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Overexpression, Purification, Characterization, and Pathogenicity of *Vibrio harveyi* Hemolysin VHH

Yingbin Zhong,[1] Xiao-Hua Zhang,[1]* Jixiang Chen,[1] Zhenghao Chi,[1] Boguang Sun,[1] Yun Li,[1] and Brian Austin[2]

Department of Marine Biology, Ocean University of China, Qingdao, China,[1] and School of Life Sciences, John Muir Building, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, United Kingdom[2]

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***Vibrio harveyi* VHH hemolysin is a putative pathogenicity factor in fish. In this study, the hemolysin gene *vhhA* was overexpressed in *Escherichia coli*, and the purified VHH was characterized with regard to pH and temperature profiles, phospholipase activity, cytotoxicity, pathogenicity to flounder, and the signal peptide.**

Vibrio harveyi is a luminous gram-negative marine bacterium that is widely distributed in the marine environment (23, 24). The organism is a major pathogen of cultured penaeid shrimp (1, 15, 30) and has also been associated with fish diseases (1, 6, 7, 10, 31). However, comparatively little is known about the pathogenicity mechanisms of *V. harveyi*. Liu et al. (16) considered that proteases, phospholipases, or hemolysins might well be important for pathogenicity. In this respect, cysteine protease has been reported as the major exotoxin for penaeid shrimp (13, 14, 17). In contrast, Montero and Austin (20) suggested that lipopolysaccharide might constitute the lethal toxin of *V. harveyi* E2 for penaeid shrimp. Also, pathogenicity has been associated with the presence of a bacteriophage (3).

Previously, we examined a large number of well-characterized *V. harveyi* strains and found that the hemolysin activity in the extracellular product (ECP) was involved in pathogenesis in salmonids (34). The most pathogenic isolate, VIB 645, contained two closely related hemolysin genes (designated *vhhA* and *vhhB*), which were cloned and sequenced. Conversely, the majority (19/20) of the other cultures possessed only single hemolysin genes, or none at all (35). The open reading frames of the *vhhA* and *vhhB* genes were both 1,257 nucleotides, the same size as the open reading frame of the *tlh* gene of *V. parahaemolyticus* (26). Data revealed that the nucleotide sequence identities of *vhhA* and *vhhB* to *tlh* were 77.5% and 77.2%, respectively (35). In the present study, we expressed the *vhhA* gene of *V. harveyi* in *Escherichia coli*, purified the recombinant VHH hemolysin, investigated its biological characteristics, and detected the hemolysin in the ECP of *V. harveyi* VIB 645 by Western blotting.

The *vhhA* gene (GenBank accession no. AF293430), encoding *V. harveyi* hemolysin VHH, was obtained by PCR amplification using p645H1-1 (35) as a template. The forward primer (5′-CGGAATTCATGAATAAAACTATTACGTT-3′) begins from the initiation codon and adds an EcoRI site at the 5′ end of the gene, and the reverse primer (5′-CGCTCGAGGAAA GGATGGTTTGACAATT-3′) ends before the stop codon and adds an XhoI site. The PCR product (1.27 kb) was excised and inserted into the EcoRI/XhoI-cut expression vector pET-24d(+) (Novagen, Madison, WI). The ligated plasmid was transformed into chemically competent *E. coli* JM109 cells (New England Biolabs, Beverly, MA), and the transformed *E. coli* was plated onto LB-Kan medium (LB supplemented with 50 μg of kanamycin ml^{-1}). The sequence of the cloned gene in the pET-24d(+) vector was confirmed by DNA sequencing. One clone was selected and transformed into *E. coli* strain BL21(DE3) (Novagen) for expression of the full-length *vhhA* gene.

A single colony of *E. coli* BL21(DE3) transformed with pET-24d(+)-*vhhA* was inoculated into 5 ml of LB-Kan medium and incubated overnight at 37°C with shaking at 200 rpm. This culture was inoculated into 495 ml of the same medium and allowed to grow for another 2.5 h, attaining an optical density at 600 nm of 0.6. Expression of VHH was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, with incubation for an additional 6 h at 25°C with vigorous shaking.

