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Protection of *Procambarus clarkii* against white spot syndrome virus using inactivated WSSV

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ABSTRACT

White spot syndrome virus (WSSV) is a highly pathogenic and prevalent virus infecting shrimp and other crustaceans. The potentiality of binary ethylenimine (BEI)-inactivated WSSV against WSSV in crayfish, *Procambarus clarkii*, was investigated in this study. Efficacy of BEI-inactivated WSSV was tested by vaccination trials followed by challenge of crayfish with WSSV. The crayfish injected with BEI-inactivated WSSV showed a better survival ($P < 0.05$) to WSSV on the 7th and 21st day post-vaccination (dpv) compared to the control. Calculated relative percent survival (RPS) values were 77% and 60% on the 7th and 21st dpv for 2 mM BEI-inactivated WSSV, and 63%, 30% on 7th and 21st dpv for 3 mM BEI-inactivated WSSV. However, heat-inactivated WSSV did not provide protection from WSSV even on 7th dpv. In the inactivation process WSSV especially their envelope proteins may be changed as happened to 3 mM BEI and heat-inactivated WSSV particles. These results indicate the protective efficacy of BEI-inactivated WSSV lies on the integrity of envelope proteins of WSSV and the possibility of BEI-inactivated WSSV to protect *P. clarkii* from WSSV.

1. Introduction

Electron microscopy (EM) showed that white spot syndrome virus (WSSV) is enveloped, bacilliform in shape and have a tail-like appendage at one end [1]. The virus contains double stranded DNA with an estimated size of 292967 bp, with 181 open reading frames consisting of 39 structural proteins [2–4]. However, different genome sizes have been reported from diverse virus isolates and large genome sequences have been reported from virus isolates from China and Thailand [5]. White spot syndrome virus has a broad host range within Decapoda crustaceans, including penaeid shrimp and crayfish [6–8]. White spot syndrome virus (WSSV) belongs to the new virus family *Nimaviridae*, genus *Whispovirus* [9]. In China, production losses of 80% of farmed shrimp were attributed to WSSV [10,11].

Crustaceans do not possess an adaptive immune system, but now it is doubted for some investigation [12,13]. A recent study in vaccinated crayfish surviving from experimental WSSV infections showed that it possess a resistance against WSSV [14–18]. Of the viral structural proteins, envelope proteins often play vital roles in virus entry and assembly [19–21]. Vaccination using viral proteins, especially VP28, has been reported to offer shrimp protection against WSSV infection [22–24]. However, a neutralization assay with the combination of antibodies against different envelope proteins showed that a combination of VP36B and VP31 antibodies could strongly inhibit WSSV infection in crayfish. It revealed that multiple envelope proteins are involved in WSSV infection in crayfish during this process [25].

Furthermore, immunostimulation of shrimp with inactivated vibrio have been reported to provide some protection [26]. The shrimp intramuscularly vaccinated with formalin-inactivated WSSV can induce a resistance to the virus of intramuscular (IM) injection on the 10th day post-vaccination [27]. These reports suggest that some of envelope proteins can induce an immune response and protect shrimp against WSSV. Thus inactivated WSSV will be a good vaccine to shrimp but inactivating with formalin and heat are not good method. Binary ethylenimine (BEI) is a kind of aziridines and formed by the cyclization of 2-chloroethylamine hydrochloride (BEA). BEI is known to alkylate nucleic acids but do not damage the protein of inactivated virus in the concentration of 1 mM [28,29]. In veterinary medicine BEI is the preferred inactivating agent for producing vaccines containing animal viruses with DNA or RNA genomes [30–32]. Although chemical agents and physical methods have been studied on the inactivation of WSSV

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[33,34], but envelope proteins of inactivated virus was damaged when WSSV was inactivated. Our study was carried out to explore the possibility of protecting *Procambarus clarkii* from WSSV infection by vaccination with BEI-inactivated WSSV.

2. Materials and methods

2.1. Crayfish

Crayfish *P. clarkii*, approximately 20 g and 8 cm each, were reared at 25 ± 1 °C. They were kept in tanks with sand-filtered, ozone-treated and flow-through freshwater and fed with commercial pellet feed at 5% of body weight per day. Walking legs from randomly selected individuals were subjected to PCR assays to ensure that the crayfish were WSSV-free before experimental challenge.

