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Characterization and Pathogenicity of the Zinc Metalloprotease EmpA of *Vibrio anguillarum* Expressed in *Escherichia coli*

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Abstract. The extracellular zinc metalloprotease (EmpA) is a putative pathogenic factor involved in the invasive process of the fish pathogen *Vibrio anguillarum*. It is synthesized as a 611–amino-acid prepproprotease. The gene encoding EmpA (*empA*) has already been cloned and sequenced. In this study, *empA* was inserted into pET24d(+) and expressed in *Escherichia coli* BL21(DE3). Recombinant EmpA with His-tag was purified in a single step with a His-binding Ni-affinity column to a purity >95%. In addition, proteolytic activity, cytotoxicity, fish pathogenicity, and solubility of the recombinant protein were determined.

The marine bacterium, *Vibrio anguillarum*, is the causative agent of vibriosis, a systemic fish disease characterized by hemorrhagic septicemia [2]. Outbreaks of vibriosis result in high mortality of infected fish. Vibriosis has evolved into a major obstacle to the spread of commercial aquaculture [2]. *V. anguillarum* secrets a variety of products involved in colonization, adhesion, virulence, and pathogenesis. These products include functioning in iron-uptake system [8], hemolysin [18], lipopolysaccharide [3, 20], and cytotoxin [13]. In addition, the extracellular zinc metalloprotease (EmpA) has also been identified as an important virulence factor that may assist the pathogen to enter host cells [5, 7, 10, 12, 17, 19]. We purified and characterized the EmpA [5] of *V. anguillarum* W-1, originally isolated from sea bass (*Lateolabrax japonicus*) [23]. This protein possesses high proteolytic activity and can cause death in turbot (*Scophthalmus maximus*) and flounder (*Paralichthys loivaceus*) when injected intraperitoneally. It is interesting that the symptoms of injected fish are similar to those of fish infected by *V. anguillarum* [5–6, 19]. The empA-encoding gene (*empA*), of *V. anguillarum* has been cloned and sequenced [6, 17]. The deduced amino-acid sequence is highly similar to that of other bacteria. EmpA synthesized by *V. anguillarum* W-1 is 611 amino acids in length. It is actually a precursor containing a 25–amino-acid signal peptide, a 174–amino-acid propeptide, and a mature protease corresponding section [17].

In this study, EmpA was expressed at high levels in *E. coli* and purified in a single step. The final purity of the enzyme was >95%. In addition, the proteolytic activity, cytotoxicity, and fish pathogenicity of the expressed EmpA were also studied.

Materials and Methods

Construction of the expression vector. Plasmid pUCm-*empA* [6] containing the *V. anguillarum* EmpA-encoding gene (GenBank accession NO. AY046320) was used as to reamplify with primers (5′-CGCGGATCCATGAAAAAAGTACAAC-3′ and 5′-CCGCTCGAGATCCAGTCTTAACGTTAC-3′ [*Bam*HI and *Xho*I restriction sites are underlined]). The polymerase chain reaction product was digested with *Bam*HI and *Xho*I and inserted into pET24d(+) expression vector (Novagen, Madison, WI) digested with the same enzymes. The final construct, pET24d(+)-*empA*, was transferred into *E. coli* BL21(DE3) (Novagen). The sequence integrity of final construct was confirmed by DNA sequencing (Bioasia, Shanghai, China).

Expression of the recombinant protein. Optimum temperature conditions, concentration of isopropyl β-D-thiogalactoside (IPTG), and induction time were determined to achieve the highest proteolytic activity of the enzyme. Induced cells were sonicated, and the supernatant (20 μl) of the cell lysates was assayed for proteolytic activity. Whole-cell

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lysates and soluble and insoluble fractions were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [16] so the solubility of rEmpA could be determined.

Proteolytic activity assay. Proteolytic activity with azocasein as substrate was determined according to Kreger and Lockwood [15].

