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

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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.

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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

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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.

2.6. Cytotoxicity analysis

Cytotoxicity analysis was performed as described previously [35]. In brief, FG cells, a cell line established from Japanese flounder gill tissue [36] were cultured at 22 °C in minimum essential medium with Earle's Balanced Salt Solution (Thermo Scientific, Beijing, China) and 2 mM L-glutamine as described by Tong et al. [36]. Purified Vhp1 or PBS was added to the wells. After incubation at 20 °C for various times, the cells were used either for the determination of viability using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Beijing, PR China) or for microscopic observation.

2.7. Fish

Japanese flounder (*Paralichthys olivaceus*, ~10 g) were purchased from a local fish farm and acclimatized in the laboratory for two weeks before experimental manipulations. Fish were kept at ~23 °C in 325-l tanks containing re-circulating and aerated seawater that was changed twice daily. Fish were fed daily with commercial dry pellets (purchased from Shandong Sheng-suo Fish Feed Research Center, Shandong, China) with the following content (%): protein, ≧45; fat, ≧10; fiber, ≦4; calcium, ≧1.5; phosphate, ≧1.2; lysine, ≧2.2; ashes, ≦17. Before experimental manipulation, the health status of the fish was examined. For this purpose, fish were randomly sampled and sacrificed with an overdose of tricaine methanesulfonate (Sigma, USA). Blood, liver, spleen, and kidney were taken aseptically from the sacrificed fish. The tissues were homogenized in PBS; the homogenates and blood were plated on TSAYE medium [32]. The plates were incubated at 28 °C for 48–60 h. Fish were considered as healthy only when no bacteria could be detected from any of the examined tissues and blood.

2.8. Bacterial cell number determination

Plate count was used to determine the number of viable bacterial cells corresponding to an OD_{600} of 1. In subsequent vaccination experiments, the numbers of bacterial cells were all estimated based on OD_{600} measurements.

2.9. Vaccination

All vaccination experiments were performed in replica with a group size of 20. For vaccination with purified recombinant Vhp1, *Bacillus* sp. B187, an avirulent strain isolated from the gut of healthy Japanese flounder [31], was used as an adjuvant as described previously [31]. Briefly, B187 was grown in LB medium to an OD_{600} of 0.6; the cells were harvested by centrifugation, washed with PBS, and resuspended in PBS to 1×10^8 CFU/ml (named B187-PBS). Purified recombinant Vhp1 was resuspended in B187-PBS to a concentration of 200 μg/ml, and the mixture was named B187-PBS-Vhp1. Japanese flounder were divided randomly into two groups designated A and B. Fish in group A were each injected i.p. with 100 μl of B187-PBS-Vhp1, while fish in group B were each injected i.p. with 100 μl of B187-PBS. At three weeks post-immunization, fish of group A were boosted with 20 μg of purified Vhp1 diluted in PBS without B187, while fish of group B were sham boosted with PBS. At the 15th day post-boost, the fish were challenged with 100 μl of T4D1 that had been cultured in LB medium to mid-logarithmic phase, washed, and resuspended in PBS to 2×10^8 CFU/ml.

For vaccination with live bacteria, the cells were cultured in LB medium to an OD_{600} of 0.8 and resuspended in PBS to 2×10^8 CFU/ml. Japanese flounder were divided randomly into three groups designated A–C. Fish of groups A and B were injected i.p. with 100 μl of DH5α/pTVP1M and DH5α/pBT3, respectively, while fish of group C were each injected i.p. with 100 μl of PBS. The fish were boosted three weeks after immunization and challenged with T4D1 as described above.

For all vaccinations, mortality was monitored over a period of 20 days post-challenge, and dying fish were randomly selected for the examination of bacterial recovery from the liver, kidney, and spleen as described above. The genetic identities of the recovered bacteria were examined by PCR analysis using primers specific to T4D1 and sequence analysis of the 16S rRNA gene. The relative percent of survival (RPS) was calculated according to the following formula: RPS = {1 − (% mortality in vaccinated fish/% mortality in control fish)} × 100 [37]. The accumulated mortalities and RPS given in the results are means of replica experiments.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Sera were collected from unvaccinated and vaccinated fish (five at each time point) at different times post-vaccination. Sera were diluted 10-fold in PBST (0.1% Tween-20 in PBS) containing 2% bovine serum albumin (BSA) and used for ELISA as described previously [38]. Color development was performed with the TMB Kit (Bios, Beijing, PR China). The plates were read at 450 nm with a Precision microplate reader (Molecular Devices, Canada).

