and approved by Ethics Committee for Animal Experiments of School of Marine Sciences of Ningbo University (approval number 2025-0028). All mouse immunization procedures for polyclonal antibody production were performed following the laboratory animal welfare guidelines of Ningbo University School of Medicine and the national standards for laboratory animals. The animal experiments were under the supervision of the Institutional Animal Care and Use Committee of Ningbo University School of Medicine.

Consent for publication

Not applicable.

Availability of data

Data are available upon reasonable request from the corresponding author.

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