Vaccine 28 (2010) 1041–1047

Contents lists available at ScienceDirect

Vaccine

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

Evaluation of the vaccine potential of a cytotoxic protease and a protective immunogen from a pathogenic *Vibrio harveyi* strain

Shuang Cheng[a,b,1], Wei-wei Zhang[a,c,1], Min Zhang[a], Li Sun[a,*]

[a] Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, PR China
[b] Graduate University of the Chinese Academy of Sciences, Beijing 100049, PR China
[c] Yantai Institute of Coastal Zone Research for Sustainable Development, Chinese Academy of Sciences, Yantai 264003, PR China

ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form 17 October 2009
Accepted 20 October 2009
Available online 6 November 2009

Keywords:
Cytotoxin
Immunoprotective
Protease
Vaccine
Vibrio harveyi

ABSTRACT

Vibrio harveyi is an important aquaculture pathogen that can infect a number of fish species and marine invertebrates. A putative protease, Vhp1, was identified from a pathogenic *V. harveyi* strain isolated from diseased fish as a protein with secretion capacity. Vhp1 is 530 amino acids in length and shares high sequence identities with several extracellular serine proteases of the *Vibrio* species. In silico analysis identified a protease domain in Vhp1, which is preceded by a subtilisin-N domain and followed by a bacterial pre-peptidase C-terminal domain. Purified recombinant protein corresponding to the protease domain of Vhp1 exhibited apparent proteolytic activity that was relatively heat-stable and reached maximum at pH 8.0 and 50 °C. The activity of purified recombinant Vhp1 protease was enhanced by Ca^{2+} and inhibited by Mn^{2+} and ethylenedinitrilotetraacetic acid. Cytotoxicity analyses indicated that recombinant Vhp1 protease was toxic to cultured Japanese flounder cells and could cause complete cell lysis. Immunoprotective analysis using Japanese flounder as an animal model showed that purified recombinant Vhp1 in the form of a denatured and proteolytically inactive protein was an effective subunit vaccine. To improve the vaccine potential of Vhp1, an *Escherichia coli* strain that expresses and secrets a cytotoxically impaired Vhp1 was constructed, which, when used as a live vaccine, afforded a high level of protection upon the vaccinated fish against lethal *V. harveyi* challenge. Taken together, these results demonstrate that Vhp1 is a cytotoxic protease and an effective vaccine candidate against *V. harveyi* infection.

1. Introduction

Vibrio harveyi is a Gram-negative bacterium that is widespread in marine environments and can exist commensally in the gut and skin microflora of marine animals. For aquaculture industries, *V. harveyi* is considered as a severe opportunistic pathogen that can infect a wide range of marine species, including both vertebrates and invertebrates [1,2]. Penaeid shrimp is particularly susceptible to *V. harveyi*, and, upon *V. harveyi* infection, develops a disease called luminous vibriosis that can result in heavy economic losses [3–8]. In addition to shrimp, fish, lobster, and abalone are also known to be affected by *V. harveyi*-related vibriosis [9–12].

Studies on the pathogenic mechanism of *V. harveyi* have identified a number of virulence-associated factors, notably haemolysin [13], exotoxins [14–17], bacteriocin-like substance [18], and quorum sensing system, which may participate in pathogenesis by regulating the production of certain proteases and the virulence-associated type III secretion system [19,20]. It is known that some pathogenic *V. harveyi* strains exhibit higher levels of protease activity in the extracellular products than non-pathogenic strains [6]. Several extracellular proteases have been identified from disease-associated *V. harveyi* isolates [16,21], one of which, a cysteine protease, was demonstrated to be toxic to prawn [15].

Control of *V. harveyi* infection relies mainly on the use of antibiotics and vaccines [22]. Currently, vaccines that have been tried against *V. harveyi* are mostly bacterins consisting of inactivated bacterial cells [23,24]. Extracellular products of some disease-related *V. harveyi* strains are also found to be immunoprotective [25–27]. Several *V. harveyi* recombinant subunit vaccine candidates have been reported by different research groups and demonstrated to be effective in laboratory trials. These protein-based vaccines include the outer membrane protein OmpK, glyceraldehyde-3-phosphate dehydrogenase, and haemolysin [28–30]. In addition, our recent study has identified a chaperon protease from *V. harveyi* and found it highly immunoprotective when used as a secreted vaccine [31].

In this report, we describe the identification and characterization of a peptidase Vhp1 from a pathogenic *V. harveyi* strain isolated from diseased fish. We found that purified recombinant Vhp1 is a serine protease with cytotoxic property and that recombinant

* Corresponding author. Tel.: +86 532 82898834; fax: +86 532 82898834.
E-mail address: lsun@ms.qdio.ac.cn (L. Sun).
[1] These two authors contributed equally to this work.

