Mutational analysis of the zinc metalloprotease EmpA of *Vibrio anguillarum*

Hui Yang, Jixiang Chen, Guanpin Yang, Xiao-Hua Zhang & Yun Li

Department of Marine Biology, College of Marine Life Sciences, Ocean University of China, Qingdao, P.R. China

Correspondence: Jixiang Chen, Department of Marine Biology, College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao 266003, P.R. China. Tel.: +86 532 82032767; fax: +86 532 82032266; e-mail: betcen@ouc.edu.cn

Received 9 August 2006; revised 11 October 2006; accepted 23 October 2006.
First published online 28 November 2006.

DOI:10.1111/j.1574-6968.2006.00533.x

Editor: Klaus Hantke

Keywords
Vibrio anguillarum; zinc metalloprotease EmpA; site-directed mutagenesis.

Abstract

The extracellular zinc metalloprotease, EmpA, is a putative virulence factor involved in pathogenicity of the fish pathogen *Vibrio anguillarum*. The 611-amino acid precursor of this enzyme is encoded by the *empA* gene. The residues His^{346}, His^{350}, Glu^{370}, Glu^{347}, His^{429}, Tyr^{361} and Asp^{417} are highly conserved and putatively function together at the active site of the enzyme. In this study, *empA* was inserted into pET24d(+) and expressed in *Escherichia coli* strain BL21(DE3) as a 6 × His tagged protein (r-EmpA). All the conserved residues of EmpA mentioned above were individually mutated by site-directed mutagenesis and the mutants were also expressed (m-r-EmpAs). r-EmpA and m-r-EmpAs were purified, and assayed for their proteolytic activities with azocasein as the substrate and cytotoxicities on a flounder gill cell line. m-r-EmpAs that had been mutated at His^{346}, His^{350}, Glu^{370} and Glu^{347} almost completely lost their proteolytic activity and cytotoxicity, pointing towards the essential roles played by these residues. In contrast, those mutated at Tyr^{361}, His^{429} and Asp^{417} still retained a partial proteolytic activity and cytotoxicity. Our results indicate that these conserved residues play important roles in enzymatic activity and that the proteolytic activity of the enzyme is involved in the pathogenesis of *V. anguillarum*.

Introduction

Vibrio anguillarum is a Gram-negative, halophilic bacterium which causes vibriosis or hemorrhagic septicemia in both marine and freshwater fish and other aquatic animals (Austin & Austin, 1999). Vibriosis causes a high mortality in infected fish and is responsible for great losses in aquaculture (Egiduis, 1987). The diverse products secreted by *V. anguillarum* have been reported as being associated with its pathogenesis, such as the iron-uptake system (Crosa, 1980), hemolysin (Kodama *et al.*, 1984), lipopolysaccharide (LPS) (Norqvist & Wolf-Watxtz, 1993; Boesen *et al.*, 1999) and cytotoxin (Kodama *et al.*, 1985). In addition, the extracellular zinc metolloprotease, EmpA, was also identified as an important factor in virulence, as it helps bacterial pathogens to enter host cells (Inamura *et al.*, 1985; Norqvist *et al.*, 1990; Farrell & Crosa, 1991). The EmpA of *V. anguillarum* W-1 – originally isolated from diseased sea perch (*Lateolabrax japonicus*) (Xiao *et al.*, 1999) and which had previously been purified and characterized (Chen *et al.*, 2002a) – showed a high proteolytic activity. It can cause symptoms similar to those of turbot (*Scophthalmus maximus*) and flounder (*Paralichthys loivaceus*) infected by *V. anguillarum* in nature, and even their death when injected intraperitoneally (Chen *et al.*, 2002a). These observations were identical to those reported by Norqvist *et al.* (1990). Unfortunately, little is known on the molecular basis of its enzymatic activity.

Vibrio anguillarum EmpA is synthesized from the *empA* gene as a 611-amino-acid precursor of ~66.7 kDa, which is processed to a protein of ~44.6 kDa during maturation (Milton *et al.*, 1992). *empA* has already been cloned and sequenced (Milton *et al.*, 1992; Chen *et al.*, 2002b), and its deduced amino acid sequence was found to be very similar to those of other bacteria, especially at the conserved regions putatively involved in zinc-binding, activation and substrate-binding (Milton *et al.*, 1992; Rawlings & Barrett, 1995). However, the role of the conserved regions in its enzymatic activity had not been clarified.

In this study, the EmpA of *V. anguillarum* W-1 was expressed in *Escherichia coli* strain BL21(DE3) as a 6 × His tagged protein (r-EmpA). Putative zinc-binding sites (His^{346}, His^{350}, Glu^{370}), putative substrate-binding sites (Tyr^{361}, His^{429}, Asp^{417}) and a putative active center (Glu^{347}) were selected as the targets for site-directed mutagenesis, and the mutants were also expressed in *E. coli* (m-r-EmpAs). In addition, the proteolytic activities and cytotoxicities of r-EmpA and m-r-EmpAs were assayed and

compared in order to determine the effects of single-point substitutions of these residues on the enzymatic activity and the role of the enzyme in the pathogenesis of *V. anguillarum*.

