

Purification and characterization of a novel alkaline serine protease secreted by *Vibrio metschnikovii*

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Abstract. A novel extracellular alkaline serine protease secreted by *Vibrio metschnikovii* (*V. metschnikovii*) ATCC700040 cells was purified by three chromatographic steps and characterized in terms of enzymatic kinetics and substrate specificity. The purified enzyme (named AKP-Vm) was composed of a single polypeptide with an apparent molecular weight of 50 kDa on 12% SDS-polyacrylamide gel in the presence of CuCl₂. The optimal temperature and the pH for the enzyme were found to be 37°C and 9.5, respectively. However, the enzyme activity was inhibited by inhibitors such as PMSF and aprotinin. AKP-Vm could hydrolyze a peptide bond at the carboxyl side of the arginine residue, as revealed by its amidolytic activity toward a chromogenic substrate, Boc-Val-Pro-Arg-pNA. The kinetic parameters of the enzyme were as follows: $K_M=0.91$ mM, $k_{cat}=0.8$ sec⁻¹ and $k_{cat}/K_M=0.88$ mM⁻¹sec⁻¹. AKP-Vm protease could cleave various blood coagulation-associated proteins, including fibrinogen, prothrombin and thrombin. In particular, the enzyme showed powerful fibrinogenolytic and fibrinolytic activities, as it could cleave all major chains of fibrinogen and also digest cross-linked fibrin. The results obtained suggest that AKP-Vm is a novel alkaline serine protease that can actively cleave fibrinogen and cross-linked fibrin.

Introduction

The pathogenic bacteria *Vibrios* produce various virulent factors, including adhesin, polysaccharide, enterotoxin,

cytotoxin, hemolysin and proteases (1). In particular, extracellular proteases produced by *Vibrio vulnificus* (*V. vulnificus*), *V. mimicus*, and *V. cholerae* have been known to play various roles in bacterial infection. For example, they enhance vascular permeability through the activation of the plasma kallikrein-kinin system (2-4). An extracellular protease from *V. fluvialis* exhibits haemagglutinating and haemorrhagic activities (5). A metalloprotease (named vEP) secreted by *V. vulnificus* affects the human blood coagulation system through prothrombin activation and fibrinolysis (6).

V. metschnikovii is a gram-negative alkalophilic human pathogenic bacterium (1,7,8). This facultative bacterium is often found in natural aquatic environments such as seawater (9). In addition, the bacterium has been known as one of causative agents that can induce dyspneic symptoms (10), diarrhea (11,12), wound infection (13), pneumonia (1) and leg ulcer (9). There have been brief reports on alkaline proteases (AprB, AprJ1, VapT and VapK) from a few *V. metschnikovii* strains. AprB, VapT and VapK proteases show SDS-resistance activity (8,14-16) and AprJ1 exhibits a stability toward non-ionic surfactant and oxidizing agents (17). However, there are no detailed studies on the biochemical properties and fibrin(ogen)olytic activity of the alkaline proteases secreted by *V. metschnikovii*.

In this study, we purified and characterized a novel extracellular alkaline serine protease, AKP-Vm (alkaline protease of *V. metschnikovii*) from *V. metschnikovii* ATCC700040 cells. We describe the biochemical properties of AKP-Vm in terms of its enzyme kinetics and substrate specificity toward various human blood coagulation-associated proteins including plasminogen, plasmin, fibrinogen, fibrin polymer and cross-linked fibrin.

