Purification and characterization of a putative virulence factor, serine protease, from *Vibrio parahaemolyticus*

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Abstract

A protease (protease A) was successfully purified from the extracellular proteins of *Vibrio parahaemolyticus* no. 93, a clinical strain carrying neither *tdh* nor *trh* genes, using phenyl-Sepharose CL-4B hydrophobic interaction chromatography. The molecular mass of protease A was 43 kDa using gel filtration, which was in agreement with the results obtained from SDS-PAGE, suggesting that protease A was a monomeric protein. Additionally, the isoelectric point of this protein was 5.0. The optimum temperature and pH of protease A ranged from 40°C to 50°C and pH 8, respectively. Protease A activity was inhibited by serine protease inhibitors, such as phenylmethylsulfonyl fluoride and soybean trypsin inhibitor; moreover, the activity could be blocked by treatment with 20 mM of 1,10-phenanthroline, but could not be restored by adding metal ions. These results indicated that protease A is a serine protease that requires metal. The 12 N-terminal residues of protease A showed a high degree of identity (81%) to the sequence of *Vibrio metschnikovii* VapT serine protease. The purified protease had significant effects on the growth of Chinese hamster ovary, HeLa, Vero and Caco-2 cells and its cytotoxic activity was not blocked by gangliosides. Protease A lysed erythrocytes well but its hemolytic activity was unstable after heat treatment, indicating that protease A is able to cause hemolysis but is a heat-labile protein. The purified protease caused tissue hemorrhage and death in mice when injected both intraperitoneally and intravenously. In conclusion, this is the first report of a serine protease purified directly from the supernatant of *V. parahaemolyticus* and identifying it as a potential virulence factor. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Purification; Characterization; Serine protease; Putative virulence factor; *Vibrio parahaemolyticus*

1. Introduction

Vibrio parahaemolyticus is an important diarrheal agent that causes outbreaks of seafood poisoning in Taiwan [1]. Many extracellular proteins have been reported as virulence factors, such as thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), lethal toxin and urease [2–5]. Among them, TDH is considered an important factor contributing to the enteropathogenesis of *V. parahaemolyticus*. It produces β -type hemolysis on Wagatsuma blood agar, a special blood agar, and this observation is called the Kanagawa phenomenon.

Many extracellular bacterial proteases are suggested to play important roles in virulence. The metalloprotease of *Vibrio vulnificus* is reported to induce hemorrhagic reactions through disorganization of the basement membrane layer after injection into the dorsal skin [6]. The elastase of *V. vulnificus* is demonstrated to be a potential virulence factor in animal models. Many symptoms, such as dermonecrosis and edema, of diseases caused by *V. vulnificus* were observed after the injection of purified elastase [7]. Finkelstein et al. proposed that hemagglutinin/protease of *Vibrio cholerae* may act as a detachase, which allows the bacterium to detach from host-cell membranes and disseminate to other hosts [8]. Furthermore, the metalloprotease of *Aeromonas salmonicida* produces lesions after injection into trout and is suggested to be a virulence factor [9].

V. parahaemolyticus no. 93 is a clinical isolate from a patient with gastroenteritis in Taiwan. We found that it carried neither the *tdh* nor *trh* genes, suggesting no pro-

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Fig. 3. Morphological changes of four cell lines induced by extracellular protease A of *V. parahaemolyticus* no. 93. Monolayers of CHO cells (A), HeLa cells (B), Caco-2 cells (C) and Vero cells (D) were observed using the Diaphot Phase Contrast-2 microscope (Nikon, Tokyo, Japan). a: Control; b: treated with purified protease A.

ultrafiltration using a hollow fiber apparatus with a 10-kDa cut-off membrane (A/G Technology Corp, Needham, MA, USA). The concentrated sample was fractionated by sequential ammonium sulfate at saturations of 20, 40, 60 and 80%. The active fraction was precipitated at a saturation of 40–60%, and then was applied to a Phenyl-Se-

pharose CL-4B column (Pharmacia, Uppsala, Sweden) pre-equilibrated with Tris-HCl (pH 8.0) buffer containing ammonium sulfate at 40% saturation. Finally, the pooled active fractions were dialyzed against 5 mM Ca^{2+} /50 mM Tris-HCl (pH 8) and concentrated by ultrafiltration with Macrosept (10-kDa cut-off, Filtron, Northborough, MA,

USA). The purified proteins were used for further characterization.

2.4. Electrophoresis techniques

The proteins were separated on a 7.5% SDS–polyacrylamide gel, which comprised a stack of 4% (w/v) acrylamide gel over a 7.5% resolving gel, and isoelectric focusing was performed as described previously [12]. For detection of protease activity in SDS–polyacrylamide gels, 0.2% gelatin was copolymerized in the 10% (w/v) polyacrylamide matrix, and the procedure was done as described previously [13].