Materials and methods

Recombination and site-directed mutagenesis of DNA

The open reading frame of the *V. anguillarum* EmpA encoding gene (*empA*, 1836 bp in length) was re-amplified using pUCm-*empA* (GenBank accession no. AY046320) (Chen *et al.*, 2002b) as a template, using as primers: P11 (5′-CGCGGATCCATGAAAAAAGTACAACGTC-3′ with *Bam*HI restriction site) and P12 (5′-CCGCTCGAGATC CAGTCTTAACGTTACAC-3′ with *Xho*I restriction site). The PCR product was double-digested with *Bam*HI and *Xho*I, inserted into pET24d(+) (Novagen, Madison, WI) digested with the same restriction endonucleases and expressed in *E. coli* BL21(DE3) (Novagen), yielding the recombinant EmpA (r-EmpA) with a 6 × His tag at its C-terminus, which facilitates its purification using Ni-NTA His-Bind Resin (Novagen).

Site-directed mutagenesis was carried out with a QuickChange Site-Directed Mutagenesis Kit (Stratagene Inc., La Jolla, CA) following the manufacturer's instructions. The thermocycling program was optimized as follows: 1 cycle of 95 °C for 30 s; 12 cycles of 95 °C for 30 s, 52 °C for 1 min and 68 °C for 7 min. pET24d(+)-*empA* was used as a template. The codons corresponding to each of the conserved amino acid residues were mutated. The mutated plasmids were named pET24d(+)-*empA*1 to pET24d(+)-*empA*15 respectively, and verified by DNA sequencing. The oligonucleotides used for mutation are listed in Table 1.

Expression and purification of the r-EmpA and m-r-EmpAs

The expression vectors for r-EmpA and m-r-EmpAs were used to transform competent *E. coli* BL21(DE3) cells. The expression of the recombinant proteins was induced by isopropyl β-D-thiogalactoside (IPTG) (0.5 mM) following recently described procedures (Zhong *et al.*, 2006).

r-EmpA and m-r-EmpAs were purified on a metal chelating affinity chromatography column packed with 5 mL of Ni-NTA His-Bind Resin according to a one-step protocol (Zhong *et al.*, 2006). Only proteins secreted into the medium were purified. The purification steps were monitored by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and the purified proteins were quantified by the Bradford method (Bradford, 1976).

Protein electrophoresis and Western blotting

SDS-PAGE on 12% acrylamide gels was performed as described by Laemmli (1970). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Millipore) using semidry Western transfer apparatus (Bio-Rad Laboratories, CA, USA). A Western blot analysis was carried out with a rabbit anti-EmpA antibody (1:1000 dilution), followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG. Dianilinobenzene substrate was used for detection.

Proteolytic activity assay

Proteolytic activity with azocasein as the substrate was measured using a modified method from Inamura *et al.* (1985). Briefly, the reaction solution containing 0.25 mL of azocasein solution (5 mg mL^{-1}) and 0.25 mL of the enzyme dissolved in 50 mm Tris-HCl (pH 8.0) was incubated at

Table 1. Oligonucleotides used to mutate the condons corresponding to different conserved amino acid residues

Mutated proteins	Mutations	Oligonucleotides used (5′–3′)*
m-r-EmpA1	His^{346}–Leu	ATCAACGTCAGCGCGCTTGAAGTGAGCCACGGC
m-r-EmpA2	His^{346}–Pro	ATCAACGTCAGCGCGCCTGAAGTGAGCCACGGC
m-r-EmpA3	His^{350}–Leu	GCGCATGAAGTGAGCCTCGGCTTTACCGAGCAG
m-r-EmpA4	His^{350}–Asp	GCGCATGAAGTGAGCGACGGCTTTACCGAGCAG
m-r-EmpA5	His^{350}–Pro	GCGCATGAAGTGAGCCCCGGCTTTACCGAGCAG
m-r-EmpA6	Glu^{347}–Ala	AACGTCAGCGCGCATGCAGTGAGCCACGGCTTT
m-r-EmpA7	Glu^{347}–Lys	AACGTCAGCGCGCATAAAGTGAGCCACGGCTTT
m-r-EmpA8	Glu^{370}–Ala	TGTCTGGTGGTATCAATGCAGCGTTCTCTGATATTGC
m-r-EmpA9	Glu^{370}–Lys	TGTCTGGTGGTATCAATAAAGCGTTCTCTGATATTGC
m-r-EmpA10	Tyr^{361}–Phe	GCAGAACTCAGGGCTTGTTTTTCAAAATATGTCTGGTGGTA
m-r-EmpA11	Tyr^{361}–Cys	GCAGAACTCAGGGCTTGTTTGTCAAAATATGTCTGGTGGTA
m-r-EmpA12	His^{429}–Leu	CTACAATGGATTAAATGTTCTCTATTCAAGTGGTGTGTTTA
m-r-EmpA13	His^{429}–Pro	CTACAATGGATTAAATGTTCCCTATTCAAGTGGTGTGTTTA
m-r-EmpA14	Asp^{417}–Ala	AAAAGATGGCCGTTCTATCGCTCACGCTTCTCAATACTACA
m-r-EmpA15	Asp^{417}–His	AAAAGATGGCCGTTCTATCCATCACGCTTCTCAATACTACA

