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碩士學位論文

Molecular cloning and site-directed mutagenesis of an extracellular protease gene from *Vibrio vulnificus*

朝 鮮 大 學 校 大 學 院

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指導敎授 李 正 燮

이 論文을 理學碩士學位 論文으로 提出함

2006年 10月

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ABSTRACT

Molecular cloning and site-directed mutagenesis of an extracellular protease gene from *Vibrio vulnificus*

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Bacterial secreted proteases have important physiological roles in the regulation of bacterial life cycle. The proteases secreted from pathogenic microorganisms can also act as toxic factors against the host. Many of these proteases are metalloproteases having a zinc(II) ion in their catalytic site. The zinc metalloproteases produced by pathogenic bacteria show a wide variety of pathological properties. For example, a zinc metalloprotease produced by *Vibrio vulnificus* (*V. vulnificus*), which is an opportunistic pathogen, can enhance vascular permeability and causes tissue damage through degradation of a wide variety of host proteins. In this laboratory a broad specificity metalloprotease referred to as vEP has been purified previously from the culture supernatant of *V. vulnificus* ATCC 29307 and characterized with respect to prothrombin activation and fibrinolytic activity. Based on the sequence of *vEP* gene, the entire coding region was obtained by polymerase chain reaction (PCR), cloned into a

periplasmic-directed expression vector (pFLAG-ATS), and expressed in *Escherichia coli* (*E. coli*). The active recombinant vEP (named rvEP) was purified from the periplasmic proteins of the transformant *E. coli* cells. The purified rvEP enzyme appeared both as 45- and 35-kDa forms on SDS-polyacrylamide gel. It has been shown that the intact 45-kDa protease can be autoprocessed to make 35-kDa in size by the removal of its C-terminal region. The purified enzyme was characterized with respect to the effects of temperature, pH-dependence, substrate specificity and inhibitors on enzyme activity. Two mutants proteases designated as ΔC100 and G202D were constructed and their properties were studied by comparing with those of at the wild type vEP enzyme. One mutant protease ΔC100, which had a deletion of the C-terminal 100 amino acids from vEP showed reduced specific activity at the level of 80%, compared to that of wild type enzyme. Another mutant protease G202D that has a substitution of glycine^{202} to aspartate^{200} exhibited approximately 58% level of specific activity, compared that of wild type enzyme. Previous data obtained in this laboratory have shown that vEP can activate prothrombin and hydrolyze fibrin as well as cross-linked fibrin by proteolysis. The activity of the two mutants on prothrombin activation and fibrin hydrolysis was also investigated in this study. The data obtained showed that ΔC100 and G202D proteases could also activate prothrombin to have actual thrombin activity at the levels of 58% and 33.1%, respectively, compared that of wild type vEP. The mutants also showed lesser fibrinolytic activity than wild type enzyme. The mutant protease G202D was less thermal stable than wild-type with ΔC100 showing no change in thermal stability. There were noticeable differences in substrate specificity between wild type and ΔC100 enzymes, as shown by both the extent of cleavage of protein substrates and the pattern of cleavage, especially with the cleavage of γ-globulin whereby

ΔC100 exhibited markedly decreased proteolysis. All three enzymes had similar sensitivity towards metal chelator-type inhibitors, but ΔC100 was less sensitive than wild-type vEP to inhibition by Ni^{2+} while G202D was more sensitive than wild-type vEP to inhibition by Cu^{2+}. Taken together, the data obtained in this study show that the C-terminal domain of vEP may play a role in the binding of certain substrate proteins to enable better cleavage. However, it does not appear to be involved in the processing of the propeptide of the enzyme during maturation process. The change in thermal stability of G202D shows that Gly^{202} may play a role in the stabilization of the enzyme at high temperature.

I. INTRODUCTION

Proteolytic enzymes play various physiological roles and are essential factors for homeostatic control in both eukaryotes and prokaryotes. However, the enzyme produced by pathogenic microorganisms, particularly by opportunistic pathogens can also act as toxic factors to the host (Travis *et al*., 1995). Many of these bacterial proteases are zinc-containing metalloproteases (Hase & Finkelstein, 1993).

V. vulnificus is a gram-negative halophilic marine bacterium that is endemic in the warm coastal waters. It is an opportunistic human pathogen that causes wound infection and septicemia (Janda *et al*., 1988; Tacket *et al*., 1984; Ulusarac & Carter, 2003). It secretes a 45-kDa zinc metalloprotease that has been reported to have many biological functions. These include the degradation of various plasma proteins and vascular permeability enhancement through the generation of inflammatory mediators. The true biological function of this enzyme is still unknown and direct evidence for its role during infection has yet to be demonstrated. However, the wide spectrum of its activities in an *in vitro* system has made it a very interesting protease. Whether these biological activities of the enzyme contribute to the pathogenecity exerted by v*ibrio* have not been conclusively demonstrated, but injection of the purified enzyme into animal reproduced some of the pathology observed with v*ibrio* infection (Miyoshi *et al*., 1993; Miyoshi & Shinoda, 2000).

In this laboratory, the purification and characterization of an extracellular metalloprotease (vEP) produced by *V. vulnificus* strain ATCC 29307 has previously been reported (Chang *et al.*, 2005). vEP exhibits prothrombin

activation and fibrinolytic activities. It is a broad specificity protease that does not seem to have a specific cleavage sites, but could cleave at sequence containing hydrophobic residues. Although the sequence of the gene encoding vEP has previously been reported (Jeong *et al*., 2000), the enzyme has not been expressed and purified. A zinc-dependent metalloprotease from *V. harveyi* has also been cloned and characterized (Teo *et al.*, 2003). The amino acid sequence of vEP predicted from the DNA sequence shows that it belongs to a neutral family of metalloproteases having the characteristic zinc binding motif of HEXXH at the active center. Of these proteases, the enzyme from *Bacilus thermoproteolyticus* (thermolysin) is best characterized. vEP and thermolysin has about 60% sequence homology.