Materials and methods

Materials. *V. metschnikovii* ATCC700040 cells were obtained from the Korean Culture Center of Microorganism (KCCM; Seoul, Korea). The columns HiPrep 16/10 Q FF, Source 15 Q 4.6/100 PE, Superdex 75 10/300 GL, and PD-10 were purchased from Amersham Biotech (Uppsala, Sweden). Ammonium sulfate [(NH₄)₂SO₄] was from Daejung Chemicals (Siheung, Korea). The YM-10 membranes were purchased from Millipore (Billerica, MA, USA). Plasmin was from Roche Applied Science (Mannheim, Germany). Prothrombin and thrombin were obtained from Calbiochem (Daemstadt,

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Abbreviations: 1,10-PT, 1,10-phenanthroline; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; LB, Luria-Bertani medium; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Key words: *Vibrio metschnikovii*, extracellular alkaline serine protease, fibrinolytic enzyme, fibrinogenolytic enzyme, alkaline protease

Table I. Summary for the purification of AKP-Vm protease secreted by *V. metschnikovii* ATCC700040 cells.

Purification step	Total protein (mg)	Total activity (units) ^a	Specific activity (U/mg)	Yield (%) ^b
Ammonium sulfate	257.4	18,412	71.5	100
HiPrep 16/10 Q FF	21.6	2,922	135.3	8.4
Source 15 Q 4.6/100 PE	3.8	1,726	454.4	1.5
Superdex 75 10/300 GL	0.2	240	1,000	0.1

^aOne unit (U) was defined as the amount of enzyme that catalyzes the release of 1 μ mol of *p*NA per min. ^bTotal activity of total extracellular proteins was assigned the value of 100%.

Germany). Human factor XIIIa was from Haematologic Technologies, Inc. (Essex Junction, VT, USA). Human fibrinogen, aprotinin, bestatin, EDTA, EGTA, 1,10-phenanthroline (1,10-PT), β -mercaptoethanol, PMSF, diisopropyl fluorophosphate (DFP) and DTT were from Sigma (St. Louis, MO, USA). Protein molecular weight size markers were from Fermentas GmbH (St. Leon-Rot, Germany). Synthetic chromogenic substrates, Boc-Leu-Gly-Arg-*p*NA and Boc-Val-Pro-Arg-*p*NA were from Seikagaku (Tokyo, Japan). Other chromogenic substrates, including Ile-Glu-(*-OR*)-Gly-Arg-*p*NA (S-2222), H-D-Phe-Pip-Arg-*p*NA (S-2238), H-D-Val-Leu-Lys-*p*NA (S-2251), H-D-Ile-Pro-Arg-*p*NA (S-2288), Pyro-Glu-Gly-Arg-*p*NA (S-2444) and N- α -Z-D-Arg-Gly-Arg-*p*NA (S-2765) were from Chromogenix (Milan, Italy).

Purification of protease. *V. metschnikovii* ATCC700040 cells were cultivated in LB medium (pH 7.5) containing 3% NaCl at 30°C, and extracellular proteins contained in the culture supernatant were fractionated by 70% ammonium sulfate in a saturation concentration. The precipitated proteins were harvested by centrifugation at 16,000 \times g for 30 min at 4°C and dissolved in 25 mM Tris-HCl buffer (pH 7.5). The proteins were desalted on a PD-10 column and applied onto a HiPrep 16/10 Q FF column equilibrated with 25 mM Tris-HCl (pH 7.5). The bound proteins were eluted with a linear gradient of NaCl (0-0.8 M) at a flow rate of 2.0 ml/min. Fractions showing proteolytic activities on a casein plate were pooled and concentrated by YM-10 membrane. The concentrated proteins were then applied onto a Source 15 Q 4.6/100 PE column equilibrated with 25 mM Tris-HCl (pH 7.5) and the bound proteins were eluted with a linear gradient of NaCl (0-0.5 M) at a flow rate of 1.0 ml/min. Active fractions were pooled and further fractionated on a Superdex 75 10/300 GL column using 25 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl. Active fractions were pooled, concentrated, and used as a purified enzyme. In each purification step, protein concentrations were determined with the Bradford reagent according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (18). Samples to be analyzed were mixed with an equal volume of 2X SDS-PAGE sample buffer, boiled at 100°C for 2 min, and then loaded onto the gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie Brilliant Blue as described elsewhere.