2.11. Western and immunoblotting analysis

Bacterial cells were grown in LB medium to an OD_{600} of 1 and harvested by centrifugation at 4 °C. Preparations of the extracellular, cytoplasmic, and periplasmic proteins were carried out as described previously [39]. The proteins obtained were subjected to SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham, UK). Immunoblotting was performed as described previously [31] using anti-His monoclonal antibody (Tiangen, Beijing, PR China).

2.12. Statistical analysis

All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., USA). Differences in cytotoxic effect were analyzed using one-way analysis of variance; differences in mortality and antibody titers were analyzed with Chi-square test and Student's *t*-test, respectively. In all cases, the significance level was defined as $P < 0.05$.

2.13. Database search

Database search was conducted using the BLAST programs at the National Center for Biotechnology Information (NCBI). Subcellular localization and signal peptide predictions were performed with PSORTb 2.0.4 and SignalP 3.0, respectively. Structural analyses were performed with SMART sequence analysis tools and the NCBI Conserved Domain Search server.

2.14. Nucleotide sequence accession numbers

The nucleotide sequence of *vhp1* has been deposited in GenBank database under the accession number GQ857067.

3. Results

3.1. Sequence characterization of Vhp1

vhp1 was cloned from the pathogenic *V. harveyi* strain T4D1 via the pBU signal sequence trapping system as a gene coding for a secretory protein [32]. *vhp1* encodes a putative protein of 530 amino acid residues that shares the highest sequence identity (99%) with an extracellular protease of *V. harveyi* GYC1108-1

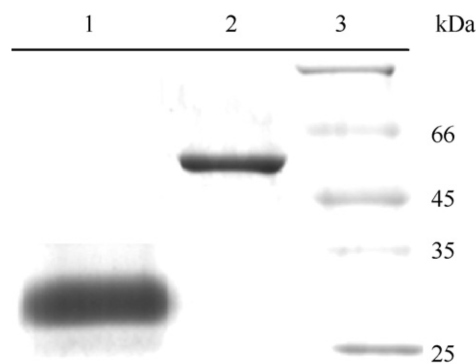

Fig. 1. SDS-PAGE analysis of purified recombinant Vhp1. Recombinant Vhp1 protease (lane 1) and complete Vhp1 (lane 2) were purified from *Escherichia coli* as histidine-tagged proteins. The purified proteins were analyzed by SDS-PAGE and viewed after staining with Coommassie brilliant blue. Lane 3: protein markers.

(GenBank accession no. EF126129). The next closest homologues of Vhp1 are alkaline serine exoproteases from two *Vibrio alginolyticus* strains (GenBank accession nos. DQ097160 and VIBPROA), which share ~89% overall sequence identity with Vhp1. Subcellular localization prediction using the PSORTb server indicated that Vhp1 is an extracellular protein. Structural analyses identified a protease domain (residues 150–410) that is preceded at the N-terminus by a subtilisin-N domain (residues 52–131) found in a number of precursor subtilisins. The protease domain is followed at the C-terminus by a bacterial pre-peptidase C-terminal (PPC) domain (residues 448–517), which is typically found at the C-terminus of secreted peptidases. A putative signal peptide sequence, consisting of the first 23 residues, was identified in Vhp1. Taken together, these data suggest that Vhp1 is probably an extracellular protease of the subtilisin family.