Purification of recombinant EmpA. To purify rEmpA from bacterial cells, induced cells from a 500-ml culture were harvested by centrifuging at 10,000 × *g* for 10 minutes at 4°C. The cell pellet was resuspended in 50 ml Ni-NTA bind buffer (20 mM Tris-HCl [pH 8.0], 10 mM imidazole, and 0.5 mM NaCl), and then sonicated on ice water for 30 minutes with a microsonicator (Sonics Inc., CA). Cellular debris was removed by centrifuging at 10,000 × g for 30 minutes at 4°C. The supernatant was filtered through a 0.22-μm porosity filter (Millipore, MA). To purify rEmpA secreted into the medium, the culture supernatant was collected by centrifuging at 10,000 × g for 30 minutes at 4°C. Solid ammonium sulfate was added to 80% saturation (591 g L^{-1}). After being kept at 4°C overnight, the precipitate was collected by centrifugation at 10,000 × *g* for 30 minutes at 4°C, then dissolved in 50 ml Ni-NTA bind buffer. The suspension was centrifuged at 10,000 × g for 10 minutes at 4°C to remove insoluble materials and dialysed against Ni-NTA bind buffer for 12 hours at 4°C. The supernatant from the sonicated *E. coli* cells and the dialyzed material from the culture medium were loaded onto Ni-NTA His-Bind Resin column (Novagen), which was pre-equilibrated with Ni-NTA bind buffer. rEmpA was purified according to the manufacturer's instructions, and the purified rEmpA was quantitated by Bradford method [4].

Gel-filtration chromatography. The oligomeric state of rEmpA was analyzed by gel-filtration chromatography on a Sephadex G-200 column (96 × 1.5 cm) (Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl [pH 7.5] and 100 mM KCl according to the directions described in Sigma Technical Bulletin (MW-GF-20). rEmpA (0.5 mg) in 500 μl 50 mM Tris-HCl (pH 7.5), and 100 mM KCl was loaded onto the column. The effluent was collected in 2-ml fractions and monitored for proteolytic activity according to absorbance at 280 nm. Peak fractions with highest activity were pooled.

Electrophoresis and Western blotting. SDS-PAGE on 12% acrylamide gels was performed as described by Laemmli [16]. After electrophoresis, proteins were transferred to nitrocellulose membrane (Millipore) using semidry Western transfer apparatus (Bio-Rad Laboratories, Hercules, CA), and the blot was blocked with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (blocking solution). The nitrocellulose filters were probed with the first antiserum diluted in blocking solution (1:4,000). A horseradish peroxidase–conjugated goat antirabbit immunoglobulin G (Wuhan BoShiDe Biotechnology, Wuhan, China) was used as the secondary antibody (1:2,000 ratio). The specific binding was detected with H_2O_2 and dianilinobenzene as chromogenic substrate.

Cytotoxicity assay. Cytotoxicity of purified rEmpA was investigated by measuring the amounts of mitochondrial dehydrogenase (MDH) released from the flounder gill (FG) cells in tissue culture. FG cells were grown at 22°C in Eagle's minimum essential medium (MEM) (Sigma) supplemented with 10% fetal calf serum and harvested with trypsin–ethylenediaminetetraacetic acid. The cells were collected by centrifuging and resuspended at a concentration of 100,000 cells/ml Eagle's MEM containing 1% fetal calf serum. A 90-μl aliquot of the cell suspension was inoculated to each well in a 96-well microtiter plate. After incubation for 4 hours at 22°C, 10 μl of the rEmpA samples at various concentrations were added and incubated as previously described. The cells were regularly inspected under light microscopy for the development of cytopathic effect (CPE)-like morphologic damage. Cytotoxicity was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell viability/cytotoxicity assay kit (Beyotime Biotechnology, Jiangsu, China) after 60 hours, with cytotoxicity calculations based on the manufacturer's instructions. Cultures of the control group were left untreated. Absorbance was measured with a microtiter plate reader (Labsystems Multiskan MS, Finland) at wavelength 570 nm. Percent viability was calculated as follows:

optical density (OD) of control group − OD of treated group / OD of control group × 100%.