Protease activity assay. Azocasein assay was routinely used for determining protease activity in purification steps (6). In this assay, a reaction mixture (200 μ l) consisting of enzyme to be tested and 0.25% azocasein in 50 mM Tris-HCl buffer (pH 9.5) was incubated at 37°C for 15 min and the reaction was stopped by the addition of 100 μ l of 10% trichloroacetic acid. After centrifugation at 13,000 \times g for 10 min, the resulting supernatant was withdrawn and the absorbance was measured at 440 nm. Protease activity assayed with synthetic chromogenic substrates was performed in a 96-well plate reader (Molecular Devices). Reaction mixture (total 100 μ l) containing enzyme to be assayed, 0.4 mM chromogenic substrate, 20 mM Tris-HCl (pH 9.5), 0.1 mg/ml BSA and 0.9% NaCl was incubated at 37°C and the absorbance at 405 nm was continuously monitored over a period of 30 min. One unit of protease activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of *p*NA per min. The following buffer systems were used for pH requirement of enzyme: 20 mM sodium acetate (pH 4.0 to 5.0); 20 mM sodium phosphate (pH 6.0 to 7.5); 20 mM Tris-HCl (pH 8.0 to 9.0); 20 mM glycine-NaOH (pH 9.5 to 11.0); 20 mM sodium carbonate (pH 11.5 to 12.0).

Effect of divalent cations on heat-induced degradation of AKP-Vm. AKP-Vm enzyme were mixed with 10 mM each of various divalent cations (CaCl₂, MnCl₂, MgCl₂, ZnCl₂, NiCl₂ and CuCl₂) in 6X reducing sample buffer (300 mM Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, and 0.012% bromphenol blue) and boiled for 2 min at 100°C. The protein samples were then electrophoresed on 12% polyacrylamide gel and the protein bands were visualized by staining the gel with Coomassie Brilliant Blue as described elsewhere.

Prothrombin activation by AKP-Vm. For the detection of prothrombin activation, 200 μ l of reaction mixture (25 mM Tris-HCl, pH 9.5, 0.4 mg/ml prothrombin, 0.5 μ g enzyme to be tested) was incubated at 37°C and 24 μ l aliquots were withdrawn at different time intervals. The reaction was stopped by the addition of 1 μ l of 24 mM CuCl₂. To measure thrombin activity, 10 μ l of sample was assayed with 0.4 mM of Boc-Val-Pro-Arg-*p*NA as a substrate in 25 mM Tris-HCl, pH 7.5, 0.9% NaCl, 1 mM CuCl₂ and 0.1 mg/ml BSA in a 100 μ l reaction volume at 37°C using a 96-well plate reader. The increase in absorbance at 405 nm was recorded over a period of 10 min. For the detection of fibrin polymerization, 10 μ l of the prothrombin digest was mixed with 190 μ l of 25 mM Tris-HCl

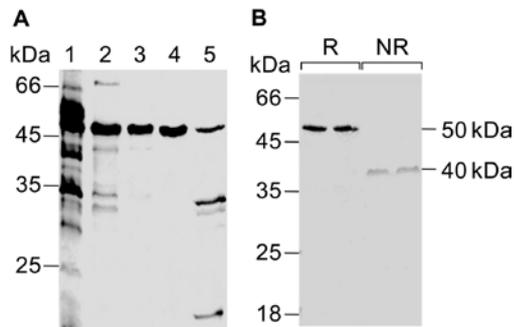


Figure 1. SDS-PAGE of purified AKP-Vm. (A) The active fraction obtained from each chromatographic step was subjected to SDS-PAGE under reducing condition. Protein samples were prepared in the presence (lanes 1-4) or absence (lane 5) of 1 mM of CuCl_2 . Lanes 1, total extracellular proteins; 2, HiPrep 16/10 Q FF; 3, Source 15 Q 4.6/100; 4 and 5, Superdex 75 10/300 GL. (B) The purified enzyme was sampled under reducing (R) and non-reducing (NR) conditions as described in Materials and methods, electrophoresed on 12% polyacrylamide gel, and then stained with Coomassie Brilliant Blue for visualization.

buffer (pH 7.5) containing 100 μg fibrinogen and 1 mM CuCl_2 , and the change in turbidity was measured at 350 nm.