2.2. Measurement of viral infectivity (LD_{50})

The infectivity (lethal dose 50%: LD_{50}) of WSSV was used as the criterion for the virus inactivation tests. White spot syndrome virus-infected shrimp, *Fennerpenaeus chinensis*, were collected from shrimp farms located near Ningbo, China. Ten grams of infected tissues (gills and tail muscle) were homogenized in 500 ml TNE buffer (50 mM Tris HCl, 400 mM NaCl, 5 mM EDTA, pH 7.5) containing a combination of protease inhibitors (1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, and 1 mM $Na_2S_2O_5$), and then centrifuged at 10,000 × g for 10 min at 4 °C. After filtering by nylon net (400 mesh), this homogenate was centrifuged at 6000 × g for 25 min at 4 °C and filtrated using a Millipore filter (pore size 0.45 μm). This filtrate was the original viral fluid that was used for the inactivation tests afterwards. To measure the infectivity of this virus fluid, 10-fold serial dilutions of the fluid were made from 10^4 to 10^9 and filtrated using a Millipore filter (pore size 0.45 μm). These diluted fluids were injected into each of 30 healthy crayfish (average body weight 20.0 g) at the dose of 0.1 mL/crayfish. Mortality and clinical signs were observed daily for two weeks.

2.3. Inactivated WSSV

0.2 M binary ethylenimine (BEI) was prepared by cyclization of 0.2 M 2-bromoethylamine-HBr in 0.2 M NaOH at 37 °C for 1 h. The β-naphthol violet (a pH indicator) was added to the solution to check the formation of BEI which causes a change in colour from violet to orange. This solution was added to 10^1 WSSV dilution as 1:100 (v/v) to a concentration of 2 mM. The solution was incubated at 37 °C with continuous stirring for 6 h, 12 h, 18 h, 24 h and the reaction was stopped by addition of sodium thiosulphate. And 0.3 M BEI solution prepared by the same way was added to 10 diluted WSSV solution to a concentration of 3 mM and incubated at 37 °C with continuous stirring for 24 h and the reaction was stopped by addition of sodium thiosulphate.

For preparation of heat-inactivated WSSV, the viral suspension was diluted 10-fold in TNE and inactivated for 15 min at 65 °C.

For in vivo injection experiment the healthy crayfish collected from stock were divided into nine groups (30 crayfish per group per tank). For test the safety of BEI and heat-inactivated WSSV six groups of 30 crayfish were intramuscularly (IM) injected with 0.1 mL inactivated WSSV solution. A group of 30 crayfish was IM injected with 0.1 mL of WSSV (10 dilution) which were heated at 37 °C for 24 h to test its effects on WSSV. The positive control groups were injected with 0.1 mL of WSSV (10 dilution) and the negative control was injected with 0.1 mL of TNE solution (Table 1).

Table 1
Effect of BEI and various temperature tested on WSSV infectivity in *Procambarus clarkii*.

Group	Treatment	Dead/tested	Mortality (%)
2 mM BEI			
6	.	2	3

2.4. Electron microscopy and SDS-PAGE

BEI-inactivated WSSV and heat-inactivated WSSV particles were negatively stained with 2% sodium phosphotungstate (PTA, pH 7.0) on collodion-carbon coated grids. All observations were made with a JEOL 1230 transmission electron microscope (JEOL, Japan) operating at 70 kV. The two kinds of inactivated WSSV were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [35]. The gels were stained with Coomassie brilliant blue (0.1% Coomassie Blue R-250 in 1% acetic acid and 40% methanol). A premixed protein molecular weight marker (Fermentas), with proteins ranging from 14.4 to 116 kDa, was co-electrophoresed to determine the molecular weights of the WSSV proteins.

2.5. PCR analysis for WSSV

Total DNA was extracted from walking legs of crayfish with an animal tissue genomic DNA mini-prep kit (Sangon, Shanghai). The samples were tested with one primer set VP28-FW (5′-CGCACA GACAATATCGAGAC-3′) and VP28-RV (5′-GTCTCAGTGCCAGAGTAG GT-3′), amplifying part of WSSV VP28 gene, was used to screen for WSSC-positive animals. PCR was performed with the VP28 primer pair using the following protocol: 5 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The PCR products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized by ultraviolet transilluminator.