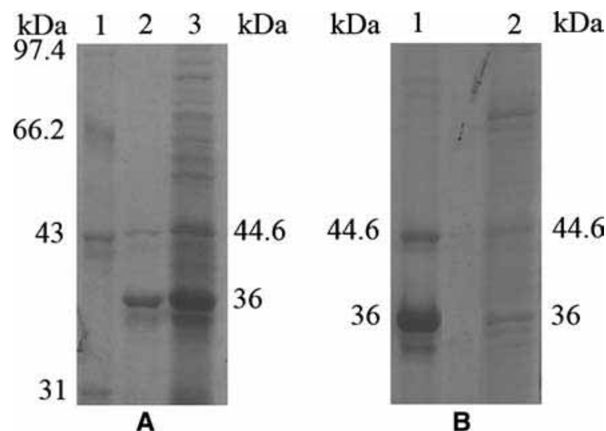

Fig. 1. SDS-PAGE analysis of expressed rEmpA. (A) Whole-cell lysates: lane 1, molecular weight markers; lane 2, control; lane 3, BL21(DE3)/pET24d(+)-*empA* induced with IPTG. (B) Solubility of rEmpA: lane 1, soluble fraction of the whole-cell lysates; lane 2, insoluble fraction.

Fish pathogenicity. Turbot used as models to assess pathogenicity were obtained from commercial fish farms in China and maintained in aerated seawater at 20°C (half of the water was changed daily). The health status was examined immediately on arrival in the aquaria and at 1- to 2-week periods thereafter [1]. Fish individuals (average weight 7.5 g, 8 fish/group) were infected by intraperitoneal (IP) injection of 0.1 ml two-fold dilutions of the rEmpA. Controls were injected with 0.1 ml PBS. Challenged animals were maintained for up to 7 days as described previously. Dead and moribund fish were removed, and disease characteristics were recorded. The LD_{50} values were calculated using the probit method described by Wardlaw [22].

Results and Discussion

Expression and purification of rEmpA. The encoding sequence of *V. anguillarum* EmpA was inserted into pET24d(+), yielding pET24d(+)-*empA*. rEmpA included an additional short noncleavable 6× His-tag at C-terminus. The optimized expression of rEmpA was induced with 1 mM IPTG at 25°C for 6 hours. Most of the rEmpA was soluble (60% to 70% of total soluble proteins) (Fig. 1B). Intriguing profiles on SDS-denaturing gel were repeatedly observed for the samples prepared from the whole-cell lysates of induced BL21(DE3)/pET24d(+)-*empA*. Except for one major band with an estimated molecular weight of

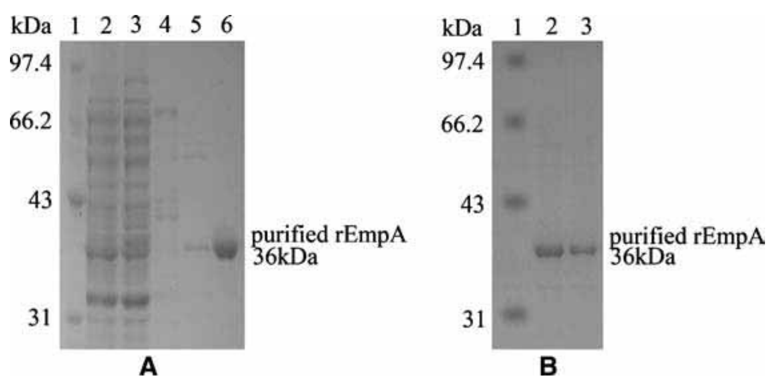

Fig. 2. SDS-PAGE analysis of purified rEmpA. (A) Analysis of different purification steps: lane 1, molecular weight markers; lane 2, crude extract; lane 3, 10 mM imidazole elutant; lane 4, 20 mM imidazole elutant; lane 5, 50 mM imidazole elutant; lane 6, rEmpA eluted by 250 mM imidazole. (B) Analysis of purified rEmpA from the medium and from bacterial cells: lane 1, molecular weight markers; lane 2, rEmpA purified from the medium; lane 3, rEmpA purified from the bacterial cells.

Table 1. Purification steps of rEmpA from *E. coli* BL21(DE3)/pET24d(+)-*empA* cells

