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Overexpression and Characterization of *Vibrio mimicus* Metalloprotease

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Abstract To investigate the biochemical properties of *V. mimicus* metalloprotease, whose gene was isolated previously from *Vibrio mimicus* ATCC33653, overexpression and purification were attempted. The 1.9 kb of open reading frame was amplified by PCR from pVMC193 plasmid which ligated the *vmc* gene with pUC19 and introduced into *Escherichia coli* BL21 (DE3) using the overexpression vector, pET22b (+). The overexpressed metalloprotease (VMC) was purified with Ni-NTA column chromatography and characterized with various protease inhibitors, pHs, temperatures, and substrates. The purified VMC showed the proteolytic activity against gelatin, soluble and insoluble collagens, and synthetic peptides. Unlike the observations made with all metalloproteases originated from other *Vibrio* sp., the VMC did not hydrolyze the casein. The proteolytic activity was critically decreased when the VMC was treated with metal chelating reagents, such as EDTA, 2,2-bipyridine, and 1,10-phenanthroline. In particular, the 71 kDa VMC exhibited the hemagglutinating activity against human erythrocyte. As the purified VMC was treated with $CuCl_2$ and $NiCl_2$ for the chemical modification of metal binding, the proteolytic activity and hemagglutinating activity were profoundly influenced. The multialignment analysis made on the reported *Vibrio* metalloproteases showed the difference of amino acid sequence similarity between the two distinctive classes of *Vibrio* metalloproteases.

Key words: *Vibrio mimicus*, metalloprotease, hemagglutination, zinc-binding motif

Vibrio mimicus is a causative agent of human diarrhea. Clinical studies on *V. mimicus* infection to human revealed that diarrhea was accompanied by vomiting and abdominal cramps [11, 29]. In a minority of patients, fever and bloody diarrhea can also be seen. *V. mimicus* is mainly isolated from cases of gastroenteritis, but different strains have also been isolated. Gastroenteritis due to *V. mimicus* has been caused by ingestion of seafoods because the reservoir of this strain is an aquatic environment [8].

Several pathogenic factors including cholera toxin [30], cholera toxin-related enterotoxin [12], heat-stable enterotoxin [26], and two types of hemolysins (a thermolabile hemolysin and a thermostable hemolysin) [17, 25] have been reported from *V. mimicus*. It has been previously observed that *V. mimicus* has the ability to colonize rabbit intestinal mucosa [3, 31] and to produce some other extracellular factors, such as protease [6], hemagglutinin, and siderophore [27]. Although adherence to the intestinal mucosa is an important step in a diarrheal disease, there is little information on potential colonization factors of *V. mimicus*. In *V. cholerae*, cholera toxin has been shown to act as a powerful mucosal adjuvant [22] and a metalloprotease has been considered to be a causative agent to enhance the activity of cholera enterotoxin in intestinal loops by changing the protective layer of epithelial mucus [9]. Hemagglutinin (HA)/protease of *V. cholerae* has been shown to activate the cholera toxin A subunit [4] and to destroy the host cell receptors for a putative *V. cholerae* adhesin [13]. Moreover, HA/protease has been demonstrated to degrade the host defending proteins against cholera, such as mucin, fibronectin, lactoferrin, and secretory immunoglobulin A [14, 32]. These degrading activities have been suggested to be important for the evasion of the host immune response and the penetration of mucous layers to colonize the lower intestine.

Hemagglutinating and protease activities were suspected as the key factor for the colonization and invasion of host cell in pathogenic bacteria. Some of these metalloproteases were shown to be a bifunctional molecule capable of

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mediating hemagglutinating and protease activities [19]. A 31 kDa metalloprotease from *V. mimicus* E-33 enhanced the vascular permeability in skin and the fluid accumulation in rabbit ileal loops [7], and also had a hemagglutinating activity [6]. This metalloprotease was shown to immunologically cross-react with the HA/protease (32 kDa) of *V. cholerae*. We have previously isolated the metalloprotease gene (*vmc*) from *V. mimicus* ATCC 33653 and demonstrated that the *vmc* gene contained 1,884 nucleotides with a conserved zinc-binding motif (His-Glu-Tyr-Thr-His) [18]. However, the molecular weight of the VMC (71 kDa) predicted from the primary amino acid sequences was much larger than that of the HA/protease (31 kDa) purified from an environmental *V. mimicus* E33. This finding suggests that the VMC may act as another virulence factor *in vivo*. Although the VMC is expected to participate in the pathogenesis and physiological function to host cell, its biochemical characteristics have not yet been defined.