2.6. Vaccination experiments

For vaccination experiment 12 groups of 30 inter-molt crayfish with an average weight of 20 g were selected. Before starting the vaccination experiment the crayfish were tested for the presence of WSSV by one step PCR. Six groups of crayfish were vaccinated by IM injection with 0.1 mL of 2mM BEI, 3mM BEI and heat-inactivated WSSV and the controls were IM injected with 0.1 mL of 2mM BEI, 3mM BEI and TNE solution. Seven days after the initial vaccination, three groups and their control groups were IM injected with 0.1 mL of WSSV dilution (1×10^7) for the challenge test. Another three groups and their control groups were challenged by the same way at 21st day post-vaccination (dpv) (Table 2).

2.7. Statistical analysis

The mortalities of the tested and control groups were compared statistically using the chi-square test (χ^2) at a significance level of 5%. The relative percent survival (RPS) values were calculated

Tab 2
Resistance against e perimental WSSV infection in *Procambarus clarkii* vaccinated with BEI or heat-inactivated WSSV.

Time of challenge	Dead/tested	Mortalit (%)	RPS (%)	*P*-value
7 dpv				
2 mM BEI-inactivated WSSV	7/30	23	77	0.000*
Control	30/30	100		
3 mM BEI-inactivated WSSV	11/30	37	63	0.000*
Control	30/30	100		
Heat-inactivated WSSV	27/30	90	10	0.609
Control	30/30	100		
21 dpv				
2 mM BEI-inactivated WSSV	12/30	40	60	0.000*
Control	30/30	100		
3 mM BEI-inactivated WSSV	21/30	70	30	0.021*
Control	30/30	100		
Heat-inactivated WSSV	29/30	97	3	0.831
Control	30/30	100		

Control group cra sh were injected with TNE solution mi ed with BEI or not. Signi cant difference (5% level) compared with the corresponding unvaccinated group in indicated b *.

according to Amend [36]. Cumulative mortalities, RPS values and *P*-values were determined at the termination (24th da) of challenge test made seven da s after the last vaccination.

3. R

3.1. Measurement of iral infecti it (LD$_{50}$)

The results of viral infectivit (LD$_{50}$) as measured b the mortalit of injected cra sh showed 100% in all test groups until 10^7 of the serial dilutions, but showed 55% for the 10^8 dilution group. Therefore, the LD$_{50}$ value of the original viral uid was estimated to be 10^8 dilution. So we used 1×10^7 dilution of WSSV as the challenge dose in the challenge test.

3.2. Inacti ation of WSSV

IM injection e periment showed that WSSV had been inactivated b BEI completel and the solution was safet for oral vaccination (Table 1). 2 mM BEI resulted in mortalities of 100% in 6 and 12 h, 90% in 18 h and 100% in 24 h. The result reveals that BEI can inactivate WSSV in temperature of 37 °C and the proper concentration of 2 mM and 3 mM BEI also inactivated WSSV completel No mortalit was recorded in the negative control and the positive control resulted in mortalities of 100% on the 17th da The cumulative mortalities in the 37 °C heated WSSV group indicated WSSV could keep the infectivit even e posed to 37 °C over 24 h. So onl BEI as the inactivant contributes to the completel inactivation of WSSV. WSSV could be completel inactivated b either 20 min e posure to 60 °C or 10 min e posure to 70 °C, but WSSV was not inactivated b even 30 min e posure to 50 °C [33]. Although aquatic viruses receive thermal protection from the natural aqueous environment as water temperature is seldom higher than 35 °C, WSSV showed a resistance to high temperature under 60 °C and the resistance is as stronger as in lower temperature. In this stud WSSV could be completel inactivated b 65 °C for 15 min.

3.3. Electron microscop

The TEM microphotograph showed that the envelopes of 2 mM and 3 mM BEI-inactivated WSSV seem to have a little changed compared with the puri ed WSSV (Fig. 1), but the virus particles are intact basicall But the envelope of WSSV was destro ed badl and the nucleocapsid was e posed to the outer in heat-inactivated WSSV (Fig. 1).

