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Activation mechanism of human Hageman factor–plasma kallikrein–kinin system by *Vibrio vulnificus* metalloprotease

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Vibrio vulnificus, an opportunistic human pathogen, secretes a metalloprotease (VVP). The VVP inoculated into a guinea pig is known to generate bradykinin through activation of the Hageman factor–plasma kallikrein–kinin system. VVP was shown to possess the ability to activate the human system through the same mechanism as that clarified in the guinea pig system, namely, VVP converted both human zymogens (Hageman factor and plasma prekallikrein) to active enzymes (activated Hageman factor and plasma kallikrein), and the then generated kallikrein liberated bradykinin from high-molecular-weight kininogen. However, in the presence of plasma α_2-macroglobulin (α_2M), the VVP action was drastically decreased. This finding suggests that the human system might be activated only at the interstitial-tissue space which contains negligible amounts of α_2M or in the bloodstream of the individuals whose plasma α_2M level is extremely reduced.

Kinin generation; Bacterial metalloprotease; *Vibrio*

1. INTRODUCTION

Vibrio vulnificus is an opportunistic human pathogen, and the infections are characterized by edema and ulcer formation on the skin [1–4]. We previously found that exocellular metalloprotease [5] (*V. vulnificus* protease: VVP) contributed to the formation of the edema [6] because VVP inoculated into the dorsal skin of a guinea pig enhanced vascular permeability through activation of the Hageman factor–plasma kallikrein–kinin system [7]. The first step in the activation of this system had been believed to be the activation of the Hageman factor, and then the activated Hageman factor converts plasma prekallikrein to kallikrein [8]. Plasma kallikrein thus generated has two actions: activation of Hageman factor, and liberation of bradykinin from high-molecular-weight kininogen [9]. However, Molla et al. [10] reported the activation of both guinea pig zymogens by VVP, indicating that VVP activated the guinea pig system through a novel mechanism.

Recently, the actions of two metalloproteases from *Pseudomonas aeruginosa* (elastase and alkaline proteinase), which are known to activate the guinea pig system through the activation of Hageman factor [10], on the human system were studied [11,12]. It was confirmed that both metalloproteases also activated the human system but the activation mechanisms are different from those in the guinea pig system. Namely, the elastase activated both Hageman factor and plasma prekallikrein, whereas alkaline proteinase activated prekallikrein very poorly.

Although VVP has also been reported to activate the human system [7], the direct action of this metalloprotease on the human zymogen(s) remains to be clarified. Thus, we investigated the mode of action of VVP on the human system and found that VVP activated the system through the same mechanism as in the guinea pig.

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2. MATERIALS AND METHODS

2.1. *Substances*

VVP (45 kDa) was purified from *V. vulnificus* strain L-180 [5]. Human Hageman factor (74 kDa), plasma prekallikrein (85 kDa) and high-molecular-weight kininogen (78 kDa) were purchased from Enzyme Research Laboratories Inc. (South Bend, IN USA). All of these human plasma proteins were demonstrated to be homogeneous by SDS-PAGE. Bovine plasma α_2-macroglobulin (α_2M; 720 kDa) was obtained from Boehringer-Mannheim (Germany), and the amount of active α_2M was determined by measuring the inhibitory effect on trypsin [13]. Normal and coagulation factor-deficient human plasma were purchased from George-King Bio-Medical Inc. (Overland Park, KS USA). Carboxy-phenylalanyl-arginine-4-methyl-coumaryl-7-amide (Z-FR-MCA) and *t*-butyloxycarbonyl-glutaminyl-glycyl-arginine-MCA (Boc-QGR-MCA) were obtained from Peptide Institute Inc. (Minoh, Japan).

IgG antibody against human plasma α_2M (anti-α_2M IgG) was prepared as follows: 1 mg of purified human plasma α_2M (Sigma) emulsified with an equal volume of Freund's complete adjuvant was injected subcutaneously into the back skin of a rabbit with 2-week intervals, and the antiserum was obtained 7 days after the third injection. The IgG fraction of the antiserum was prepared by ammonium sulfate fractionation followed by affinity-column chromatography on a Protein A Superose HR 10/2 column (Pharmacia LKB, Uppsala, Sweden), and this preparation was used as anti-α_mM IgG. 1 mg of this antibody was found to neutralize an equivalent of 70 pmol of the

purified human plasma α_2M. To prepare the control rabbit IgG, saline emulsified with the adjuvant was injected subcutaneously.

