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Characterization of Metalloprotease from *Vibrio mimicus* and Determination of Substrate Binding Site

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To investigate the enzymatic properties of *Vibrio mimicus* metalloprotease, the mature metalloprotease gene (*vmc*) was overexpressed in *Escherichia coli* and the recombinant protein (rVMC61) was purified by metal affinity chromatography. The rVMC61 showed maximum activity at about 37℃, pH 8. The purified rVMC61 was very specific toward collagen substrates, such as gelatin, type I, II, and III collagens and synthetic peptides (Cbz-GPLGP and Cbz-GPGGPA). To examine the role of the C-terminal region of rVMC61, the 3' end of the *vmc* gene was digested serially with exonuclease III. We demonstrate that deletion of 100 amino acids, but not 67 amino acids, from the C-terminus of the intact VMC protein (VMC61) abolished the collagenase activity. The intervening 33-amino acid region contains a repeated FAXWXXT motif that is essential for insoluble type I collagen binding. The isolated 33-amino acid peptide bound to insoluble type I collagen, while a peptide containing only the first FAXWXXT motif did not. Compared to the VMC61, the 33-amino acid peptide corresponding to the C-terminus exhibited a similar binding affinity and a lower binding capacity.

Key words : *Vibrio mimicus*, Metalloprotease, Collagen biding site

Introduction

Vibrio mimicus is a pathogenic bacterial strain similar to *V. cholerae* non-01 strain in its enteropathic properties. *V. mimicus* produces a number of toxic substances such as phospholipase, hemolysin, cholera toxin, heat stable enterotoxin, hemagglutinin, and protease [1-6]. The extracellular metalloprotease of *V. mimicus* has been shown to be important for the enhancement of vascular permeability in skin and fluid accumulation in rabbit ileal loops[7].

Collagens are major protein constituents of the extracellular matrix and the most abundant proteins in all higher organisms. A significant number of collagenolytic organisms associated with pathogenic processes involving the destruction of collagen-containing tissues have been described[8]. Mammalian matrix metalloproteases (MMPs), which include the mammalian collagenases, are involved in maintaining the correct collagen homeostasis in normal tissues. The MMPs share a common overall structure that includes a propeptide domain, a catalytic domain, and a hemopexin C domain. The C-terminal hemopexin C domain is known to be a collagen-binding domain (CBD)[9]. In contrast to mammalian collagenases, the functional domains of bacterial collagenases have not been well investigated.

Several metalloproteases have been isolated from various strains of *Vibrio* sp., such as *V. alginolyticus* (VAC), *V. anguillarum* (EmpA), *V. cholerae* (Hap and VCC), *V. fluvialis* (VFP), *V. parahaemolyticus* (VPPRT and VppC), *V. proteolyticus* (Nprv), and *V. vulnificus* (VVP)[10-18]. In addition, an atypical strain of *V. cholerae*, *V. mimicus*, produces two metalloproteases, the 31-kDa VMP protein and the 61-kDa VMC protein[6,19]. Previously, we showed that the intact VMC protein (VMC61) has a specific collagenase activity and that its amino acid sequence is similar to those of VCC of *V. cholerae*, VAC of *V. alginolyticus*, and VPPRT of *V. parahaemolyticus*[20]. VMP protein of V. *mimicus* has biological activities similar to those of Hap of *V. cholerae*. Several previous studies have addressed the roles

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of the metalloproteases of *Vibrio* sp. as virulence factors. To our knowledge, however, no description of their substrate-binding domains has been reported.

In this study, we examined the collagen-binding activity using recombinant proteins with various C-terminal truncations and glutathione S-transferase (GST) fusion proteins. The collagen-binding activity of the purified fusion proteins and the untagged thrombin-digested peptide segment was measured. We also found that a repeated motif in the C-terminal region of VMC61 play a critical role for the collagen-binding capacity.

