Fish & Shellfish Immunology 30 (2011) 77–83

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

WSSV: VP26 binding protein and its biological activity

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ARTICLE INFO

Article history:
Received 14 April 2010
Received in revised form
17 August 2010
Accepted 19 September 2010
Available online 29 September 2010

Keywords:
Binding protein
Genes
Neutralization assay
Recombinant protein
White spot syndrome virus (WSSV)
Shrimp

ABSTRACT

White spot syndrome virus (WSSV) is one of the major causes of disease in the shrimp culture industry causing enormous economic losses. In this study, we displayed peptides from a cDNA library obtained from the hemolymph of shrimp infected with WSSV, on the surface of phage and screened for the peptides that interacted with the WSSV. One WSSV binding protein (WBP) gene was found to consist of 171 bp that had no matches in the NCBI database. This WBP was shown to bind to the VP26 protein of the WSSV by Western blotting. In addition, WBP reduced the binding of WSSV to shrimp haemocytes from 2.0×10^7 copies in the control to 6.0×10^2 after treatment with 80 μg of WBP. The survival rate of shrimp after WSSV were mixed with WBP at 80 μg, was 89% and the binding of WBP remained unchanged for at least 24 h. Therefore, the results indicate that the WBP can bind to VP26 and inhibit the invasion of WSSV into host cells. This finding may introduce another future way to try to fight this disease in shrimp culture.

1. Introduction

White spot syndrome virus (WSSV), is one of the major causative agents of significant economic losses for the shrimp farming industry [1,2]. Prevention of the disease such as by vaccinating against the virus or by feeding immunostimulants would be highly desirable. It is well known that crustaceans rely on a variety of innate immune response systems to rapidly and efficiently recognize and destroy "non-self" materials [3]. Viral neutralizing factors in the hemolymph of immune kuruma shrimp have been reported [4]. Although, several immunostimulants have been reported [2,5,6] finding alternative ways to prevent the virus from causing disease in the host cell is a big challenge.

Phage display technology is known as a powerful method to identify partners of protein–protein interactions. This technique is widely used to produce antibodies quickly from phage display libraries [7]. The technique is based on the display of peptides or proteins fused to phage coat proteins with the phage being selected by interaction with a partner protein from its host [8].

It is well known that viral structural proteins, are enveloped proteins involved in the recognition and attachment of the virus to its host cell surface during the infection process [9]. Even though, more than 40 structural protein genes have been reported [10–13] and a host cell surface molecule, integrin, involved in the mechanism of viral infection has been characterized as being able to bind to the WSSV envelope protein VP187 [10], other host cell surface molecules that interact with other major viral proteins such as VP26, VP24, VP15, VP28 and VP19 have not been identified. Therefore, it is important to establish how these major viral proteins are involved in infecting the host cell.

In this work, we have explored the host cell surface of WSSV for binding proteins using phage display technology and identified a protein partner for VP26. This viral binding protein was detected by using a neutralization assay both *in vivo* and *in vitro* to determine if VP26 plays any role in shrimp infections.

2. Materials and methods

2.1. Preparation of WSSV binding protein (WBP) from infected hemolymph

A cDNA library prepared from WSSV-infected shrimp hemolymph (Stratagene, California, USA) was constructed and the cDNA was incorporated into pCANTAB 5E phage by PCR-based methods (Pharmacia, Buckinghamshire, UK). The display phage was used for panning with WSSV. Briefly, WSSV (10 μg/mL) suspended in phosphate buffered saline (PBS) was coated onto a 96 well microtiter plate for 1 h, the wells were then blocked using skimmed milk for 1 h. The display phage library was added after washing the well

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doi:10.1016/j.fsi.2010.09.008

3 times with PBS containing 0.05% Tween 20 (PBS–Tween) and incubated for 2 h at room temperature. The non-specifically bound display phages were removed by washing 20× with PBS–Tween, and the remaining bound phage was used to infect *Escherichia coli* TG1. Selected WSSV binding protein clones (WBP) were sequenced and expressed in *E. coli* HB2151 as described in the instruction manual (Pharmacia, Buckinghamshire, UK). Briefly, *E. coli* (TG1) containing pCANTAB-WBP was grown in LB broth with 80 μg ampicillin for 16–18 h at 37 °C, then 5 mL was transferred into 500 mL LB broth, cultured for 1 h with added ampicillin, to a final concentration of 100 mg/mL and 4×10^{10} pfu of M13K07, while shaking at 37 °C for 1 h. The cultured cells were centrifuged at 1000 rpm for 10 min, the pellet was suspended in LB broth (80 mg/mL), cultured for 16–18 h at 37 °C and centrifuged at 1000 rpm for 20 min. The supernatant containing the recombinant phage was mixed with 10 mL of PEG/NaCl (0.025 M polyethylene glycol-8000, 2.5 M NaCl), chilled on ice for 30–60 min and centrifuged at 10,000 rpm at 4 °C for 20 min. The phage pellet was suspended in 16 mL of LB broth and stored at 4 °C until used.