In this study, we present the purification of a His-tagged recombinant protein using the overexpression system and the characterization of biochemical properties including the hemagglutinating activity. The enzyme activity of a purified protein was also compared to those of the chemically modified proteins which bound different metal ions instead of zinc ion.

MATERIALS AND METHODS

Construction of the Overexpression Plasmid

We previously reported the sequence of the metalloprotease gene (*vmc*) of *V. mimicus* ATCC33653 [18]. To construct the overexpression plasmid, two specific primers based on the *vmc* gene sequence were designed; meta 1 (forward, 5'-GGCCCATATGGTGT ACTCTCAACC-3'; *Nde*I site is underlined) and meta 2 (reverse, 5'-GGCCGGATCCCCTGT-AAAGATCGGCGTGG-3'; *Bam*HI site is underlined). PCR was performed with meta 1 and 2 primers to amplify the 1.9 kb open reading frame of the metalloprotease gene from pVMC193 which was constructed by ligation of the *vmc* gene and pUC19. The PCR product was digested with *Nde*I and *Bam*HI, and subcloned into the overexpression vector, pET22b(+). The recombinant plasmid was named pET-META and transformed into *Escherichia coli* BL21(DE3).

Purification of the Recombinant VMC

E.coli BL21 (DE3) harboring a pET-META plasmid was cultivated in 500 ml of Luria-Bertani broth supplemented with 50 µg/ml of ampicillin at 37°C until an OD_{600} of 0.6 was reached. Five hundred µl of 1 M IPTG was added to induce the *vmc* gene expression and the transformants were further incubated for 4 h. The cells were harvested and disrupted by sonification. After centrifugation (10,000 ×g) at 4°C for 30 min, the insoluble pellet was suspended in 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 6 M guanidine hydrochloride, which was incubated at 4°C for 1 h. The dissolved solution was applied directly to Ni-NTA resin (Qiagen, Germany) equilibrated with 50 mM $NiSO_4$. The proteins were eluted by 20 mM imidazole buffer. To recover the enzyme activity, renaturation was carried out by the method of Ahn *et al.* [1]. The purified fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol (V/V) at 4°C for 12 h. The protein concentrations during the enzyme purification were determined by the method of Lowry *et al.* [21]. All purification procedures were carried out at 4°C. The purity of the enzyme was analyzed by SDS-PAGE.

Assay for Protease Activity

Determination of the protease activity using gelatin or insoluble type I collagen as a substrate was carried out by the ninhydrin method. Ten mg of insoluble collagen was added to 0.8 ml of 50 mM Tris-HCl (pH 8.0) containing 4 mM $CaCl_2$ and 0.2 ml of enzyme solution. The reaction mixture was incubated at 30°C with shaking, and the reaction was stopped at various incubation times by the addition of 1.0 ml of 0.1 N acetic acid. The initial rate of increase of free amino groups was measured by the ninhydrin method, and the specific activity was expressed as 1 µmol of leucine equivalent per min per mg protein.

Hydrolysis of the synthetic peptides such as Z-GPLGP (carbobenzoxy-glycyl-L-prolyl-L-leucyl-glycyl-L-proline, Sigma, U.S.A.) and Z-GPGGPA (carbobenzoxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine, Sigma, U.S.A.) was assayed as described by Sasagawa *et al.* [27]. The reaction mixture contained 0.1 ml of 5 mM substrate solution, 0.3 ml of 50 mM Tris-HCl, pH 8.0, containing 4 mM $CaCl_2$, and 0.1 ml of enzyme solution (15 µg/ml). The reaction mixture was incubated at 30°C for 60 min and the reaction was stopped by the addition of 0.5 ml of 0.1 N HCl. The initial rate of increase of free amino groups was measured by the ninhydrin method. Caseinolytic activity was measured by using azocasein [16].

pH and Thermostability of Protease

To investigate the protease activity at various temperatures and pHs, protease solution containing collagen type I was incubated at temperatures from 15–60°C in 50 mM Tris-HCl (pH 8.0) and 4 mM of $CaCl_2$ for 30 min or in citrate-phosphate (pH 4.0–7.5), potassium-phosphate (pH 6.0–8.0), Tris-HCl (pH 7.0–9.0), and glycine-NaOH (pH 9.0–11.0) buffers for 30 min, respectively, and the protease activity was measured by the ninhydrin method. The stability of protease activity was measured by incubating the VMC alone for 30 min at various elevated temperatures or pHs and further incubating with type I collagen as a substrate for 30 min at 37°C. For the pH stability of the protease, the remaining activity was measured at pH 8.0.