Fibrin(ogen)olytic activity assay of Vm-AP. To examine the fibrinogenolytic activity of AKP-Vm, human fibrinogen (30 μg) was dissolved in 25 mM Tris-HCl buffer (pH 9.5) containing 150 mM NaCl and incubated with AKP-Vm enzyme (62.5 ng) at 37°C for various time periods. The reaction products were analyzed by 12% SDS-polyacrylamide gel electrophoresis. The spontaneous polymerization of fibrin monomers derived from AKP-Vm-cleaved fibrinogen was assayed by turbidity assay. In this assay, 90 μl of 1 mg/ml fibrinogen in 25 mM Tris-HCl buffer (pH 7.5) was mixed with 10 μl each of thrombin (10 U/ml) and AKP-Vm (50 $\mu\text{g}/\text{ml}$), and the increase in absorbance at 350 nm was then recorded with a 96-well plate reader (Molecular Devices). For the detection of cross-linked fibrin cleavage by AKP-Vm, fibrinogen (10 μg), thrombin (0.02 units) and factor XIIIa (0.03 units) were mixed in 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1 mM CaCl_2 , and the mixture was incubated at room temperature for 2 h. AKP-Vm (0.5 μg) was then added to the resulting cross-linked fibrin and further incubated at 37°C for 5 min. The resulting products were analyzed by 12% SDS-PAGE. Fibrinolytic activity was also measured using a fibrin plate as previously described (6).

Results

Purification of an extracellular protease from *V. metschnikovii* ATCC700040 cells. An extracellular alkaline serine protease was purified from the culture supernatant of *V. metschnikovii* ATCC700040 cells. To obtain the extracellular proteins, *V. metschnikovii* ATCC700040 cells were cultivated overnight in LB medium containing 3% NaCl at 30°C, and the proteins contained in the culture medium were precipitated with ammonium sulfate in a saturation concentration of 70%. The fractionated proteins were subjected to HiPrep 16/10 Q FF, Source 15 Q 4.6/100 PE, and Superdex 75 10/300 GL columns in order (data not shown), and the finally purified enzyme was named AKP-Vm. The summary of the purifica-

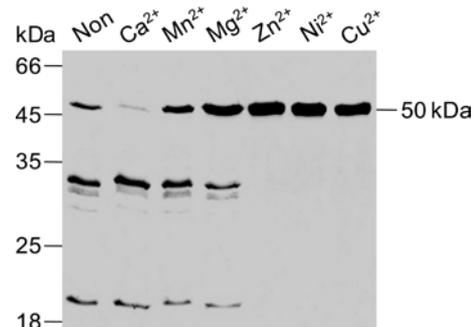


Figure 2. Effects of various divalent cations on the heat-induced degradation of AKP-Vm. Five micrograms of purified AKP-Vm enzyme were boiled at 100°C for 2 min in the absence or presence of CaCl_2 , MnCl_2 , MgCl_2 , ZnCl_2 , NiCl_2 and CuCl_2 as indicated and electrophoresed on 12% polyacrylamide gel under reducing condition. Non, AKP-Vm enzyme without any cation.

