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Functional Domains of a Zinc Metalloprotease from *Vibrio vulnificus*

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***Vibrio vulnificus*, an opportunistic human pathogen causing wound infection and septicemia, secretes a 45-kDa metalloprotease (*V. vulnificus* protease; VVP). A plasmid which carries the entire *vvp* gene subcloned into pBluescriptIIKS+ was transformed into *Escherichia coli* DH5α for overproduction of the protease. The 45-kDa recombinant protease (rVVP) was isolated from the periplasmic fraction of the transformant by ammonium sulfate precipitation followed by column chromatography on phenyl Sepharose. Biochemical characterization of the isolated rVVP showed that the recombinant protease was identical to that produced by *V. vulnificus*. When rVVP was incubated at 37°C, a 35-kDa fragment was generated through autoproteolytic removal of the C-terminal peptide. This 35-kDa fragment (rVVP-N) was found to have sufficient proteolytic activity toward oligopeptides and soluble proteins but had markedly reduced activity toward insoluble proteins. Lineweaver-Burk plot analysis indicated increased K_m values of rVVP-N for all of the protein substrates. rVVP, but not rVVP-N, was shown to agglutinate rabbit erythrocytes, bind to the erythrocyte ghosts, and digest the ghost membrane proteins. These results strongly suggest that rVVP (and VVP) consists of at least two functional domains: an N-terminal 35-kDa polypeptide mediating proteolysis and a C-terminal 10-kDa polypeptide which may be essential for efficient attachment to protein substrates and erythrocyte membranes.**

Metalloproteases, in which zinc is an essential metal ion for catalytic activity, are elaborated by various human pathogenic bacteria, as well as by nonpathogenic ones (5). They can be classified into three families: thermolysins, serralysins, and neurotoxins (7). The members of the thermolysin family are synthesized as inactive precursors, with a large N-terminal propeptide acting as a specific inhibitor for the mature protease and/or an intramolecular chaperone to control the folding of the protease (10, 14, 20, 24). Since the N-terminal propeptide is cleaved by autoproteolysis (9, 13, 24), a transformant carrying a cloned metalloprotease gene also produces a correctly processed metalloprotease (2, 13). On the other hand, studies of metalloproteases from vibrios (4, 6, 15) have demonstrated that, although these also belong to the thermolysin family, their precursors have C-terminal propeptides as well as N-terminal ones. The biochemical function of the C-terminal propeptide is currently unknown.

Vibrio vulnificus is an opportunistic human pathogen causing wound infection and septicemia (8, 22). Like other vibrios, this pathogen secretes the 45-kDa zinc metalloprotease (*V. vulnificus* protease; VVP) since it belongs to the thermolysin family (18). VVP has been reported to have many biological functions: proteolytic degradation of a wide variety of host proteins, such as plasma proteins, involved in coagulation or complement action, induction of hemorrhagic tissue damage, and enhancement of vascular permeability through the generation of inflammatory mediators (18). Recently, cloning and sequencing of the *vvp* gene were carried out by two research groups (3, 21). These studies indicated that the VVP precursor was a 609-amino-acid polypeptide and that VVP generated by removal of the N-terminal propeptide consisted of 413 amino acids and had a molecular mass of 44,745 Da. It was proposed that VVP underwent maturation without processing of the C-terminal peptide, so that VVP is ca. 10 kDa larger than other vibrio proteases (11, 16). However, we observed that, when the plasmid carrying the *vvp* gene was transformed into *Escherichia coli*, the transformant produced the 35-kDa metalloprotease as well as the 45-kDa one and that the 35-kDa metalloprotease was also neutralized by the antibody against the 45-kDa VVP purified from *V. vulnificus* (data not shown).

In the present study, we report that the 45-kDa recombinant protease (rVVP) isolated from the periplasmic fraction of the *E. coli* transformant generates autocatalytically the 35-kDa N-terminal fragment (rVVP-N) during incubation at 37°C, indicating autoproteolytic conversion to the 35-kDa form. In addition, we show that rVVP-N has decreased affinity for protein substrates and erythrocyte membranes, suggesting that rVVP may consist of two functional domains: the N-terminal 35-kDa polypeptide mediating the proteolytic action and the C-terminal 10-kDa polypeptide mediating efficient association with the target substance.

