Developmental and Comparative Immunology 53 (2015) 47–54

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Recombinant expression and functional analysis of an isoform of anti-lipopolysaccharide factors (FcALF5) from Chinese shrimp *Fenneropenaeus chinensis*

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form
23 June 2015
Accepted 24 June 2015
Available online 26 June 2015

Keywords:
Anti-lipopolysaccharide factors
Antibacterial
Antiviral
VP24
WSSV

ABSTRACT

Antimicrobial peptides (AMPs) have a great potential to be used as a substitute for antibiotics since AMPs don't lead to bacteria's drug resistance. Anti-lipopolysaccharide factors (ALFs) are one type of AMPs and exist in crustaceans. In the present study, we produced a recombinant protein (rFcALF5) of an ALF isoform (FcALF5) from Chinese shrimp *Fenneropenaeus chinensis* through a prokaryotic expression system. The rFcALF5 exhibited varied antibacterial activities against different bacteria. Besides its antibacterial activities, it could also inhibit the infection of white spot syndrome virus (WSSV) to shrimp after pre-incubation with this virus. In order to learn the antiviral mechanism on how rFcALF5 influences WSSV infection, the interaction between the total proteins of WSSV and rFcALF5 was analyzed and the data showed that rFcALF5 had direct interaction with the envelope protein VP24 of WSSV. The LPS binding domain (LBD) of FcALF5 also showed direct interaction with VP24 of WSSV. Therefore we inferred that the antiviral activity of FcALF5 might be achieved through the binding of its LBD to VP24 of WSSV. These findings provided more information to develop new strategies for the control of shrimp disease in aquaculture.

1. Introduction

Antimicrobial peptides (AMPs), as effectors of immune response, are a kind of cationic peptides which play important roles in killing the infected pathogens directly (Bachère, 2003). They displayed multiple functions in the immunity of animals, including microbicidal activities and selective immune modulatory roles (Yount et al., 2006). AMPs are recognized as the key components of the innate immune reactions in both invertebrates and vertebrates (Bulet et al., 1999; Hancock and Diamond, 2000). They are receiving more and more attentions in recent years for their antimicrobial activities without causing any drug resistance of the bacteria.

Anti-lipopolysaccharide factors (ALFs), firstly isolated from the hemocytes of horseshoe crab, are one type of AMPs (Tanaka et al., 1982). It can bind and neutralize the activity of lipopolysaccharides (LPS) and lipoteichoic acid (LTA) to inhibit the growth of Gram-positive and Gram-negative bacteria (Morita et al., 1985; Zhao and Wang, 2008). Since ALF was found for its broad-spectrum antimicrobial activity, it had been widely isolated from different crustaceans. Till present, multiple isoforms of ALFs have been identified and characterized in crustaceans. Seven ALF isoforms were identified from the swimming crab *Portunus trituberculatus* which showed varied tissue expression profiles (Liu et al., 2012, 2011) and six isoforms of ALF were reported in *Penaeus monodon* (Ponprateep et al., 2012; Supungul et al., 2004). Although the similarities of different ALF isoforms were very low (Li et al., 2013), they showed a common feature with the existence of a disulfide loop formed by two conserved cysteine residues, termed as the LPS-binding domain (LBD), which was regarded as the functional domain for their antibacterial activities (Hoess et al., 1993; Supungul et al., 2004). Both the synthesized peptide of LBD and recombinant ALF proteins produced in bacteria and yeast from different ALF isoforms exhibited different antimicrobial activities against Gram-positive or Gram-negative bacteria (Liu et al., 2014;

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http://dx.doi.org/10.1016/j.dci.2015.06.015
0145-305X/

Nagoshi et al., 2006). Knock down of some ALF isoforms by RNA interference could increase the mortality of shrimp caused by infection of *Vibrio penaeicida, Vibrio harveyi* or *Fusarium oxysporum* (de la Vega et al., 2008; Kadowaki et al., 2011). Pre-injection of recombinant protein rALFPm3 from *P. monodon* produced by the yeast *Pichia pastoris* could reduce the cumulative mortality of shrimp caused by *V. harveyi* infection (Ponprateep et al., 2009). Besides the antibacterial activity of ALFs, the antiviral activities were also reported. The cyclic synthetic fragment of ALF from *P. monodon* could agglutinate the nervous necrosis virus (NNV) and exhibit the antiviral activity (Chia et al., 2010). A synthetic LPS-binding domain of *Limulus polyphemus* could block the entry of some human virus into cells, like HIV-1, HCV and HSV1 (Krepstakies et al., 2012). The recombinant protein ALFPm3 produced in *P. pastoris* showed apparent antiviral activities against herpes simplex virus type 1 and could reduce the propagation of white spot syndrome virus (WSSV) in haematopoietic (Hpt) cells (Carriel-Gomes et al., 2007; Tharntada et al., 2009).

In our previous research, seven isoforms of ALFs (FcALF1-ALF6 and ALFFc) were identified and their expressions at transcriptional level in shrimp were apparently up-regulated at the acute infection stage of WSSV, which suggested that ALFs might play important roles in shrimp during WSSV infection (Liu et al., 2005; Li et al., 2013). The synthetic LBD peptides of two isoforms of ALFs from *Fenneropenaeus chinensis*, ALFFc and FcALF2, showed different antibacterial spectrum and antiviral activities to WSSV which suggested that different isoforms of ALF might play coordinate roles in the immune response of shrimp during pathogen infection (Guo et al., 2014; Li et al., 2014). Although synthetic peptide of ALFs showed antibacterial and antiviral activities, considering the high cost of chemical synthesis, it was not regarded as a proper way to produce peptides at large scale in aquaculture.