To purify the recombinant VHH from the bacterial cells, the induced cells from a 500-ml culture were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, and the pellet was resuspended in 50 ml of nickel-nitrilotriacetic acid (Ni-NTA) bind buffer (20 mM Tris-HCl [pH 8.0], 10 mM imidazole, 0.5 mM NaCl). Lysozyme was added at a final concentration of 1 kU ml^{-1}, and the mixture was incubated for 15 min at 30°C. Then the cell suspension was sonicated in ice-water for a total of 15 min with a microsonicator (Sonics Inc., CA), the lysed cells were centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was filtered through a 0.22-μm-pore-size filter. To purify the recombinant VHH secreted into the medium, the culture supernatant was collected by centrifugation at 10,000 × *g* for 30 min. Solid ammonium sulfate was added to 80% saturation (591 g $liter^{-1}$). After overnight incubation at 4°C, the resulting precipitate was collected by centrifugation at 10,000 × *g* for 30 min at 4°C and dissolved in 50 ml of Ni-NTA bind buffer. The suspension was centrifuged at 10,000 × *g* for 10 min at 4°C to remove insoluble residue. Dialysis was performed for 12 h at 4°C against Ni-NTA bind buffer in dialysis tubing with a molecular weight cutoff from 8,000 to 14,000.

The supernatant from the sonicated *E. coli* cells and the

* Corresponding author. Mailing address: Department of Marine Biology, Ocean University of China, 5 Yushan Road, Qingdao 266003, People's Republic of China. Phone and fax: 0086-532-82032266. E-mail: xhzhang@ouc.edu.cn.

FIG. 1. SDS-PAGE and N-terminal amino acid sequence of VHH. (A) SDS-PAGE of purified VHH from transformed *E. coli* cells and culture supernatant. Lane 1, molecular marker; lane 2, purified VHH from transformed *E. coli* cells; lane 3, purified VHH from the culture supernatant of transformed *E. coli* cells. (B) N-terminal amino acid sequence of VHH secreted into the medium. (a) 5′ nucleotide sequence of *vhhA*; (b) deduced N-terminal amino acid sequence of the protein encoded by *vhhA*; (c) N-terminal amino acid sequence of VHH secreted into the medium.

dialyzed material from the culture medium were applied to a metal chelating affinity chromatography column packed with 5 ml of Ni-NTA His-Bind resin (Novagen), and VHH was purified according to the manufacturer's instructions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified VHH from transformed cells revealed a single band with an estimated molecular mass of ~46.2 kDa (pro-VHH) (Fig. 1A). VHH in the culture supernatant was also purified, albeit with a slightly smaller molecular mass of ~45.6 kDa (mature VHH) (Fig. 1A). However, the VHH yield in the culture supernatant was lower than that in bacterial cells.

The molecular mass of the VHH purified from the culture supernatant of transformed *E. coli* was estimated as ~45.9 kDa by gel filtration on a Sephadex G-200 column (Amersham Biosciences, United Kingdom) by the method outlined in the Sigma Technical Bulletin (MW-GF-200). Since the molecular masses of the mature VHH determined by SDS-PAGE (45.6 kDa) and gel filtration (~45.9 kDa) were comparable, it is evident that mature VHH is a monomeric polypeptide when expressed in *E. coli*. In contrast, the molecular mass of the dimeric *V. parahaemolyticus* thermostable direct hemolysin (Vp-TDH) was estimated as ~42 kDa by gel filtration and 21 kDa by SDS-PAGE (29).