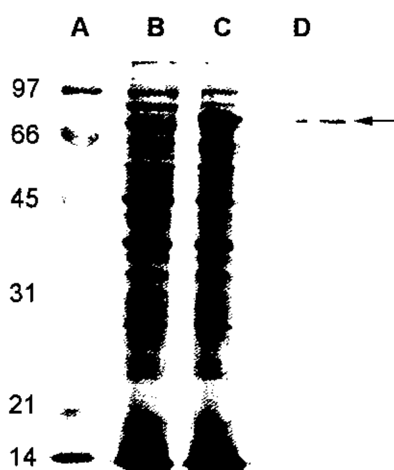

Fig. 1. SDS-PAGE of the purified VMC.

Lane A, Molecular mass markers (low-range marker; Bio-Rad, USA); lane B, pET22b(+)/BL21(DE3); lane C, pET-META/BL21(DE3); lane D, purified VMC. The arrow on the right indicates the position of the expressed metalloprotease in *E. coli*.

Table 2. Substrate specificity of VMC.

Substrate[a]	Proteolytic activity (U/μg)
Type I collagen	0.33
Type III collagen	0.18
Gelatin	0.4
Casein	n.d.[b]
Synthetic peptides[c]	
Z-GPLGP	0.37
Z-GPGGPA	0.33

[a]The standard reaction mixture containing 0.3 ml of 0.2% (w/v) substrate solution, 0.2 ml of 50 mM Tris-HCl (pH 8.0) containing 4 mM $CaCl_2$ and 0.1 ml of VMC. The mixture was incubated at 37°C for 30 min. The protease activity was assayed by measuring the absorbance rate at 570 nm using the ninhydrin method.

[b]n.d. is not detected.

[c]The reaction mixture containing 0.1 ml of 5 mM substrate solution, 0.3 ml of 50 mM Tris-HCl (pH 8.0) containing 4 mM $CaCl_2$ and 0.1 ml of VMC. Activity was measured under the same conditions as described above.

Hemagglutination Assay

Hemagglutinating activity of the purified VMC was determined according to the method of Lewis *et al.* [20]. Briefly, 0.1 ml of phosphate buffered saline (PBS) was added to each well of the round bottomed microplate. Then, 0.1 ml of the protease solution containing 40 μg of the purified enzyme was added to a single well and diluted serially across several wells. Finally, 0.1 ml of 1% human erythrocytes which were washed three times with PBS was added to each well. Plates were incubated overnight at 4°C. Hemagglutination was assessed visually, and the reciprocal of the highest dilution displaying a positive agglutination of erythrocytes was recorded.

Chemical Modification of the VMC

To substitute metal ion in the catalytic site of VMC, chemical modification was performed by the method of Miyoshi *et al.* [24]. One mg of the purified enzyme in TBS buffer (20 mM of Tris-HCl, 0.15 M NaCl, pH 8.0) was dialyzed against 2 l of TBS buffer containing 10 mM TEP (tetraethylene pentamine) at 4°C for 24 h. Second dialysis was completed with 2 l of TBS buffer containing $CuCl_2$ or $NiCl_2$ at 4°C for 24 h. Thereafter, free metal ions from enzyme were removed by dialysis against TBS buffer.

RESULTS

Overexpression of the VMC and Purification

In order to investigate the biochemical properties of *V. mimicus* metalloprotease, we overexpressed its gene in *E. coli*. After the *vmc* gene expression was induced with IPTG, we could detect a distinct overexpressed band which was not present in *E. coli* cells harboring only the expression vector pET22b(+). The expressed VMC encodes a 6x His-tag for convenient purification using a metal chelate affinity column. The His-tagged protein was purified as described in Materials and Methods. The purification scheme of the overexpressed VMC is summarized in Table 1. The molecular weight of the purified VMC was estimated to be about 71 kDa by SDS-PAGE (Fig. 1). This value is coincident with the molecular weight calculated from the predicted amino acid sequences.