3.2. Proteolytic activity of purified recombinant Vhp1 protease

Recombinant protein corresponding to the middle region of Vhp1 containing the protease domain but lacking the subtilisin-N domain and the PPC domain was purified from *E. coli*. SDS-PAGE analyses showed that the molecular size of the purified protein was comparable to the calculated molecular mass of the recombinant protein (32.6 kDa; Fig. 1, lane 1). Enzymatic analyses indicated that the purified protein exhibited apparent proteolytic activity (11,000 U/ml), which was completely abolished in the presence of the serine protease inhibitor phenylmethanesulfonyl fluoride, suggesting that the purified recombinant protein was a serine protease.

3.3. Effect of pH and temperature on the activity of purified recombinant Vhp1 protease

The optimum temperature and pH of recombinant Vhp1 protease were determined to be 50 °C and pH 8.0 respectively. The enzyme exhibited more than 60% of the maximum activity over the pH range of 6–10 and the temperature range of 30–60 °C (Fig. 2). Thermostability analyses showed that recombinant Vhp1 protease was stable over the temperature range of 10–60 °C and retained 60% of the maximum activity after incubation at 60 °C for 1 h.

3.4. Effect of metal ions on the activity of purified recombinant Vhp1 protease

The effect of K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, and EDTA on the proteolytic activity of purified recombinant Vhp1 protease was determined. The results showed that Ca^{2+} was the most potent activator of recombinant Vhp1 protease and increased the activity of the latter by ~7-fold, whereas Mn^{2+} exhibited an inhibitory effect,

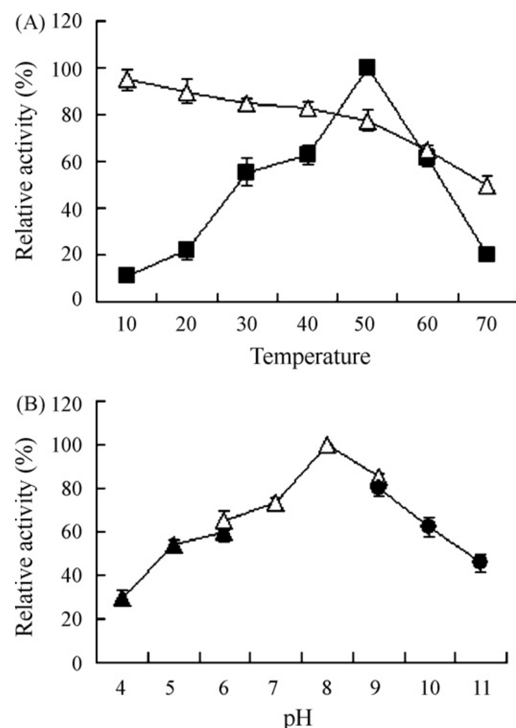

Fig. 2. Effects of temperature (A) and pH (B) on the activity of purified recombinant Vhp1 protease. (A) The effect of temperature (■) was determined using azocasein as a substrate. Thermostability (△) was determined by preincubating the enzyme in the assay buffer at the indicated temperature for 1 h before initiating enzymatic reaction by the addition of azocasein. (B) The effect of pH was determined in three different buffers: 50 mM citric acid-sodium phosphate (pH 4–6; ▲), 50 mM sodium phosphate (pH 6–9; △), and 50 mM glycine–NaOH (pH 9–11; ●). In each panel, the enzymatic activities were expressed as percentages of the maximum activity. Data are means for three independent assays and presented as the means ± SD.

which becomes stronger as the concentration of Mn^{2+} increased from 1 to 10 mM (Table 2). The presence of EDTA completely inactivated Vhp1 protease, whereas the presence of K^+ and Mg^{2+} had no apparent effect on the activity of Vhp1 protease.

3.5. Cytotoxic effect of purified recombinant Vhp1 protease

Cytotoxicity analyses showed that treatment of cultured Japanese flounder FG cells with purified recombinant Vhp1 protease reduced the viability of the cells in a dose-dependent manner (Fig. 3A). Consistently, microscopic observation indicated that incubation of FG cells with purified Vhp1 protease caused time-dependent destruction of cellular structures, which eventually led to complete cell lysis (Fig. 3 B and C).

Table 2
Effects of cations and EDTA on the activity of the purified recombinant Vhp1 protease.