2.2. *Generation of plasma kallikrein and activated Hageman factor in human plasma by VVP*

Human plasma (0.1 ml) preincubated with control IgG (3.0 mg) or anti-α_2M IgG (3.0 mg) at 4°C for 20 min was diluted with 0.9 ml of the assay buffer (50 mM Tris-HCl buffer supplemented with 0.9% NaCl, 0.02% NaN_3, 0.01% bovine serum albumin and 0.05% polybrene; pH 8.0). This 10-fold diluted plasma was incubated with an appropriate concentration of VVP at 30°C in the presence of 0.2 mM Z-FR-MCA (the substrate for plasma kallikrein [14]) of Boc-QGR-MCA (the substrate for activated Hageman factor [15]). The increase in A_{370} due to the liberation of 7-amino-4-methyl-coumarine (AMC) was measured with a spectrophotometer, and the amount of AMC liberated was calculated.

2.3. *Activation of purified human plasma prekallikrein by VVP*

2.3.1. Hydrolysis of Z-FR-MCA by VVP-generated plasma kallikrein

Human plasma prekallikrein (36 nM) in 1 ml of the assay buffer was incubated with VVP (0–20 nM) at 30°C in the presence of Z-FR-MCA, and the generation of amidase activity toward the substrate was measured. To test the effect of α_2M on the VVP action, VVP (20 nM) was allowed to act on prekallikrein in the presence of 40 nM of bovine plasma α_2M.

2.3.2. Release of bradykinin from high-molecular-weight kininogen by VVP-generated plasma kallikrein

VVP (4 nM) and prekallikrein (36 nM) were allowed to react at 30°C for 20 min, and then, VVP was inactivated by the addition of 0.1 mM phosphoramidon and 10 mM *o*-phenanthroline. Thereafter, this solution (0.25 ml) containing VVP-generated kallikrein was mixed with human high-molecular- weight kininogen (100 pmol in 0.25 ml of the assay buffer) or 0.25 ml of heat-treated (60°C for 30 min) human plasma. After incubation at 30°C for 10 min, the reaction was stopped with trichloroacetic acid solution (final concentration of 6%), and the bradykinin content in the supernatant was determined by enzyme-immunoassay with MARKIT-A Bradykinin (Dainippon Pharmaceutical Co., Suita, Japan) according to the manual.

2.4. *Activation of purified human Hageman factor by VVP*

2.4.1. Hydrolysis of Boc-QGR-MCA by VVP-activated Hageman factor

Human Hageman factor (68 nM) in 1 ml assay buffer was mixed with VVP (0–20 nM). After the addition of Boc-QGR-MCA, generation of the hydrolytic activity toward the substrate was measured at 30°C. To test the effect of α_2M, VVP (20 nM) was incubated with Hageman factor in the presence of 40 nM of bovine plasma α_2M.

2.4.2. Bradykinin generation through conversion of prekallikrein to kallikrein

VVP (4 nM) and Hageman factor (68 nM) were allowed to react at 30°C for 20 min, and then, VVP was inactivated. This solution (0.25 ml) containing VVP-activated Hageman factor was added to 0.25 ml of the mixture of prekallikrein (3.6 pmol) and kininogen (100 pmol) or heat-treated human plasma (0.25 ml) supplemented with 3.6 pmol of prekallikrein. After the incubation at 30°C for 10 min, the reaction was stopped, and the bradykinin content in the supernatant was determined.