Substrate Specificity and Effects of Inhibitors

The substrate specificity of the VMC was examined using various collagen related proteins or synthetic peptides, and the result is summarized in Table 2. The VMC had its highest activity towards gelatin. When type III collagen was used as a substrate, the hydrolytic activity was 45% of its activity with gelatin. Caseinolytic activity was not detectable. It is intriguing that the VMC did not hydrolyze casein, although all metalloproteases reported from *Vibrio* sp. have

Table 1. Purification of VMC.

Purification step	Total protein (mg)	Total activity (units)[a]	Specific activity (U/mg)	Yield (%)	Purification fold
Cell homogenate	31.0	3373	109	100.0	1.0
Inclusion body isolation	3.0	760	253	22.5	2.3
Ni-NTA column chromatography	0.8	267	334	7.9	3.1

[a]One unit of protease activity was defined as μmol of leucine equivalent per min per mg protein.

Table 3. Effect of various protease inhibitors and metal-chelating agents on proteolytic activity of VMC.

Inhibitors	Concentration (mM)	Residual activity (%)[a]
None	-	100
PMSF[b]	1	90
TLCK[c]	1	100
SBTI[d]	1	84
Aprotinin	1	91
TPCK[e]	1	95
2-2'-Bipyridine	1	12
1,10-Phenanthroline	1	15
EDTA	5	6

[a]VMC of 0.1 ml was incubated with various reagents in the final concentrations indicated at 37°C for 30 min before assaying. Residual activity is expressed as a percentage of the activity of an untreated sample on type I insoluble collagen hydrolysis.

[b]PMSF (phenylmethylsulfonyl fluoride), [c]TLCK (*p*-toluenesulfonyl-L-lysine chloromethyl ketone), [d]SBTI (soybean trypsin inhibitor), [e]TPCK (*p*-toluenesulfonyl-L-phenyl alanine chloromethyl ketone).

exhibited hydrolytic activities toward casein. The hydrolytic activity against synthetic peptides, such as Z-GPLGP and Z-GPGGPA, were 0.37 and 0.33 U/μg, respectively.

The various protease inhibitors were added to the standard reaction mixture, and the relative activity was measured. As shown in Table 3, 95% of the enzyme activity was inhibited by EDTA at 5 mM. 2,2-Bipyridine and 1,10-phenanthroline, which are also potent inhibitors of metalloprotease, significantly affected the activity at 1 mM. However, PMSF, SBTI, and aprotinin slightly inhibited the enzyme activity, and TLCK and TPCK had almost no effect on the activity.

Effects of pH and Temperature on the Enzyme Activities

The purified enzyme was found to be a typical neutral protease, displaying its activity predominantly in the neutral region (pH 7.0-8.0) (Fig. 2A). When the VMC was incubated in various buffers over a broad pH range (pH 4.0-11.0) for 3 h at 37°C, the protease showed a maximum stability at pH 8.0 (Fig. 2B). The enzyme activity decreased, and about 30% and 20% of the initial activity were retained at pH 6.0 and 9.0, respectively. The purified enzyme exhibited maximum activity against type I collagen at 30-40°C. At lower temperatures, the enzyme activity gradually decreased by as much as 50% of its maximum activity (Fig. 2C). The enzyme activity was highly stable at temperatures between 15-40°C, whereas a rapid loss of enzyme activity was shown at temperatures above 50°C (Fig. 2D). Based on these observations, it