tion procedure is shown in Table I. About 0.2 mg of enzyme could be obtained from 1 liter of culture supernatant. The purified enzyme showed a specific activity of 1,000 U/mg protein, which represented ~14-fold increase over the culture supernatant (Table I). Fig. 1A shows the results of SDS-PAGE with proteins obtained from each chromatographic step. The purified enzyme appeared as a single band on SDS-PAGE, having an apparent molecular mass of 50 kDa in the presence of 1 mM of CuCl_2 (Fig. 1A, lanes 2-4). However, the enzyme was degraded into several fragments in the absence of the divalent cation (Fig. 1A, lane 5). This result suggests that the purified enzyme can be degraded by its own proteolytic activity at the boiling step for SDS-PAGE, and this heat-induced degradation can be inhibited by CuCl_2 . The molecular weights of purified AKP-Vm could be estimated to 50 and 40 kDa under reducing and non-reducing conditions, respectively, in the presence of 1 mM CuCl_2 (Fig. 1B). These results suggest that disulfide bonds located in the enzyme may play an important role in maintaining the conformation of enzyme.

Effects of divalent cations on the heat-induced degradation of AKP-Vm. As described in the previous section, AKP-Vm seemed to be degraded into several fragments at the heating step for SDS-PAGE, which could be suppressed by the addition of CuCl_2 . Therefore, it was necessary to examine the effects of various divalent cations on the heat-induced degradation of AKP-Vm. When the purified enzyme was incubated for 2 min at 100°C in the absence or presence of various divalent cations, the heat-induced degradation could be completely blocked by the addition of ZnCl_2 , NiCl_2 and CuCl_2 , but not by CaCl_2 , MnCl_2 and MgCl_2 (Fig. 2). It seemed to be closely related to the proteolytic activity of the enzyme because the event did not occur when the enzyme was inhibited by CuCl_2 and NiCl_2 (Table II). Interestingly, the event also did not occur when the enzyme was incubated with ZnCl_2 , although the divalent cation showed no inhibitory effect on the enzyme activity (Table II). These results suggest that the heat-induced degradation occurs by the enzyme's own proteolytic activity and can be suppressed by inhibiting the enzyme activity. In addition, Zn^{2+} may block the suicidal activity of the enzyme by stabilizing the enzyme's conformation under high temperature to maintain its intrinsic enzyme specificity.

Table II. Effects of various divalent cations, protease inhibitors and chemical reagents on AKP-Vm activity.

Additive	Concentration (mM)	Activity (%) ^a
Control	0	100.0
Ni ²⁺	1	74.6
Mg ²⁺	1	109.4
Mn ²⁺	1	80.4
Cu ²⁺	1	29.9
Zn ²⁺	1	96.4
DFP	1	70.9
PMSF	1	27.4
Aprotinin	1	10.3
Bestatin	1	83.7
1,10-PT	1	81.6
EDTA	1	82.1
EGTA	1	88.0
β-mercaptoethanol	500	42.6
DTT	500	45.1

^aAKP-Vm activity was assayed with Boc-Val-Pro-Arg-pNA as a substrate with or without the corresponding additive at 37°C for 30 min in 20 mM Tris-HCl (pH 9.5) as described in Materials and methods.

Amidolytic activity and kinetic parameters of AKP-Vm protease.

The amidolytic activity of AKP-Vm protease was examined with various synthetic peptide substrates. Of the eight chromogenic substrates examined (Boc-Val-Pro-Arg-pNA, S-2222, S-2238, S-2251, S-2288, S-2444, S-2765, Boc-Leu-Gly-Arg-pNA), Boc-Val-Pro-Arg-pNA and S-2238 (H-D-Phe-Pip-Arg-pNA) could be efficiently hydrolyzed by the enzyme (data not shown). However, the amidolytic activity of AKP-Vm toward S-2238 was 38% lower than that to Boc-Val-Pro-Arg-pNA (data not shown). These results suggest that AKP-Vm can hydrolyze a peptide bond at the carboxyl sides of arginines in those peptide substrates, and the degree of cleavage may be affected by the sequence around the arginine residue. When Boc-Val-Pro-Arg-pNA was used as a substrate, the kinetic values of AKP-Vm were found to be as follows: $K_M=0.91$ mM, $k_{cat}=0.8$ sec⁻¹ and $k_{cat}/K_M=0.88$ mM⁻¹sec⁻¹.

