Fish & Shellfish Immunology 26 (2009) 685–690

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

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

Fei Zhu [a], Huahua Du [a], Zhi-Guo Miao [b], Hai-Zhi Quan [a], Zi-Rong Xu [a,*]

[a] Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Feed Science Institute, Zhejiang University, Hangzhou 310029, PR China
[b] School of Animal Science, Henan Institute of Science and Technology, XinXiang 453003, PR China

ARTICLE INFO

Article history:
Received 1 January 2009
Received in revised form
5 February 2009
Accepted 21 February 2009
Available online 4 March 2009

Keywords:
White spot syndrome virus
Procambarus clarkii
Vaccination
BEI
Inactivate

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 maybe 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

* Corresponding author. Tel.: +86 571 8609 1820; fax: +86 571 8699 4963.
E-mail address: zrxu@zju.edu.cn (Z.-R. Xu).

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.02.022

[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			
1	37 °C/6 h	30/30	100
2	37 °C/12 h	30/30	100
3	37 °C/18 h	27/30	90
4	37 °C/24 h	0/30	0
3 mM BEI	37 °C/24 h	0/30	0
37 °C	37 °C/24 h	30/30	100
65 °C	65 °C/15 min	0/30	0
Positive control	–	30/30	100
Negative control	–	0/30	0

No mortality indicated WSSV was inactivated completely and this treatment could be used to produce vaccine.

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