2010年 8月
碩士學位 論文

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

朝 鮮 大 學 校　大 學 院
生 命 工 學 科
朴 在 泳

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

Vibrio metschnikovii 균주로부터 분비되는 새로운 알칼리성
세린계열 단백질분해효소의 정제 및 특성 연구

2010年 8月 25日

朝 鮮 大 學 校　大 學 院
生 命 工 學 科
朴 在 泳

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

指圖敎授　李 正 燮

이 論文을 理學碩士 學位申請 論文으로 제출함

2010年 4月

朝 鮮 大 學 校　大 學 院

生 命 工 學 科

朴 在 泳

朴在泳의 碩士學位 論文을 認准함

委員長　朝鮮大學校　敎授　　유 호 진　　印

委　員　朝鮮大學校　敎授　　이 건 호　　印

委　員　朝鮮大學校　敎授　　이 정 섭　　印

2010年 5月

朝 鮮 大 學 校　大 學 院

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

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

Park, Jae Yeong

Advisor : Prof. Lee, Jung Sup, Ph.D

Department of Biotechnology

Graduate School of Chosun university

The marine bacterium *Vibrio metschnikovii* (*V. metschnikovii*) evokes serious symptoms, including pneumonia, leg ulcer, and diarrheal disease when it infects a wound. In this study, a novel extracellular alkaline serine protease was purified from culture supernatant of *V. metschnikovii* ATCC700040 and its biochemical features and effects on human blood coagulation-associated proteins were investigated. The maximal protease activity could be obtained when the bacterium was cultured in LB medium (pH 7.5) containing 3% NaCl at 30℃. To purify a proteolytic enzyme, total extracellular proteins from *V. metschnikovii* cells cultivated in LB medium containing 3% NaCl at 30℃ were precipitated by the addition of 70% ammonium sulfate [$(NH_4)_2SO_4$]. The precipitated proteins were applied onto Hiprep 16/10 Q FF, Source 15 Q 4.6/100 PE, and Superdex 75 10/300 GL columns in order, and Vm-AP (stands for *V. metschnikovii* alkaline

protease) could be purified at the final purification step with near homogeneity. The purified Vm-AP seemed to be degraded by the heating step for SDS-PAGE. However, the degradation could be inhibited by the addition of divalent cations such as $CuCl_2$, $ZnCl_2$, and $NiCl_2$. Vm-AP was composed of a single polypeptide and its apparent molecular weight was found to be approximately 50 kDa under reducing condition in the presence of $CuCl_2$ and about 40 kDa under non-reducing condition on 12% SDS-polyacrylamide gel. Among synthetic chromogenic substrates tested, Boc-VPR-*p*NA that is known as a typical chromogenic substrate for thrombin was the most favorable one for the enzyme. When the chromogenic substrate was used as a substrate, kinetic values for Vm-AP were as follows: K_M = 0.91 mM, k_{cat} = 0.8 s^{-1}, and k_{cat}/K_M = 0.88 $mM^{-1}s^{-1}$. The optimal temperature and the pH for Vm-AP enzyme activity were 37℃ and pH 9.5, respectively. In addition, the maximal protease activity could be found under alkalic condition ranging from pH 8.0 to 12.0, suggesting that Vm-AP can be included in alkaline protease family. Approximately 81.1% of Vm-AP activity could be inhibited by the addition of serine protease inhibitors such as PMSF and aprotinin. These results suggest that Vm-AP is a member of serine protease. Vm-AP could cleave various blood coagulation-associated proteins, including plasminogen, plasmin, prothrombin, and thrombin. In addition, the enzyme showed a powerful fibrin(ogen)olytic activity, as it could cleave all kinds of chains of fibrinogen, even with ɣ-chain of fibrinogen, fibrin polymer, and cross-linked fibrin. Taken together, the results obtained by the present study suggest that the pathogenic bacterium *V. metschnikovii* might disturb the blood coagulation system by secreting an alkaline Vm-AP protease during the course of bacterial infection.

I. INTRODUCTION

The bacterium in the genus *Vibrio* is a curved, rod-shaped, and motile microorganism with a single flagellum. Among more than 70 *Vibrio* species, 11 species are known to be pathogenic to humans (Janda *et al*., 1988; Miyoshi, 2006). Alkalophilic *V. metschnikovii* is a member of the human pathogenic *Vibrios* and shares common features of the genus *Vibrio* (Kwon *et al*., 1994). This facultative anaerobic Gram-negative bacterium is ubiquitous in natural aquatic environments including marine waters and sewage, as well as shellfish, birds, poultry, and domestic ungulates (Pariente Martin *et al*., 2008). The first case of human pathogenesis induced by *V. metschnikovii* has been reported from a 82-year-old female who suffered from abdominal pain and cholecystitis (Jean-Jacques *et al*., 1981). Later then, the bacterium was reported as a causative agent to induce dyspneic symptoms (Hansen *et al*., 1993), diarrhea disease (Magalhaes *et al*., 1996; Dalsgaard *et al*., 1996), wound infection (Linde *et al*., 2004), pneumonia (Wallet *et al*., 2005), and leg ulcer (Pariente Martin *et al*., 2008).

Human pathogenic *Vibrios* can use various virulent factors to induce various symptoms, including flagellum, adhesin, polysaccharide, enterotoxin, cytotoxin, hemolysin, protease, and several factors during the course of its infection. Among these virulent factors, the protease(s) can act as a major factor for vibrionic diseases. Extracellular proteases produced from *V. vulnificus*, *V. mimicus*, and *V. cholerae* can enhance vascular permeability through activation of the plasma kallikrein-kinin system (Miyoshi *et al*., 1988; Chowdhury *et al*., 1991; Sakata *et al*., 1996). An extracellular metalloprotease named vEP secreted from *V. vulnificus* can affect on human blood coagulation system by prothrombin activation and fibrinolysis (Chang *et al*., 2005). Collagenases secreted from *V. vulnificus* (Smith *et al*., 1982; Miyoshi *et al*., 2008) and *V. parahaemolyticus* (Miyoshi *et al*., 2008) have been reported to induce tissue-damage potentially. A protease secreted from *V. fluvialis* shows haemagglutinating, haemorrhagic activities in addition to proteolytic

activity toward oligopeptide, casein or elastin (Miyoshi *et al*., 2002).

Until now, four kinds of alkaline proteases secreted from *V. metschnikovii* have been studied in terms of their biochemical properties. Alkaline serine proteases named AprB, VapT, and VapK from RH530 strain show SDS-resistible activity (Kwon *et al*., 1992; Kwon *et al*., 1994; Kwon *et al*., 1995; Chung *et al*., 2001), and other alkaline serine protease named AprJ1 secreted from J1 strain exhibits a stability toward non-ionic surfactant and oxidizing agents (Jellouli *et al*., 2009).

In this study, a novel alkaline protease (designated to as Vm-AP) was purified and characterized from the culture supernatant of *V. metschnikovii* in terms of its biochemical properties, including enzyme kinetics and substrate specificity toward various human blood coagulation-associated proteins including plasminogen, plasmin, prothrombin, thrombin, fibrinogen, fibrin polymer, and cross-linked fibrin catalyzed by factor XIIIa.

II. MATERIALS AND METHODS

II-1. Materials

V. metschnikovii ATCC700040 (KCCM 41681) was obtained from Korean Culture Center of Microorganism (KCCM). Bacto tryptone, bacto yeast extract, and sodium chloride were purchased from Becton Dickinson (Franklin lake, USA), Gellix (Chungbuk, Korea), Junsei (Kyoto, Japan), and Difco (Detroit, USA). Ammonium sulfate [$(NH_4)_2SO_4$] was obtained from Daejung Chemicals (Siheung, Korea). Hiprep 16/10 Q FF, Source 15 Q 4.6/100 PE, and Superdex 75 10/300, and PD-10 columns were from Amersham Biotech Corp. (Uppsala, Sweden). The synthetic chromogenic substrates used in this study are listed in Table 1. EDTA (ethylenediamintetraacetic acid), EGTA (ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid), TLCK (tosyl-L-lysine chlomethyl ketone), TPCK (tosyl-L-phenylalanine chloromethyl ketone), 1,10-PT (1,10-phenanthroline), β-mercaptoethanol, PMSF (phenylmethanesulphonylfluoride), and DTT (dithiothreitol) were purchased from Sigma (St. Louis, Mo, USA). Prothrombin and thrombin were obtained from CalBiochem (Daemstadt, Germany). Plasminogen and plasmin were from Roche Applied Science (Mannheim, Germany). Human factor XIIIa was purchased from Haematologic Technologies Inc. (Essex, USA). Human fibrinogen and other chemicals were obtained from Sigma (St. Louis, Mo, USA).

Table 1. Synthetic chromogenic substrates used in this study.

Substrate	Sequence	Susceptibility
S-2222	Co-Ile-Glu-(OR)Gly-Arg-*p*NA	Factor Xa
S-2238	H-D-Phe-Pip-Arg-*p*NA	Thrombin
S-2251	H-D-Val-Leu-Lys-*p*NA	Plasmin and plasminogen
S-2288	H-D-Ile-Pro-Arg-*p*NA	t-PA and broad serine proteases
S-2444	Glu-Gly-Arg-*p*NA	Urokinase
S-2765	Z-D-Arg-Gly-Arg-*p*NA	Factor Xa
LGR	Boc-Leu-Gly-Arg-*p*NA	Factor Xa
VPR	Boc-Val-Pro-Arg-*p*NA	Thrombin

* S-2222, S-2238, S-2251, S-2288, S-2444, and S-2765 were obtained from Chromogenix Corp. (Washington, USA), and LGR and VPR were obtained from Seikagaku Corp. (Tokyo, Japan).