3.4. SDS-PAGE anal sis

The SDS-PAGE anal sis showed that more than ten bands in the puri ed WSSV and 2 mM BEI-inactivated WSSV, but onl ve bands in the 3 mM BEI-inactivated WSSV (Fig. 2). In the puri ed WSSV and 2 mM BEI-inactivated WSSV, the bands of VP15, VP19, VP28 and VP281 obviousl e ist. In 3 mM BEI-inactivated WSSV onl the bands of VP26 and VP28 were obvious. No protein was found in linear heat-inactivated WSSV (Fig. 2).

3.5. Vaccination e periment

The time mortalit relationships in the vaccination e periment are shown in Fig. 3. The cumulative mortalit percentage (CMP) of the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV and challenged at 7 dpv was 23%, 37% and 90% respectivel (Table 2, Fig. 3a). Calculated RPS values were 77%, 63% and 10% for the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV (Table 2). Signi cantl lower mortalit ($P < 0.05$) in the 2 mM BEI-inactivated WSSV (23%) and 3 mM BEI-inactivated WSSV (37%) vaccinated group compared with its control group (100%). But no signi cant differences in cumulative mortalities were observed between the groups injected with heat-inactivated WSSV and its control group.

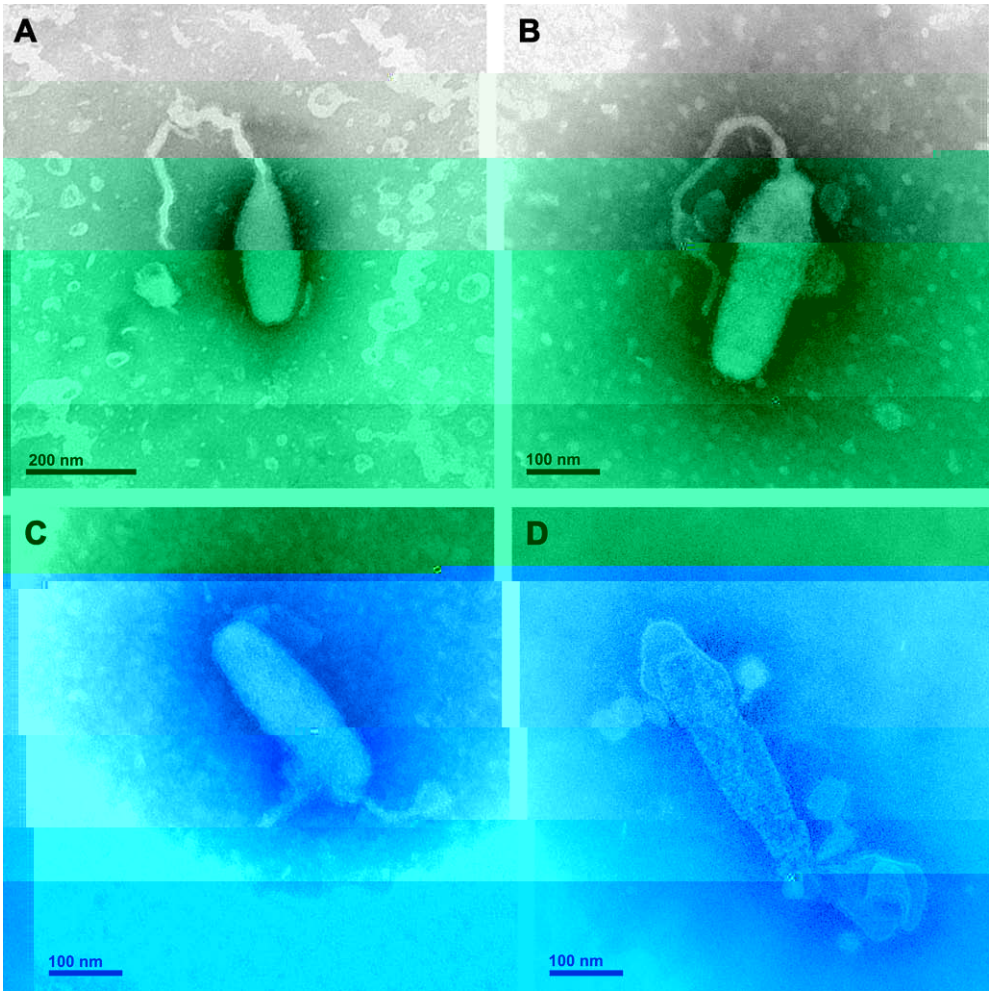
The CMP of the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV vaccinated groups challenged at 21 dpv were 40%, 70% and 97% respectivel (Table 2, Fig. 3b). Calculated RPS values were 60%, 30% and 3% for the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV (Table 2). Signif-icantl lower mortalities ($P < 0.05$) were observed in the 2 mM BEI-inactivated WSSV (40%) and 3 mM BEI-inactivated WSSV (70%) vaccinated group compared with its control group (100%).