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.10.122

Vhp1, both as a purified subunit vaccine and as a secreted vaccine delivered by a bacterial carrier, can induce strong protective immunity in Japanese flounder against *V. harveyi* infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are described in Table 1. *V. harveyi* T4D1 is genetically isogenic to *V. harveyi* T4, which was isolated from diseased Japanese flounder using TSAYE medium and demonstrated to be pathogenic to flounder in live animal infection studies [31,32]. T4D1 was obtained as described previously [33]. Briefly, T4 was cultured in Luria–Bertani broth (LB) containing 1% NaCl [34] at 28 °C to an OD_{600} of 0.6 and resuspended in phosphate-buffered saline (PBS) (pH 7.4) to 5×10^7 CFU/ml. 100 μl of T4 suspension was injected intraperitoneally (i.p.) into Japanese flounder. At four-day post-infection, liver was taken aseptically from the fish and homogenized in PBS. The homogenate was plated on LB plates, and the plates were incubated at 28 °C for 48 h. One of the colonies appeared on the plates was cultured in LB at 28 °C to an OD_{600} of 0.6 and used for the second round of infection as described above. After five rounds of infection, the recovered T4 derivative was named T4D1, which exhibited higher virulence potential than the parental T4 [33].

All strains were cultured in LB medium at 37 °C (for *Escherichia coli*) or 28 °C (for *V. harveyi*) for 15 h (for liquid culture) or 24 h (for plate culture). When appropriate, ampicillin and kanamycin were added at 100 and 50 μg/ml, respectively. Cell cultures were maintained at 4 °C for short-term storage (one to three days) and at −80 °C in culture medium containing 10–15% (v/v) glycerol for long-term storage.

2.2. Cloning of vhp1

T4D1 genomic DNA was digested with Sau3A1, and the fragments between 4 and 6 kb were recovered and ligated into pBU (Table 1) at the BamHI site. DH5α was transformed with the ligation mix, and the transformants were selected as described previously [32]. One of the transformants was found to harbor a recombinant plasmid carrying a partial *vhp1* truncated at the protease domain. The complete sequence of *vhp1* was subsequently obtained by genome walking as described previously [32].

2.3. Plasmid construction

The plasmids and primers used in this study are listed in Table 1. To construct pEVP1 and pEVP1P, the coding sequences of the signal peptide-less Vhp1 and the Vhp1 protease domain were amplified by PCR with the primer pairs S5F5/S5R3 and S5F12/S5R9, respectively; the PCR products were ligated with the T-A cloning vector pBS-T, and the recombinant plasmids were digested with NdeI/XhoI; the fragments containing *vhp1* were inserted into plasmid pET258 between NdeI/XhoI sites. To construct pTVP1M, the coding sequence of a truncated Vhp1 lacking the C-terminal 136 residues was amplified by PCR with primers S5F11 and S5R8; the PCR products were inserted into pBT3 between NdeI/XhoI sites.

2.4. Purification of recombinant proteins

E. coli BL21(DE3) was transformed with pEVP1 and pEVP1P, resulting in transformants BL21(DE3)/pEVP1 and BL21(DE3)/pEVP1P, respectively. Recombinant Vhp1 protease was purified from BL21(DE3)/pEVP1P and reconstituted as described previously on nickel–nitrilotriacetic acid (Ni–NTA) columns (GE Healthcare, USA) [35]. Recombinant Vhp1 was purified from BL21(DE3)/pEVP1 using Ni-NTA agarose beads (Qiagen, USA) under denatured conditions as recommended by the manufacturer. Purified proteins were dialyzed in PBS and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, USA). The purity of purified proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coommassie brilliant blue R-250.

2.5. Proteolytic analysis

The protease activity of purified recombinant Vhp1 was determined in the standard assay buffer as described previously using azocasein (0.5%; Sigma, USA) as a substrate [31]. One unit (U) of enzyme activity was defined as the amount of enzyme that caused an increase of 0.001 at 350 nm absorbance in 1 min. The effects of temperature, pH, metals ions, and ethylenedinitrilotetraacetic acid (EDTA) were determined as described previously [31].

Table 1
Bacterial strains, plasmids, and primers used in this study.

Strain or plasmid or primer	Relevant characteristic(s)[a]	Source or reference
Strain		
E. coli BL21(DE3)	Host strain for protein expression	Tiangen (Beijing, PR China)
E. coli DH5α	Host strain for general cloning	Takara (Dalian, PR China)
V. harveyi T4D1	Fish pathogen	31, 32
Plasmid		
pBS-T	Ap^r; T-A cloning vector	Tiangen (Beijing, PR China)
pBT3	Ap^r; cloning vector	31
pBU	Kn^r; signal sequence trap	32
pET258	Kn^r; cloning vector	32
pEVP1	Kn^r; pET258 carrying *vhp1*	This study
pEVP1P	Kn^r; pET258 carrying the coding sequence of *vhp1* protease	This study
pTVP1M	Ap^r; pBT3 carrying truncated *vhp1*	This study
Primer	Sequences (5′ → 3′)[b]	
S5F5	CATATGCAATCGACAGAACTCCCAAA (NdeI)	
S5F11	CCCGGGCATATGTTAAAGAAACTACTAAGTTGT (NdeI)	
S5F12	GCATATGCCAATAGTCTCGGCAGAA (NdeI)	
S5R3	CTCGAGGTAACTCGCTTGGATGCTT (XhoI)	
S5R8	CTCGAGGATATCACTGGTTGCGCGCT (XhoI)	
S5R9	CTCGAGCCCTGTTGAACCGCT (XhoI)	

[a] Ap^r: ampicillin resistant; Kn^r: kanamycin resistant.
[b] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.