II-2. Cell growth and protease production

To determine correlation between cell growth and protease(s) production, *V. metschnikovii* cells were cultivated at various growth temperatures (25, 30, or 37℃). A single colony was cultivated in 5 ml of LB (pH 7.5) medium containing 3% NaCl at 30℃ until OD_{600} value reached to 1.0 and then 1 ml of the pre-cultured cells was inoculated in 50 ml of the same fresh medium. The culture was continued at 25, 30, or 37℃ for 20 h and the cell density was monitored by observing OD_{600} value at every 2 h interval using UV visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

V. metschnikovii cells were also cultivated in 3 ml of various kinds of growth media such as LB containing 1% or 3% NaCl, or 2YT media. In addition, to examine effects of various pHs of media on protease production, the cells were cultivated in pH 6.8, 7.5, 9.5, or 10.5 of LB media containing 3% NaCl at 30℃. The proteolytic activities contained in the culture supernatants were assayed with azocasein as a substrate according to the method described in section II-3.

II-3. Proteolytic activity assay from culture supernatant

The culture supernatants described above were collected by centrifugation at 16,000 x*g* for 10 min and proteolytic activities contained in the culture supernatants were assayed with azocasein as a substrate. Each 100 μl of reaction mixture was contained 60 μl of the culture supernatant and 40 μl of 1% azocasein dissolved in 50 mM Tris-HCl (pH 9.0), and these mixtures were incubated at 37℃ for 1 h. The reactions were terminated by the addition of 100 μl of 10% trichloroacetic acid solution. After centrifugation at 16,000 x*g* for 15 min, 100 μl of the supernatant was withdrawn and the absorbance was measured at 440 nm with 96-well plate reader (Molecular Device Corporation, California, USA).

II-4. Purification of a novel alkaline protease

V. metschnikovii ATCC700040 cells were cultivated at 30℃ for 20 h in 6 liters of LB media containing 3% NaCl. The culture supernatant was collected by centrifugation at 6,000 x*g* and 4℃ for 30 min, and ammonium sulfate was added to 70% saturation (474 grams per 1 liter of the culture supernatant). The precipitates were collected by centrifugation at 16,000 x*g* and 4℃ for 30 min. The pellet was dissolved in appropriate volume of 25 mM Tris-HCl (pH 7.5) and desalted on PD-10 columns equilibrated with the same buffer. The desalted sample was applied onto a Hiprep 16/10 Q FF column pre-equilibrated with 25 mM Tris-HCl (pH 7.5). The bound proteins were eluted with a linear gradient of NaCl from 0 to 0.8 M at the flow rate of 2.0 ml/min. The fractions having proteolytic activities on azocasein were pooled and concentrated by YM-10 membrane (Millipore, MA, USA). The concentrated proteins were applied onto a Source 15 Q 4.6/100 PE column pre-equilibrated with 25 mM Tris-HCl (pH 7.5) and the bound proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M at the flow rate of 1.0 ml/min. The eluted proteins were pooled and further fractionated on a Superdex 75 10/300 GL column pre-equilibrated with 25 mM Tris-HCl (pH 7.5) containing 200 mM NaCl. The active fractions from the final purification step were pooled and concentrated, and used as the purified enzyme.

II-5. SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed according to the method of Laemmli (1970). Protein samples taken from each purification step were heated at 100℃ for 2 min with 6x reducing sample buffer and 1 mM of $CuCl_2$, and then loaded onto 12% SDS-polyacrylamide gel. After electrophoresis, the protein bands were visualized by staining with Coomassie brilliant blue. The protein molecular weight marker was used as follows: β -galactosidase of *E. coli* (116 kDa), ovalbumin of chicken egg white (45 kDa),

lactate dehydrogenase of porcine muscle (35 kDa), restriction enzyme Bsp98I of *E. coli* (25 kDa), β-lactoglobulin of bovine milk (18.4 kDa), lysozyme of chicken egg white (14.4 kDa).

II-6. Effects of divalent cations on heat-degradation of Vm-AP

Five micrograms of the purified Vm-AP were mixed with 10 mM of various divalent cations ($CaCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $NiCl_2$, or $CuCl_2$) and 6x reducing sample buffer. The enzyme samples were heated at 100℃ for 2 min and then electrophoresed onto 12% SDS-polyacrylamide gel.

II-7. Zymographic assay

Total extracellular proteins (2.5 μg) and purified Vm-AP (0.3 μg) were loaded onto the zymographic gels containing 1% casein and 1% gelatin with non-reducing sample buffer. These assays were proceeded on ice to abolish proteolytic activities of the proteases during those migrations on the zymographic gels. After the electrophoresis, SDS contained in the gels were removed by shaking gently in 5% Triton X-100 solution for 30 min and 2 times. The proteases were activated by incubating at 37℃ for 30 min in 50 mM Tris-HCl (pH 9.0) and the gels were stained with Coomassie brilliant blue.

II-8. Substrate specificity of Vm-AP

The substrate specificity of Vm-AP was determined in 100 μl of reaction mixture composed of 20 mM Tris-HCl (pH 9.0) and 0.4 mM chromogenic substrate in a 96-well plate. The synthetic chromogenic substrates were used as follows: Boc-VPR-*p*NA and S-2238 for thrombin; Boc-LGR-*p*NA, S-2222 and S-2765 for factor Xa; S-2251 for plasmin and plasminogen; S-2288 for t-PA and broad serine

proteases; S-2444 for urokinase. The reaction mixtures were incubated at 37℃ for 30 min and the hydrolysis of chromogenic substrates was detected by measuring the absorbance at 405 nm with 96-well plate reader.

II-9. Effects of temperature and pH on Vm-AP enzyme activity

To determine temperature dependency of Vm-AP activity, one microgram of purified Vm-AP was incubated with 0.4 mM Boc-VPR-*p*NA as a substrate in 20 mM Tris-HCl (pH 9.0) for 30 min at various temperature (4, 10, 20, 30, 37, 50, 60, and 70℃). pH dependency of the enzyme was also investigated with following various buffer systems: 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). The reaction mixtures were contained 1 μg of Vm-AP and 0.4 mM Boc-VPR-*p*NA as a substrate in the various buffer systems, and incubated 37℃ for 30 min. Vm-AP activities were determined by measuring the increased absorbance at 405 nm induced by hydrolysis of Boc-VPR-*p*NA.

II-10. Effects of various inhibitors and divalent cations on Vm-AP activity

One microgram of Vm-AP was pre-incubated with 1 mM of various protease inhibitors (DFP, TLCK, TPCK, EDTA, 1,10-PT, bestatin, aprotinin, PMSF, or EGTA), 500 mM of reducing reagents (DTT or β-mercaptoethanol), or 1 mM of various divalent cations ($CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, or $ZnCl_2$) in 20 mM Tris-HCl (pH 9.0) at 37℃ for 10 min. To determine effects of these additives on Vm-AP enzyme activity, 0.4 mM Boc-VPR-*p*NA was added on the reaction mixtures as a substrate, and then the reaction mixtures (100 μl) were incubated at 37℃ for 30 min. Residual activities of Vm-AP were determined by measuring the

absorbance at 405 nm and compared to that of control without additive.

II-11. Effects of detergents on Vm-AP activity

To analyze effects of detergents on Vm-AP activity, 1 μg of the enzyme was pre-incubated with various concentrations of SDS (1, 5, or 10%) or urea (1, 2.5, or 5 M) in 20 mM Tris-HCl (pH 9.0) at 37℃ for 10 min, and then 0.4 mM Boc-VPR-*p*NA was added on the reaction mixtures as a substrate. The reaction mixtures (100 μl) were incubated at 37℃ for 30 min and the residual activities of Vm-AP in the presence of detergents were determined by measuring the absorbance at 405 nm.

II-12. Susceptibility of Vm-AP activity to plasma proteins

To determine the effect of Vm-AP activity on plasminogen, Vm-AP (0.3 μg) was incubated with plasminogen (5 μg) at 37℃ for 0, 10, 30, and 60 min in 25 mM Tris-HCl (pH 7.5). The reaction mixtures were electrophoresed on 12% SDS-polyacrylamide gel to visualize cleavage pattern of plasminogen. Vm-AP (0.3 μg) was also pre-incubated with plasminogen or plasmin (each 5 μg) at 37℃ for 60 min in the same buffer, and then 0.4 mM of S-2251 were added in the reaction mixtures as a substrate for plasminogen and plasmin. The reaction mixtures (100 μl) were incubated at 37℃ for 30 min and the amidolytic activities of plasminogen and plasmin were detected by measuring the absorbance at 405 nm.

The effects of Vm-AP activity on prothrombin was investigated. Vm-AP (0.3 μg) was incubated with prothrombin (5 μg) at 37℃ for 0, 0.1, and 5 min in 25 mM Tris-HCl (pH 7.5), and then the reaction mixtures were electrophoresed on 12% SDS-polyacrylamide gel. To determine effect of Vm-AP activity on thrombin, the enzyme was pre-incubated with thrombin at 37℃ for 10 min in the same

buffer, and then 0.4 mM of S-2238 was added in the reaction mixtures as a substrate for thrombin. The reaction mixtures were incubated at 37℃ for 30 min and the amidolytic activity of thrombin was determined by measuring hydrolysis of S-2238 substrate at 405 nm.

II-13. Susceptibility of Vm-AP activity to fibrinogen

Vm-AP (0.0625 μg) was incubated with human fibrinogen (30 μg) dissolved in 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at 37℃ for 0, 1, 20, and 360 min. The reaction mixtures were electrophoresed on 12% SDS-polyacrylamide gel to examine the cleavage pattern of fibrinogen. To determine fibrin polymerization by Vm-AP activity, Vm-AP (1 μg) was incubated with fibrinogen (90 μg) dissolved in 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at 37℃ for 60 min. Turbidity increase of the fibrin polymer was detected by measuring the absorbance at 350 nm every 2 min interval. Effects of various inhibitors and divalent cations on the fibrinogenolytic activity of Vm-AP were analyzed. Vm-AP (0.01 μg) was incubated with fibrinogen (10 μg) at 37℃ for 10 min in 25 mM Tris-HCl (pH 7.5) containing 1 mM of various protease inhibitors (DFP, PMSF, aprotinin, bestatin, 1,10-PT, EDTA, or EGTA), 500 mM of reducing reagents (DTT or β-mercaptoethanol), or 1 mM of various divalent cations ($NiCl_2$, $ZnCl_2$, $MgCl_2$, $CuCl_2$, or $CaCl_2$). The reaction mixtures were mixed with 6x reducing sample buffer and electrophoresed on 12% SDS- polyacrylamide gel.