Materials and Methods

Construction of plasmid DNA

The plasmid pVMC193, carrying a 2-kb *Eco*RI, *Sac*I fragment of the *vmc* gene from *V. mimicus* ATCC 33653, was used as a template for polymerase chain reaction (PCR)[6]. To construct the expression vector for the mature VMC protein, the primer meta 6 (5'-GGCCCATATGGCAGAACAAGCCCAACGC-3'; the underlined bases encode an *Nde*I site) and the primer meta 2 (5'-GGCCGGATCCCCTGTAAAGATCGGCGTGG-3'; the underlined bases encode a *BamH*I site) were designed from the nucleotide sequence of the *vmc* gene from A92 to T628 and incorporated into the PCR reaction. The resultant 1.6-kb PCR product was digested with *Nde*I and *BamH*I and cloned into a pET22b(+) vector which was digested with the same endonucleases. The resultant plasmid was named pETMETA61.

Purification of extracellular recombinant VMC (rVMC)

The plasmid pETMETA61 was introduced into *E. coli* BL21 (DE3). The cell containing plasmid pETMETA61 was cultivated in Luria-Bertani broth until an optical density (OD_{600}) of 0.3. Isopropyl-L-D-thiogalactose (IPTG) was added to the medium at a final concentration of 1 mM and incubated at 37℃ for 5 h. The culture was harvested by centrifugation at 10,000×g for 20 min at 4℃ and the cell pellet was dissolved in 20 mM Tris-HCl (pH 8.0). The cell suspension was disrupted by sonication in an ice bath and then centrifuged at 10,000×g for 20 min at 4℃ to remove insoluble materials. The clear supernatant was loaded into a column packed with Ni-NTA, which was charged with 50 mM $NiSO_4$. After unbound proteins had been washed off, the rVMC61 protein was eluted with 0.5 M imidazole in 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. The eluted rVMC61 was dialyzed in 20 mM Tris-HCl (pH 8.0) for 12 h at 4℃ and lyophilized for further characterization.

Assay of protease activity

The protease activity of rVMC61 was identified on gelatin (0.5%) containing agar plated with Frazier solution. The liquid assay was performed as described by Sasagawa *et al*[21]. One unit of activity was defined as the amount of enzyme that released 1 μmol of leucine. Cbz-GPLGP (carbobenzoxy-glycyl-L-prolyl-L-leucyl-glycyl-Lproline) and Cbz-GPGGPA (carbobenzoxy-glycyl-L-prolyl-glycyl-glycyl-L-prolyl-L-alanine) were used as synthetic substrates.

Deletion of the C-terminus of VMC

To construct C-terminally truncated metalloproteases, the plasmid pETMETA61 was digested by exonuclease III at the 3' end. The exact deleted sites were confirmed by nucleotide sequencing with ABI Prism 377 (Applied Biosystems). The resultant plasmids were transformed into *E. coli* BL21 (DE3) and expression induced with IPTG. The cells were disrupted and the protease activities were checked with the ninhydrin method or using an agar plate containing 0.5% gelatin.

Construction of plasmids for expression of GST-CBD fusion proteins and purification

DNA fragments encoding peptides from the putative C-terminal CBD region of VMC61 were cloned in-frame with the GST gene to produce constructs for the expression of GST-CBD fusion proteins using PCR amplification with the primers as described in Table 1. The PCR reactions were performed and the purified amplicons were digested with *Bam*HI and *Xho*I, whose recognitions sites were incorporated in the forward and reverse primers, respectively. The digested fragments were inserted into the pGEX-4T-1 vector to generate a series of pVMCBD plas-

Table 1. Characteristics of the primers used in the construction of pVMCBD expression plasmids and the encoded GST-CBD fusion proteins