2.5. Determination of molecular mass and N-terminal amino acid sequence

The molecular mass of the native enzyme was determined by gel filtration using TSK G-3000 SW column (Tosoh, Tokyo, Japan), and the standard proteins were purchased from Bio-Rad (Hercules, CA, USA). The purified protein was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), and the N-terminal sequence was determined on a protein sequencer (Model 477A; Applied Biosystems, Foster City, CA, USA).

2.6. Effects of temperature, pH and inhibitors on enzyme activity

All experiments were done using azocoll as the enzyme substrate, and the details of the procedures were as previously described [12].

2.7. Detection of cytotoxic effects on mammalian cells and ganglioside binding assay

The test cells were CHO, HeLa, Vero and Caco-2 cells. The growth conditions and CT activity assay, which was performed using the methyl-thiazol-diphenyl-terazolium method, were described previously [14]. For analyzing ganglioside binding [15], protease A (0.25–2 µg) was incubated with two gangliosides, G_{M1} and G_{D1a} (1 mg ml⁻¹; Sigma, St. Louis, MO, USA), at 37°C for 30 min, respectively, and the CT assay was done on CHO cells.

2.8. Determination of hemolytic activity

In our preliminary study, it was reported that *V. parahaemolyticus* no. 93 showed weak hemolytic activity on Wagatsuma blood agar [16]. In order to quantify hemolytic activity, a liquid blood assay was used [17]. In brief, a 120-µl aliquot of sample was incubated with an equal volume of 4% (v/v) human erythrocytes suspended in 0.9% NaCl/10 mM Tris–HCl (pH 7.0) at 35°C for 42 h. Hemolysis was determined by measuring the OD₅₂₀ of the super-

natant, and the hemolytic activity was expressed as a percentage of the distilled water control, which showed 100% hemolysis. For the hemoglobin hydrolysis test, the ninhydrin method was used to detect enzyme activity [14]. 1 U of enzyme activity was defined as the amount that liberated 1 µmol of leucine, as a standard, equivalent per min.

2.9. Virulence in mice

Groups of six adult ICR mice (6 weeks old; average weight, 21 g) were injected intravenously (i.v.) and intraperitoneally (i.p.) with serial 10-fold dilutions of protease A, respectively, as described previously [18,19]. For examining the virulence of *V. parahaemolyticus* no. 93, groups of eight ICR suckling mice were fed with 100 µl of serial 10-fold dilutions of bacterial suspension; phosphate-buffered saline (PBS) was used as the negative control. The 50% lethal dose (LD₅₀) was calculated using the method of Reed and Muench [20].

3. Results

3.1. Purification of protease A

Approximately 1 mg of purified protein was obtained from the 50 l of culture medium containing the extracellular proteins. The specific activity of the purified enzyme was 126 U mg⁻¹ of protein; the final purification fold and recovery rate were 70 and 3.5%, respectively. The homogenized protease A (with a molecular mass of 40 kDa) was detected on 10% SDS–PAGE and 10% native PAGE, respectively (Fig. 1). In addition, the hydrolytic activity was

Fig. 4. Hemolytic activity of the crude extracellular protein from various *V. parahaemolyticus* strains. The value of the x-axis is the total protein (mg) at a constant OD of bacteria culture. Some calculated values exceeded 100%, suggesting that the test samples destroyed human erythrocytes more efficiently than the control.

Fig. 5. Histopathology of lung tissue damaged by i.p. injection of purified protease A. A: Lung; B: alveolar cells.

Table 1
Cytotoxic dose of protease A on the viability of various mammal cells

Cell line	Cytotoxic dose of protease A ^a (μ g; mean $\pm$ S.D.)
CHO	0.67 ± 0.01
HeLa	5.5 ± 0.12
Vero	6.6 ± 0.43
Caco-2	7.1 ± 0.06

^aCytotoxic dose: the amount of enzyme that affected the growth of cells and brought about 50% remaining viability as detected by the methyl-thiazol-diphenyl-tetrazolium method.

Values are the means of three determinations.

detected as a single band corresponding to the 40-kDa enzyme in gelatin zymography (Fig. 1B).

3.2. N-terminal sequence of protease A

The N-terminal sequence of protease A, SETVPWGY-NAVN, was analyzed for homology to other sequences in the GenBank. The results, aligned by the BLAST program, showed that the 12-residue fragment had a high degree of identity (81%) with extracellular alkaline serine protease from *Vibrio metschnikovii* RH530 (Fig. 2). Furthermore, it also revealed 70% identity with both the alkaline serine protease and the serine elastase of *Bacillus* sp. We also found that the TXPWG residues were highly conserved in protease A and the other related proteins.