Cation/EDTA	Relative activity (%) of Vhp1 protease at cation/EDTA concentration of:	
	1 mM	10 mM
K^+	96 ± 3	114 ± 5
Mg^{2+}	94 ± 2	107 ± 4
Ca^{2+}	678 ± 9	733 ± 7
Mn^{2+}	74 ± 8	36 ± 6
Zn^{2+}	152 ± 4	99 ± 3
EDTA	13 ± 1	0

The activities are expressed as percentages of the enzyme activity measured in 50 mM citric acid-sodium phosphate. Data are the means of three independent experiments and presented as the means ± SD.

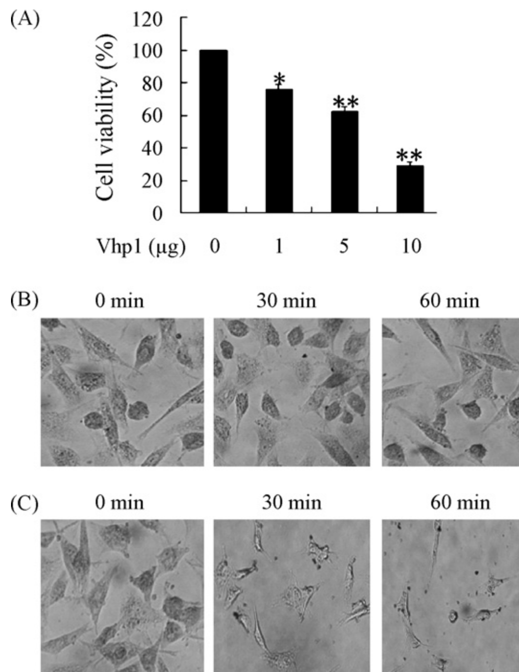

Fig. 3. Examination of the toxic effect of purified recombinant Vhp1 protease on FG cells by cell viability analysis (A) and microscopy (B and C). (A) FG cells were treated for 30 min with different amounts of Vhp1 protease, and viabilities of the cell were determined by the MTT method. Data are the means of three independent assays and presented as the means ± SD. *$P < 0.05$; **$P < 0.01$. (B and C) FG cells were treated with PBS (B) or Vhp1 protease (C) for different times and observed under an inverted microscope. Images were taken at 120× magnification.

3.6. Immunoprotective effect of recombinant Vhp1 as a purified subunit vaccine

Since recombinant Vhp1 protease possesses cytolytic activity and thus cannot be used directly as a vaccine, to examine the immunoprotective potential of Vhp1, the protein corresponding to the complete Vhp1 was purified in a denatured form from *E. coli* (Fig. 1, lane 2). Purified recombinant Vhp1 exhibited no detectable proteolytic or cytotoxic activity. Japanese flounder were immunized with purified recombinant Vhp1 and challenged with the pathogenic *V. harveyi* strain T4D1. The fish were monitored for mortality, which showed that the accumulated mortalities of the fish vaccinated with recombinant Vhp1 was 22.5%, whereas the accumulated mortality of the fish vaccinated with PBS was 75%. Hence, the immunoprotective efficacy of purified recombinant Vhp1 was 70% in terms of RPS. Bacterial recovery analyses indicated that T4D1 was the single type of bacterial strain that was recovered from the liver, kidney, and spleen of moribund fish, suggesting that mortality was the result of T4D1 infection.

3.7. Serum antibody response induced by vaccination with purified recombinant Vhp1

ELISA analysis showed that Vhp1-specific antibodies were produced in the sera of Vhp1-vaccinated fish during five to eight weeks post-vaccination. The amounts of antibodies were relatively highest at seven weeks post-vaccination and lowest at five weeks post-vaccination (Fig. 4).