Purification of 45-kDa rVVP. A cloned 4.1-kb DNA fragment from *V. vulnificus* L-180 (21) was digested with *Dra*I, and the resultant 2.6-kb fragment carrying the entire *vvp* gene was inserted into pBluescriptIIKS+. This recombinant plasmid, designated pVVP7-1, was transformed into *E. coli* DH5α. *E. coli* DH5α (pVVP7-1) was inoculated into Luria-Bertani broth and cultivated at 37°C for 14 h with shaking (120 cycles/min). After cultivation, the bacterial cells were harvested by centrifugation (7,000 × *g*, 40 min) and washed with 2% NaCl, and the periplasmic fraction containing 45-kDa rVVP was collected by the osmotic shock method (19). The periplasmic preparation was adjusted to pH 8.5 with 20 mM Tris-HCl, and ammonium sulfate was added to 70% saturation. The resulting precipitate was collected, dissolved in distilled water, and dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 M NaCl. The crude rVVP preparation thus obtained was applied to a phenyl Sepharose HP 10/10 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl supplemented with 1 mM $CaCl_2$ and 1 M NaCl (pH 7.5). Under these conditions,

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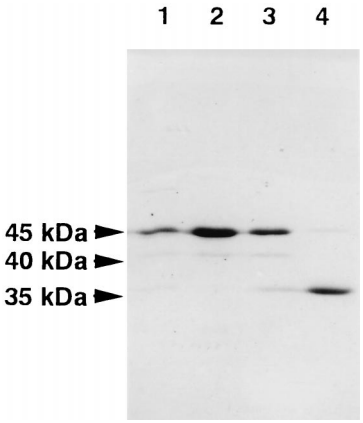

FIG. 1. SDS-PAGE profiles of the protease samples. Each of the samples (1.5 μg) was inactivated by 5% trichloroacetic acid, treated with 1% SDS in the presence of 5% 2-mercaptoethanol at 100°C for 3 min, and subjected to SDS-PAGE with 10% acrylamide gel. After electrophoresis, the gel was stained with 0.5% Coomassie brilliant blue. Lanes: 1, crude rVVP obtained by ammonium sulfate fractionation; 2, rVVP obtained by phenyl Sepharose column chromatography; 3, VVP purified from *V. vulnificus* L-180; 4, rVVP-N obtained by incubation of rVVP at 37°C for 24 h.

the 35-kDa metalloprotease did not associate with the gel. The bound 45-kDa rVVP was eluted with 20 mM Tris-HCl containing 1 mM $CaCl_2$ and 30% ethylene glycol (pH 7.5). The eluted rVVP was diluted to 1.0 mg/ml with 20 mM Tris-HCl containing 1 mM $CaCl_2$ (pH 7.5) and stored at −20°C.

The rVVP was inactivated by the addition of 5% trichloroacetic acid, treated with 1% sodium dodecyl sulfate (SDS)–5% 2-mercaptoethanol at 100°C for 3 min, and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12). The SDS-PAGE analysis revealed a protein band with a molecular mass of 45 kDa (Fig. 1). Native PAGE also showed a protein band corresponding to the proteolytic activity (data not shown). These results indicate the homogeneity of the final rVVP preparation, which was used in the experiments discussed below. From 3.6 liters of the culture of the transformant, 35 mg of rVVP was obtained (Table 1). This yield was about 30 times higher than that from the culture supernatant of *V. vulnificus* L-180 (16).

The purified rVVP, diluted serially, was allowed to act on 1 mg of azocasein (Sigma Chemical, St. Louis, Mo.) at 30°C for 15 min, and digestion of the substrate was quantified by measuring the liberation of the dye from the substrate (16). One unit of activity was defined as the amount of the enzyme which digested 1 μg of azocasein in 1 min (16). rVVP showed activity comparable to that of VVP. In particular, the specific activities of rVVP and VVP were determined to be 15,100 U/mg (680 U/nmol) and 13,800 U/mg (620 U/nmol), respectively. Also, the K_m value of rVVP (0.85 mg/ml) was very similar to that of VVP (0.91 mg/ml). Like VVP (18), rVVP injected intradermally elicited edematous and hemorrhagic tissue damage in a dose-dependent manner. In an immunodiffusion test using the antibody against purified VVP, rVVP and VVP formed a single fused precipitation line. We conclude that rVVP isolated from *E. coli* DH5α (pVVP7-1) is identical to VVP from *V. vulnificus* L-180. Furthermore, it is most likely that VVP is also processed according to the general rule for the thermolysin family, namely, the N-terminal propeptide is removed by autoproteolysis.

TABLE 1. Purification of rVVP

Purification step	Total protein (mg)	Total activity (PU[a])	Sp act (PU/mg)	Relative activity	Yield of activity (%)
Periplasmic preparation	284	1,290,000	4,550	1.0	100
Ammonium sulfate fraction	63	709,000	11,300	2.5	55
Phenyl Sepharose HP column	35	521,000	15,100	3.3	40

[a] PU, proteolytic units.

TABLE 2. Peptidase activities of rVVP and rVVP-N[a]

Substrate	k_{cat} (s^{-1})		K_m (mM)	
	rVVP	rVVP-N	rVVP	rVVP-N
Z-Gly-Phe-NH_2	62	130	1.3	1.3
Z-Gly-Leu-NH_2	5.8	14	9.1	9.5

[a] The extent of hydrolysis of the substrate upon incubation at 30°C for 30 min was measured by the ninhydrin method. The k_{cat} and K_m values were determined by Lineweaver-Burk plot.