II-14. Susceptibility of Vm-AP activity to fibrin polymer and cross-linked fibrin catalyzed by factor XIIIa

Effect of Vm-AP activity on fibrin polymer was determined on fibrin plate. The fibrin plate was prepared by incubating 2 ml of 2% fibrinogen dissolved in 25 mM Tris-HCl (pH 7.5), 2 ml of 1% agarose, and 100 μl of 100 U/ml thrombin.

The plate was allowed to set for 2 h at room temperature. Vm-AP (1 μg) and plasmin (0.5 U) were applied into 3 mm diameter of wells made on the plate, and the fibrin plate was incubated at 37℃ for 12 h. Effect of Vm-AP activity on cross-linked fibrin was determined with 12% SDS-polyacrylamide gel. Cross-linked fibrin was formed by incubating fibrinogen (10 μg), thrombin (0.02 U), and factor XIIIa (0.003 U) in 25 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1 mM $CaCl_2$ at room temperature for 2 h. Vm-AP (0.5 μg) was incubated with the cross-linked fibrin at 37℃ for 5 min and then the reaction mixtures were electrophoresed on 12% SDS-polyacrylamide gel.

III. RESULTS AND DISCUSSION

III-1. Cell growth and protease production

It has been reported that the production of extracellular protease(s) from *V. metschnikovii* is affected by both of growth temperature and pH of the culture medium (Kwon *et al*., 1992; Kwon *et al*., 1994).

In this study, correlation of cell growth rate and protease(s) production of *V. metschnikovii* ATCC700040 was investigated. The cells were cultivated in LB medium under different temperatures (Fig. 1A) and the proteolytic activities contained in the culture supernatants were measured using azocasein method as described in Materials and Methods (Fig. 1B). As shown in Fig. 1. the maximal growth rate (Fig. 1A) and protease(s) production (Fig. 1B) could be obtained when the cells were cultivated at 30℃.

Effects of various kinds of growth media for protease(s) production were also investigated. As shown in Fig. 2A, the highest proteolytic activity could be observed from the culture supernatant cultivated in LB medium containing 3% NaCl at 30℃ (Fig. 2A). When the cells were cultivated in various pHs of media (6.8, 7.5, 9.5, or 10.5), the culture supernatant cultivated in pH 7.5 of medium showed the maximal proteolytic activity (Fig. 2B). These results suggest that the optimal growth condition for protease(s) production is 30℃ in LB medium containing 3% NaCl under pH 7.5 and the growth condition is similar with the used condition for protease(s) production from *V. metschnikovii* J1 in a previous report (Jellouli *et al*., 2009).

III-2. Purification of a novel alkaline protease

A novel extracellular alkaline protease was purified from culture supernatant of *V. metschnikovii* ATCC700040 by sequential chromatographic procedures using

Hiprep 16/10 Q FF, Source 15 Q 4.6/100 PE, and Superdex 75 10/300 GL columns (Fig. 3). A single peak containing proteolytic activity was found in the fractions collected from the Hiprep 16/10 Q FF column when azocasein was used as a substrate (Fig. 3A). Further chromatography of the active fractions was performed on the Source 15 Q 4.6/100 PE (Fig. 3B) and the fractions containing proteolytic activities were also applied to the Superdex 75 10/300 GL (Fig. 3C). The purified protease designated as to Vm-AP was collected by pooling and concentrating from the active fractions obtained from the final chromatographic step. The purification summary of Vm-AP is shown in Table 2. The proteins obtained from each purification step were analyzed on 12% SDS-polyacrylamide gel (Fig. 4). Vm-AP seemed to be degraded in the heating step of sampling for SDS-PAGE (Fig. 4, lane 5), however, the addition of above 1 mM of $CuCl_2$ could protect the enzyme from heat-degradation (Fig. 4, lane 4). The apparent molecular weights of Vm-AP was corresponded to 50 kDa and 40 kDa under reducing condition with 1 mM $CuCl_2$ and non-reducing condition (Fig. 5). The purification yield and the specific activity of the enzyme were 0.1% and 1,000 U/mg, respectively. K_M, k_{cat} and k_{cat}/K_M of Vm-AP were determined with Boc-VPR-*p*NA as a substrate and the kinetic values were as follows: K_M = 0.91 mM, k_{cat} = 0.8 s^{-1} and k_{cat}/K_M = 0.88 mM/s^{-1} (Table 3). These kinetic values of Vm-AP were compared with those of an extracellular alkaline serine protease named AprJ1 secreted from J1 strain, K_M = 0.158 mM, k_{cat} = 1.9 x 10^3 s^{-1} and k_{cat}/K_M = 0.88 mM/s^{-1} with *N*-succinyl-L-Ala-L-Ala-L- Pro-L-Phe-*p*-nitroanilide as a substrate (Jellouli *et al*., 2009).

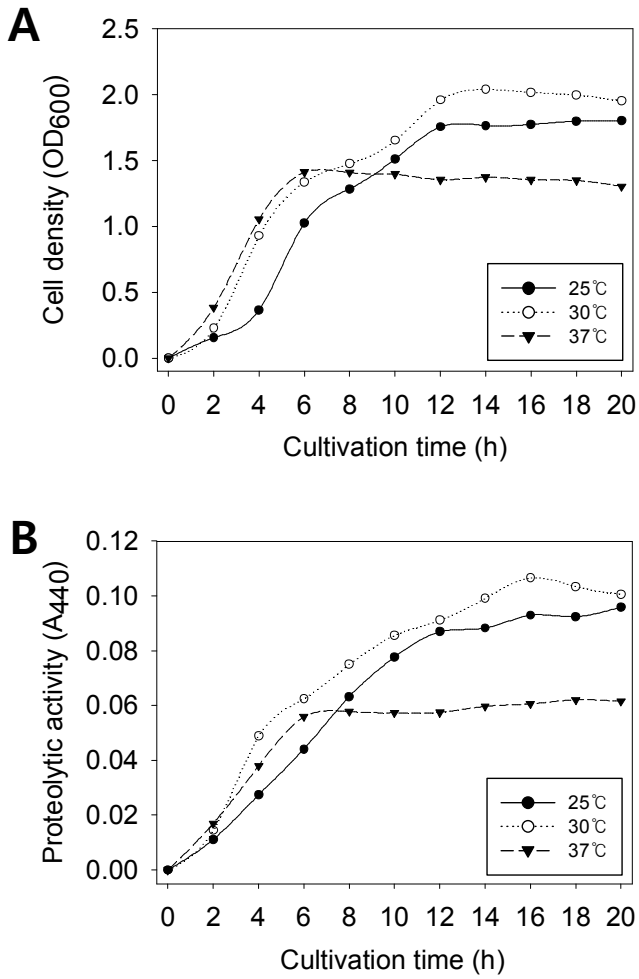

Fig. 1. Correlation of cell growth rate and proteolytic activity from culture supernatant of *V. metschnikovii*. *V. metschnikovii* cells were cultivated at various growth temperatures (25, 30, or 37℃) for 20 h. Cell density was monitored by observing OD_{600} value at every 2 h interval (A) and proteolytic activity contained in the culture supernatant was assayed with azocasein as a substrate by measuring the absorbance at 440 nm (B).

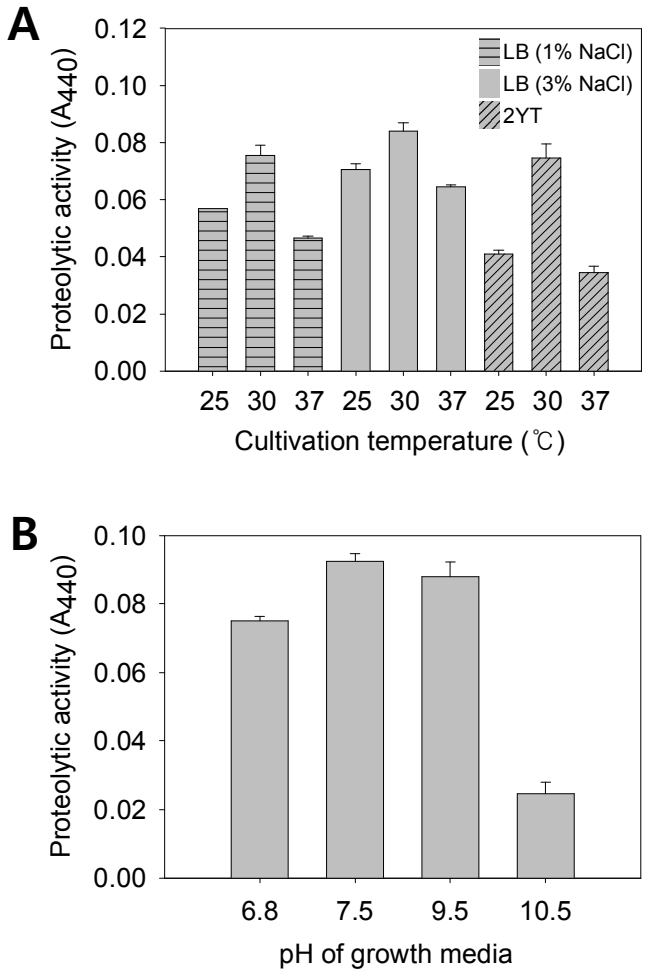

Fig. 2. Effects of growth media and pHs on protease production. *V. metschnikovii* cells were cultivated in LB containing 1% or 3% NaCl, or in 2YT media at 25, 30, or 37℃ for 12 h (A). The cells were also cultivated in various pHs (6.8, 7.5, 9.5, or 10.5) of media at 30℃ for 12 h (B). Proteolytic activity contained in the culture supernatant was assayed with azocasein as a substrate by measuring the absorbance at 440 nm.