VHH purified from the culture supernatant of transformed *E. coli* was subjected to SDS-PAGE. The protein bands were transferred to a polyvinylidene difluoride (PVDF) (Amersham Biosciences) sheet by electric transfer. The N-terminal amino acid sequence on the excised PVDF membrane was determined to be Ala-Glu-Pro-Thr-Leu-Ser (AEPTLS) (Fig. 1B) by Edman degradation using a protein sequencer (Applied Biosystems, Foster City, CA). Thus, mature VHH was a protein with 20 N-terminal amino acids deleted from pro-VHH. Analysis of these 20 amino acids revealed that they are strongly hydrophobic and have a positively charged lysine residue, which means that they constitute a typical signal peptide sequence.

Mature VHH is 20 amino acid residues shorter than pro-VHH. The inferred molecular masses of pro-VHH and mature VHH by DNA sequencing were 47.3 kDa and 45.1 kDa, respectively. Thus, the difference between them was 2.2 kDa. However, the difference in estimated molecular mass between mature VHH and pro-VHH was only ~600 Da by SDS-PAGE (Fig. 1A). This may be caused by the His tag of mature VHH and pro-VHH. Indeed, it has been reported that a deviation often results in determining the molecular weight of the His tag fusion protein by SDS-PAGE. However, the causes leading to this deviation are unclear (4, 21).

Among gram-negative bacteria, there are five types of secretion pathways: types I, II, III, IV, and V. The type I and type II pathways are most commonly used for recombinant protein secretion in *E. coli* strain K-12 (19). The type II secretion system is a two-step process for the extracellular secretion of proteins mediated by periplasmic translocation. The signal peptide of recombinant proteins was usually cleaved during secretion across the cytoplasmic membrane using the type II secretion system (19). From our results, it is likely that the signal peptide of VHH was cleaved during secretion across the cytoplasmic membrane into the periplasm. VHH may use the SecB-dependent pathway of the type II secretion system when secreted into the medium, because the signal peptide of VHH was very similar to that of the SecB-dependent pathway. This situation is similar to that of *V. fluvialis* hemolysin, which is secreted into the extracellular environment as a 79-kDa protein after cleavage of 25 residues in the N-terminal region (5). However, VHH is different from the hemolysin of *V. cholerae*, which is secreted into the culture medium by two-step processing. The latter is initially synthesized as an 82-kDa protein, and the signal peptide is cleaved during secretion

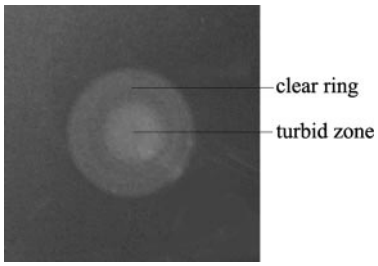

FIG. 2. Phospholipase activity of purified VHH on a 1% egg yolk emulsion plate.

through the inner membrane, yielding a 79-kDa protoxin. Finally, this protein becomes the 65-kDa active mature hemolysin during transport to the culture medium (33).

Hemolytic activity against turbot erythrocytes was determined by a previously published procedure (35). For determination of the optimal temperature, VHH (8 hemolysin units) was diluted twofold in Tris-buffered saline (TBS) in 96-well microtiter plates, and equal volumes of 2% (vol/vol) turbot erythrocytes were added. The plates were incubated for 1 h at 4, 15, 25, 37, and 45°C, and the hemolytic activity was recorded. At temperatures above 55°C, the erythrocytes were destroyed. For determination of thermostability, volumes (100 μl) of the VHH sample were preincubated for 30 min at 4, 25, 37, 45, 55, and 75°C, respectively, and the residual hemolytic activity was recorded. The results showed that the optimal temperature for the hemolytic activity of purified VHH was 37°C. Overall, there was no detectable decline in hemolytic activities after treatment for 30 min at 4, 25, and 37°C. Treatment for 30 min at 55°C resulted in a 75% loss of activity. The hemolytic activity was destroyed entirely following 30 min at >65°C, indicating that the hemolysin was heat labile, like the thermolabile hemolysin of *V. parahaemolyticus* (25, 26) and the β-hemolysin produced by *V. mimicus* (8).