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification-fold
Cell homogenate	296.5	1.49×10^6	5.04×10^3	100	1.00
Ni-NTA resin	1.7	4.32×10^5	2.54×10^5	29	50

approximately 36 kDa, another much less prominent band (approximately 44.6 kDa) appeared as well (Fig. 1), and both bands were much smaller than the deduced molecular weight of approximately 66.7 kDa. Interestingly, we found that the band with the molecular weight of approximately 44.6 kDa became prominent, although the band of approximately 36 kDa disappeared if the sample was not heated in boiling water (data not shown). This indicated that a general incomplete proteolysis depending on EmpA seemed to occur during preparation of the sample. It has been proposed that this 611–amino-acid protein contains a putative signal sequence (Met1 to Ala25/Ala26) [17, 21] and a putative leader peptide [21]. The mature protein consists of 411 amino acids, with a calculated molecular mass of approximately 44.6 kDa. The band of approximately 36 kDa may correspond to a degraded form of the mature protein, which is caused by self-cleaving an approximately 9-kDa peptide at C-terminus during heating [19]. A similar mechanism has been suggested for the synthesis of metalloproteases of *V. vulnificus* [14], *V. proteolyticus* [9], and *V. cholerae* [11].

As shown in Fig. 1B, both soluble and insoluble fractions of cell lysates contained rEmpA, but the soluble fraction contained a much larger amount. Accordingly, recombinant protein from the bacterial cells was purified from this fraction (Fig. 2A). The specific activities of the purified protein could reach approximately 10^5 U mg^{-1} protein (Table 1), which was a little higher than that of the purified EmpA from the secreted fractions of *V. anguillarum* W-1 [5]. Proteins purified from both bacterial cells and the medium showed a single band with the molecular weight of approximately

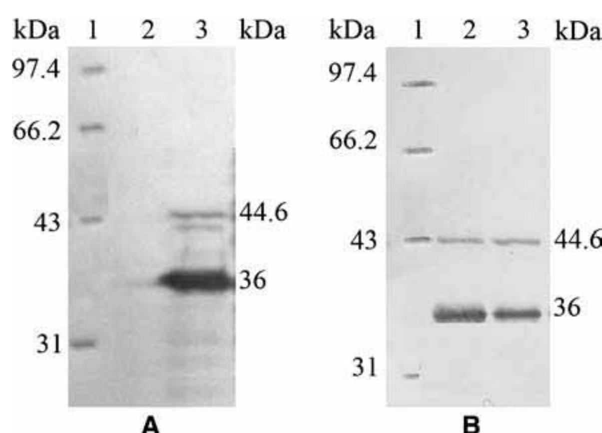

Fig. 3. Western blot analysis of purified rEmpA. (A) Analysis of the whole-cell lysates: lane 1, molecular weight markers; lane 2, control; lane 3, BL21(DE3)/pET24d(+)-*empA* induced with IPTG. (B) Analysis of purified proteins from the medium and the bacterial cells: lane 1, purified rEmpA from the medium; lane 2, purified rEmpA from the bacterial cells.

36 kDa on SDS-PAGE (Fig. 2B). The yield of rEmpA from the medium was a little higher than that of rEmpA from the bacterial cells (data not shown).

The expression of rEmpA was further proved by its reaction with anti-EmpA serum. As shown in Fig. 3A, the antiserum recognized rEmpA from the whole-cell lysates, mostly of the approximately 36-kDa band, less prominently of the approximately 44.6-kDa band when the samples were heated to 100°C before loading (Fig. 3A, lane 3). Therefore, the anti-EmpA antibody specifically recognized rEmpA. Interestingly, only one prominent polypeptide of approximately 44.6 kDa was

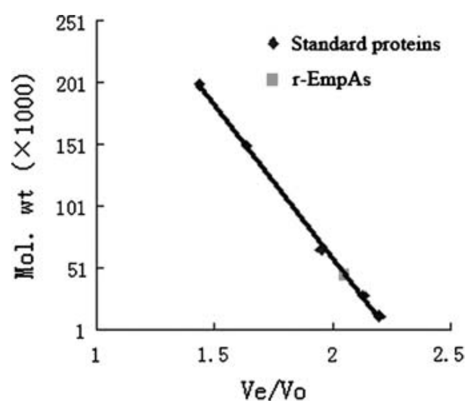

Fig. 4. The standard curve of gel filtration on a Sephadex G-200 column. From the curve, the apparent mass of rEmpAs from the medium and the bacterial cells were both estimated to be approximately 44.6 kDa.