*Only sense primer sequences are listed. Underlined bases indicate mismatches to the original *empA* sequence.

25 °C for 20 min, and then 1.75 mL of 5% trichloroacetic acid was added. After centrifuging at 2000 ***g*** for 5 min, the supernatant was mixed with 2.25 mL of 0.5 N NaOH. The absorbance at 440 nm was recorded. One unit of activity was defined as the amount of enzyme causing the optical density to increase by 0.001 units min^{-1} under assay conditions.

Cytotoxicity assay

The cytotoxicities of the purified r-EmpA and m-r-EmpAs were measured by recording the amounts of mitochondrial dehydrogenase (MDH) released from the flounder gill (FG) cells (Tong *et al.*, 1997) in a tissue culture. FG cells were grown at 22 °C in Eagle's Minimum Essential Medium (MEM) (Sigma, St Louis, MO) supplemented with 10% fetal calf serum and sub-cultured at 7-day intervals. When the cells formed a monolayer, the cell sheets were dispersed with trypsin-EDTA. The cells were collected by centrifuging and re-suspending at a concentration of 100 000 cells mL^{-1} of the Eagle's MEM containing 1% fetal calf serum. A 90-μL aliquot of the cell suspension was inoculated into each well in a 96-well microtiter plate. After a 4-h incubation at 22 °C, 10 μL of the samples of r-EmpA and m-r-EmpAs at various concentrations were added, and incubated as previously described. The control group was left untreated. The cells were regularly inspected under light microscopy for the development of any 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 h according to the manufacturer's instructions. Absorbance was then measured with a microtiter plate reader (Labsystems Multistank, MS, Finland) at 570 nm. Percentage viability was calculated as:

$$\frac{\text{OD of control group} - \text{OD of treated group}}{\text{OD of control group}} \times 100\%$$

Results

Site-directed mutagenesis of empA

Several amino acid residues of EmpA were selected for site-directed mutagenesis, based on sequence alignments with the proteases of other bacteria, including metalloproteases of *Vibrio* spp., *Bacillus thermoproteolyticus* thermolysin (Matthews *et al.*, 1974) and *Pseudomonas aeruginosa* elastase (Bever & Iglewski, 1988) (data not shown). They were highly conserved at the sites putatively functioning in zinc-binding, activation and substrate-binding. However, the substrate-binding sites were variable to some extent, which implies that these sites are specific for different substrates. It has been found that EmpA has an active site with a 346-HEXXH-350 motif, followed by a few conserved amino acid residues. According to the predictions of Milton *et al.* (1992), these residues are putatively involved in interactions with ligands and are essential for protease activity: His^{346}, His^{350}, Glu^{370} (zinc-binding sites); Glu^{347} (the active center); Tyr^{361}, His^{429}, Asp^{417} (substrate-binding sites). All these residues were substituted respectively, yielding m-r-EmpA1 to m-r-EmpA15, which were expressed, purified and compared for their proteolytic activities and cytotoxicities.

Expression and purification of r-EmpA and m-r-EmpAs

The overproduction of r-EmpA and m-r-EmpAs was assessed by analyzing protein profiles of the whole-cell lysate preparations. As shown in Fig. 1a, both r-EmpA and m-r-EmpAs are expressed as a single band with an estimated molecular mass of ~36 kDa on SDS-PAGE. We noted that this observed molecular mass was smaller than that of the mature protein (~44.6 kDa). Furthermore, if the samples