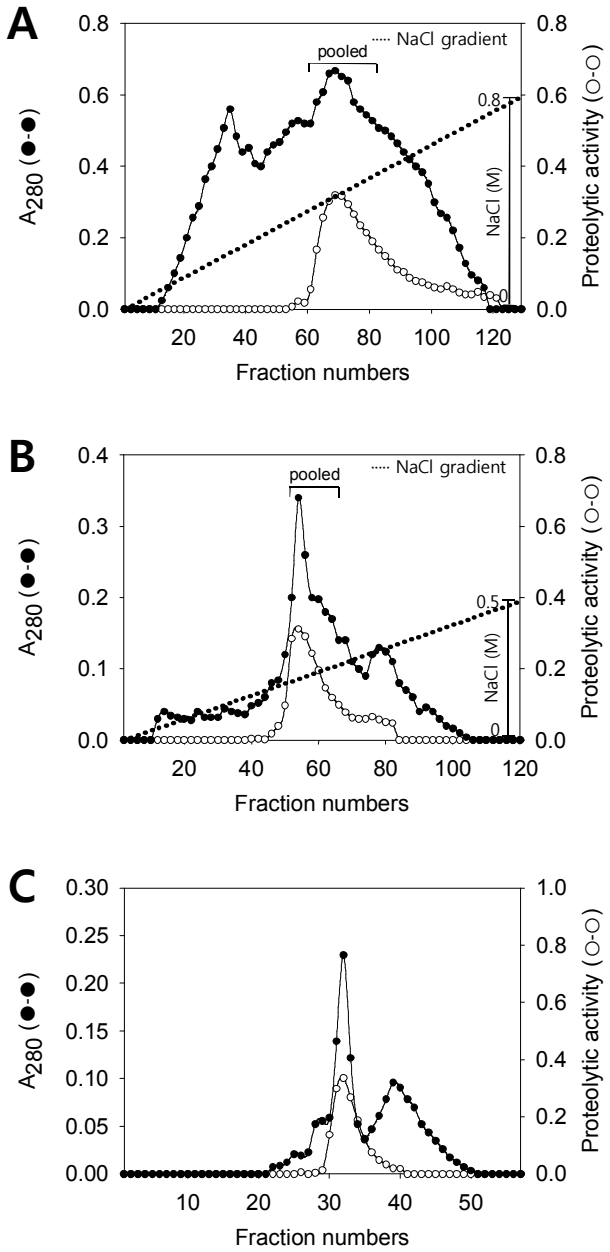

Fig. 3. Purification profiles of a novel alkaline protease. A novel alkaline protease was purified from total extracellular proteins of *V. metschnikovii* by sequential chromatographic steps using Hiprep 16/10 Q FF (A), Source 15 Q 4.6/100 PE (B), and Superdex 75 10/300 GL columns (C). The brackets indicate the pooled fractions that showed proteolytic activities when the azocasein was used as a substrate.

Table 2. Purification summary of Vm-AP.

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

* One unit of Vm-AP activity was defined as the amount of enzyme that hydrolyzes 0.18 mM Boc-VPR-*p*NA in 20 mM Tris-HCl (pH 9.0) at 37℃ for 30 min.

** Total activity of total extracellular proteins was assigned the value of 100%.

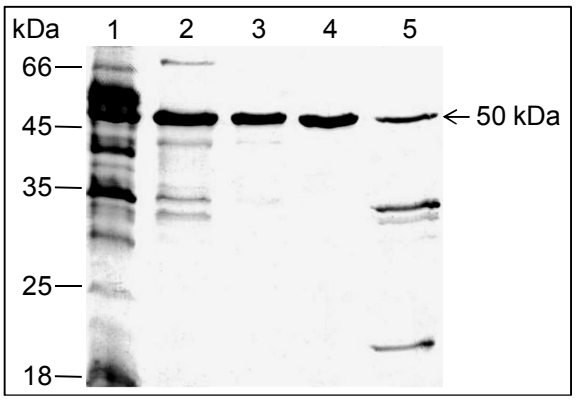

Fig. 4. SDS-PAGE of the active fractions obtained from each chromatographic step. The active fractions from each chromatographic step were electrophoresed on 12% SDS-polyacrylamide gel under reducing conditions with (lanes 1~4) or without (lane 5) $CuCl_2$, and the gel was stained with Coomassie brilliant blue. Lanes 1, total extracellular proteins; 2, Hiprep 16/10 Q FF; 3, Source 15 Q 4.6/100; 4, Superdex 75 10/300 GL with $CuCl_2$; 5, Superdex 75 10/300 GL without $CuCl_2$.

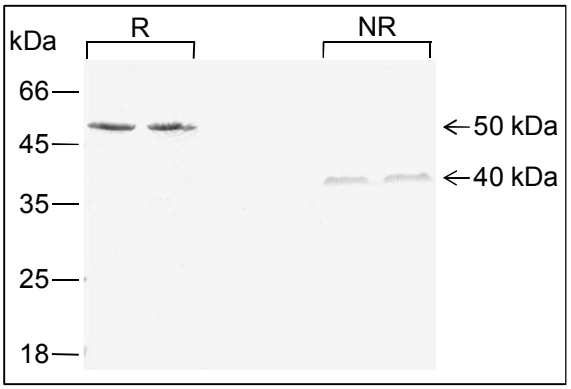

Fig. 5. SDS-PAGE of purified Vm-AP under reducing and non-reducing conditions. Purified Vm-AP enzymes were electrophoresed on 12% SDS- polyacrylamide gel under reducing condition (R) with 1 mM of $CuCl_2$ and non-reducing condition (NR) without $CuCl_2$, and stained with Coomassie brilliant blue.

Table 3. Kinetic values of Vm-AP enzyme.

K_M (mM)	k_{cat} (s^{-1})	k_{cat} / K_M (mM / s^{-1})
0.91	0.80	0.88

* These kinetic values of Vm-AP were determined using Boc-VPR-*p*NA as a substrate.

III-3. Effects of divalent cations on heat-degradation of Vm-AP

As shown in Fig. 4, the addition of $CuCl_2$ could protect Vm-AP enzyme from heat-degradation. To determine protective effects of various divalent cations ($CaCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $NiCl_2$, or $CuCl_2$) on degradation of Vm-AP, the enzyme was heated at 100℃ for 2 min in the presence of the various divalent cations, and then electrophoresed on 12% SDS-polyacrylamide gel. As shown in Fig. 6, Vm-AP was stable against the heating and appeared as an intact single band on the gel by addition of 10 mM $ZnCl_2$, $NiCl_2$, or $CuCl_2$. These results suggest that Vm-AP might be unstable at high temperature around 100℃ and cleaved by its own proteolytic activity. In addition, these results suggest that $ZnCl_2$, $NiCl_2$, or $CuCl_2$ can inhibit the suicidal activity of Vm-AP.

This type of protease degradation induced by heating have not been reported in previous studies about proteases, however, a similar effect of high temperature on protease have been investigated in a study about extracellular metalloprotease secreted from *V. vulnificus* (Chang *et al.*, 2005). The enzyme disappeared completely on polyacrylamide gel when the enzyme was pre-incubated at 75℃ for 20 min (data not shown). This result suggests that the extracellular metalloprotease was autodegraded by incubating at 75℃. However, the enzyme could be stable on polyacrylamide gel by heating step of SDS-PAGE at 100℃ for 1 min (data not shown) not like Vm-AP.

III-4. Zymographic analysis of total extracellular proteins and purified Vm-AP

A major and six minor gelatinolytic proteases were identified on zymographic gels containing gelatin using total extracellular proteins secreted from *V. metschnikovii* RH530 (Kwon *et al.*, 1992).

In this study, proteolytic activities of protease(s) contained in the total extracellular proteins were investigated using 10% SDS-polyacrylamide gel

containing 1% casein or 1% gelatin. As shown in Fig. 7, each single proteolytic band was appeared on the gels by both samples of total extracellular proteins and purified Vm-AP. The sizes of proteolytic bands were corresponded to 40 kDa on gel containing casein and 37 kDa on gel containing gelatin. Other significant proteolytic band(s) could not be identified on the zymographic gels (Fig. 7). These results suggest that purified Vm-AP is the major protease secreted from *V. metschnikovii* having caseinolytic and gelatinolytic activities, and minor protease(s) could not be detected from this zymographic assay.

III-5. Substrate specificity of Vm-AP

The substrate specificity of Vm-AP was determined by observing cleavage of various synthetic chromogenic substrates listed in Table 1. As shown in Table 4, Vm-AP showed the most strong susceptibility for Boc-VPR-*p*NA, thrombin specific substrate, giving rise to 100%. However, S-2238 for thrombin was not susceptible as below as 38%. Vm-AP could not show significant specificities for the following substrates: Boc-LGR-*p*NA, S-2222, and S-2765 for factor Xa, S-2251 for plasmin and plasminogen, S-2288 for t-PA and broad serine proteases, S-2444 for urokinase, and S-2238 for thrombin. These results indicate that Vm-AP enzyme can hydrolyze Boc-VPR-*p*NA as a specific substrate, suggesting similar proteolytic activity of the enzyme like that of thrombin.

III-6. Effects of temperature and pH on Vm-AP activity

The effects of temperature and pH on Vm-AP activity were assayed with Boc-VPR-*p*NA as a substrate. Temperature dependency of the enzyme activity was examined at various temperatures in pH 9.0 buffer. Optimal temperature for Vm-AP was determined to be 37℃ (Fig. 8A). The effect of pH on Vm-AP activity was also determined in various pH buffer systems described in Materials and Methods.