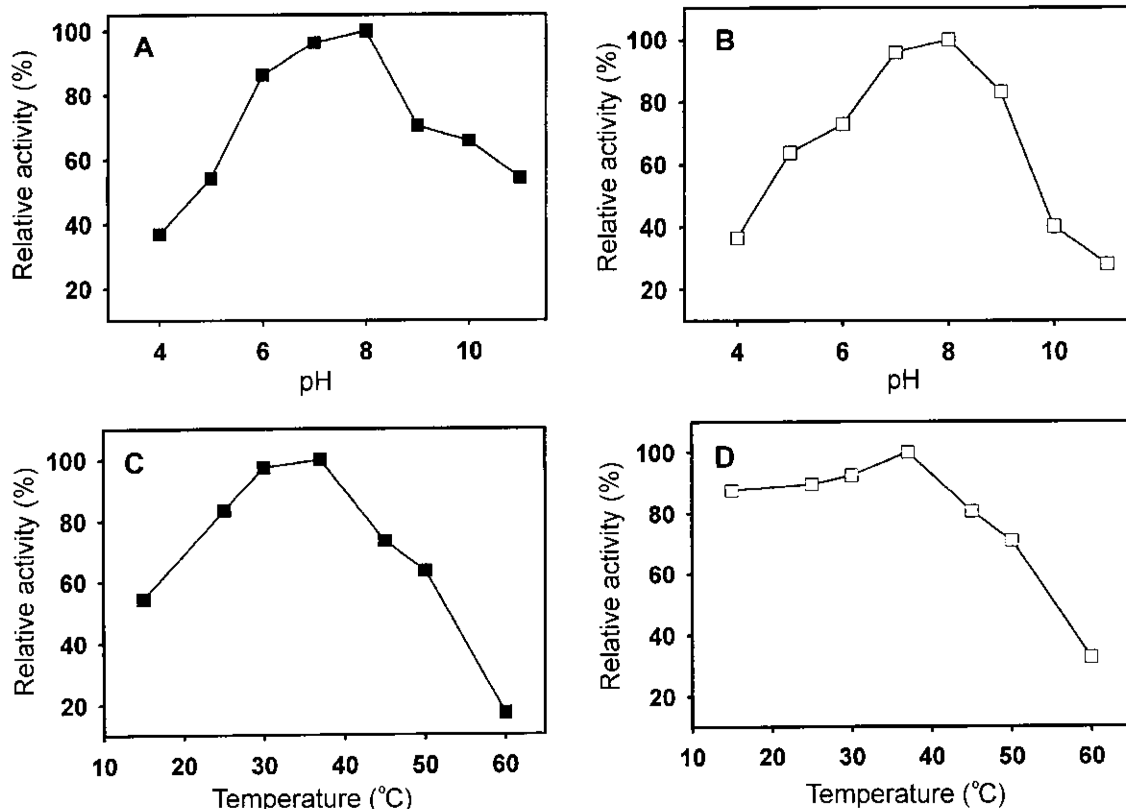

Fig. 2. Effect of pH and temperature on protease activity (■) and stability (□).

Relative activity is expressed as the percentage of the maximum activity under the experimental condition. A, Optimal pH; B, pH stability; C, Optimal temperature; D, Temperature stability.

Table 4. Effect of chemical modification on VMC activity.

Protease	Proteolytic activity (U/μg)	
	Gelatin	Type I collagen
Unchanged VMC	0.40	0.33
Cu^{2+}-treated VMC	0.32	0.25
Ni^{2+}-treated VMC	0.18	0.10

suggests that the optimum conditions on the VMC activity might be related to habitable conditions in an aquatic environment.

Effect of Chemical Modification

Many bacterial metalloproteases from pathogenic organisms have been shown to require a divalent ion for their activity. Most metalloproteases are zinc-containing proteins, although other divalent ion such as Ca^{2+} was found to be involved in enzyme stability. The conserved sequence for zinc binding is His-Glu-X-X-His, where X represents any amino acid. This motif is important to transfer electron with zinc for the hydrolysis of peptide bonds. To examine the importance of zinc binding for enzyme activity, we modified the zinc binding site with a chemical method as described in Materials and Methods.

As shown in Table 4, the proteolytic activity of VMC treated with $CuCl_2$ was slightly reduced: When compared with unchanged VMC, the corresponding activity was 80% towards gelatin and 75% towards type I collagen, respectively. However, there was a significant reduction of the enzyme activity when the VMC was treated with $NiCl_2$: The proteolytic activity showed 0.18 U/μg (45%) towards gelatin and 0.1 U/μg (30%) towards type I collagen, respectively. These results are consistent with the studies of Miyoshi *et al.* [24], who reported the change of proteolytic activity of the VVP metalloprotease purified from *V. vulnificus* by chemical modification.

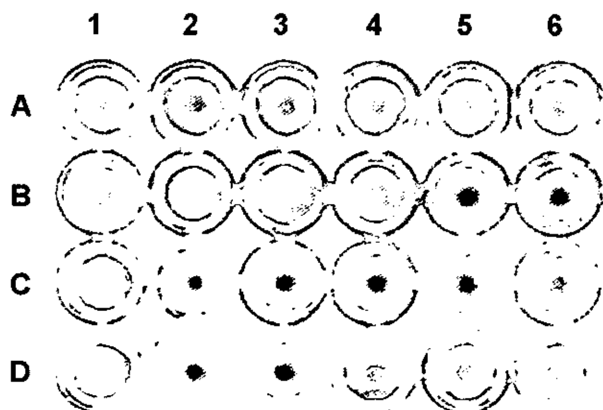

Fig. 3. Hemagglutination activity of VMC.
Numerals in the figure represents amount of protease; 1, 40 μg; 2, 20 μg; 3, 10 μg; 4, 5 μg; 5, 2.5 μg; 6, 1.25 μg. A, PBS buffer; B, purified recombinant VMC; C, Cu^{2+}- treated VMC; D, Ni^{2+}- treated VMC.