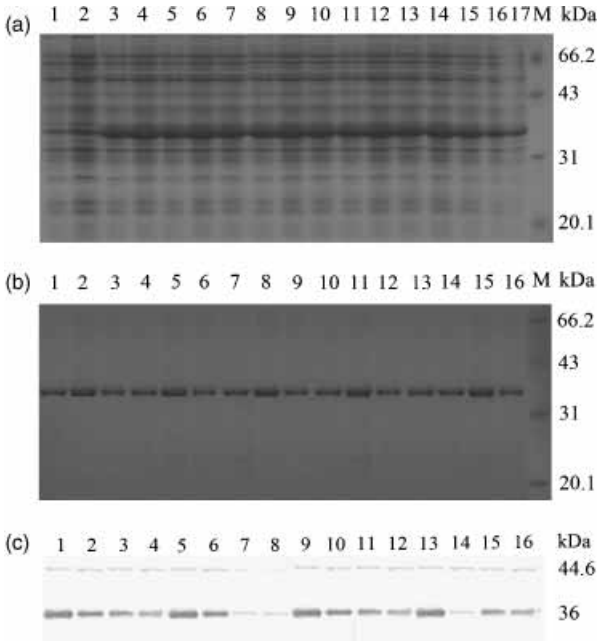

Fig. 1. SDS-PAGE and Western blot analysis of the expressed and purified r-EmpA and m-r-EmpAs. (a) SDS-PAGE analysis of the whole-cell lysates of *E. coli* BL21(DE3) expressing r-EmpA and m-r-EmpAs. Lane 1, BL21(DE3) containing pET24d(+)-*empA*, uninduced; lane 2, BL21(DE3) containing pET24d(+)-*empA*, induced; lanes 3–17, BL21(DE3) expressing m-r-EmpA1 to m-r-EmpA15, respectively, induced; lane 18, protein markers. (b) SDS-PAGE analysis of purified r-EmpA and m-r-EmpAs. Lane 1, purified r-EmpA; lanes 2–6, purified m-r-EmpA1 to m-r-EmpA15, respectively; lane 17, protein markers. (c). Western blot analysis of purified r-EmpA and m-r-EmpAs. The recombinant proteins (0.49 μg for each) were loaded and separated in 12% SDS – polyacrylamide gel. Lane 1, purified r-EmpA; lanes 2–16, purified m-r-EmpA1 to m-r-EmpA15, respectively.

Table 2. Purification steps of r-EmpA from the medium

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Medium	55.2	3.56×10^5	6.49×10^3	100	1.00
Condensate	9.38	2.35×10^5	2.50×10^4	66	4
Ni-NTA Resin	0.22	1.26×10^5	5.74×10^5	35	88

Table 3. Proteolytic activities of r-EmpA and m-r-EmpAs from the medium and the whole-cell lysates*

	Medium		Whole-cell lysates	
Proteins	Specific activity (U mg^{-1})	Relative activity (%)	Specific activity (U mg^{-1})	Relative activity (%)
r-EmpA	574 000	100	3372.09	100
m-r-EmpA1	5454.55	1.0	23.26	0.7
m-r-EmpA2	3636.36	0.6	25.64	0.8
m-r-EmpA3	5454.55	1.0	20.83	0.6
m-r-EmpA4	No activity	0	No activity	0
m-r-EmpA5	3636.36	0.6	21.73	0.6
m-r-EmpA6	No activity	0	No activity	0
m-r-EmpA7	No activity	0	No activity	0
m-r-EmpA8	1818.18	0.3	19.85	0.6
m-r-EmpA9	No activity	0	No activity	0
m-r-EmpA10	76 363.64	13.3	493.00	14.6
m-r-EmpA11	410 909.09	71.6	2356.08	69.9
m-r-EmpA12	160 000	27.9	1053.78	31.3
m-r-EmpA13	92 727.27	16.2	525.37	15.6
m-r-EmpA14	83 636.36	14.6	413.42	12.3
m-r-EmpA15	47 272.72	8.2	310.91	9.2

*To establish that the relative trends observed were reproducible, values for the specific activities were determined using at least four independently grown cultures and are the averages of four measurements that did not differ more than 20%.

were loaded directly onto the gels without prior heating at 100 °C, a dominant band with the molecular mass of ~44.6 kDa was observed (data not shown). These results indicate that the recombinant proteins became active following the rupture of cells and the thermal treatment. As was reported for endogenous synthesis in *V. anguillarum*, the ~36 kDa polypeptide might well be derived from thermo-induced self-proteolysis of the EmpA precursor (Milton *et al.*, 1992).

The culture supernatants of the induced cells were condensed and 0.2–0.3 mg of secreted proteins from 1 L of the culture supernatants were purified by Ni-NTA Resin. The r-EmpA and m-r-EmpAs showed comparable expression and purification levels in affinity chromatography (data not shown), indicating that single-point mutations did not greatly influence the amount of the mutated proteins that was expressed. Only the purification results of r-EmpA are presented (Table 2).