Plasmid	Primer	Primer sequence[a]	Restriction endonuclease	Amino acids encoded	Expressed fusion protein
pVMCBD-A	GSTF1	5'-CCGCGTGGATCCTTGGTACTGTCTCGACCA-3'	*Bam*HI	Leu529-Thr628	GSTCBD-A
	GR1	5'-GGGCCGCTCGAGTGTAAAGATCGGCGTCGC-3'	*Xho*I		
pVMCBD-B	GSTF1	(As above)	*Bam*HI	Leu529-Thr576	GSTCBD-B
	GRII	5'-GGGCCGCTCGAGAGTTTCTGGCTCTGAAGG-3'	*Xho*I		
pVMCBD-C	GSTF1	(As above)	*Bam*HI	Leu529-Thr561	GSTCBD-C
	GRIII	5'-GGGCCGCTCGAGTGTATCAAGCCAGACTGC-3'	*Xho*I		
pVMCBD-D	GSTF1	(As above)	*Bam*HI	Leu529-Gln549	GSTCBD-D
	GIV	5'-GGGCCGCTCGAGTTGTTCACCTAAGTTTTT-3'	*Xho*I		

[a] The underlined letters in the primer sequences indicate the recognition sites for the restriction endonucleases used in the construction of the plasmids.

mids encoding GST-CBD fusion proteins. The fusion proteins were purified on GST-sepharose columns (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions.

Collagen-binding assay

The collagen-binding assay was performed following the methods of Matsushita *et al.* [22].

Random mutagenesis of the CBD region

Random mutagenesis of the CBD region of vmc was conducted by random mutagenesis PCR according to the methods of Song and Rhee[23]. Two oligonucleotides flanked by *Nde*I and *Bam*HI restriction sites, 5'-GGCCCATATGG CAGAACAAGCCCAACGC-3' and 5'-GGCCGGATCCGCG GC-CGCTGTATCAAGCCAGACT-3', were used as forward and reverse primers, respectively.

Results

Purification and properties of rVMC61

The purification of rVMC61 protein was performed by Ni-NTA metal affinity chromatography and verified by SDS-PAGE (Fig. 1). The rVMC61 was purified 5.3-fold with an activity recovery of 4.8% through the single purification step. From 50 mg of total cell lysates, about 0.5 mg of the pure metalloprotease was recovered. The specific activity toward gelatin as a substrate was 1.6 U mg^{-1}. The rVMC61 had maximal activity at pH 8.0 and was stable over a range of pH 6-10, retaining at least 80% of its original activity. The optimal temperature for enzyme activity ranged from 30 to 40℃, preincubation at temperatures above 45℃ resulted in a decrease of enzyme activity, indicating that the rVMC61 is a thermally unstable enzyme. Most metalloproteases isolated from different *Vibrio* sp. also showed decreased activities at temperatures above 50℃. Gelatin had the lowest Km value (3.0 mg ml^{-1}) followed by type I collagen. However, the rVMC61 did not degrade type V collagen. This result is in good agreement with two metalloproteases from *V. parahaemolyticus*[16,24]. The rVMC metalloprotease also degraded synthetic peptides such as Cbz-GPGGPA and Cbz-GPLGP, which are known as substrates for collagenase.

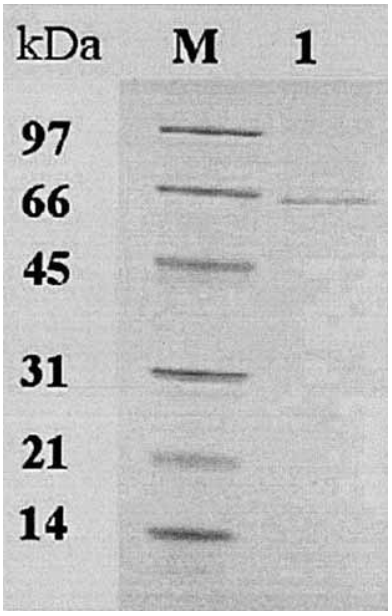

Fig. 1. SDS-PAGE of purified rVMC61 from Ni-NTA chromatography. Lane M: Molecular marker; lane 1: purified rVMC61. 10 μg of purified rVMC61 was loaded on the 12% slab gel and the proteins were visualized with Coomassie R-250.