Hemagglutination Assay

We assessed the hemagglutinating activity of the unchanged control and modified VMC. A minimal concentration of the unchanged VMC for hemagglutination with human erythrocytes was 5 μg (8-fold dilution, Fig. 3), while the hemagglutinating capabilities of each modified VMC were significantly reduced. At a minimal concentration of 40 μg (1-fold dilution), each chemically modified VMC induced the hemagglutination.

DISSCUSION

In the previous report [18], we described the isolation of a metalloprotease gene from *V. mimicus* ATCC33653. The isolated gene consisted of 1,884 nucleotides and the calculated molecular weight from the deduced amino acid sequences was about 71 kDa. We expressed the *vmc* gene by using a pUC19 vector, but an efficient expression was not achieved in *E. coli* [18]. In this study, we constructed a new plasmid for overexpression. Significant production of the metalloprotease was detected from cells harboring the overexpression plasmid after induction by IPTG. The proteolytic activity of purified VMC was strongly inhibited by the metal ion chelators, such as EDTA, 2,2'-bipyridine, and 1,10-phenanthroline. In contrast, serine and trypsin protease inhibitors did not affect the VMC. These results indicated that the VMC could be regarded as a metalloprotease.

To-date, several metalloproteases from *Vibrio* sp. have been investigated. These metalloproteases are considered to play an important role in pathogenesis. Since primary amino acid sequences of metalloproteases from *Vibrio* sp. have been reported, it may be possible to compare sequences with each other. We previously proposed that metalloproteases from *Vibrio* sp. could be divided into two classes, based on the amino acid sequence homology within the limits of the zinc-binding motif [18], and designated them as class I and class II metalloproteases. Class I includes metalloproteases isolated from *V. cholerae*, *V. proteolyticus*, *V. anguillarum*, and *V. vulnificus*. The metalloproteases from *V. alginolyticus*, *V. mimicus*, and *V. parahaemolyticus* belong to class II. The zinc-binding motif of class I shows the sequence of HEXXH-E, whereas class II (HEXXH) does not involve an extra glutamate residue in the region for zinc binding.

To confirm whether the adequacy of this classification based on the amino acid sequence similarities of the conserved zinc-binding motif is apparent, the whole amino acid sequence and the cleavage site of extracellular metalloproteases from various *Vibrio* sp. were compared. As shown in Fig. 4, multiple alignment for class I metalloprotease sequences isolated from various *Vibrio* sp. revealed a strong similarity. The HA/protease *V. cholerae* showed amino acid sequence identities of 68–71% to

A) Class I

B) Class II

Fig. 4. The multi-alignment of deduced amino acid sequences from *Vibrio* metalloproteases.
The related sequences were primarily divided by the BLASTP program and the similarl sequences were realigned by the Clustal W program. The black boxes represent the identical amino residues to the amino acid residues of *V. cholerae* (for class I) and *V. mimicus* (for class II). Asterisks indicate the HEXXH motif for conserved zinc binding sequences. The accession numbers are as follows: 1: *V. cholerae* (M59466), 2: *V. anguillarum* (L02528), 3: *V. proteolyticus* (M64809), 4: *V. vulnificus* (U48780), 5: *V. mimicus* (AF004832), 6: *V. parahaemolyticus* (Z46782), 7: *V. cholerae* (AF109145), 8: *V. alginolyticus* (X62635).