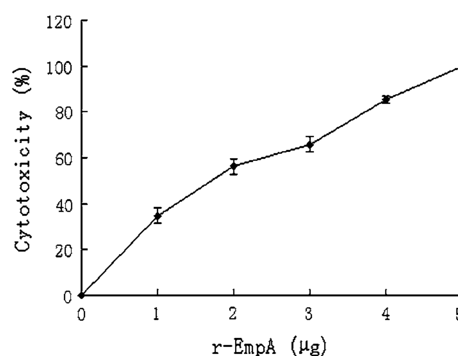

Fig. 5. Cytotoxicity of rEmpA on the FG cell line with 48 hours incubation. The effects of different concentrations (from 0 to 5 μg) of rEmpA were examined by measuring the MDH amount released from the lysed cells using the MTT cell viability/cytotoxicity assay kit.

Table 2. LD_{50} dose of rEmpA determined by IP injection of 0.1 ml volume in turbot.[a] LD_{50} was calculated to be 8.1 μg protein g^{-1} of fish

r-EmpA (μg protein $fish^{-1}$)	No. in each group	Death (/8)	Mortality (%)	Time to death (h)	LD_{50} (μg protein $fish^{-1}$)
7.6	8	1	12.5	132	8.1
15.2	8	1	12.5	108	
30.4	8	2	25	48–72	
60.8	8	4	50	16–24	
121.6	8	6	100	8–12	
Control	8	0	0	–	

detected when the protein samples were not subject to heating to 100°C (data not shown). Fortunately, the same results were observed in Western blotting analysis of the purified proteins (Fig. 3B). These findings were consistent with the results obtained using SDS-PAGE analysis (Fig. 1). Our findings support the hypothesis that the approximately 36-kDa polypeptide is derived from thermoinduced proteolysis of the EmpA precursor. Taken together, the rEmpA synthesized in *E. coli* accumulates in approximately 44.6-kDa processed form, and heating the samples resulted in proteolysis of rEmpA into an approximately 36-kDa stable derivative.

Estimation of oligomeric state and molecular mass of rEmpA. The elution volume for rEmpAs from the bacterial cells and the medium were 140.3 and 140.1 ml, respectively. From the standard curve (Fig. 4), the apparent molecular mass of rEmpAs from the two sources was both estimated to be approximately 44.6 kDa, which was comparable with the estimated molecular mass of purified rEmpAs on SDS-PAGE when the samples were prepared without heating, evidencing that the rEmpA was a monomer when expressed in *E. coli*. As expected, the polypeptide with the molecular mass of approximately 36 kDa was not observed through the assay, and the rEmpAs, both from the bacterial cells and the medium, showed high proteolytic activity. This result further confirmed that the rEmpA synthesized in *E. coli* exists in approximately 44.6-kDa active form. Heating will result in the proteolysis of rEmpA into an approximately 36-kDa stable derivative.

Cytotoxicity and fish pathogenicity of rEmpA. The development of CPE-like morphologic damage to flounder gill cells was visible by light microscopy within 12 hours of incubation of the cells with 5 μg purified rEmpA. Release of a quantity of MDH from host cells was measured, and this reflects the cytotoxicity of rEmpA. To examine the effects of different concentrations of rEmpA on the FG cell line, we added rEmpA, approximately 5 μg, and incubated for 48 hours. As shown in Fig. 5, the level of cytotoxicity increased by 32.1% at 1 μg and 100% at 5 μg, indicating that cytocoxicity of rEmpA was dose dependent.

Moreover, rEmpA was harmful to turbot when injected intraperitoneally. Death of turbot occurred between 8 and 132 hours after IP injection. Most injected fish showed hemorrhage in the peritoneal cavity and hemorrhagic and necrotic signals at the site of the

injection. The undiluted rEmpA, standardized at approximately 1.2 mg protein ml^{-1}, killed all of the fish when 0.1 ml was injected intraperitoneally into turbot. In comparison, the 2-, 4-, 8-, and 16-fold diluted rEmpA killed 50%, 25%, 12.5%, and 12.5% of fish, respectively. Thus, the LD_{50} dose of rEmpA to turbot was established as 8.1 μg protein/g^{-1} fish (Table 2), which was a little different from that (10 μg protein/g^{-1} fish) of the EmpA of *V. anguillarum* W-1 [5]. These results indicate that, as a virulence factor of *V. anguillarum*, EmpA may cause tissue damage by directly degrading host tissues and may promote the invasion process in bacterial pathogenesis.

ACKNOWLEDGMENTS

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