4. D c n

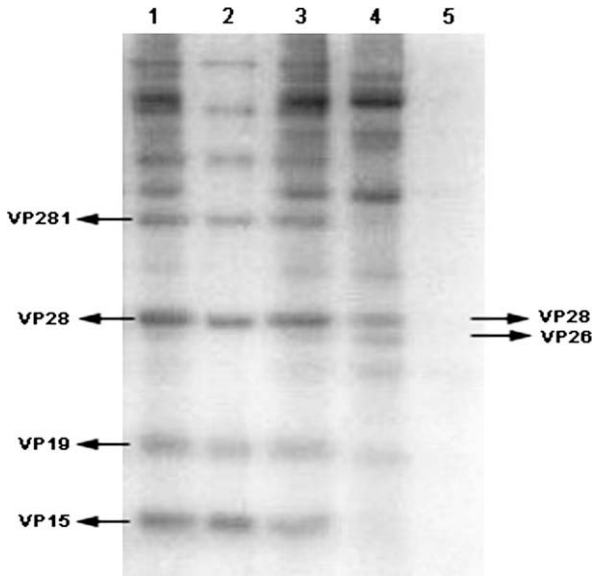
Recentl various agents, i.e. inactivated WSSV, antibacterial components, and subunit recombinant envelope proteins tried so far against WSSV have shown encouraging results [14 18,22 24,27,37,38]. Now more and more report indicated that envelope proteins, especiall VP28, could offer shrimp protection against WSSV infection [22 25]. In this investigation we found that the protection ef cac of inactivated WSSV is dependent on the integrit of envelope proteins as previous report in the vaccination trial of veterinar virus [30,32,39,40]. We also found that the protection will decrease along with da after last vaccination like previous reports [23,27].

The Electron microscop showed that ma be the envelope of WSSV is changed a little in 2 mM and 3 mM BEI (Fig. 1). And the SDS-PAGE anal sis indicated that higher than 2 mM concentration of BEI ma change the envelope proteins of WSSV more (Fig. 2). The loss of envelope protein in 3 mM BEI-inactivated WSSV ma e plain the signi cant difference among the RPS of 2 mM BEI, and heat-inactivated WSSV. Because envelope proteins have been con rmed that could protect the crustaceans against WSSV. In this stud we think that 2 mM BEI can inactivate WSSV completel and preserve the integrit of envelope proteins in optimum. The signi cant differences ($P < 0.05$) between BEI and heat-inactivated WSSV ma be e plained b the loss of envelope proteins, which provided the protection against WSSV.

No mortalit was recorded in vaccinated cra sh before the challenge test indicated that the inactivated WSSV vaccine was safet for vaccine cra sh as con rmed in the inactivation e periment. The challenge test made on the 7th and 21st dpv resulted in signi cantl lower mortalit ($P < 0.05$) in the 2 mM and 3 mM BEI-inactivated WSSV vaccinated group compared with its control



Fi . 1. Electron microscopic view of negativel stained, normal WSSV particle (A), 2 mM BEI- inactivated WSSV particle (B), 3 mM BEI-inactivated WSSV particle (C) and heat-inactivated WSSV (D). (A. bar = 200 nm; B D. bar = 100 nm).