Deletion of C-terminal region

The 3' end of plasmid pETMETA61 was deleted serially and truncates were identified with DNA electrophoresis (data not shown). The exact deleted sequence was con-firmed by nucleotide sequencing. The truncated proteins showed the removal of 34, 67 and 100 amino acid residues from the C-terminus (Fig. 2 A). The predicted truncated proteins ended at V592 to L528 of the mature extracellular protease. The mutant proteins were overexpressed in *E. coli* BL21 (DE3) and analyzed by SDS-PAGE. The molecular masses of VMC61, VMC57, VMC53 and VMC50 were respectively estimated to be 61, 57, 53and 50 kDa from SDS-PAGE, which coincided with those calculated from their amino acid sequences. The enzymatic activities of these mutant proteins were further confirmed by a plate assay. The gelatinolytic activity could be found from *E. coli* cells carrying plasmids pETMETA61-I (VMC57) and -II (VMC53), whereas the gelatin degrading activity disappeared with deletion of 100 amino acids. These observations indicate that the deletion of the C-terminal region affects the protease activity. The expressed His-tagged proteins were purified by Ni-NTA affinity chromatography (Fig. 2 B).

Collagen-binding activity of the recombinant C-terminal truncated VMC proteins

Although the VMC50 retains the catalytic region, including the HEXXH motif required for the binding of metal ligands by metalloproteases, it did not exhibit collagenolytic activity[25]. Based on this result, we speculated that the loss of enzymatic activity in the VMC50 may be attributable to a loss of the capacity for collagen-binding. To investigate this hypothesis, the purified VMC61, VMC57, VMC53, and VMC50 were incubated with insoluble type I