Generation of 35-kDa rVVP-N. When the 45-kDa rVVP was incubated at 37°C, the 35-kDa fragment was generated; after a 24-h incubation, most of the 45-kDa rVVP was processed to the 35-kDa protein (Fig. 1). However, the putative 10-kDa fragment was not detected. It should be noted that the final rVVP preparation contained a small amount of a 40-kDa protein (Fig. 1). This 40-kDa protein, which has its proteolytic activity neutralized by the anti-VVP antibody, was also generated during incubation of the 45-kDa rVVP to convert the 45-kDa form to the 35-kDa form. Therefore, the 35-kDa protein may be generated by two-step proteolysis, with the 40-kDa protein being an intermediate.

In order to remove the small fragment(s), the autodigested preparation containing the 35-kDa protein was diluted with 20 mM Tris-HCl containing 1 mM $CaCl_2$ (pH 7.5) and filtered through a YM 30 membrane (Amicon, Beverly, Mass.). Thereafter, the protein concentration was adjusted to 1.0 mg/ml.

N-terminal amino acid sequencing of the 35-kDa protein was carried out with an Applied Biosystems 477 automatic sequencer. The sequence, determined to be NH_2-Ala-Gln-Ala-Asp-Gly-Thr-Gly-Pro, was identical to the N-terminal sequence of the 45-kDa form (3, 11, 21). Hence, the 35-kDa protein was generated by removal of the C-terminal peptide, so we termed this 35-kDa fragment rVVP-N. The cleavage of the C-terminal peptide has also reportedly been achieved by heating in the presence of SDS; however, the molecular mass of the product was slightly higher than 35 kDa (4, 11).

Proteolytic activity. The peptidase activities of rVVP and rVVP-N toward carbobenzoxy-glycyl-leucine amide (Z-Gly-Leu-NH_2) and Z-glycyl-phenylalanine amide (Z-Gly-Phe-NH_2) were compared. An appropriate amount of the protease was allowed to act at 30°C for 30 min on a 2.5 to 7.0 mM concentration of the peptide substrate (Peptide Institute, Minoh, Osaka, Japan). Thereafter, the extent of hydrolysis of the substrate was determined by the ninhydrin method, and the k_{cat} and K_m values were estimated by Lineweaver-Burk plot. The estimated K_m value of rVVP was very similar to that of rVVP-N and was independent of the substrates used (Table 2). However, rVVP showed a markedly lower k_{cat} value (Table 2), suggesting that the C-terminal peptide of rVVP may interfere with the access of either of the peptide substrates.

As shown in Table 3, rVVP-N was found to have significant proteinase activity toward azocasein and azoalbumin (Sigma Chemical). However, it showed reduced activity toward insoluble proteins such as collagen and elastin (Table 3). rVVP or rVVP-N was serially diluted and incubated with 2 mg of azo

TABLE 3. Proteinase activities of rVVP and rVVP-N[a]

Substrate	Sp act (U/nmol)[b]		K_m (mg/ml)[c]	
	rVVP	rVVP-N	rVVP	rVVP-N
Soluble protein				
Casein	680	840	0.85	2.6
Albumin	180	170	4.3	17
Insoluble protein				
Collagen	170	50	18	110
Elastin	14	0.50	17	150

[a] The extent of hydrolysis of the substrate upon incubation at 30°C for 15 min (soluble protein) or 3 h (insoluble protein) was measured spectrophotometrically.
[b] One unit of activity was defined as the amount of the protease which digested 1 μg of the substrate in 1 min.
[c] The K_m value was determined by Lineweaver-Burk plot.

dye-impregnated collagen (Sigma Chemical) at 30°C with shaking, and liberation of the dye was measured after a 3-h incubation. The collagenolytic activity of rVVP was determined to be 170 U/nmol, while that of rVVP-N was 50 U/nmol. The elastolytic activities of both proteases were also measured with elastin-Congo red (Sigma Chemical) as described previously (17). It was found that 1 nmol of rVVP was capable of the digestion of 14 μg of elastin in 1 min but that an equal amount of rVVP-N could digest only 0.5 μg of the substrate. The K_m values of both proteases for each protein substrate were estimated (Table 3). The K_m value of rVVP-N was higher than that of rVVP, suggesting that removal of the C-terminal peptide might be responsible for the lower affinity to the proteins. Therefore, the C terminus is likely to be necessary for the efficient association of rVVP with the protein substrates.