Optimal pH of Vm-AP was determined to be 9.5. In addition, more than 65% of enzyme activity remained in alkalic pH ranging from pH 8.0 to 12.0 (Fig. 8B). These results suggest that the enzyme is a member of alkaline protease family. Microbial alkaline proteases are interested in their activities and stability at alkaline pH to use for detergent additives, therapeutic applications, food industry, and waste treatment as well as chemical industry (Kumar *et al*., 1999). Purified Vm-AP also have high activities and stability in alkalic pH as a microbial alkaline protease, suggesting the potential applications.

III-7. Effects of various inhibitors and divalent cations on Vm-AP activity

The effects of various protease inhibitors and divalent cations on Vm-AP activity were investigated with Boc-VPR-*p*NA as a substrate. As shown in Fig. 9, approximately 90% and 72% of Vm-AP activities could be inhibited by the addition of serine protease inhibitors such as aprotinin and PMSF (Fig. 9A). However, other protease inhibitors showed no significant effect on Vm-AP activity. These results suggest that Vm-AP might be a member of serine protease family. Effects of divalent cations on the enzyme activity was shown in Fig. 9B. More than 70% of Vm-AP activity could be inhibited by the addition of $CuCl_2$.

III-8. Effects of detergents on Vm-AP activity

Two extracellular alkaline proteases secreted from *V. metschnikovii* RH530 retain more than 80% of their activities in the presence of 5% SDS or 8 M urea (Kwon *et al*., 1994). Several SDS-resistant proteases were also reported from *V. alginolyticus* (Dean *et al.*, 1987) and *Pyrococcus furiosus* (Blumental *et al.*, 1990).

To investigate the resistancy of Vm-AP, Vm-AP activities were measured with Boc-VPR-*p*NA as a substrate in the presence of detergents such as SDS and

urea. As shown in Fig. 10, 98% of the enzyme activity was abolished by the presence of more than 1% SDS, however, 60% of Vm-AP activity retained in the presence of 1 urea. These results suggest that Vm-AP might be a stable in the presence of 1 M urea. These differences of Vm-AP and the alkaline proteases secreted from RH530 strain on SDS-resistancy also suggest that these enzymes share low relativities on those activities though both enzymes are extracellular alkaline proteases secreted from *V. metschnikovii* strains.

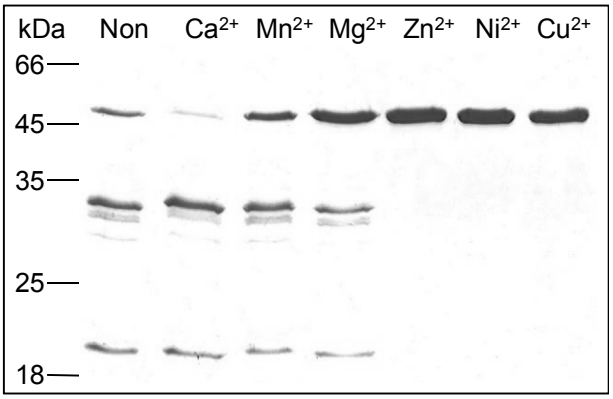

Fig. 6. Effects of various divalent cations on heat-degradation of Vm-AP. To determine protective effects of divalent cations on heat-degradation of Vm-AP, 5 μg of the enzyme was heated at 100℃ for 2 min in the presence of $CaCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $NiCl_2$, or $CuCl_2$. The enzyme samples were electrophoresed on 12% polyacrylamide gel under reducing condition. Non, heat-treated Vm-AP without cation.

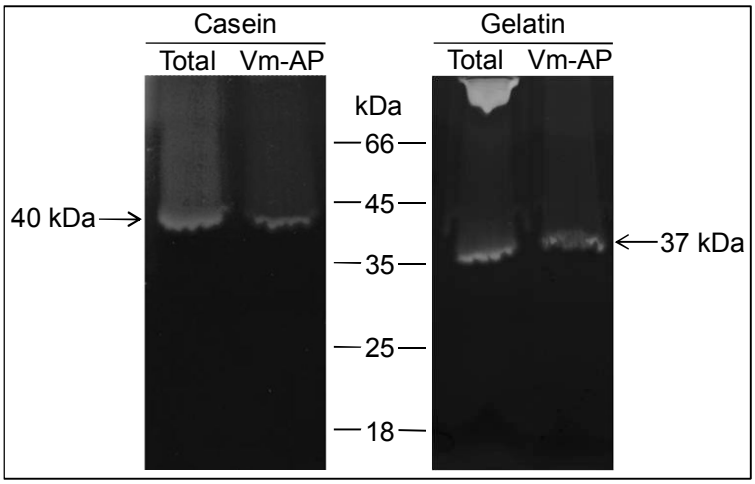

Fig. 7. Zymographic assays of total extracellular proteins and purified Vm-AP. Zymographic assays were carried out using total extracellular protein (2.5 μg) and purified Vm-AP (0.3 μg) on 10% SDS-polyacrylamide gels containing 1% casein or 1% gelatin. SDS contained in the zymographic gels was removed by incubating in 5% Triton X-100 solution and then the protease(s) were activated in pH 9.0 buffer at 37℃ for 30 min. The gels were stained with Coomassie brilliant blue to visualize proteolytic bands on the gels. Total, total extracellular proteins.

Table 4. Substrate specificity of Vm-AP.

Substrate*	Susceptibility	Relative activity (%)**
VPR	Thrombin	100
S-2222	Factor Xa	11.3
S-2238	Thrombin	37.9
S-2251	Plasmin and plasminogen	18.0
S-2288	t-PA and broad serine proteases	12.4
S-2444	Urokinase	25.1
S-2765	Factor Xa	13.9
LGR	Factor Xa	18.9

* Vm-AP was reacted with various kinds of chromogenic substrates at 37℃ for 30 min in 20 mM Tris-HCl (pH 9.0).

** Relative activities were expressed as a relative value compared to that of Boc-VPR-*p*NA.

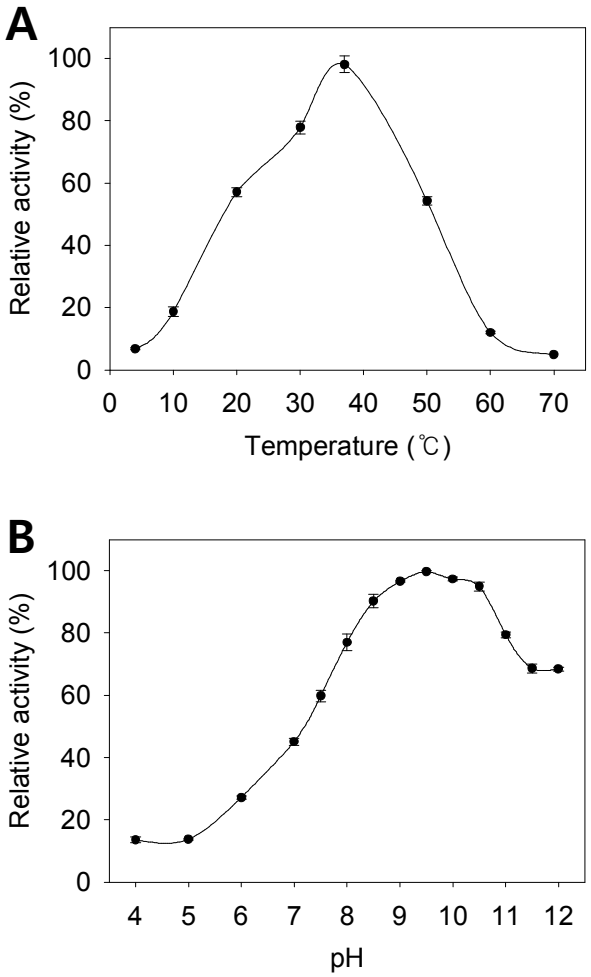

Fig. 8. Effects of temperature and pH on Vm-AP activity. Vm-AP activities were assayed with Boc-VPR-*p*NA as a substrate under various temperatures (A) or pH conditions (B).

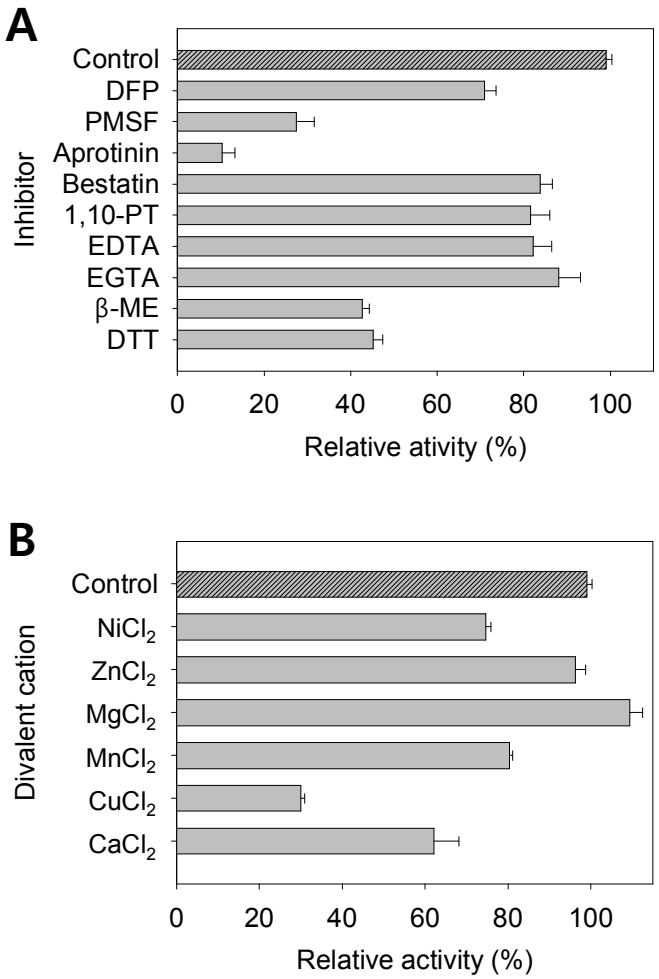

Fig. 9. Effects of various protease inhibitors and divalent cations on Vm-AP activity. Vm-AP activities were assayed with Boc-VPR-*p*NA as a substrate in the presence of various protease inhibitors (A) or divalent cations (B). Concentrations of the inhibitors and divalent cations were described in Materials and Methods. The enzyme activities were expressed as a relative value compared to that of non-treated control. β-ME, β-mercaptoetanol.