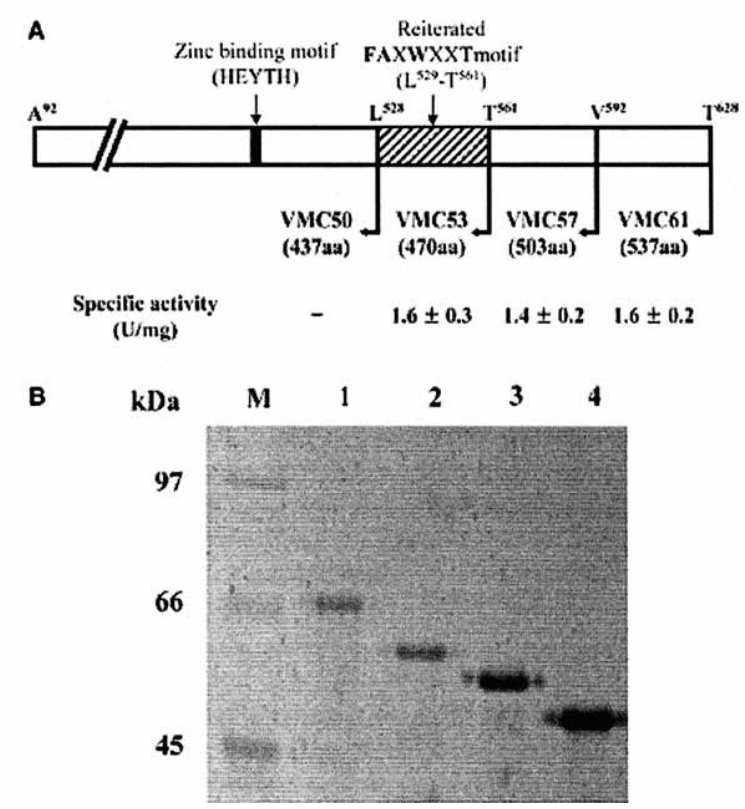

Fig. 2. Collagenolytic activity of the VMC61 and C-terminal truncated VMC proteins. (A) Graphical representation and collagenolytic activities of the C-terminal truncated VMC proteins. The numbered amino acids indicate the sites of the C-terminal truncations. HEYTH is the conserved zinc binding motif of *Vibrio* metalloproteases. The VMC61 and truncates were purified with Ni-NTA affinity chromatography and the protease activities were detected with gelatin as substrate. The protease activities were measured by ninhydrin method. The data are means ± standard deviations for at least three independent experiments. (B) SDS-PAGE analysis of the purified C-terminal truncated VMC proteins. M, molecular weight marker (BioRad low range marker); lane 1, VMC61; 2, VMC57; 3, VMC53; 4, VMC50.

collagen and filtered to retain the collagen and any bound proteins. As shown in Fig. 3, the VMC61, VMC57, and VMC53 were not detected in the filtrates, indicating that these proteins had bound to the collagen. However, the VMC50 was abundant in the filtrate, indicating that it had not bound to the collagen. These results imply that the VMC50 could not bind to insoluble collagen, which was consistent with the absence of protease activity exhibited by this truncated protein.

Collagen-binding properties of GST-CBD fusion proteins

We also performed a collagen-binding assay using GST fusion proteins containing peptides derived from the putative CBD of the C-terminal region of VMC61. Interestingly, the 33-amino acid stretch (Leu529-Thr561) that is not present in VMC50 (as compared to VMC53) contains a repeated motif, FAXWXXT. In order to examine the importance of this repeated motif in collagen-binding activity, we generated GST-CBD fusion proteins containing one or two FAXWXXT motifs. The DNA sequences corresponding to Leu529-Thr628, Leu529-Thr576, Leu529-Thr561, and Leu529-Gln549 were amplified by PCR and incorporated into the pGEX-4T-1 vector to generate plasmids expressing the GST fused with peptides: GSTCBD-A, -B, -C and -D, respectively (Fig. 4 A). The expressed GST-CBD fusion proteins were purified on glutathione-sepharose columns, and the relative molecular weights of the purified GSTCBD-A, -B, -C, and -D proteins were estimated as 37, 35, 31, and 29 kDa, respectively, by SDS-PAGE (Fig. 4 B and C). When the collagen-binding assays were performed using the purified fusion proteins, the GSTCBD-A and -B proteins bound to insoluble collagen (Fig. 5 A). Likewise, GSTCBD-C protein, which contains the 33-amino acid segment with the repeated FAXWXXT motif, bound to collagen, whereas GSTCBD-D protein, which contains only one FAXWXXT motif, did not (Fig. 5 B). There was no binding at GST protein alone to collagen (Fig. 5 B, lanes 5, 6).

To further define the collagen-binding properties of the 33-amino acid peptide alone, the GSTCBD-C fusion protein was treated with thrombin to separate the GST protein and the VMCBD peptide. After thrombin digestion of GSTCBD-C protein (Fig. 6, lane 1), three bands corresponding to thrombin (37 kDa), GST protein (26 kDa), and the 33-amino acid VMCBD peptide (37 kDa) were detected. When the thrombin-treated sample was incubated with collagen, the VMCBD-C peptide was not detected in the unbound fraction, confirming that the peptide itself bound to collagen (Fig. 6, lane 2).