The purified proteins were determined by SDS-PAGE and Western blotting. According to Fig. 1b, single bands with a molecular mass of ~36 kDa were observed for all proteins on SDS-PAGE, which is in good agreement with that of the expressed proteins from the whole-cell lysate preparations. A Western blotting analysis (Fig. 1c) of the purified r-EmpA and m-r-EmpAs showed that the antibody against native EmpA from secreted fractions of *V. anguillarum* W-1 reacted mainly with a ~36 kDa protein. However, it was found that the antibody also recognized proteins of ~44.6 kDa, which could only very weakly be visualized on SDS-PAGE. This result might have arisen from some incomplete degradation of the ~44.6 kDa proteins to ~36 kDa ones. In addition, the interactions between the antibody and m-r-EmpA13 (His^{429}–Pro), m-r-EmpA 6 (Glu^{347}–Ala) and m-r-EmpA7 (Glu^{347}–Lys) were relatively weak (Fig. 1c). Since equal amounts of the purified proteins were subjected to SDS-PAGE, the results further demonstrate that these mutations might have changed the structure of the protein and decreased their affinities with the antibody.

Proteolytic activities of r-EmpA and m-r-EmpAs

As shown in Table 3, the specific activity of r-EmpA was about 574 000 units mg^{-1}, which is higher than that of the native EmpA from *V. anguillarum* W-1 (Chen *et al.*, 2002a). The activities of all the m-r-EmpAs were lower than that of r-EmpA, to different extents. m-r-EmpA4 (His^{350}–Asp), m-r-EmpA6 (Glu^{347}–Ala), m-r-EmpA7 (Glu^{347}–Lys) and m-r-EmpA9 (Glu^{370}–Lys) almost completely lost their

proteolytic activities. m-r-EmpA1 (His^{346}–Leu), m-r-EmpA2 (His^{346}–Pro), m-r-EmpA3 (His^{350}–Leu), m-r-EmpA5 (His^{350}–Pro) and m-r-EmpA8 (Glu^{370}–Ala) also showed a significant reduction in their specific activities (1000–6000 units mg^{-1}, a 100–400-fold reduction). However, the Tyr^{361}, His^{429} and Asp^{417} mutants still maintained high specific activities of 40 000–400 000 units mg^{-1}, only with a 1–20-fold reduction.

To determine whether or not the reduction in proteolytic activities of the culture supernatants might be due to an unrecognized secretion defect of m-r-EmpAs, we further evaluated the proteolytic activities of the whole-cell lysate preparations of the strains expressing m-r-EmpAs. We also found that m-r-EmpA4 (His^{350}–Asp), m-r-EmpA6 (Glu^{347}–Ala), m-r-EmpA7 (Glu^{347}–Lys) and m-r-EmpA9 (Glu^{370}–Lys) did not express a proteolytic activity with azocasein as substrate, and the whole-cell lysates of other mutants presented activities similar to those of their corresponding culture supernatants (Table 3).

Cytotoxicities on a flounder gill (FG) cell line

As shown in Fig. 2, the r-EmpA and m-r-EmpAs express different cytotoxic activities towards FG cells. To determine the effects of different concentrations of r-EmpA and m-r-EmpAs on the FG cell line, we added 0–5 μg of purified proteins and incubated them for 60 h. For r-EmpA, cytotoxicity and an apparent change in cell morphology were visible within 24 h of incubation of the cells with 5 μg of the purified protein (results not shown). After incubation for 60 h, its cytotoxicity increased by 32% at 1 μg and by 100% at 5 μg. In contrast, m-r-EmpAs with mutated Glu^{347}, His^{346}, His^{350} and Glu^{370} completely lost their cytotoxicities to FG cells, and there was no detectable cytotoxicity after 60 h of incubation of the cells with 5 μg of the mutated proteins. While the m-r-EmpAs with mutated Tyr^{361}, His^{429}, Asp^{417} still retained partial cytotoxicities under the same assay conditions with r-EmpA, especially for m-r-EmpA11 (Tyr^{361}–Cys), which retained a much higher level of cytotoxicity than other mutations. These findings were consistent with those of the proteolytic activity assay.

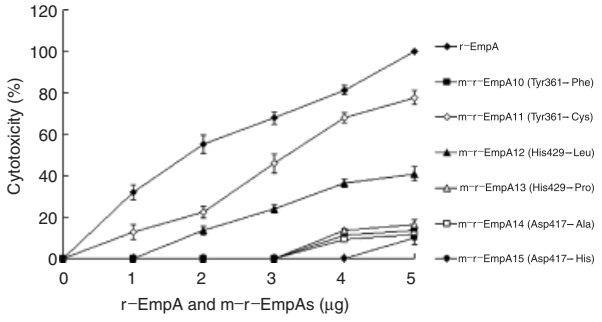

Fig. 2. Cytotoxicities of r-EmpA and m-r-EmpAs on a FG cell line with 60 h incubation. The effects of different concentrations (from 0 μg to 5 μg) of r-EmpA and m-r-EmpAs were examined by measuring the MDH amounts released from the lysed cells using the MTT cell viability/cytotoxicity assay kit. m-r-EmpAs without cytotoxicities were not included in the figure.