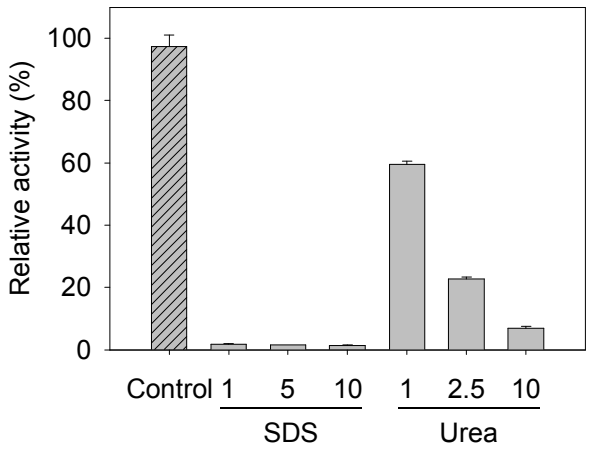

Fig. 10. Effects of detergents (SDS and urea) on Vm-AP activity. Vm-AP activities were assayed with Boc-VPR-*p*NA as a substrate in the presence of various concentrations of SDS (1, 5, or 10%) or urea (1, 2.5, or 5 M). The Vm-AP activities were expressed as a relative value compared to that of non-treated control.

III-9. Susceptibility of Vm-AP to plasma proteins

Several proteases secreted from *Vibrios* can affect on various blood clotting-associated proteins. Among these proteases, metalloproteases secreted from *V. vulnificus* can induce cleavages of plasminogen, factor Xa, and fibrinogen as well as activation of prothrombin (Chang *et al.*, 2005; Kwon *et al.*, 2007).

The effects of Vm-AP activity on plasminogen, plasmin, prothrombin, and thrombin were investigated. As shown in Fig. 11A, Vm-AP could cleave plasminogen, generating approximately 50, 33, and 20 kDa of fragments. To determine effects of Vm-AP on plasminogen and plasmin activities, S-2251 was added as a substrate in pre-incubated plasminogen or plasmin with Vm-AP. As shown in Fig. 11B, both activities of plasminogen and plasmin decreased by incubating with Vm-AP enzyme. Prothrombin was also cleaved by Vm-AP very quickly (Fig. 12A) and the effect of Vm-AP on thrombin activity was determined by the addition of S-2238 as a substrate. The activity of thrombin on S-2238 decreased by pre-incubating with Vm-AP (Fig. 12B). These results suggest that Vm-AP can degrade various blood coagulation-associated proteins such as plasminogen, plasmin, prothrombin, and thrombin.

III-10. Susceptibility of Vm-AP activity to fibrinogen, fibrin polymer, and cross-linked fibrin catalyzed by factor XIIIa

Fibrino(geno)lytic enzymes have been reported from food bacteria (Hwang *et al.*, 2007; Kim *et al.*, 2009), pathogenic bacteria (Balaraman *et al.*, 2006; Fricke *et al.*, 1999; Imamura *et al.*, 2007), and mushroom (Kim *et al.*, 2006) as well as snake venom (Bernardes *et al.*, 2007; Koh *et al.*, 2000). The fibrinogenolytic protease secreted from *V. vulnificus* could rapidly digest α-chain of fibrinogen (Miyoshi *et al.*, 1995) and metalloprotease from *V. cholerae* could cleave three major chains (α, β, and ɣ) of fibrinogen (Vaitkevivius *et al.*, 2007).

To determine fibrinogenolytic activity of Vm-AP, fibrinogen was incubated

with Vm-AP at 37℃ in pH 7.5 buffer. As shown in Fig. 13, α- and β-chains of fibrinogen could be degraded by Vm-AP within 1 min and 20 min, respectively. In addition, most of ɣ-chain of fibrinogen could be also degraded by Vm-AP for 360 min (Fig. 13A). However, the fibrinogenolytic activity of Vm-AP could not induce the polymerization of fibrin compared to that induced by thrombin (Fig. 13B). Effects of various inhibitors (Fig. 14A) and divalent cations (Fig. 14B) on fibrinogenolytic activity of Vm-AP were determined on 12% SDS-polyacrylamide gel. PMSF, aprotinin, and $CuCl_2$, the effective inhibitory additives of Vm-AP, also inhibited fibrinogenolytic activity of the enzyme.

In addition, Vm-AP also showed significant fibrinolytic activities. When Vm-AP was applied on fibrin plate, the enzyme could make a halo zone on the plate like in plasmin, suggesting the degradation of fibrin polymer (Fig. 15A). Vm-AP was also incubated with cross-linked fibrin catalyzed by factor XIIIa. The enzyme could cleave the cross-linked fibrin including α-α and ɣ-ɣ chains (Fig. 15B).

As shown above, Vm-AP can cleave all kinds of chains (α, β, and ɣ) of fibrinogen and fibrin polymer as well as cross-linked fibrin, suggesting the powerful fibrin(ogen)olytic activity of the enzyme. These same results have been obtained from *V. vulnificus* (Chang *et al*., 2005; Kwon *et al*., 2007). Taken together, this study suggest that the normal human blood coagulation cascade might be interrupted by a secreted Vm-AP enzyme from *V. metschnikovii*.

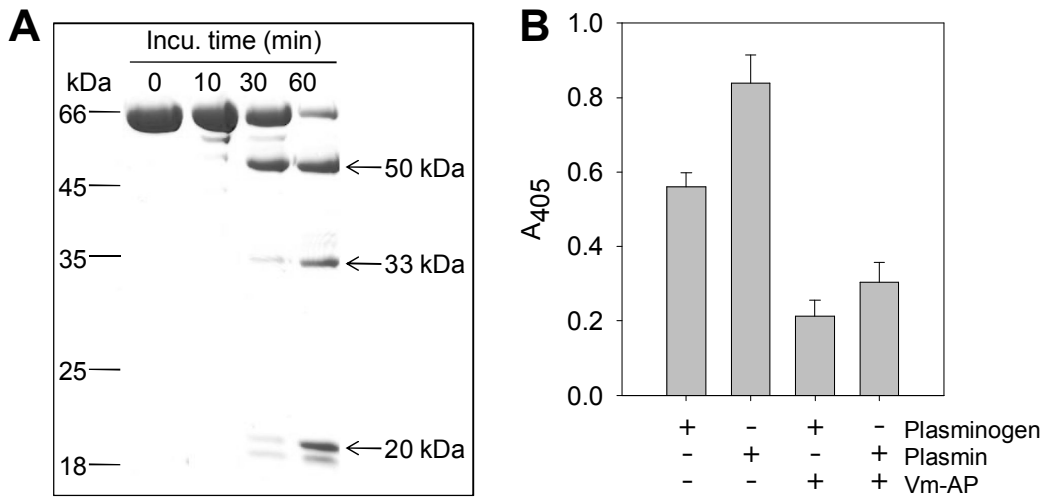

Fig. 11. Susceptibility of Vm-AP activity to plasminogen and plasmin. Plasminogen (5 μg) was incubated with Vm-AP (0.3 μg) at 37℃ for indicated time periods in pH 7.5 buffer. The cleavage of plasminogen by Vm-AP activity was determined on 12% SDS-polyacrylamide gel (A). Plasminogen or plasmin (each 5 μg) was pre-incubated with Vm-AP (0.3 μg) at 37℃ for 1 h in pH 7.5 buffer and 0.4 mM of S-2251 was added as a substrate for plasminogen and plasmin. Hydrolysis of S-2251 was detected by measuring the absorbance at 405 nm (B).

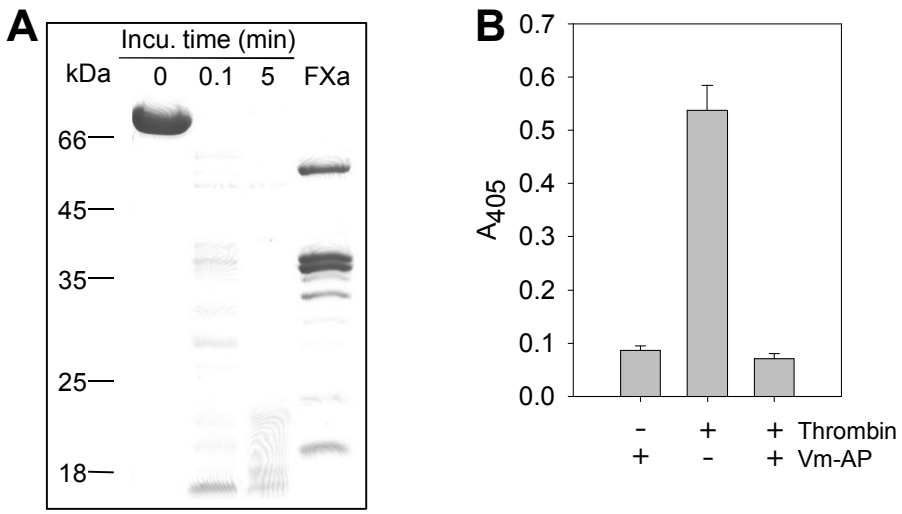

Fig. 12. Susceptibility of Vm-AP activity to prothrombin and thrombin. Prothrombin (5 μg) was incubated with Vm-AP (0.3 μg) at 37℃ for indicated time periods in pH 7.5 buffer. The cleavage of prothrombin by Vm-AP activity was determined on 12% SDS-polyacrylamide gel (A). FXa, prothrombin treated with factor Xa (0.1 U) at 37℃ for 1 h in pH 7.5 buffer. Thrombin (5 μg) was pre-incubated with Vm-AP (0.3 μg) at 37℃ for 10 min in pH 7.5 buffer and 0.4 mM of S-2238 was added as a substrate for thrombin. Hydrolysis of S-2238 was detected by measuring the absorbance at 405 nm (B).