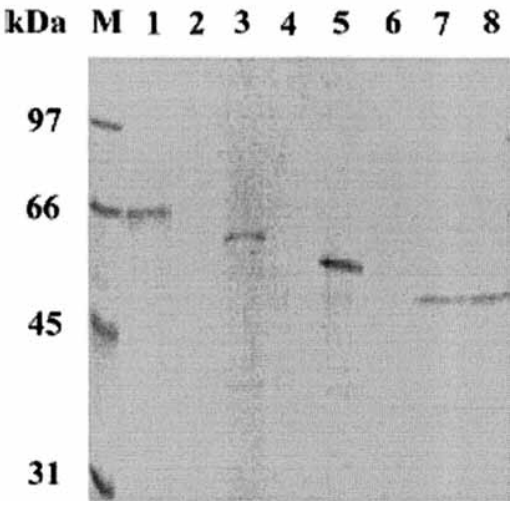

Fig. 3. Collagen-binding activity of C-terminal truncated VMC proteins. The VMC61 and various C-terminal truncated VMC proteins were incubated with or without collagen at room temperature, and the filtrates were analyzed on SDS-PAGE gels stained with Coomassie brilliant blue G250. M, molecular weight marker; lane 1, VMC61 alone; 2, VMC61 with collagen; 3, VMC57 alone; 4, VMC57 with collagen; 5, VMC53 alone; 6, VMC53 with collagen; 7, VMC 50 alone; 8, VMC50 with collagen.

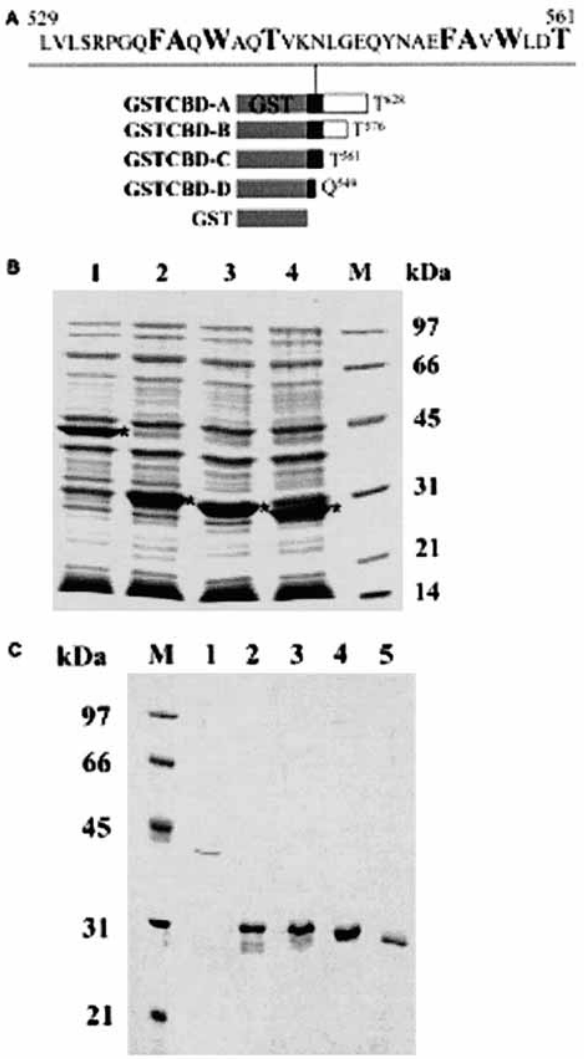

Fig. 4. Graphical representation of the GST fusion proteins containing peptides corresponding to the C-terminal CBD region of VMC61, and SDS-PAGE analysis of the overexpressed GST-CBD fusion proteins. (A) The sequences encoding peptides corresponding to the C-terminal region from Leu529 to Thr628 were amplified by PCR with the primers listed in Section 2 and cloned into the pGEX-4T-1 expression vector to generate four GST-CBD fusion proteins. The black boxes indicate the Leu529-Thr561 region containing the repeated FAXWXXT motif. The numbered amino acids indicate the C-terminal amino acid. (B) The GST-CBD fusion proteins were overexpressed in *E. coli* and analyzed by SDS-PAGE. M, molecular weight marker; lane 1, GSTCBD-A; 2, GSTCBD-B; 3, GSTCBD-C; 4, GSTCBD-D. (C) The overexpressed GST-CBD fusion proteins were purified on glutathione-sepharose columns and the purified proteins were analyzed by SDS-PAGE. M, molecular weight marker; lane 1, GSTCBD-A; 2, GSTCBD-B; 3, GSTCBD-C; 4, GSTCBD-D; 5, GST.