Fi . 2. Fifteen percent Coomassie brilliant blue-stained SDS-PAGE gel of puri ed WSSV and BEI-inactivated WSSV. Lane 1. Puri ed WSSV. Lane 2. Protein marker (14.4 kDa, 18.4 kDa, 25 kDa, 35 kDa, 45 kDa, 66.2 kDa and 116.0 kDa). Lane 3. 2 mM BEI-inactivated WSSV. Lane 4. 3 mM BEI-inactivated WSSV. Lane 5. Heat-inactivated WSSV.

group (100%). However, the RPS on 21st dpv showed a signi cant difference between 2 mM and 3 mM BEI-inactivated WSSV. The difference ma be e amined b the damages in the envelope proteins of WSSV that showed in Electron microscop and SDS-PAGE. Electron microscop showed that a little damage happened to the envelope proteins of WSSV in 2 mM and 3 mM BEI-inactivated WSSV but heat at 65 °C for 15 min destro ed the envelope proteins completel (Fig. 2). The anal sis of SDS-PAGE indicated that in the linear of 3 mM BEI-inactivated WSSV the band of VP28 was not obvious and the band of VP15, VP19 and VP281 could not be found compared within the linear of 2 mM BEI-inactivated WSSV. The envelope of heat-inactivated WSSV particle was destro ed ma e plained that no proteins was found in the linear of heat-inactivated WSSV (Fig. 1d, Fig. 2). The high cumulative mortalit in the heat-inactivated WSSV vaccinated group indicated that heat-inactivated WSSV did not provide a protection from WSSV, and Namikoshi et al. also reported the same result [27]. So we think that the protection provided b inactivated WSSV is dependent on the integrit of envelope proteins of WSSV.

Our results showed that vaccinating cra sh with BEI-inactivated WSSV would protect the cra sh against WSSV seven da s after the last vaccination. Cra sh *P. clarkii* is susceptible to WSSV as penaeid shrimp and WSSV caused more than 90% mortalit in cra sh through oral challenge and IM injection [15]. Cra sh injected with 3 mM BEI-inactivated WSSV showed no bad signs and

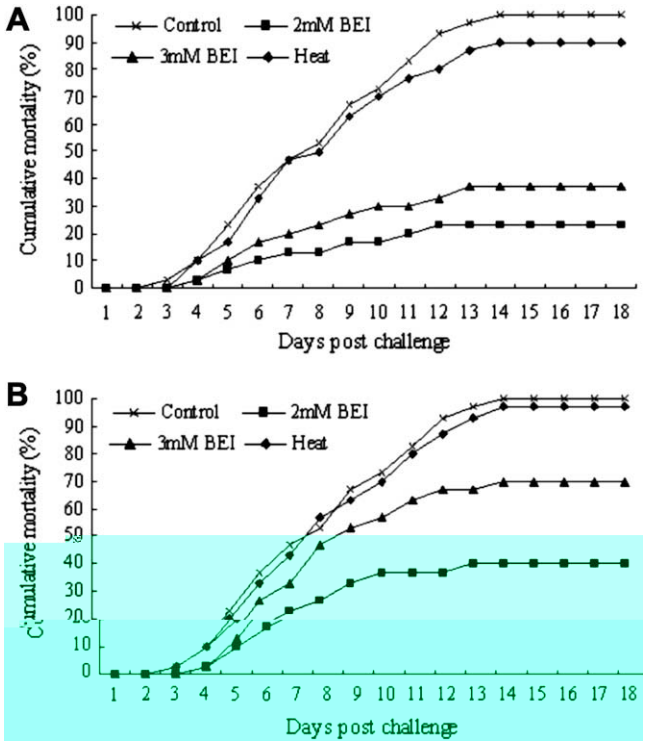


Fig. 3. Time mortalit relationship of e periment 1. Juvenile cra sh were vaccinated inactivated WSSV followed b viral challenge at (A) 7 da s and (B) 21 da s post-vaccination. Cumulative mortalit rates of shrimp from the e perimental groups, as indicated in Table 2 are plotted against time (18-da period) after challenge.

be active like normal cra sh in this stud . We believe that vaccination with BEI-inactivated WSSV was successful in protecting cra sh against WSSV, similar ndings were also obtained b Namikoshi et al. using formalin-inactivated WSSV [27]. In conclusion, cra sh can be induced a resistance to WSSV b BEI-inactivated WSSV, but BEI in concentration of above 2 mM will damage the envelope proteins of WSSV and reduce the protective ef cac of BEI-inactivated WSSV. These results con rm that the protective ef cac of vaccine made of BEI-inactivated WSSV lies on the integrit of envelope proteins of WSSV and reveal the possibilit of vaccination of *P. clarkii* with BEI-inactivated WSSV.

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