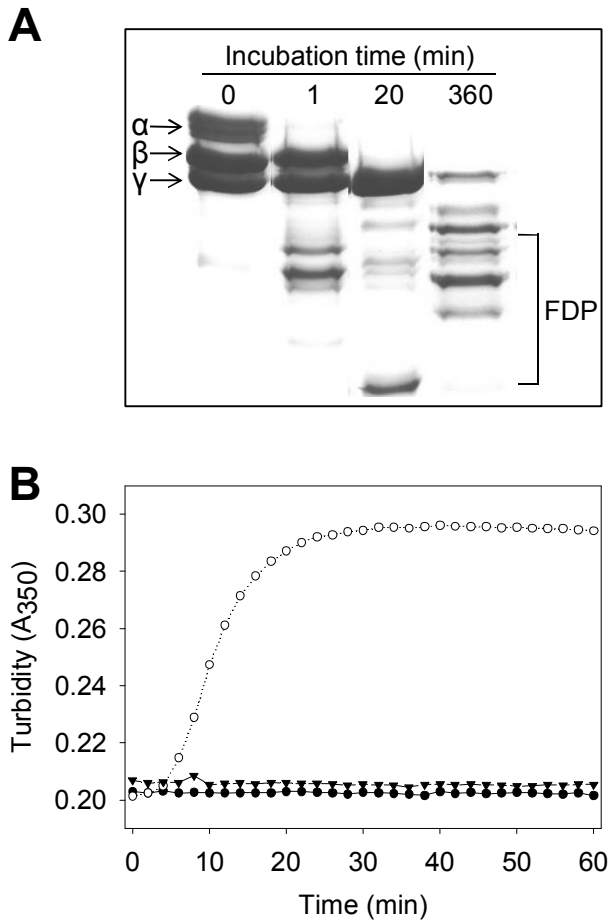

Fig. 13. Fibrinogen cleavage and formation of fibrin polymer by Vm-AP activity. Human fibrinogen (30 μg) was incubated with Vm-AP (0.0625 μg) enzyme at 37℃ for indicated time periods in pH 7.5 buffer and the resulting products were electrophoresed on 12% SDS-polyacrylamide gel. FDP, fibrinogen degradation products (A). Fibrinogen (90 μg) was also incubated with Vm-AP (1 μg) or thrombin (0.1 U) in pH 7.5 buffer at 37℃ to induce fibrin polymerization. Turbidity of fibrin polymer was detected by measuring the absorbance at 350 nm (B).

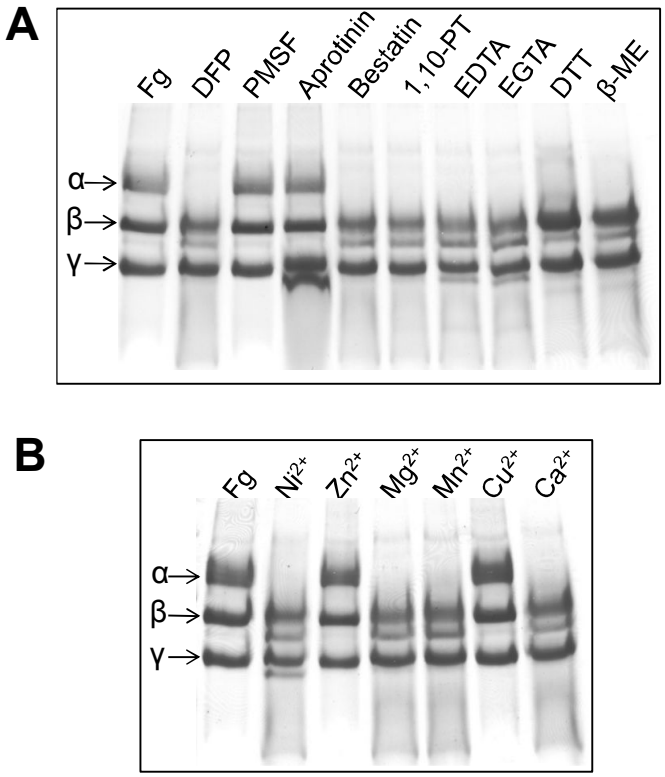

Fig. 14. Effects of various protease inhibitors and divalent cations on fibrinogenolytic activity of Vm-AP. Vm-AP (0.01 μg) was incubated with fibrinogen (10 μg) at 37℃ for 10 min in pH 7.5 buffer containing various inhibitors (A) or divalent cations (B). Effects of the inhibitors and divalent cations on fibrinogenolytic activity of Vm-AP were determined on 12% SDS-polyacrylamide gel. β-ME, β-mercaptoetanol.

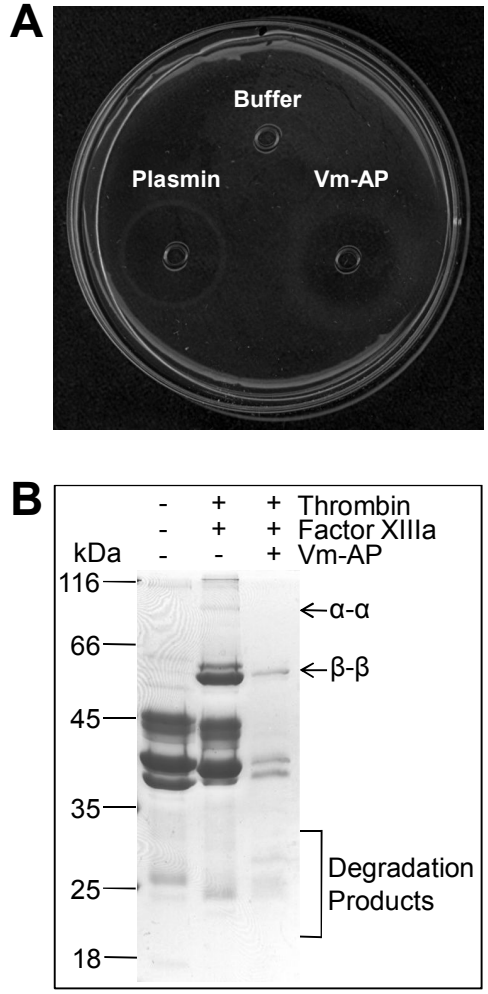

Fig. 15. Susceptibility of Vm-AP activity to cross-linked fibrin. Fibrinolytic activity of Vm-AP was assayed on fibrin plate. Vm-AP (1 μg) and plasmin (0.5 U) were applied on the fibrin plate and the plate was incubated at 37℃ for 12 h (A). Vm-AP (0.5 μg) was incubated with cross-linked fibrin catalyzed by factor XIIIa at 37℃ for 5 min. The samples were electrophoresed on 12% SDS-polyacrylamide gel (B).

IV. 적 요

Vibrio metschnikovii 균주로부터 분비되는 새로운 알칼리성 세린계열 단백질분해효소의 정제 및 특성 연구

박 재 영
지도 교수 : 이 정 섭
조선대학교 대학원
생명공학과

해양성 박테리아인 *Vibrio metschnikovii* (*V. metschnikovii*)는 상처를 통해 침투하여 폐렴, 궤양 및 설사 등의 심각한 증상을 유발하는 것으로 알려져 있다. 본 연구에서는, *V. metschnikovii* ATCC700040 균주의 배양상층액으로부터 새로운 알칼리성 세린계열 단백질분해효소를 분리·정제 하였으며, 그 생화학적 특성 및 인간혈액응고단백질들에 미치는 영향을 연구하였다. 이 균주를 3% NaCl이 포함된 pH 7.5의 LB배지에서 배양하였을때, 최대의 단백질분해활성이 나타는 것을 확인하였다. 새로운 단백질분해효소를 분리하기 위하여, 모든 세포외단백질들을 70%의 황산암모늄 [$(NH_4)_2SO_4$] 처리과정을 통해 침전시켰고, 이 단백질들을 Hiprep 16/10 Q FF, Source 15 Q 4.6/100 PE 및 Superdex 75 10/300 GL 컬럼크로마토그래피를 순차적으로 사용하여 단백질분해효소를

분리하였다. 순리 분리한 이 효소는 Vm-AP (*V. metschnikovii* alkaline protease)라 명명하였다. Vm-AP는 SDS-PAGE의 열처리 과정에서 분해되었는데, 이러한 분해는 $CuCl_2$, $ZnCl_2$ 및 $NiCl_2$를 첨가함으로써 억제할 수 있었다. 이 효소는 단일 폴리펩타이드로 구성되어 있었으며, $CuCl_2$가 존재하는 환원상태 하에서 50 kDa, 비환원상태 하에서 40 kDa이 분자량을 지님을 12% SDS-폴리아크릴아미드 겔 상에서 확인하였다. 발색성기질을 이용한 실험에서, 트롬빈의 대표적인 기질로 알려진 Boc-VPR-*p*NA가 이 효소에 대해 가장 특이적인 것으로 확인하였다. 이 기질을 이용하여 Vm-AP의 K_M, k_{cat} 및 k_{cat}/K_M 값이 각각 0.91 mM, 0.8 s^{-1} 와 0.88 $mM^{-1}s^{-1}$임을 확인하였다. Vm-AP의 효소활성에 대한 최적온도와 pH는 각각 37℃ 와 pH 9.5이었으며, pH 8.0~12.0인 알칼리 환경에서 상대적으로 강한 활성을 나타내는 것으로 보아 이 효소가 알칼리성 단백질분해효소라는 사실을 알 수 있었다. 세린단백질 분해효소의 억제제로 알려진 PMSF와 aprotinin를 첨가하였을 때, 약 81.1%의 Vm-AP 활성이 감소하였는데, 이러한 결과는 본 효소가 세린계열 단백질 분해효소라는 사실을 나타낸다. Vm-AP 효소는 플라스미노겐, 플라스민, 프로트롬빈 및 트롬빈과 같은 다수의 혈액응고단백질들을 절단하였으며, 또한 강력한 피브리노겐 절단활성을 보였다. 이 효소는 γ-사슬을 포함한 피브리노겐의 모든 폴리펩타이드 사슬을 분해하였고, 피브린 중합체 및 교차-연결된 피브린까지도 분해하는 것을 확인하였다. 이러한 모든 결과를 종합하여, 병원성 균주인 *V. metschnikovii*는 감염 시 Vm-AP를 분비하여 혈액응고시스템을 교란시킬 가능성을 시사하는 것이다.