Hemagglutinating activity. Since VVP is known to agglutinate both rabbit and chicken erythrocytes (1), the hemagglutinating activities of rVVP and rVVP-N for rabbit erythrocytes were also compared. Each of the proteases (50 μl), diluted serially with KRT buffer (128 mM NaCl, 5.1 mM KCl, 1.34 mM $MgSO_4$, 2.7 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5), was mixed with an equal volume of 1.5% erythrocytes, and the mixture was then incubated at room temperature for 1 h. The activity was defined as the reciprocal of the highest dilution of the sample causing visible hemagglutination (1). rVVP showed a high level of activity (1,200 U/nmol), while rVVP-N showed negligible activity.

Subsequently, 500 μg of rabbit erythrocyte ghosts prepared by the method of Tomoda et al. (23) was incubated with 30 μg of rVVP or rVVP-N in 100 μl of KRT buffer at room temperature for 1 h. After incubation, the ghosts were collected and rinsed, and an aliquot was treated with SDS and subjected to SDS-PAGE. As shown in Fig. 2A, most of the proteins on the ghost membranes were digested by rVVP but were resistant to rVVP-N. After SDS-PAGE, the samples were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, Calif.), and the VVP antigen was detected with the rabbit immunoglobulin G antibody against purified VVP (Fig. 2B). rVVP was detected on the PVDF membrane, while the band corresponding to rVVP-N was not. Since the sensitivity of rVVP-N to the antibody was comparable to that of rVVP, this result indicates that rVVP can associate more tightly with the ghost membranes.

In summary we conclude that the C-terminal peptide of rVVP is essential for rVVP to induce hemagglutination of rabbit erythrocytes, to bind tightly to the erythrocyte ghosts, and to proteolyze the proteins on the ghost membranes.

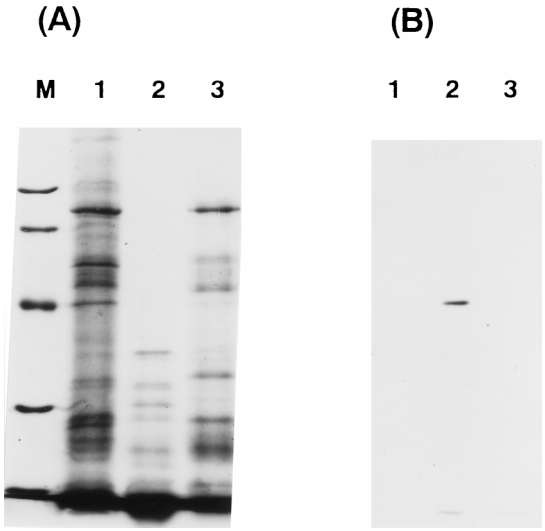

FIG. 2. (A) SDS-PAGE profiles of membrane proteins of rabbit erythrocyte ghosts. Ghosts (500 μg) were incubated with 30 μg of either rVVP or rVVP-N at room temperature for 1 h. After incubation, an aliquot of each sample was treated with SDS and subjected to SDS-PAGE. Thereafter, the gel was stained with 0.5% Coomassie brilliant blue. Lanes: M, molecular mass markers (97, 68, 43, and 31 kDa); 1, ghosts incubated without the protease; 2, ghosts incubated with rVVP; 3, ghosts incubated with rVVP-N. (B) Western blot analysis with the anti-VVP immunoglobulin G antibody. After SDS-PAGE, the samples were transferred to a PVDF membrane, and the VVP antigen was detected with the antibody against purified VVP. Lanes: 1, ghosts incubated without the protease; 2, ghosts incubated with rVVP; 3, ghosts incubated with rVVP-N.

Exocellular metalloproteases in the thermolysin family are known to be synthesized as inactive precursors with several domains: a signal peptide, an N-terminal propeptide acting as an inhibitor for the mature protease and/or as an intramolecular chaperone, and an endoproteinase domain (9, 10, 13, 20, 24). Studies of several vibrio metalloproteases have shown that vibrio enzymes belong to the thermolysin family, with precursors that have a C-terminal propeptide (4, 6, 15). VVP was recently reported to undergo maturation without processing of the C-terminal propeptide (3, 21). The data presented herein clearly indicate that the C-terminal propeptide of rVVP (and VVP) was also autodigested gradually during incubation at 37°C. Although it is not clear why only VVP has a highly stable C-terminal propeptide, this proregion may be important for extraintestinal infection by *V. vulnificus*. Unlike other pathogenic vibrios, *V. vulnificus* primarily causes wound infection and septicemia in immunocompromised hosts, and the infections are characterized by the formation of edema, necrotic ulcer, and cellulitis on the skin (8, 22). Because VVP is a crucial factor eliciting such skin damage, the C-terminal peptide may mediate the binding of VVP to the intradermal target substance(s). In conclusion, we propose that the C-terminal peptide of VVP may act as a substrate binding domain.

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