3.8. Construction of an E. coli strain that expresses and secrets a cytotoxically defective Vhp1

The working principle of the pBU system is that the reporter protein, a secretion-defective β-agarase, can only be transported extracellularly (and thus be detected) by fusion with a secretion-competent protein that acts as a carrier for the agarase. Since Vhp1 was identified via the pBU system, recombinant Vhp1 must be capable of secretion in *E. coli*. This fact suggested to us the idea of constructing an *E. coli* strain that can express and produce a cytotoxically impaired Vhp1 extracellularly. For this purpose, the plasmid pTVP1M was created, which constitutively expresses a His-tagged Vhp1 mutant lacking the C-terminal 136 residues containing the PPC domain and 16 amino acids of the protease domain. DH5α harboring pTVP1M (DH5α/pTVP1M) was examined for Vhp1 production by western immunoblotting, which showed that Vhp1 was detected in the supernatant, but not in the periplasm and cytoplasm, of DH5α/pTVP1M (Fig. 5 and data not shown). In contrast, no Vhp1 was detectable in the supernatant of DH5α harboring the control plasmid pBT3 (DH5α/pBT3). Cytotoxic analyses showed that the culture supernatant of DH5α/pTVP1M exhibited no apparent toxic effect on FG cells, suggesting that the mutant Vhp1 produced by DH5α/pTVP1M was defective in cytotoxicity.

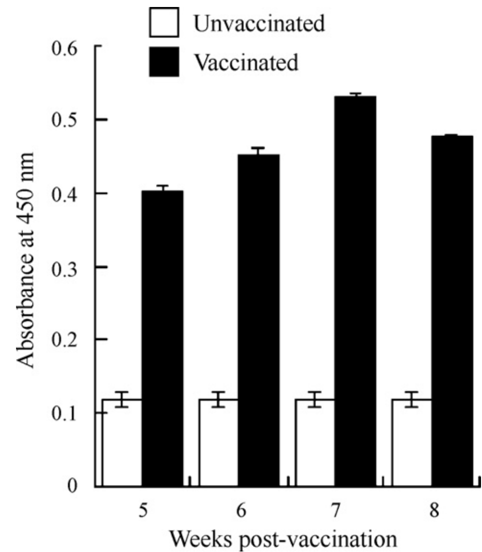

Fig. 4. Serum antibody production in fish vaccinated with purified recombinant Vhp1. Sera were collected from unvaccinated and Vhp1-vaccinated Japanese founder at five to eight weeks post-vaccination. Serum antibodies against Vhp1 were determined by ELISA. Data are means for five assays and presented as the means ± SD.

3.9. Immunoprotective effect of secreted Vhp1 delivered by DH5α/pTVP1M

To examine the vaccine potential of DH5α/pTVP1M, Japanese flounder were immunized via i.p. injection with live DH5α/pTVP1M, DH5α/pBT3, or PBS and monitored for mortality following T4D1 challenge. The results showed that the accumulated mortalities of DH5α/pTVP1M-, DH5α/pBT3-, and

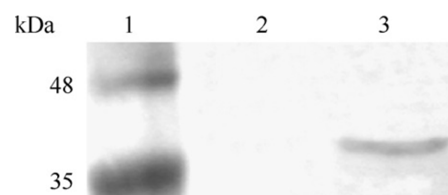

Fig. 5. Western immunoblotting analysis of the subcellular localization of Vhp1 in DH5α/pTVP1M. Extracellular proteins were prepared from DH5α/pBT3 (lane 2) and DH5α/pTVP1M (lane 3). The proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-His antibody. Lane 1: protein markers.

PBS-vaccinated fish were 7.5%, 75%, and 77.5%, respectively. Hence, compared to vaccination with DH5α/pBT3, vaccination with DH5α/pTVP1M produced a RPS of 90%. ELISA analysis indicated that DH5α/pTVP1M-vaccinated fish produced Vhp1-specific serum antibodies, which were comparable in levels to those produced by fish vaccinated with purified recombinant Vhp1.