Enzymatic properties of AKP-Vm. The optimal temperature and the pH for AKP-Vm activity were found to be 37°C (Fig. 3A) and pH 9.5 (Fig. 3B), respectively, when Boc-Val-Pro-Arg-pNA was used as a substrate. In addition, the enzyme was relatively active even at pH 12.0 (Fig. 3B), in which ~65% of the enzyme activity was retained, compared to that at pH 9.5. These results suggest that AKP-Vm appears to be a typical alkaline protease. Various divalent cations and protease inhibitors were also tested for their effects on AKP-Vm activity (Table II). Divalent cations such as Mg²⁺ and Zn²⁺ showed not so significant effects on AKP-Vm activity; however, Ni²⁺, Mn²⁺ and Cu²⁺ exhibited an inhibitory effect on AKP-Vm activity at different levels. Cu²⁺ showed a strong inhibitory effect on the enzyme activity with ~70.1% of the enzyme activity inhibited by 1 mM of

CuCl₂, compared to that of the non-treated control (Table II). The enzyme activity could be also inhibited by treatments with PMSF and aprotinin (Table II). However, the metalloprotease inhibitors such as EGTA and 1,10-PT had no so significant effects (Table II). These results suggest that AKP-Vm is an enzyme included in the alkaline serine protease family and is not a metalloprotease. In addition, an average of 56% of the enzyme activity could be inhibited by reducing agents such as β-mercaptoethanol and DTT (Table II), suggesting that a disulfide bond(s) located in the enzyme may play an important role in maintaining the enzyme activity.

Fibrinogenolytic and fibrinolytic activities of AKP-Vm.

AKP-Vm could cleave various plasma proteins, including fibrinogen, plasminogen, plasmin, prothrombin, and thrombin (data not shown). By cleaving prothrombin, the enzyme could make a peptide fragment corresponding to thrombin in size, but there was no detectable thrombin activity, as assayed with a typical chromogenic substrate for thrombin (data not shown). This result suggests that AKP-Vm does not have an ability to activate prothrombin, unlike vEP and the vEP-MO6 proteases of *V. vulnificus* strains (6,19). As with prothrombin, fibrinogen was one of efficient protein substrates for AKP-Vm. The enzyme could cleave all three major chains of fibrinogen, resulting in fibrinogen degrading products (FDPs) (Fig. 4A). The α₂ and β₂ chains of fibrinogen could be totally degraded by AKP-Vm within 1 and 20 min, respectively (Fig. 4A). However, the γ-chain of fibrinogen was required for a longer time to be digested by the enzyme (Fig. 4A). Although AKP-Vm could cleave fibrinogen efficiently, there was no occurrence of spontaneous polymerization of fibrin monomers that might be produced from fibrinogen cleavage by AKP-Vm, as judged by turbidity assay (Fig. 4B). In addition, AKP-Vm exhibited a strong protease activity to cleave cross-linked fibrin that was catalyzed by thrombin in the presence of factor XIIIa and fibrinogen (Fig. 4C and D). Both α chain polymers and γ-γ chains of fibrins were susceptible to cleavage by AKP-Vm, as shown by the fibrin plate assay (Fig. 4D).

Discussion

In this study, we have purified an alkaline serine protease (named AKP-Vm) from the culture filtrate of *V. metschnikovii* ATCC700040 cells and characterized the biochemical properties of purified enzyme in terms of substrate specificity and enzyme kinetics.