Discussion

Many bacterial zinc metalloproteases have been classified as belonging to either clan MA or MB (Hase & Finkelstein, 1993; Rawlings & Barrett, 1995; Stocker & Bode, 1995; Bode *et al.*, 1996). Sequence analysis suggests that *Vibrio anguillarum* EmpA belongs to peptidase family M4 of the MA clan. Family M4 is also referred to as the thermolysin-like proteases. *Bacillus thermoproteolyticus* thermolysin was the first zinc metalloprotease for which a three-dimensional structure was determined (Colman *et al.*, 1972). Typically, the thermolysin-like metalloproteases contain the HEXXH and GXXNEAFSD motifs. EmpA shares an homology with the preproenzymes (Thayer *et al.*, 1991) including *Pseudomonas aeruginosa* LasB (Bever & Iglewski, 1988), *B. thermoproteolyticus* thermolysin (Matthews *et al.*, 1974) and *Vibrio cholerae* hemagglutinin/protease (Hase & Finkelstein, 1991). It also has the conserved HEXXH and GXXNEAFSD motifs that are found in the proteases of the M4 family (Milton *et al.*, 1992; Rawlings & Barrett, 1995). The two histidine residues in the 346-HEXXH-350 motif and the glutamic acid in the GXXNEAFSD motif putatively act as the zinc ligands. A water molecule typically provides the fourth zinc ligand. The glutamic acid in the HEXXH active site motif and a histidine further downstream are putatively required for proteolytic activity. However, the roles of these conserved regions have not thus far been extensively studied.

We studied the structure – function relationship of EmpA using various EmpA mutants obtained from the *Escherichia coli* expression system. We selected the conserved sites (His^{346}, His^{350}, Glu^{370}, Glu^{347}, Tyr^{361}, His^{429} and Asp^{417}) which putatively function with zinc-binding, activation and substrate-binding as the targets for site-directed mutagenesis, in order to determine the effects of single-point substitutions of these residues on enzymatic activity. Initially, we constructed the recombinant plasmids pET24d(+)-*empA* and then pET24d(+)-*empA*1 to pET24d(+)-*empA*15, which encode His-tagged r-EmpA and m-r-EmpAs from *E. coli*. It has been reported that the endogenous EmpA of *V. anguillarum* (611 amino acids in length, ~66.7 kDa) will cut away a putative signal sequence (Met1 to Ala25/Ala26) and a putative leader peptide (von Heine, 1986; Norqvist *et al.*, 1990) during secretion and maturation, as other proteases do. The mature protein is thought to consist of 411 amino acids, with a deduced molecular mass of ~44.6 kDa. As the EmpA secreted by *V. anguillarum*, r-EmpA expressed in *E. coli* accumulates in a 44.6-kDa processed form and heating treatment before loading onto

the gels on SDS-PAGE resulted in proteolysis of EmpA into a 36-kDa derivative (Fig. 1). It is possible that the degraded form of the mature protein results from self-processing by cleaving an additional 9-kDa peptide at the C-terminus of the protein. Similar explanations have been proposed for other metalloproteases of *V. vulnificus* (Kothary & Kreger, 1987), *V. proteolyticus* (David *et al*., 1992), and *V. cholerae* (Hase & Finkelstein, 1991). The profiles of m-r-EmpAs observed on SDS-denaturing gels were similar to that of r-EmpA (Fig. 1).

By stable expression and subsequent one-step purification of the r-EmpA and m-r-EmpAs, we were able to perform proteolytic activity and cytotoxicity studies on the effects of single-point mutations of the conserved regions. The results indicated the essential roles that histidine and glutamic acid play in this enzymatic mechanism. Mutations of His^{346}, His^{350}, Glu^{347} and Glu^{370} resulted in an almost complete loss of proteolytic activities, although they differed from each other to some extent (Table 3). Furthermore, changing His^{346} to a proline and Glu^{347} to either an alanine or a proline appeared to alter the structure or stability of the enzyme, since in Western blotting, smaller amounts of the proteins were detected for these mutants than in the wild-type or other mutants when equal amounts of the purified proteins were subjected to SDS-PAGE. These results are consistent with previous studies on *Burkholderia cenocepacia* ZmpA (Kooi *et al*., 2005), *Bacteroides fragilis* BFT (Franco *et al*., 2005), *P. aeruginosa* LasB (McIver *et al*., 1991, 1993) and *B. thermoproteolyticus* thermolysin (Toma *et al*., 1989).