For determination of the optimal pH, 10-μl (0.8-hemolysin-unit) volumes of VHH were added to pH buffers (1/9, vol/vol) ranging from pH 6 to 11, twofold dilutions were prepared in corresponding pH buffers, and the hemolytic activity was determined. The pH stability was estimated after the VHH sample was preincubated in pH 4 to 11 buffers at 4°C for 12 h, diluted in TBS (pH 7.5; 1/9, vol/vol), and the residual activity determined. The buffers used were 20 mM sodium citrate–citric acid from pH 4 to 6, 20 mM Tris-HCl from pH 7 to 9, and 20 mM sodium dicarbonate-NaOH from pH 10 to 11. Each buffer was supplemented with 0.85% (wt/vol) NaCl. The results showed that the optimal pH was 8 to 9, slightly alkaline. Only 50% of the hemolytic activity was retained at pH 7 and 10. At pH values lower than 6, the erythrocytes were destroyed. Overall, VHH was stable at pH 7 to 10, but stability decreased sharply at pH values of <6.

Phospholipase and lipase activities were determined as previously described (20). Fifty microliters of VHH was pipetted into 5-mm-diameter Oxford cups, which were placed on 2% (wt/vol) agar prepared in TBS (pH 7.5) supplemented with 1% (vol/vol) egg yolk emulsion or 1% Tween 80 and were incubated at 37°C overnight. On the egg yolk emulsion plate, the VHH formed concentric circles comprising a clear outer ring and a turbid inner zone (Fig. 2). This indicated that the VHH had strong phospholipase activity. However, the VHH had no lipase activity on the Tween 80 plate (data not shown). We attempted to determine the phospholipase type of the VHH. For this purpose, we used the specific substrate *p*-nitrophenylphosphorylcholine (*p*-NPPC; Sigma) to investigate whether the VHH had phospholipase C (PLC) activity. The PLC of *Clostridium perfringens* (Sigma) was used as the control. The results revealed that there was no PLC activity in the VHH. However, the formation of concentric circles on the egg yolk emulsion plate by VHH was very similar to the phenomenon caused by a lecithin-dependent hemolysin encoded by a *V. parahaemolyticus* gene, which was a phospholipase A_2/lysophospholipase (25). Thus, VHH may well be a phospholipase A_2/lysophospholipase.

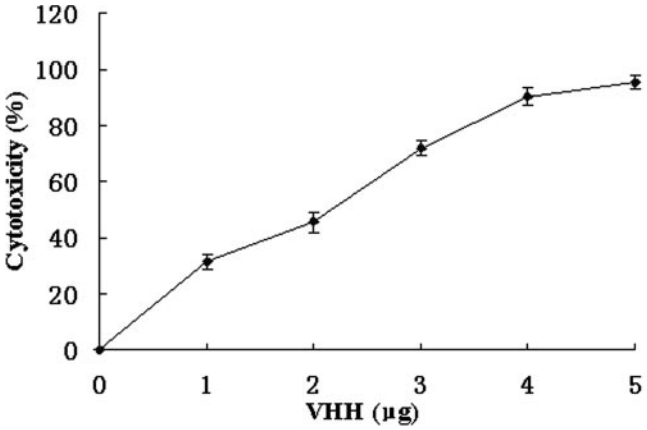

FIG. 3. Cytotoxicity effect of VHH at different concentrations on the FG cell line.

Certainly, it has been reported that some vibrio hemolysins demonstrate phospholipase activity. Thus, the lecithin-dependent hemolysins of *V. parahaemolyticus* and *V. cholerae* O139 have phospholipase A_2/lysophospholipase (25) and phospholipase C activities, respectively (22). Furthermore, some vibrio phospholipases show hemolytic activity on erythrocytes. For example, PhlA from *V. mimicus* demonstrated the highest hemolytic activity toward rainbow trout and tilapia (12). In addition, *V. vulnificus* extracellular phospholipase A_2 and lysophospholipase have hemolytic activity against sheep and mouse erythrocytes (27). These data revealed that there is some relationship between hemolysin and phospholipase.