The purified enzyme showed heat-labile properties under high temperature (Fig. 1A). This heat-induced degradation seemed to be related with enzyme activity, as the native 50 kDa enzyme remained intact when inhibited by NiCl₂ and CuCl₂ (Fig. 2; Table II). These results suggest that the enzyme can be destabilized under high temperature to acquire non-specificity that can cleave the enzyme itself. Interestingly, the event does not occur in the presence of ZnCl₂ (Fig. 2), even though the divalent cation does not inhibit enzyme activity (Table II). This result also demonstrates that Zn²⁺ may protect the enzyme from the heat-induced loss of enzymatic specificity by stabilizing the conformation of the enzyme to maintain its intrinsic enzymatic specificity. A similar heat-induced degradation can also be observed with the VSPase protease of

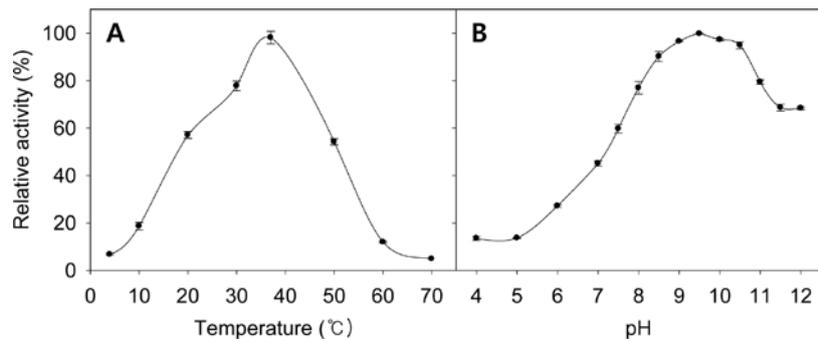


Figure 3. Effects of temperature and pH on AKP-Vm activity. AKP-Vm activity was assayed with Boc-Val-Pro-Arg-pNA as a substrate under various temperatures (A) or pHs (B) as described in Materials and methods.

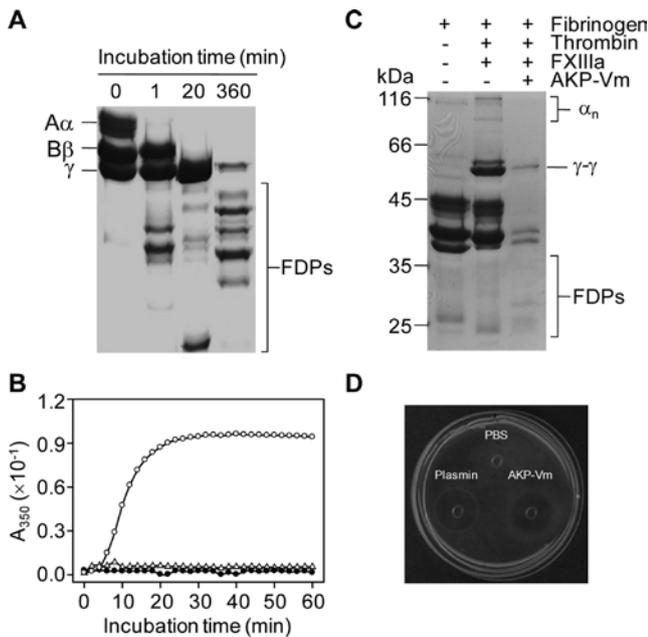


Figure 4. Fibrinogenolytic and fibrinolytic activity of the AKP-Vm enzyme. (A) Fibrinogenolytic activity assay. Fibrinogen (30 μ g) was incubated with 1 μ g of AKP-Vm at 37°C for indicated time periods, and the resulting products were electrophoresed on 12% SDS-polyacrylamide gel. FDPs means fibrinogen degradation products. (B) Turbidity assay for the spontaneous polymerization of fibrin monomers derived from AKP-Vm-cleaved fibrinogen. In this assay, 90 μ l of 1 mg/ml fibrinogen in 25 mM Tris-HCl buffer (pH 7.5) was mixed with 10 μ l each of thrombin (10 U/ml) and AKP-Vm (50 μ g/ml), and the increase in absorbance at 350 nm was then recorded with a 96-well plate reader. \circ , thrombin plus fibrinogen; Δ , AKP-Vm plus fibrinogen; \bullet , fibrinogen only. (C) Fibrinolytic activity assay. Cross-linked fibrin formed by thrombin in the presence of fibrinogen and factor XIIIa (FXIIIa) was incubated with 0.5 μ g of AKP-Vm for 5 min at 37°C, and the reaction products were electrophoresed on 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Symbols + and - represent the addition and the omission, respectively. (D) Fibrinolytic activity of AKP-Vm was also assayed on fibrin plate as described in Materials and methods. Phosphate-buffered saline (PBS), AKP-Vm (1 μ g), and plasmin (0.5 units) were applied on the fibrin plate, and the plate was incubated at 37°C for 12 h. α_n and γ - γ represent α chain polymers and γ chain dimers, respectively.