V. REFERENCES

Balaraman, K., Prabakaran. (2006) Production and purification of a fibrinolytic enzyme (thrombianse) from *Bacillus sphaericus*. *Indian J Med Res* **126**, 459-464.

Bernardes, C.P., Santos-Filho, N.A., Costa, T.R., Gomes, M.S.R., Torres, F.S., Costa, J., Borges, M.H., Richardson, M., Santos, D.M., Pimenta, A.M., Homsi-Brandeburgo, M., Soares, A.M., Oliveira, F. (2007) Isolation and structural characterization of a new fibrin(ogen)olytic metalloprotease from *Bothrops moojeni* snake venom. *Toxicon* **51**, 574-84.

Blumentals, I.I., Robinson, A.S., Kelly, R.M. (1990) Characterization of sodium dodecyl sulfate-resistant proteolytic activity in the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *Applied and Environmental Microbiology* **56**. 1992-1998.

Chang, A.K., Kim, H.Y., Park, J.E., Acharya, P., Park, I.S., Yoon, S.M., You, H.J., Hahm, K.S., Park, J.K., Lee, J.S. (2005) *Vibrio vulnificus* secretes a broad-specificity metalloprotease capable of interfering with blood homeostasis through prothrombin activation and fibrinolysis. *J Bacteriol* **187**, 6909-6916.

Chowdhury, M.A., Miyoshi, S., Shinoda, S. (1991) Vascular permeability enhancement by *Vibrio mimicus* protease and the mechanisms of action. *Microbiol Immunol* **35**, 1049-1058.

Chung, S.S., Kim, H.J., Shy, U., Jin, J.H., Lee, H.H. (2001) Molecular characterization of gene encoding an extracellular alkaline protease in *Vibrio metschnikovii*. *Biotechnol Lett* **23**, 1175-1182.

Dalsgaard, A., Alarcon, A., Lanata, C.F., Jensen, T., Hansen, H.J., Delgado, F., Gil,

A.I., Penny, M.E., Taylor, D. (1996) Clinical manifestations and molecular epidemiology of five cases of diarrhoea in children associated with *Vibrio metschnikovii* in Arequipa, Peru. *J Med Microbiol* **45**, 494-500.

Deane, S.M., Robb, F.T., Woods, D.R. (1987) Production and activation of a SDS-resistant alkaline serine exoprotease of *V. alginolyticus*. *J General Microbiology* **133**. 391-398.

Fricke, B., Parchmann, O., Kruse, Katharina, K., Rucknagel, P., Schierhorn, A., Menge, S. (1999) Characterization and purification of an outer membrane metalloproteinase from Pseudomonas aeruginosa with fibrinogenolytic activity. *Biochimica et Biophysica Acta* **1454**, 236-250.

Hansen, W., Freney, J., Benyagoub, H., Letouzey, M., Gigi, J., Wauters, G. (1993) Severe human infections caused by *Vibrio metschnikovii*. *J Clin Microbiol* **31**, 2529-2530.

Hwang, K.J., Choi, K.H., Kim, M.J., Park, C.S., Cha, J. (2007) Purification and characterization of a new fibrinolytic enzyme of *Bacillus licheniformis* KJ-31, Isolated from Korean traditional Jeot-gal. *J. Microbiol. Biotechnol* **17**, 1469-1476.

Imamura, T., Nitta, H., Wada. Y., Kobayashi, H., Okamoto, K. (2007) Impaired plasma clottability induction through fibrinogen degradation by ASP, a serine protease released from *Aeromonas sobria*. *FEMS Microbiol Lett* **284**, 35-42

Janda, J.M. (1986) Mucinase activity among selected members of the family *Vibrionaceae*. *Microbios Lett* **33**, 19-22.

Jean-Jacques, W., Rajashekaraiah, K.R., Farmer, J.J., Hickman, F.W., Morris, J.G., Kallick, C.A. (1981) *Vibrio metschnikovii* bacteremia in a patient with cholecystitis. *J Clin Microbiol* **14**, 711-712.

Jellouli, K., Bougatef, A., Manni, L., Agrebi, R., Siala, R., Younes, I., Nasri, M. (2009) Molecular and biochemical characterization of an extracellular

serine-protease from *Vibrio metschnikovii* J1. *J Ind Microbiol Biotechnol* **36**, 939-948.

Kim, G.M., Lee, A.R., Lee, K.W., Park, J.Y., Chun, J., Cha, J., Song, Y.S., Kim, J.H. (2009) Characterization of a 27 kDa fibrinolytic enzyme from *Bacillus amyloliqueefaciens* CH51 isolated from Cheonggukjang. *J Microbiol Biotechnol* **19**, 997-1004.

Koh, Y.S., Chung, K.H., Kim, D.S. (2000) Biochemical cahracterization of a thrombin-like enzyme and a fibrinolytic serine protease from snake (*Agkistrodon saxatilis*) venom. *Toxicon* **39**, 555-560.

Kumar, C.G., Takagi, H. (1999) Microbial alkaline protease : from a bioindustrial viewpoint. *Biotechnol Advances* **17**, 561-594.

Kwon, J.Y., Chang, A.K., Park, J.E., Shin, S.Y., Yoon, S.M., Lee, J.S. (2007) Vibrio extracellular protease with prothrombin activation and fibrinolytic activities. *Int J Mol Med* **19**, 157-63.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Kyo, Y.H., Rho, H.M. (1992) Characterization of extracellular protease from alkalophilic *Vibrio sp.* strain RH530. *J Microbiol Kor* **30**, 501-506.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Yoo, Y.D., Rho, H.M. (1994) Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. *Biotech Lett* **16**, 413-418.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Yoo, Y.D., Rho, H.M. (1995) Cloning and characterization of the gene encoding an extracellular alkaline serine protease from *Vibrio metschnikovii* strain RH530. *Gene* **152**, 59-63.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Linde, H.J., Kobuch, R., Jayasinghe, S., Reischl, U., Lehn, N., Kaulfuss, S., Beutin, L. (2004) *Vibrio metschnikovii*, a rare cause of wound infection. *J Clin*

Microbiol **42**, 4909-4911.

Magalhaes, V., Branco, A., de Andrade Lima, R., Magalhaes, M. (1996) *Vibrio metschnikovii* among diarrheal patients during cholera epidemic in Recife Brazil. *Rev Inst Med Trop Sao Paulo* **38**, 1-3.

Miyoshi, S. (2006) *Vibrio vulnificus* infection and metalloprotease. *J Dermatol* **33**, 589-595.

Miyoshi, S. Nitanda, Y., Fujii, K., Kawahara, W., Maehara, Y., Ramamurthy, T., Takeda, Y., Shinoda, S. (2008) Differential gene expression and extracellular secretion of the collagenolytic enzymes by the pathogen *Vibrio parahaemolyticus*. *FEMS Microbiol Lett* **283**, 176-181

Miyoshi, S., Shinoda, S. (1988) Role of the protease in the permeability enhancement by *Vibrio vulnificus*. *Microbiol Immunol* **32**, 1025-1032.

Miyoshi, S., Sonoda, Y., Wakiyama, H., Rahman, M.M., Tomochika, K., Shinoda, S., Yamamoto, S., Tobe, K. (2002) An exocellular thermolysin-like metalloprotease produced by *Vibrio fluvialis*: purification, characterization, and gene cloning. *Microb Pathog* **33**, 127-134.

Pariente Martin, M., Escribano Garaizabal, E., Liria Sanchez, P.J., Crespo Sanchez, M.D. (2008) *Vibrio metschnikovii* from a human infected leg ulcer. *Rev Inst Med Trop Sao Paulo* **50**, 311-312.

Sakata, Y., Akaike, T., Khan, M.M., Ichinose, Y., Hirayama, H., Suga, M., Ando, M., Maeda, H. (1996) Activation of bradykinin generating cascade by *Vibrio cholerae* protease. *Immunopharmacology* **33**, 377-379.

Smith, G.C., Merkel, J.R. (1982) Collagenolytic activity of *Vibrio vulnificus*: potential contribution to its invasiveness. *Infect Immun* **35**, 1155-1156.

Wallet, F., Tachon, M., Nseir, S., Courcol, R.J., Roussel-Delvallez, M. (2005) *Vibrio metschnikovii* pneumonia. *Emerg Infect Dis* **10**, 1641-1642.

Vaitkevicius, K., Rompikuntal, P.K., Lindmark, B., Vaitkevicius, R., Song, T., Wai,

S.N. (2007) The metalloprotease PrtV from *Vibrio cholerae* purification and properties. *FEBS J* **275**, 3167-3177.

저작물 이용 허락서

학 과	생명공학과	학 번	20087062	과 정	석사과정
성 명	한글 : 박재영	한문 : 朴在泳	영문 : Park Jae Yeong		
주 소	광주광역시 동구 동명동 143-43번지 105호				
연락처	e-mail : warpdrive@naver.com				
논문제목	한글 *Vibrio metschnikovii* 균주로부터 분비되는 새로운 알칼리성 세린계열 단백질분해효소의 정제 및 특성 연구				
	영문 Purification and characterization of a novel alkaline serine protease secreted from *Vibrio metschnikovii*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함(다만, 저작물의 내용변경은 금지함)
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함

동의여부 : 동의(0) 반대( )

2010 년 8 월

저작자 : 박재영 (인)

조선대학교 총장 귀하