The cytotoxicity of VHH was investigated by measuring the amount of mitochondrial dehydrogenase released from flounder gill (FG) cells (28) in tissue culture. A 90-μl aliquot (10,000 cells) of the cell suspension was inoculated into each well in a 96-well microtiter plate. After 4 h of incubation at 22°C, 10 μl of the VHH sample at various concentrations was added and incubated as before. Cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability/cytotoxicity assay kit (Beyotime Biotechnology, Jiangsu, China) after 60 h, with cytotoxicity calculations based on the manufacturer's instructions. Cytotoxic activity was visible within 12 h of incubation of the cells with 5 μg of purified VHH. To examine the effects of different concentrations of VHH on the FG cell line, we added 1 to 5 μg of VHH and incubated for 60 h. As shown in Fig. 3, the level of cytotoxicity

TABLE 1. Pathogenicity of VHH for flounder[a]

Dose (μg of protein fish^{-1})	Total mortality (n = 10)	Time to death (h)
5.9	1	144
11.8	1	120
23.6	6	12–96
47.2	8	12–36
94.4	10	10–20

[a] Groups of 10 fish (average weight, 15.3 g) were infected by i.p. injection with 0.1-ml volumes of twofold dilutions of VHH preparations. Controls were injected with 0.1-ml volumes of TBS.

increased by 31.3% at 1 μg and 95.5% at 5 μg. The cytotoxicity of VHH in tissue culture is similar to those of other bacterial hemolysins (9, 18).

Flounder from quarantined stocks recognized as disease free (2) were used as models to assess pathogenicity. Groups of 10 fish (average weight, 15.3 g) were infected by intraperitoneal (i.p.) injection with 0.1-ml volumes of twofold dilutions of the VHH preparations. Controls were injected with 0.1-ml volumes of TBS. The infected animals were maintained for as long as 7 days in covered polypropylene tanks supplied with aerated seawater (50% of the volume was changed daily) at a temperature of ~20°C. The 50% lethal doses were calculated using the probit method (32). Deaths among the flounder occurred between 10 and 144 h after i.p. injection. Most infected fish revealed hemorrhaging in the peritoneal cavity and hemorrhagic and necrotic signals at the site of injection. The undiluted VHH, standardized at 944 μg of protein ml^{-1}, killed all the fish when 0.1-ml volumes were administered. In comparison, the 2-, 4-, 8-, and 16-fold-diluted protein killed 80, 60, 10, and 10% of the flounder, respectively. Thus, the 50% lethal dose of VHH for flounder was established as 18.4 μg of protein fish^{-1} (equivalent to 1.2 μg of protein g of fish^{-1}) (Table 1).

To prepare a polyclonal antibody against VHH, 2.0 ml of a mixed emulsion of equal volumes of purified VHH solution (1 mg ml^{-1}, inactivated by 3% [vol/vol] formalin for 24 h at 28°C) and Freund's complete adjuvant (Sigma) was injected subcutaneously at two to four sites on each of two New Zealand White female rabbits (body weight, 2 kg). At 2 and 4 weeks after the first injection, 1.0 ml of a mixed emulsion of equal volumes of purified VHH solution and Freund's incomplete adjuvant (Sigma) was injected subcutaneously. On the 7th day after the last injection, antibody titers were determined by Western blotting. If the titers were >1:1,000, blood was withdrawn by venipuncture and an antiserum was prepared. The specificity of the antibody against VHH was confirmed by immunoblotting with purified and crude VHH preparations from the recombinant *E. coli*, in which the antibody reacted with a single protein (data not shown) and the antibody titer was >1:2,500.