Staphylococcus aureus (20). The VSPase protease undergoes auto-degradation when it is incubated at a temperature of 55°C or higher, resulting in a complete loss of protein within 10 min of incubation (20). There is another report that an extracellular metalloprotease called vEP from *V. vulnificus* disappears

completely on the polyacrylamide gel when it is pre-incubated at 75°C (6).

There have been a few reports on alkaline proteases secreted by several *V. metschnikovii* strains (15-17). An alkaline serine protease (named AprJ1) shows an amidolytic activity against N-succinyl-Ala-Ala-Pro-Phe-pNA, and its catalytic efficiency (k_{cat}/K_M) is estimated to 7.23x10⁸ mM⁻¹ min⁻¹ (17). The AKP-Vm enzyme exhibits substrate specificity against Boc-Val-Pro-Arg-pNA (Table II) and shows much lower catalytic efficiency (0.5x10² mM⁻¹ min⁻¹) than AprJ1. These substrate specificities of the enzymes indicate that AKP-Vm and AprJ1 can cleave peptide bonds at the carboxyl sides of arginine and phenylalanine residues, respectively. These results also suggest that the two proteases are different from each other in terms of substrate specificity and kinetic values. AKP-Vm enzyme is active under alkali condition ranging from pH 8.0-12.0, and the enzyme activity can be inhibited by inhibitors (Table II) such as PMSF and aprotinin that are known as inhibitors for serine proteases (21). These results support the fact that AKP-Vm belongs to the family of alkaline serine proteases.

Fibrinogenolytic enzymes have been reported from food bacteria (22,23), pathogenic bacteria (24-26), mushroom (27) as well as snake venom (28,29). α -fibrinogenases are often found from snake venoms, and most of them are metalloproteases, which can cleave mainly on the A α and less on both the B β and the γ chains of fibrinogen subunit chains (30,31). A fibrinogenolytic protease secreted from *V. vulnificus* can also rapidly digest the A α chain of fibrinogen (32) and a metalloprotease from *V. cholerae* has an ability to digest three major chains (α , β and γ) of fibrinogen (33). Generally, the fibrinogenolytic serine proteases preferentially cleave the B β chain of fibrinogen with lower activity towards the A α chain (34). However, an alkaline serine protease AKP-Vm enzyme shows more preference to the A α chain than to B β chain in the fibrinogen cleavage (Fig. 4A). Almost all of A α and B β chains of fibrinogen can be digested by the enzyme within 20 min (Fig. 4A). Most of α , β and γ chains of fibrinogen can be digested by the enzyme within 360 min at the mass ratio (enzyme versus fibrinogen) of 1:480. Together with the fibrinogenolytic activity, AKP-Vm is a unique enzyme, in that it has an ability to cleave cross-linked fibrin polymer and cross-linked fibrin. The enzyme can digest actively α chain polymers as well as γ - γ chains of the cross-linked fibrins (Fig. 4). Collectively, AKP-Vm can cleave all major chains of fibrinogen, fibrin polymer as well as cross-linked fibrin,

suggesting that the enzyme has a powerful fibrin(ogen)olytic activity like a few enzymes from *V. vulnificus* (6,19,33).

Acknowledgements

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