Production of recombinant proteins by prokaryotic expression system was regarded as the most effective ways to obtain functional protein at large scale (Hannig and Makrides, 1998). Our previous study showed that the synthetic LBD of FcALF5 exhibited certain antibacterial and antiviral properties, and it had low inhibition activity to *Escherichia coli* (Li et al., 2015). Therefore, we guessed that the prokaryotic expression system in *E. coli* might be used to produce recombinant protein of FcALF5 at large scale. In order to confirm this hypothesis, a prokaryotic expression system to produce recombinant protein of FcALF5 was developed, and its activities were evaluated. Furthermore, the antiviral mechanism of FcALF5 was analyzed. These data will not only widen the approach to use ALF for further application in aquaculture, but also enrich the knowledge for the function of AMPs in crustaceans.

2. Materials and methods

2.1. Construction of the plasmid expressing recombinant FcALF5 protein (rFcALF5)

A pair of gene-specific primers, ALF5expF and ALF5expR (shown in Table 1) were designed based on the sequence with accession number JX853778 to amplify the cDNA fragment encoding the mature peptide of FcALF5 (*EcoR I* and *Nco I* sites were underlined). The PCR amplification was performed using the synthesized cDNA from cephalothorax RNA as the template: 1 cycle of 94 °C for 5 min, 35 cycles including denaturation at 94 °C for 40 s, annealing at 56.5 °C for 40 s, and extension at 72 °C for 40s; followed by 1 cycle of 72 °C for 10 min. The amplified PCR products were purified and inserted into pMD19-T simple vector, digested completely by the same restriction enzymes, then subcloned into the expression vector pET30a(+) (Novagen). The recombinant plasmid was then transformed into *E. coli* BL21 (DE3) plysS.

Table 1
Information of primers used for gene cloning and real time PCR.

Primers	Sequences (5′-3′)
ALF5expF	CATGccattgAACGTCCTCAGCTCGGAG
ALF5expR	CCGgaattcTTAGAGATACTGTGCAGC
VP24expF	CCGgaattcATGCACATGTGGGGGG
VP24expR	CCGctcgagTTATTTTTCCCCAACCTT
pGEX-F	GGGCTGGCAAGCCACGTTTGGTG
pGEX-R	CCGGGAGCTGCATGTGTCAGAGG
M13-F	CGCCAGGGTTTTCCCAGTCACGAC
M13-R	CACACAGGAAACAGCTATGAC
VP28−F	AAACCTCCGCATTCCTGTGA
VP28-R	TCCGCATCTTCTTCCTTCAT

2.2. Expression, purification and western-blot detection of rFcALF5

E. coli BL21 was transformed with pET30a-FcALF5 and cultured in LB medium with 50 mg/l ampicillin, till the absorbance at 600 nm (OD600) reached 0.4–0.6. Expression of the fusion protein was induced by 1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) for 5 h at 37 °C. The cells were harvested by centrifugation at 8000 r/min for 10 min at 4 °C, resuspended in buffer (300 mM NaCl, 50 mM sodium phosphate, pH 7.0) and sonicated at 4 °C for 30 min. Then the cell lysates were centrifuged at 8000 r/min to collect the inclusion bodies and dissolved in buffer (300 mM NaCl, 50 mM sodium phosphate, 8 M urea, pH 7.0).

The His-tagged recombinant FcALF5 protein in bacterial lysates was purified according to the method described previously (Wang et al., 2013). The purified proteins were refolded in gradient urea-TBS glycerol buffer with pH 7.4, consisting of 50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 2 mM reduced glutathione, 0.02 mM oxidized glutathione, 10% glycerol, 1% glycine, and gradient urea concentration of 6, 4, 3, 2, 1, 0 M in each gradient. The purified proteins were dialyzed in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, pH 7.4). Each gradient was kept at 4 °C for 12 h. The proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R250.

Western-blot analysis was performed to detect the expressed rFcALF5 with a His-tag. After SDS-PAGE, the rFcALF5 protein was transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, USA) and blocked with 5% nonfat milk in TBST (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) for 2 h at room temperature. Then it was incubated overnight with HRP-conjugated anti His-tag mouse monoclonal antibody (1/1000 diluted in TBS). After the membrane was washed with TBST, the signal was detected using enhanced chemiluminescence detection assay kit (Tiangen, China). The purified rFcALF5 was dried in vacuum freeze-drying equipment and dissolved in PBS (pH 7.4) for further experiment. The concentration of rFcALF5 was measured by the Bradford method using Bradford Assay kit (Takara, China) based on the manufacturer's instructions.

2.3. The assay on the antimicrobial activity of rFcALF5

The antimicrobial activity of rFcALF5 was evaluated by minimal inhibitory concentration (MIC) assay. The MIC was determined using a liquid medium growth inhibition assay for each type of bacteria. It was performed according to the method described previously (Guo et al., 2014). Two Gram-negative bacteria including *E. coli* (*E. coli*) and *Vibrio alginolyticus* (*V. alginolyticus*), three Gram-positive bacteria including *Bacillus subtilis* (*B. subtilis*), *Micrococcus luteus* (*M. luteus*) and *Staphylococcus epidermidis* (*S. epidermidis*) were used for MIC assay. *B. subtilis* was cultured in LB medium at 28 °C, and the others were cultured in LB medium at 37 °C.