another class I of *Vibrio* metalloproteases. In class II, the VMC from *V. mimicus* showed amino acid sequence identities of 64% to *V. parahaemolyticus* metalloprotease and 76% to *V. cholerae* 569B exoprotease. However, the level of similarity to *V. alginolyticus* metalloprotease was considerably low with 26% of amino acid identity with the VMC. Häse *et al.* [15] determined N-terminal amino acid sequences of the purified *V. cholerae* HA/protease which represents class I metalloproteases, and estimated the molecular weight from SDS-PAGE. According to these results, the cleavage site of the polypeptide synthesized from the complete open reading frame (609 amino acids) was determined to be a peptide bord between [195]His and [196]Ala. The calculated molecular weight was 46.7 kDa. However, the molecular weight of the purified HA/protease was shown to be 32 kDa, which was substantially smaller than that predicted from the DNA sequences. The result suggests that the HA/protease may undergo a cleavage of the N-terminal sequence and an autocatalytic cleavage of the C-terminal sequence to transport a protein across the cytoplasmic

membrane. Similar mechanisms have been observed for other class I *Vibrio* sp. including *V. vulnificus*, *V. proteolyticus*, and *V. anguillarum* [5, 10, 23]. On the other hand, the molecular weights of purified proteins in class II metalloproteases were relatively larger than those of class I metalloproteases. In our previous report, we suggested the possibility that other metalloproteases which are similar to the VMC metalloprotease may exist in *V. cholerae* [18]. Recently, this suggestion was proved by the isolation of two different metalloprotease genes from *V. cholerae* in which these genes were not homologous with the 32 kDa HA/protease of *V. cholerae*. The exoprotease gene of *V. cholerae* 569B deposited under accession No. AF109145 in GenBank database showed a high sequence similarity with the VMC [McKenzie, R., unpublished data]. Therefore, the above classification seems to be not absolute, nevertheless, we consider that this is useful for determining the structural relationships among metalloproteases of *Vibrio* sp.

From strain E-33 of *V. mimicus*, a 31 kDa HA/protease was identified and characterized [6]. The HA/protease of *V. mimicus* was found to have a proteolytic activity towards several substrates, and hemagglutinating activity. At the same time, the enzyme activity of *V. mimicis* E-33 HA/protease was strongly inhibited by metalloprotease inhibitors. This metalloprotease cross-reacted with antiserum against the 32 kDa HA/protease of *V. cholerae*. Based on this observation, it is possible that the HA/protease isolated from *V. mimicus* E-33 may have a highly similar structure to *V. cholerae* HA/protease. However, we could not compare the gene structure, because the corresponding gene from *V. mimicus* has not yet been elucidated. Besides a 31 kDa HA/protease, two additional hemagglutinating substances were found in *V. mimicus* E-33 [2]. One was specific only to rabbit erythrocyte, which was designated as Vm-LPSHA, and the other was specific to chicken and rabbit erythrocytes, which was designated as Vm-OMPHA. The immunological analysis confirmed that the Vm-LPSHA contained a lipopolysaccharide moiety, whereas the Vm-OMPHA was shown to be a major outer membrane protein with a molecular weight of 39 kDa. Moreover, the hemagglutinating activity of Vm-OMPHA was enhanced by incubation with the HA/protease of *V. mimicus*. Interestingly, the 71 kDa VMC used for this study was originated from *V. mimicus* ATCC33653 which is a heterologous strain of *V. mimicus* E-33. The VMC showed a proteolytic activity only towards native or denatured collagen, as well as a hemagglutinating activity with human and rabbit erythrocytes. The amino acid sequences around the zinc-binding motif in each class were very similar, however, no consensus sequences were found between two classes except the HEXXH motif. Miyoshi *et al.* [24] reported that the proteolytic activity of VVP isolated from *Vibrio vulnificus* was reduced by treatment with Ni and showed small reduction with Cu. In this study, we found a similar pattern in treatment of VMC, with Ni or Cu. In spite of the difference on the amino acids sequences near the HEXXH motif, one of each class I metalloprotease (VVP) and class II metalloprotease (VMC) showed a similar tendency in the selection of metal ion for proteolytic activity. However, a difference was found in the hemagglutinating activity of VVP and VMC. Hemagglutinating activity of VVP was only manifested towards rabbit erythrocytes, whereas VMC showed activity towards human and rabbit erythrocytes. Moreover, hemagglutinating activity of VVP was significantly increased by Cu treatment. However, in the case of VMC, the treatment of Ni or Cu reduced hemagglutinating activity. This may imply that the Zn is essential for proteolytic activity and also an important factor for hemagglutinating activity.

Our results strongly implicate that the VMC may play an important role as a virulence factor, although it is still uncertain how the VMC contributes to the pathogenicity *in vivo*. Therefore, further studies on the VMC are needed to elucidate and to fully understand the mechanisms for the host infection.

Acknowledgments

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