The putative active sites of ZmpA thought to be necessary for the autocatalytic activity of the preproZmpA and for proteolytic activity in *B. cenocepacia*, and a putative zinc-binding site were mutagenized. They greatly reduced the proteolytic activity of ZmpA (Kooi *et al*., 2005). Changing His^{380} to either an alanine or a proline also appeared to alter the structure or stability of ZmpA. Mutagenesis of the corresponding residues in *P. aeruginosa* LasB, Glu^{338} (residue 141 on the mature LasB sequence), and His^{420} (residue 223 on the mature LasB sequence) resulted in the loss of both autoproteolytic processing of proLasB (Kawamoto *et al*., 1993) and the proteolytic activity of LasB (McIver *et al*., 1991, 1993). Mutagenesis of the corresponding residues Glu^{375} and His^{463} (Glu^{143} and His^{231} in the mature protease) of a *B. thermoproteolyticus* thermolysin homologue, *B. subtilis* neutral protease, also inactivated the enzyme (Toma *et al*., 1989). In addition, Franco *et al*. have reported on the mutagenesis of the zinc-binding metalloprotease motif of *B. fragilis* BFT, and demonstrated that the mutations did not affect BFT processing, but significantly reduced its toxin activity (Franco *et al*., 2005). Taken together, these findings strongly suggest that the conserved residues His^{346}, His^{350}, Glu^{347} and Glu^{370}, putatively involved in the zinc-binding sites, and the active center of EmpA are important for the proteolytic activity and architecture of the enzyme.

Mutations were also performed at the predicted substrate-binding sites (Tyr^{361}, His^{429} and Asp^{417}) of EmpA, and they caused smaller reductions in proteolytic activity than those in the putative zinc-binding sites and the putative active center. It is possible that these mutations only affect the substrate specificity rather than the proteolytic activity of the enzyme. As for m-r-EmpA11 (Tyr^{361}–Cys), it even retained most of its proteolytic activity as r-EmpA. This result indicates the similar function of these two amino acids in their enzymatic action and suggests an explanation for some of the amino acid variations which exist in the substrate-binding sites of the zinc metalloproteases.

Our findings from the cytotoxicity assay were consistent with those of the proteolytic activity assay. Proteolytic degradation of azocasein leads to loss of normal cell morphology in the cytotoxicity assay, as is common in other metalloproteases. This concordance between the results of the two assays indicates that bacterial proteases may cause tissue damage by directly degrading host tissues. As for *V. anguillarum*, EmpA may promote the invasion of the pathogen in its pathogenesis and the proteolytic activity of the enzyme plays an important role in this process.

In conclusion, our results from mutating the conserved sites of *V. anguillarum* EmpA demonstrate that His^{346}, His^{350}, Glu^{370} and Glu^{347} play essential roles in the proteolytic activity of EmpA and also suggest that His^{346} and Glu^{347} are important for the structural stability of the enzyme. The active sites that were predicted from a sequence comparison to other M4 thermolysin-like metalloproteases appear to be reasonable. Conservation of the residues in the active site and a similarity to other M4 zinc metalloproteases suggest that EmpA conducts its catalysis by a mechanism similar to that of other M4 zinc metalloproteases. The proteolytic activity of the enzyme is involved in pathogenesis of *V. anguillarum*.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (30371108), the Major State Basic Research Development Program of China (2006CB101803) and the National High-Technology R&D Program (2003AA622070).

References

Austin B & Austin DA (1999) *Bacterial Fish Pathogens: Diseases in Farmed and Wild Fish*, 3rd edn. Springer, London, UK.

Bever RA & Iglewski BH (1988) Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. *J Bacteriol* **170**: 4309–4314.

Bode W, Grams F, Reinemer P, Gomis-Ruth FX, Baumann U, McKay DB & Stocker W (1996) The metzincing-superfamily of zinc-peptidases. *Adv Exp Med Biol* **389**: 1–11.

Boesen HT, Pedersen K, Larsen JL, Koch C & Ellis AE (1999) *Vibrio anguillarum* resistance to rainbow trout (*Oncorhynchus mykiss*) serum: role of O-antigen structure of lipopolysaccharide. *Infect Immun* **67**: 293–301.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins using the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Chen JX, Li Y, Wang XH, Du ZJ, Yu DH, Ji WS & Xu HS (2002a) Cloning and sequenceing of metalloprotease gene of a pathogenic *Vibrio anguillarum*. *High Tech Lett* **6**: 106–110.

Chen JX, Liu S, Li Y, Wang XH, Du ZJ, Yu DH, Ji WS & Xu HS (2002b) Purification of an extracellular protease from *Vibrio anguillarum* and its physicochemical properties. *J Fish Sci China* **9**: 318–322.

Colman PM, Jansonius JN & Matthews BW (1972) The structure of thermolysin: an electron density map at 2–3 Å resolution. *J Mol Biol* **70**: 701–724.

Crosa JH (1980) A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron sequestering system. *Nature (London)* **284**: 566–568.

David VA, Deutch AH, Sloma A, Pawlyk D, Aly A & Durham DR (1992) Cloning, sequencing and expression of the gene encoding the extracellular neutral protease, vibriolysin, of *Vibrio proteolyticus*. *Gene* **112**: 107–112.

Egiduis E (1987) Vibriosis: pathogenicity and pathology. *Aquaculture* **7**: 15–28.