3. RESULTS AND DISCUSSION

3.1. *VVP action to peptide-MCA substrate*

Almost all metalloproteases including *P. aeruginosa* elastase have been known to be 'N type' proteases because these enzymes hydrolyze the peptide bond at amino group side of the P_1' amino acid residue [16,17]. However, some bacterial metalloproteases such as *P. aeruginosa* alkaline proteinase were recently reported to be 'C type' proteases which hydrolyze the peptide bond at carboxy group side of P_1 amino acid residue [18]. So, this type metalloprotease possess the amidolytic activity toward various kinds of peptide-MCA substrates. Therefore, we initially examined the action of VVP to ten kinds of peptide-MCA substrates including Z-FR-MCA and Boc-QGR-MCA. VVP (200 nM) was incubated with 0.2 mM of the substrate at 30°C for 20 min, and the liberation of AMC from the substrate was measured. VVP did not hydrolyze any of the substrates, indicating that VVP, like *P. aeruginosa* elastase, is one of the 'N type' metalloproteases.

3.2. *Generation of plasma kallikrein by VVP*

VVP was added to diluted human plasma, and generation of the amidolytic activity toward Z-FR-MCA was examined (Table I). Since diluted normal human plasma employed for the experiments contained 150 nM of active α_2M, a sole plasma inactivator for VVP [19,20], generation of the amidolytic activity was very poor when 100 nM VVP was added to control IgG-treated normal plasma, while in the study using normal plasma treated with anti-α_2M IgG, steady generation of the hydrolytic activity toward the substrate was observed after the addition of 10 nM of VVP. On the other hand, no amidolytic activity was detected when VVP was allowed to act on prekallikrein-deficient plasma, indicating that the observed amidolytic activity toward Z-FR-MCA was attributed to the activity of plasma kallikrein generated by VVP.

To confirm the generation of plasma kallikrein by VVP, a mixed solution of human Hageman factor (68 nM), plasma prekallikrein (36 nM) and kininogen (150 nM) was incubated with VVP (0–20 nM) at 30°C, and the generation of plasma kallikrein was measured by using Z-FR-MCA. VVP generated the plasma kallikrein in a dose- and time-dependent manner (data not shown).

3.3. *Direct activation of human plasma prekallikrein by VVP*

Diluted Hageman factor-deficient plasma or purified prekallikrein was incubated with VVP, and the resulting generation of the amidolytic activity toward Z-FR-MCA was measured. VVP was demonstrated to activate prekallikrein and to generate kallikrein in a dose- and time-dependent manner (Fig. 1). Then, we examined the bradykinin-releasing ability of VVP-generated plasma kallibrein. Plasma kallikrein generated by the incubation with VVP was mixed with kininogen or heat-treated human plasma (the crude kininogen preparation). This mixture was incubated at 30°C and the amount of bradykinin liberated from kininogen was determined. VVP-generated kallikrein was shown to re-

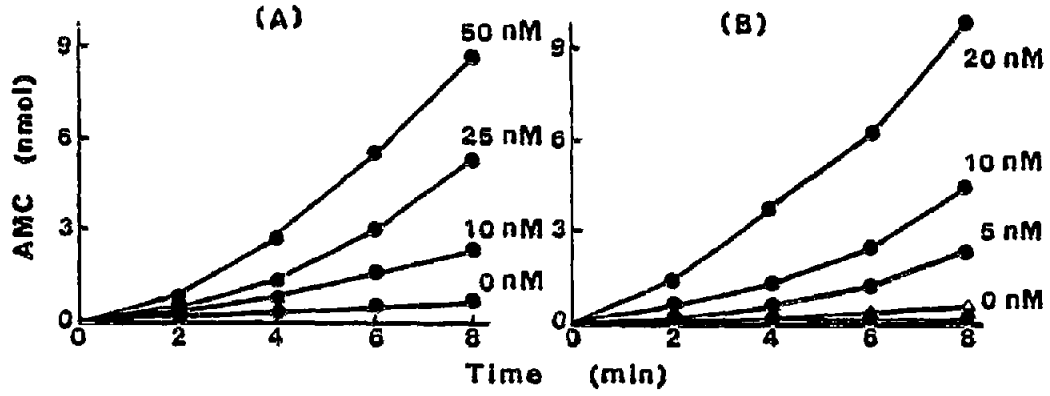

Fig. 1. Activation of human plasma prekallikrein by VVP. Two series of experiments, which yielded very similar results, were performed, and the results presented are from one series of experiments. (A) Hageman factor-deficient human plasma (0.1 ml) treated with anti-α_2M IgG was diluted to 10-fold with the assay buffer and was incubated with VVP (0–50 nM) at 30°C in the presence of Z-FR-MCA, and AMC liberation from the substrate was measured. (B) VVP (0–20 nM) was added to purified human prekallikrein (36 nM) in a total of 1 ml in the assay buffer. After the addition of Z-FR-MCA, AMC liberation from the substrate was measured at 30°C. The line with triangles denote the time-course of the prekallikrein activation observed with 20 nM VVP in the presence of 40 nM α_2M.