V. harveyi VIB 645 was cultured on ZoBell 2216E medium (Difco) at 28°C overnight, with subculturing every 7 to 14 days. The ECP of *V. harveyi* VIB 645 was prepared as previously described (34), subjected to SDS-PAGE or to native PAGE without SDS and without α-2-mercaptoethanol (11), and transferred to PVDF sheets. The antiserum against VHH was preabsorbed on *E. coli* BL21(DE3). Western blotting was performed using a commercial kit (Boster, Wuhan, China) and employing the method outlined by the manufacturer. As shown in Fig. 4, similar results were obtained using SDS-PAGE and native PAGE. A single band appeared when the antibody against VHH reacted with the ECP of VIB 645, and the molecular mass of the band (~44.3 kDa) was lower than those of the purified VHH from transformed *E. coli* cells (~46.2 kDa) and the culture supernatant of transformed *E. coli* (~45.6 kDa). We determined that the purified VHH from transformed *E. coli* was a monomeric peptide. Given the result of Western blotting, we concluded that the VHH in the ECP of VIB 645 was also a monomer.

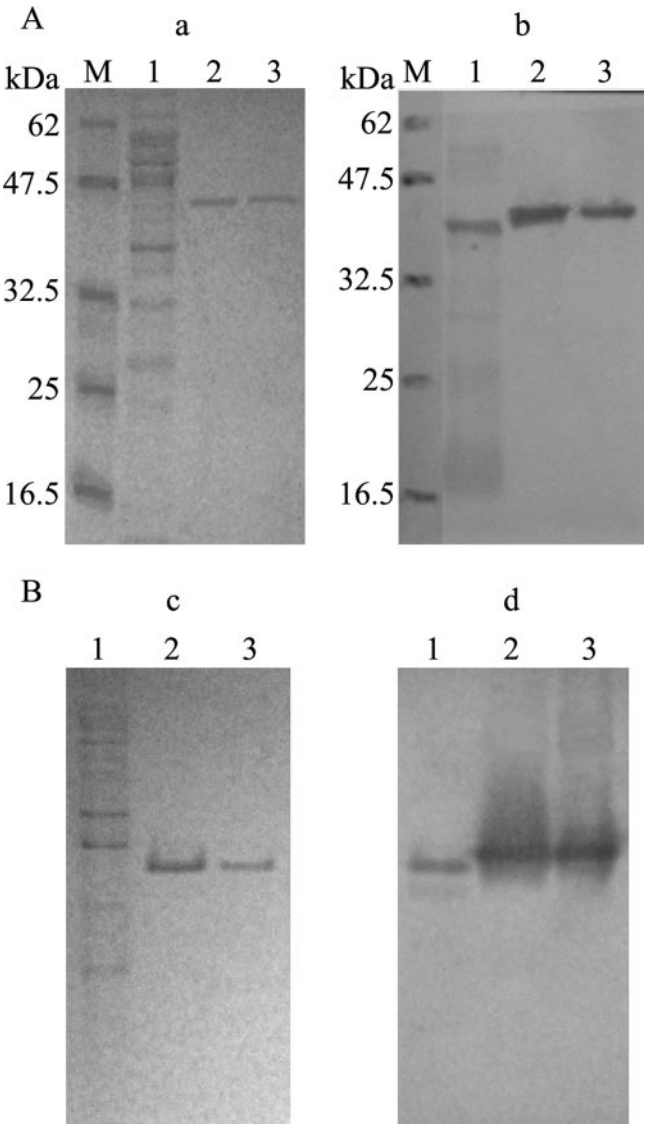

FIG. 4. Western blot profiles of the ECP of *V. harveyi* VIB 645 and purified VHH after SDS-PAGE (A) and native PAGE (B). The secondary antibody was peroxidase-labeled goat anti-rabbit immunoglobulin G at a dilution of 1:400. (a and c) SDS-PAGE and native PAGE, respectively, of the ECP of *V. harveyi* VIB 645 and purified VHH; (b and d) Western blotting of the ECP of *V. harveyi* VIB 645 and purified VHH. Lanes M, molecular marker; lanes 1, the ECP of *V. harveyi* VIB 645; lanes 2, VHH purified from the culture supernatant of transformed *E. coli*; lanes 3, VHH purified from transformed *E. coli* cells.

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