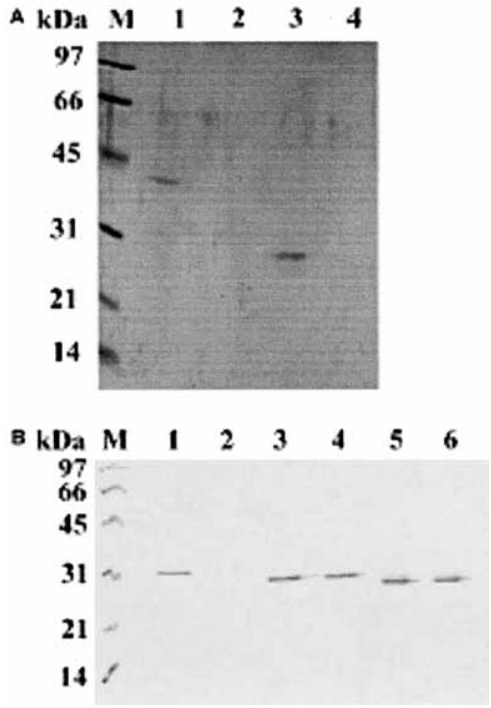

Fig. 5. Collagen-binding activity of GST-CBD fusion proteins. The purified GST-CBD fusion proteins were incubated with or without collagen and the filtrates were analyzed by SDS-PAGE. (A) Lane 1, GSTCBD-A alone; 2, GSTCBD-A with collagen; 3, GSTCBD-B alone; 4, GSTCBD-B with collagen; M, molecular weight marker. (B) M, molecular weight marker; lane 1, GSTCBD-C alone; 2, GSTCBDC with collagen; 3, GSTCBD-D; 4, GSTCBD-D with collagen; 5, GST alone; 6, GST with collagen.

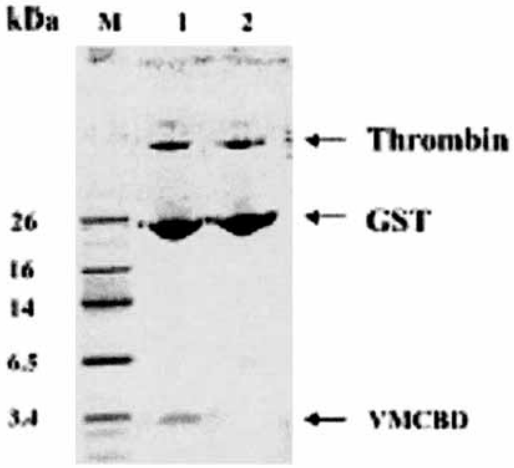

Fig. 6. Collagen-binding activity of VMCBD peptide. The untagged 33-amino acid peptide from Leu529 to Thr561, VMCBD peptide, was generated by digestion of GSTCBD-C with thrombin. The digested mixture was then incubated with collagen. The filtrates were subjected to Tricine-SDS PAGE analysis, and the gels were stained with Coomassie brilliant blue G250. M, molecular weight marker; lane 1, thrombin-digested GSTCBD-C alone; 2, thrombin-digested GSTCBDC incubated with collagen.

In addition, we performed random mutagenesis at the L528-G526 region containing CBD in VMC61, and obtained 50 colonies. Among these colonies, a plasmid was isolated from one colony that was deficient in protease activity on gelatin agar plate. Analysis of the sequence of this plasmid revealed that Phe residue in the second FAXWXXT motif of the predicted amino acid sequence was changed to Leu.

Discussion

The C-terminal regions of mammalian MMPs and some bacterial collagenases are known to be CBDs, as shown by the loss of collagenase activity after deletion of the C-terminal region[26]. *Vibrio* metalloproteases contain an N-terminal signal peptide, a cleavage site, and a catalytic domain that includes the zinc-binding motif (HEXXH)[27]. However, the role of the C-terminal region has not yet been identified. To our knowledge, the present report is the first to identify the CBD in a *Vibrio* metalloprotease.