3.3. Properties of protease A

The molecular mass of protease A, as determined using gel filtration, was 43 kDa. This was similar to the value obtained from analysis using SDS-PAGE. These results indicate that the 43-kDa protease A was monomeric in form. In addition, isoelectric focusing of protease A revealed an isoelectric point at pH 5.0. The optimal temperature of enzyme activity ranged from 40 to 50°C. Pre-incubation at temperatures above 50°C resulted in a decrease of enzyme activity, indicating that protease A is a thermally unstable enzyme. The optimal pH for protease A was pH 8, and the enzyme activity was stable over a broad range of pH 3.5–10.4, through which it retained at least 80% of its original activity.

The protease A activity was inhibited by phenylmethylsulfonyl fluoride (1 and 10 mM), soybean trypsin inhibitor (0.1 mg ml⁻¹; a serine protease inhibitor) and leupeptin (0.1 mM; a serine/cysteine protease inhibitor). However, it was not influenced by E-64 or pepstatin A, up to the concentration of 0.1 mM, which are inhibitors of cysteine and aspartic protease, respectively. In addition, a metal chelator 1,10-phenanthroline, at a concentration of 20 mM, also inhibited protease A enzyme activity. Unlike metalloproteases, adding divalent metal ions, such as Ca²⁺ and Zn²⁺, did not restore the activity of protease A. Based on these results, we conclude that protease A is a metal-requiring serine protease.

3.4. Cytotoxic effect of protease A on mammalian cells

As shown in Table 1, the CT dose on CHO cells was approximately 10-fold lower than that on HeLa, Vero or Caco-2 cells. This indicates that protease A exhibited significant CT on the CHO cells. Nonetheless, morphological changes (cell rounding) in all cell lines were observed after incubation with protease A when compared with PBS, the negative control (Fig. 3). In addition, it is reported that the hemolytic activity of TDH was blocked after incubation with ganglioside G_{T1} and G_{D1a}, which are usually located on the cell membrane and play the role of cell receptors [21]. Ganglioside G_{M1} is identified as the receptor for cholera toxin [23]. Based on our results, the cytotoxic activity of protease A was not blocked by G_{M1} or G_{D1a}.

3.5. Hemolytic activity

Compared with other *tdh*- or *trh*-containing strains, the extracellular supernatant of *V. parahaemolyticus* no. 93 showed the weakest hemolytic activity (Fig. 4). This was coincident with the results determined on the blood agar [16]. Purified protease A hemolysed 50% of blood cells at a dose of 6 µg. Additionally, the hemolytic activity was inhibited by heat treatment (95°C, 10 min). These results

indicate that protease A is able to cause hemolysis and is a heat-labile protein. Protease A also degraded hemoglobin well with an activity of 698 U mg⁻¹.

3.6. Virulence in mice

Purified protease A caused death in mice via the i.v. and i.p. routes. At 3 days post-injection, gangrenous, edematous lesions were observed in the whole tail when protease A was injected i.v. at a dose of 60 µg. The mice died when they were injected i.p.; they showed multiple organ hemorrhages in the abdominal and thoracic cavities. As shown in Fig. 5, an obvious hemorrhagic phenomenon was found on tissue sections of the lungs, and the walls of alveolar cells became thickened. In addition, the LD₅₀ of purified protease A and *V. parahaemolyticus* no. 93 bacteria ranged from 2.5 to 5 mg kg⁻¹ of mouse via the i.p. route and 1 × 10¹¹ CFU via the oral route, respectively.

4. Discussion

In our previous study, we found the extracellular proteins of *V. parahaemolyticus* no. 93 had substantial CT toward CHO cells. Moreover, the proteins were classified and termed proteases. In this report, we describe the successful attempt to purify the putative cytotoxic factor. From our results, the factor protease A was characterized, and it was demonstrated to be a serine protease.

Although protease A showed the same morphological changes in the four cell lines, from this work, CHO cells appeared to be more sensitive than others, according to the CT assay. In contrast, the *Escherichia coli* thermolabile toxin showed CT on Vero cells but had no effect on Y1 adrenal and CHO cells [22]. In addition, it has been reported that the activities of some bacterial toxins are blocked by membrane receptor gangliosides, indicating that they were gateways for toxins going to the cells. For example, G_{M1} has been characterized as a cholera toxin receptor, and can block the activity of that toxin [23]. TDH activity was inactivated by treatment with G_{T1} and G_{D1a} but not by G_{M2} [21]. Nevertheless, G_{T1} and G_{D1a} did not neutralize the CT of protease A. Based on our results, there were two possible ways for protease A to cause CT but they remain unclear. First, protease A might recognize other gangliosides that we have not investigated yet. Second, it might directly degrade the proteins located on the surface of the membrane, and then change the membrane permeability to cause cell death. As a cytotoxic factor for epithelial cells, protease A also showed hemolytic activity on blood cells and the ability to degrade hemoglobin. These characteristics of protease A might have resulted in the hemorrhagic damage that was found in the abdominal and thoracic cavities of the mice.

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