Farrell DH & Crosa JH (1991) Purification and characterization of a secreted protease from the pathogenic marine bacteria *Vibrio anguillarum*. *Biochem* **30**: 3422–3436.

Franco AA, Buckwold SL, Shin JW, Ascon M & Sears CL (2005) Mutation of the zinc-binding metalloprotease motif affects *Bacteroides fragilis* toxin activity but does not affect propeptide processing. *Infect Immun* **73**: 5273–5277.

Hase CC & Finkelstein RA (1991) Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. *J Bacteriol* **173**: 3311–3317.

Hase CC & Finkelstein RA (1993) Bacterial extracellular zinc-containing metalloproteases. *Microbiol Rev* **57**: 823–837.

Inamura H, Nakai T & Muroga K (1985) An extracellular protease produced by *Vibrio anguillarum*. *Bull Jpn Soc Sci Fish* **51**: 1915–1920.

Kawamoto S, Shibano Y, Fukushima J, Ishii N, Morihara K & Okuda K (1993) Site-directed mutagenesis of Glu-141 and His-223 in *Pseudomonas aeruginosa* elastase: catalytic activity, processing, and protective activity of the elastase against *Pseudomonas* infection. *Infect Immun* **61**: 1400–1405.

Kodama H, Moustafa M, Ishiquro S, Mikami T & Izawa H (1984) Extracellular virulence factors of fish Vibrio: relationships between toxic material, hemolysin, and proteolytic enzyme. *Am J Vet Res* **45**: 2203–2207.

Kodama H, Moustafa M, Mikami T & Izawa H (1985) Characterization of extracellular substance of *Vibrio anguillarum* toxic for rainbow trout and mice. *Microbiol Immunol* **29**: 909–920.

Kooi C, Corbett CR & Sokol PA (2005) Functional analysis of the *Burkholderia cenocepacia* ZmpA metalloprotease. *J Bacteriol* **187**: 4421–4429.

Kothary MH & Kreger AS (1987) Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. *J Gen Microbiol* **133**: 1783–1791.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**: 680–685.

Matthews BW, Weaver LH & Kester WR (1974) The conformation of thermolysin. *J Biol Chem* **249**: 8030–8044.

McIver K, Kessler E & Ohman DE (1991) Substitution of active-site His-223 in *Pseudomonas aeruginosa* elastase and expression of the mutated *lasB* alleles in *Escherichia coli* show evidence for autoproteolytic processing of proelastase. *J Bacteriol* **173**: 7781–7789.

McIver KS, Olson JC & Ohman DE (1993) *Pseudomonas aeruginosa* lasB1 mutants produce an elastase, substituted at active-site His-223, that is defective in activity, processing, and secretion. *J Bacteriol* **175**: 4008–4015.

Milton DL, Norqvist A & Wolf-Watz H (1992) Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. *J Bacteriol* **174**: 7235–7244.

Norqvist A & Wolf-Watxtz H (1993) Characterization of a novel chromosomal virulence locus involved in expression of a major surface flagellar sheath antigen of the fish pathogen *Vibrio anguillarum*. *Infect Immun* **61**: 2434–2444.

Norqvist A, Norrman B & Wolf-Watz H (1990) Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. *Infect Immun* **58**: 3731–3736.

Rawlings ND & Barrett AJ (1995) Evolutionary families of metallopeptidases. *Meth Enzymol* **248**: 183–228.

Stocker W & Bode W (1995) Structural features of a superfamily of zinc-endopeptidases: the metzincins. *Curr Opin Struct Biol* **5**: 383–390.

Thayer MM, Flaherty KM & McKay DB (1991) Three-dimensional structure of the elastase of *Pseudomonas aeruginosa* at 1.5-A resolution. *J Biol Chem* **266**: 2864–2871.

Toma S, Campagnoli S, De Gregoriis E, Gianna R, Margarit I, Zamai M & Grandi G (1989) Effect of Glu-143 and His-231 substitutions on the catalytic activity and secretion of *Bacillus subtilis* neutral protease. *Protein Engng* **2**: 359–364.

Tong SL, Li H & Miao HZ (1997) The establishment and partial characterization of a continuous fish cell line FG-9307 from

the gill of flounder *Paralichthys olivaceus*. *Aquaculture* **156**: 327–333.

von Heine G (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14**: 4683–4690.

Xiao H, Li Y, Wang XH, Ji WS & Xu HS (1999) Studies on pathogens of rotted gill and rotted caudal fins of seaperch (*Lateolabrax japonicus*) fry. *J Ocean U Qingdao* **29**: 87–93.

Zhong YB, Zhang XH, Chen JX, Chi ZH, Sun BG, Li Y & Austin B (2006) Overexpression, purification, characterization, and pathogenicity of *Vibrio harveyi* hemolysin VHH. *Infect Immun* **74**: 6001–6005.