lease significant amounts of bradykinin from both purified and crude human kininogen preparations (data not shown). Thus, it is possible to conclude that VVP directly acts on the human plasma prekallikrein and converts it to kallikrein which is able to interact with both the synthetic peptide substrate (Z-FR-MCA) and the natural protein substrate (high-molecular-weight kininogen).

3.4. *Direct activation of human Hageman factor by VVP*

Diluted prekallikrein-deficient plasma or purified Hageman factor was incubated with VVP, and the generation of the amidolytic activity toward Boc-QGR-MCA was measured. As shown in Fig. 2, significant generation of the amidase activity was observed by the addition of VVP, indicating direct activation of this human zymogen by VVP. Besides, the ability of VVP-activated Hageman factor to convert plasma prekallikrein to kallikrein was studied. VVP-activated Hageman factor was added to the mixture of prekallikrein and kininogen or heat-treated human plasma supplemented with prekallikrein. Thereafter, this solution was incubated at 30°C for 10 min, and the amount of bradykinin generated was determined. The results indicated that VVP-activated Hageman factor generated bradykinin through conversion of plasma prekallikrein to active enzyme (data not shown). These findings show that the human Hageman factor, like plasma prekallikrein, is directly activated by the VVP and the VVP-activated one possesses the capacity to act on both peptide and protein substrates.

3.5. *Inhibitory effect of α_2M*

The data shown in Table I suggests that VVP added to human plasma is immediately inactivated by plasma α_2M even in the presence of target human zymogens. Because the activity of human α_2M obtained commercially was very low, to clarify the inhibitory effect of the α_2M, the zymogen-activating ability of VVP was studied in the presence of bovine plasma α_2M. By the addition of the inhibitor, the hydrolytic action toward the peptide-MCA substrate was decreased drastically (Figs. 1 and 2). Since the plasma α_2M had no effect on the amidolytic activities of activated Hageman factor (Protogen AG, Switzerland) and plasma kallikrein (Protogen AG), these results indicate quick inactivation of VVP by this plasma inhibitor resulting in the decreased activation of human zymogens. From these findings, it can be suggested that the VVP is able to act on the human zymogens only at the interstitial-tissue space in

Table I

Generation of plasma kallikrein in human plasma by VVP*

Plasma	IgG	VVP (nM)	Hydrolysis of Z-FR-MCA (nmol of AMC liberated)
Normal plasma	Control IgG	0	1.2 ± 0.26
		50	1.2 ± 0.15
		100	2.5 ± 0.13
Normal plasma	Anti-α_2M IgG	0	1.6 ± 0.19
		10	4.2 ± 0.19
		25	7.7 ± 0.39
Prekallikrein-deficient plasma	Anti-α_2M IgG	0	0.0
		100	0.0

*Human plasma (0.1 ml) preincubated with control IgG or anti-α_2M IgG was diluted with 0.9 ml of the assay buffer. This 10-fold diluted plasma was incubated with an appropriate concentration of VVP at 30°C for 5 min in the presence of 0.2 mM Z-FR-MCA, and the extent of hydrolysis of the substrate was determined. Results are the means ± S.D. (n = 3 or 4).