4. Discussion

In this study, we described the cloning and characterization of the *V. harveyi* protease Vhp1 identified via the pBU system. Since pBU is a signal sequence trap designed for the selection of proteins with active secretion domains, Vhp1 is most likely a protein with functional secretion capacity in its native genetic background. In support of this hypothesis, in silico analyses indicated that Vhp1 is an extracellular peptidase of the subtilisin family and is highly homologous to a number of known extracellular proteases. A study on a subtilisin-like proteinase from a psychrotrophic *Vibrio* species showed that the mature enzyme is a peptide corresponding to the protease region of the protein [40,41]. Similarly, in our study, we found that the protease domain of Vhp1 possesses apparent proteolytic activity when purified as a recombinant protein. Like many subtilisin proteases that are known to be calcium-dependent [40,42–44], we found that Vhp1 was most active in the presence of calcium and completely blocked in activity by the presence of EDTA. Since other divalent metal ions failed to activate Vhp1 in a magnitude approaching that caused by calcium, the effect of calcium on Vhp1 is probably specific.

It is reported that for a subtilisin-type protease from *Thermus thermophilus*, the C-terminal PPC-like domain is involved in cellular translocation [45]. In the case of Vhp1, we found that the truncated protein devoid of the C-terminal region containing PPC was secretion competent, which is consistent with the fact that Vhp1 was identified because the Vhp1 truncated at the protease domain could direct the transport of the secretion-defective reporter of pBU. These results indicate that at least in *E. coli*, the C-terminal region of Vhp1 is not required for translocation. The lack of cytolytic activity in the truncated form of Vhp1 expressed by DH5α/pTVP1M could be due to the incompleteness of the protease domain and/or the presence of the subtilisin-N domain, which may block the residual activity remained in the truncated Vhp1.

A number of extracellular proteases of *Vibrio* species are found to be associated with bacterial virulence. For example, the extracellular metalloprotease PrtV of *Vibrio cholerae* is required for host-killing [46], and the zinc metalloprotease EmpA of *Vibrio anguillarum* is a virulence factor that participates in pathogen–host interactions [47–50]. Both PrtV and EmpA are known to be cytotoxic to cultured host cells [51,52]. In our study, we found that, like EmpA and PrtV, Vhp1 exhibited apparent cytotoxicity to cultured flounder cells, which suggests the possibility that Vhp1 may play a role in the pathogenesis of *V. harveyi* by destructing the cellular barrier of host defense. This hypothesis is in line with the observation that immunization with Vhp1 conferred effective protection upon fish against T4D1 infection, which favors the notion that Vhp1 is produced and involved in the infection process of T4D1.

Since Vhp1 possesses cytotoxic property, it cannot be used as a vaccine in the proteolytically active form. For this reason, recombinant Vhp1 purified in the denatured form was examined for vaccine potential, which showed that the inactivated Vhp1 induced the production of specific serum antibodies and elicited effective protective immunity in vaccinated fish. These results suggest that there probably exist in Vhp1 certain linear epitopes that are retained in the denatured protein and contribute to the immunogenicity of the latter. It is reasonable to speculate that cytotoxically active Vhp1, if used as a vaccine, may provoke stronger immunoprotection.

Compared to subunit vaccines in the form of purified recombinant proteins, secreted vaccines produced by live bacteria are labor-effective in preparation and usually induce higher levels of protection. In this study, we found that live DH5α/pTVP1M was highly effective as a vaccine, which suggests that recombinant Vhp1 secreted by DH5α/pTVP1M, though truncated at the C-terminus, is not affected at immunogenicity. Since fish vaccinated with DH5α/pTVP1M exhibited a survival rate that is much higher than that of the fish vaccinated with purified recombinant Vhp1, it is possible that secreted Vhp1 produced by DH5α/pTVP1M is more effective as an immunogen than purified recombinant Vhp1, the latter may have lost certain conformational epitopes due to denaturation. It is also likely that in the case of DH5α/pTVP1M vaccination, cellular components of the host strain, such as lipopolysaccharides, may induce certain immune responses that enhanced the specific immune defense elicited by Vhp1.

In conclusion, the results of this study demonstrate that purified recombinant Vhp1 possesses proteolytic and cytotoxic activities, which suggests the possibility that Vhp1 may be involved in the infection of *V. harveyi*. Given the fact that both purified recombinant Vhp1 and secreted Vhp1 delivered by *E. coli* are immunoprotective against *V. harveyi* infection, Vhp1 is promising as a vaccine candidate to combat *V. harveyi*-related vibriosis.

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