Previously, we classified the metalloproteases of *Vibrio* sp. into two distinct categories according to the amino acid sequences[6]. Class I *Vibrio* metalloproteases included Hap, VVP, VVF, and NprV, and class II *Vibrio* metalloproteases included VMC, VPPRT, and VAC. The class I *Vibrio* metalloproteases have a large signal peptide region and a zinc-binding motif (HEXXH) that includes an extra glutamic acid near the motif. The class II *Vibrio* metalloproteases have a HEXXH motif only. The two classes of *Vibrio* metalloproteases exhibit different biochemical properties. The intact VMC (VMC61) belonging to class II enzyme degrades collagen, but does not degrade other soluble protein substrates such as casein and albumin. In contrast, in class I *Vibrio* metalloproteases such as VVP and VFP, the autocatalytic removal of part of the C-terminal region results in changes in the substrate specificity. Full-length class I metalloproteases exhibit proteolytic activity with a wide range of substrates, including albumin, casein, elastin, and collagen. The autocatalytic removal of the C-terminal region has not been detected in class II *Vibrio* metalloproteases, including VMC61.

Another class II *Vibrio* metalloprotease, VPPRT, also exhibits collagen-specific activity and does not degrade albumin or casein[28]. Distinct differences in the CBD region further support the classification of *Vibrio* metalloproteases into two classes. The C-terminal regions of class I metalloproteases do not contain the FAXWXXT motif that we identified in VMCBD peptide. This result suggests that the binding mechanism of class I *Vibrio* metalloproteases is probably different with that of class II metalloproteases. As shown in Fig. 7, the CBD regions of class II *Vibrio* metalloproteases are highly homologous; in particular, the first FAXWXXT motif in the CBD region of VCC, VPPRT, and VAC shows 71%, 86%, and 57% identity with that of VMC, respectively, while the second motif is highly conserved in all class II *Vibrio* metalloproteases. This analysis indirectly supports our experimental result that the second FAXWXXT motif plays a more critical role in collagen binding.

The result of random mutagenesis in the CBD region also supported the importance of second motif in collagen binding. We found that the substitution of Leu for Phe555 in the second FAXWXXT motif of the CBD region was associated with a loss of collagenase activity. Further work using site-directed mutagenesis is in progress to investigate which amino acid residues of the 33-amino acid CBD region are important for collagen binding and how the FAXWXXT

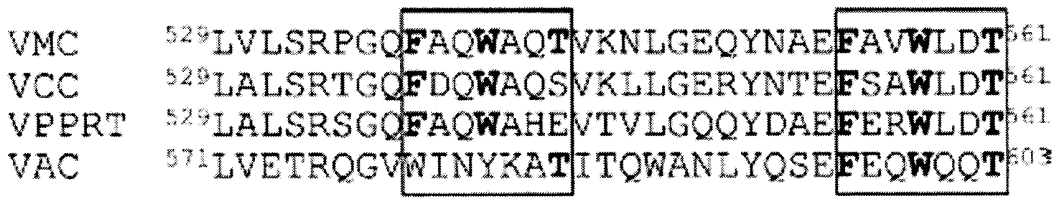

Fig. 7. Amino acids alignment of the FAXWXXT motifs in the collagen-binding region of *Vibrio mimicus* with those of other class II *Vibrio* metalloproteases. VMC, metalloprotease from *V. mimicus*, Accession No. AF004832; VCC, metalloprotease from *V. cholerae*, Accession No. AF109145; VPPRT, metalloprotease from *V. parahaemolyticus*, Accession No. Z46782; VAC, metalloprotease from *V. alginolyticus*, Accession No. X62635. The conserved amino acids are indicated in boldface type. The alignments were performed with the CLUSTAL W program.

motifs interact with collagen.

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