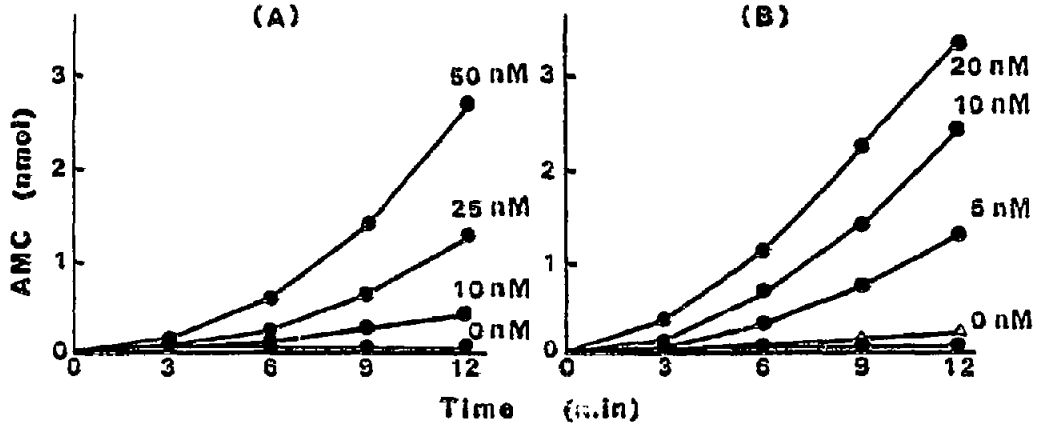

Fig. 2. Activation of human Hageman factor by VVP. Two series of experiments, which yielded very similar results, were performed, and the results presented are from one series of experiments. (A) Prekallikrein-deficient human plasma (0.1 ml) treated with anti-α_2M IgG was diluted to 10-fold with the assay buffer. This diluted plasma (1 ml) and an appropriate concentration of VVP were incubated at 30°C in the presence of Boc-QGR-MCA (the synthetic substrate for activated Hageman factor), and liberation of AMC from the substrate was measured. (B) VVP (0–20 nM) was added to purified human Hageman factor (68 nM) in a total of 1 ml in the assay buffer. After the addition of Boc-QGR-MCA, AMC liberation from the substrate was measured at 30°C. The line with triangles denotes the time-course of the prekallikrein activation observed with 20 nM VVP in the presence of 40 nM α_2M.

which negligible amount of α_2M is contained [20,21] or in the bloodstream of the individuals whose plasma level of the inhibitor is extremely reduced.

3.6. *Concluding remarks*

Recently, Molla et al. [10] studied the action modes of some bacterial metalloproteases to guinea pig Hageman factor–plasma prekallikrein–kinin system and divided them into two groups: one activates Hageman factor but not plasma prekallikrein, and the other activates both zymogens. However, this grouping may not be applicable to the human system, since two metalloproteases from *P. aeruginosa* were shown to activate human system by different mechanisms from those clarified in the guinea pig system [11,12]. In contrast to *Pseudomonas* proteases, VVP was found to activate the human system by the same mechanism as that in the guinea pig system. VVP acted directly on both human zymogens, and plasma kallikrein generated by direct and indirect actions of VVP liberated bradykinin from high-molecular-weight kininogen.

V. vulnificus is the causative agent of wound infection and primary septicemia [1–4]. The wound infection is characterized by the development of edema and erythema around a new wound exposed to seawater. The primary septicemia is often accompanied by secondary skin lesions, such as bullae and necrotic ulcer. In addition, extreme hemo concentration due to edema fluid accumulation has been reported experimentally in mice injected with living bacterial cells [22]. Therefore, the factor(s) that possess(es) the ability to enhance vascular permeability and to form edema appear(s) to be an important virulence factor of the vibrio. Previously, VVP was demonstrated to be the most probable edema-forming factor produced by the organism [6]. However, any evidence for in vivo VVP production has not been obtained. Results obtained in the present study seem to support our current hypothesis on the contribution of VVP in *V. vulnificus* infections. Namely, vibrio invades into the interstitial-tissue space and elaborates VVP, which induces the generation of mediator(s) for inflammation, enhancement of vascular permeability and leakage of plasma constituents. Since the plasma from the healthy body includes sufficient α_2M, the VVP is immediately inactivated by forming the VVP-α_2M complex, which is rapidly excluded by receptor-mediated endocytosis. However, the inflammatory activity of VVP may be prolonged in individuals whose plasma α_2M level is reduced by some underlying diseases or severe septicemia [23].

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