The phage containing pCANTAB-WBP (2 μL) was incubated with 400 μL of log phase HB2151 cells at 37 °C for 30 min, then 50 μL was spread on LB agar containing ampicillin (80 μg/mL) and incubated at 30 °C for 16–18 h. One colony was cultured in LBG (LB + 2% glucose) at 30 °C for 16–18 h, 1 mL was then transferred into 10 mL of LBG (LB + 2% glucose + 80 μg/mL of ampicillin) and incubated at 30 °C for 1 h. The culture was centrifuged at 1500 rpm at 25 °C for 20 min and the pellet was resuspended in 10 mL of LB containing 1 mM IPTG and 80 μg/mL of ampicillin and incubated at 30 °C for 24 h and again centrifuged at 1500 rpm for 20 min. The protein in the supernatant was further purified by elution from a Sephadex™ G-25 (GE Healthcare Bio-Sciences AB, Sweden) connected to an AKTAprime plus (GE Healthcare Bio-Sciences AB, Sweden). The column was equilibrated with 25 mL of phosphate buffered saline at a flow rate of 5 mL/min, then 2 mL of sample was injected and eluted after 6 min. Proteins in the fractions were checked by 14% SDS-PAGE and kept at −80 °C for a neutralization assay and for binding of WSSV antigen.

2.2. Conjugation of WBP with biotin

The WBP proteins conjugated with biotin [14] were used to detect VP26 of WSSV after separation by 14% SDS-PAGE. Briefly, 1 mg WBP protein was solubilized in 1 mL of PBK (6.67 mM K_2HPO_4 and 3.33 mM KH_2PO_4, pH 7.5), 1.5 mL of 0.15 M KCl, pH 8.8 containing 0.2 M $NaHCO_3$ and 100 μL (25 ng/mL) of biotin-N-hydroxy-succinimide ester (Sigma, USA) was added and incubated at room temperature for 3 h. The reaction was stopped by adding 750 μL of 1 M NH_4Cl. The unconjugated biotin was removed using a 3K Amicon Ultra centrifugal filter (Millipore Ireland BV, Carrigtwohill, Co. Cork) and washing with 10 × 3 mL of PBK. The WBP-biotin-labeled protein was used to detect VP26 of WSSV.

2.3. Production of glutathione S-transferase–VP26 (GST–VP26) from E. coli (BL21)

The GST–VP26 protein and the GST protein product were prepared by the standard method of growing the bacterial culture in the presence of ampicillin, followed by induction with 0.5 mM IPTG (isopropyl β-D-thiogalactopyronositol). The GST–VP26 fusion protein and GST protein were purified using the Glutathione Sepharose 4B resin (Amersham Biosciences) and analyzed for purity by 14% SDS-PAGE and stored at −80 °C until used for the binding assay.

2.4. Binding between WBP and GST–VP26 by Western blotting

Binding between WBP and GST–VP26 was examined by separating the GST–VP26 fusion protein and GST (protein control) on 14% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes then blocked with 5% skim milk in PBS/0.05% Tween 20 for 1 h. The blots were incubated with biotin conjugated WBP (1:10) for 1 h and washed 3 times for 10 min each with PBS/0.05% Tween 20. A conjugated Streptavidin-alkaline phosphatase (Promega, USA, diluted 1:1000) was added and the substrate (NBT–BCIP) in the detection buffer (0.1 M Tris–HCl, pH 7.5, 0.1 M NaCl and 0.05 M $MgCl_2$) bound alkaline phosphatase produced a blue color and when the positive bands became sufficiently intense the reaction was stopped by dipping the membrane in water. The membrane was then air-dried.

2.5. In vitro neutralization of WSSV by WBP

Shrimp hemolymph with added anticoagulant was collected with 1:1 volumes of KC199 and then centrifuged at 6500 rpm for 2 min at 4 °C to obtain haemocytes. The haemocytes were washed with DMEM and resuspended at 3.0×10^6 cells in 200 μL of DMEM for incubation with WBP.

Purified WBP protein was diluted to 10 μg, 20 μg, 40 μg and 80 μg each in 100 μL of PBS and incubated with WSSV (1.6×10^8 copies) or PBS, as a control group, for 1 h at 25 °C. The mixtures of protein and WSSV were then incubated with the haemocytes (3.0×10^6 cells) for 1 h at 28 °C. After incubation for 1 h, mixtures were centrifuged at 6500 rpm for 2 min at 4 °C, haemocytes washed with PBS, then resuspended in 50 μL of PBS before boiling for 10 min then immediately transferred to ice for 5 min and spun for 1 min to remove cell debris. The supernatant solution was used as a template for *Real-Time PCR*

2.6. Preparation of VP19 template

The hearts of infected shrimp were homogenized in TN buffer (20 mM Tris–HCl, 0.4 M NaCl, pH 7.4) [17] and centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was centrifuged at 8000 rpm for 5 min at 4 °C before boiling for 10 min and immediately transferred to ice for 5 min. the supernatant solution was used as a template for PCR. The primers used to amplify the VP19 gene were VP19-FB: 5′ CGGGATCCATGGCCACCACGACTAA 3′ and VP19-RX: 5′ GCCTCGAGCCTGATGTTGTGTTTCTATA 3′. PCR was performed in a final volume of 50 μL 0.4 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% TritonX-100 and 2.5 U Taq DNA polymerase. Thirty cycles of PCR were carried out each with 1 min of denaturation at 94 °C, 1 min of annealing at 59 °C followed by 1 min of extension at 72 °C and terminated by 5 min of incubation at 72 °C. The PCR product was purified by QIAquick® Gel Extraction Kit (QIAGEN, Germany).

2.7. The copy number of VP19 gene [15]

The number of copies of the VP19 gene was calculated according to its molecular weight and then converted into a copy number based on Avogadro's number.

$$\text{Copies Number} = \frac{\left(\text{Amount of DNA (g)} \times 6.022 \times 10^{23}(\text{molecules/mol})\right)}{(\text{The length of template (bp)} \times 650(\text{g/mol}))}$$