The schematic structure of vEP is shown in Figure 1. The precursor consists of three distinct domains: the signal peptide, the propeptide and the mature enzyme. The mature enzyme comprises of the catalytic domain and the C-terminal domain. The signal sequence targets the enzyme for secretion while the propeptide function as intra-molecular chaperone required for the folding of the polypeptide and as an inhibitor preventing premature activation of the enzyme. Tang and coworkers (2003) who studied the general function of the N-terminal propeptide of the extracellular thermolysin-like metalloprotease (PA protease) produced by *Aeromonas caviae* T-64 showed that if acts an intramolecular chaperone to assist the folding of PA protease and shows inhibitory activity toward its cognate mature enzyme. The N-terminal propeptide also inhibits the autoprocessing of the C-terminal domain. The C-terminal domain of vEP comprises of about 100 amino acids and is subjected to auto-processing during enzyme purification, and the loss of this domain does not appear to have a significant effect on enzyme activity (Miyoshi *et al.*, 1997). The function of the

C-terminal domain has been proposed to play a role in the processing of the propeptide for a related enzyme. For the genus v*ibrio*, it was previously suggested to play a role in the binding of insoluble substrates such as collagen and elastin (Miyoshi *et la*., 1997). These authors studied the recombinant enzyme from *V. vulnificus* strain L-180 reported that both the 45-kDa and 35-kDa forms have similar proteolytic activities, but the 35-kDa is less active toward insoluble proteins such as collagen and elastin.

This thesis describes the cloning, expression, purification and characterization of vEP. Two vEP mutants, one having a deletion of the C-terminal 100, residues and another having a Gly to Asp substitution at residue 202 were also made, expressed and characterized. The differences in enzyme activity and properties with respect to substrate specificity, thermal stability and sensitivity to inhibitors are discussed.

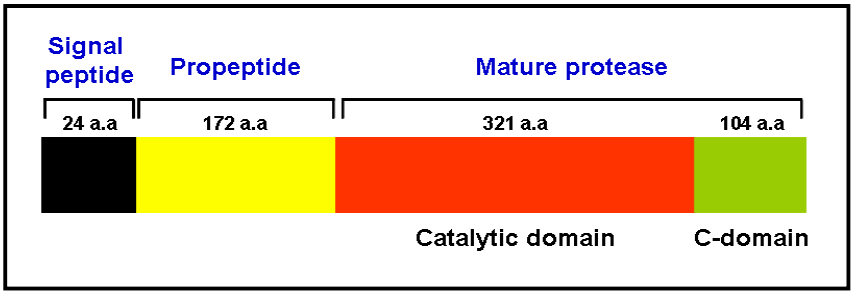

Fig. 1. Schematic representation of the structure of vEP.

II. MATERIALS AND METHODS

II-1. Materials

HiPrep 16/10 Q FF column, Source 15 Q 4.6/100 PE column and PD-10 column were purchased from Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Plasminogen and plasmin were purchases from Roche Applied Science (Mannheim, Germany). Human prothrombin was obtained from CalBiochem (Darmstadt, Germany). Protein molecular weight markers were obtained from Fermentas. (Darmstadt, Germany). Bradford protein assay kit, thermolysin, bovine serum albumin (BSA), fibrinogen, γ-globulin, elastin, collagen, azocasein, EGTA, EDTA, DEPC, phenylmethylsulfonyl fluoride (PMSF), agarose, 1,10-phenanthroline (1,10-PT), ammonium sulfate, N,N-methylene-*bis*-acrylamide SDS, TEMED, trizma base, and other chemicals used were obtained from Sigma (St. Louis, MO, USA). The plasmids pFLAG-ATS and pGEM-T vector were obtained from Sigma and Promega (Madison, USA), respectively. The synthetic chromogenic peptide substrate Boc-VPR-*p*NA was a kind gift from T. Morita (Meiji Pharmarceutical University, Tokyo, Japan). The synthetic fluorogenic substrates used in this study were purchased from Genscript Co. (New Jersey, USA).

II-2. Cultivation of *Vibrio vulnificus* and *E. coli.*

Vibrio vulnificus ATCC 29307 was cultured in LB medium containing 0.5% NaCl and *E. coli* DH5α was cultured in LB medium supplemented with 1% NaCl.

II-3. Cloning of *vEP* gene

The entire coding region of *vEP* gene was amplified by PCR from the chromosomal DNA of *V. vulnificus* ATCC 29307 using two primers; Forward primer 1 (5'-CTTCTCGAGATGAAACTCAATCAACGT-3') and Reverse primer 1 (5'-CGGGGTACCTCAATATTGCAGCTTTAA-3'). The underlined bases in the primers show the introduced restriction sites (*Xho*I in Forward primer 1, and *Kpn*I in Reverse primer 1). PCR was performed for 1 min at 94℃, 45 sec at 50℃ and 2 min at 72℃ for 30 cycles using the Applied Biosystem 9700 thermal cycler. The amplified PCR product (1.8 kb) was digested with *Xho*I and *Kpn*I and then ligated with *Xho*I/*Kpn*I-cut pFLAG-ATS to give the construct pvEP.

II-4. Construction of vEP mutants

II-4-1. Construction of vEP mutant with C-terminal deletion

The plasmid pvEP, carrying a 1.8 kb *Xho*I-*Kpn*I insert from *V. vulnificus* ATCC 29307 was used as a template for the PCR. The coding region of *vEP* gene without 3' end 900 bp was amplified from pvEP with Forward primer 1 and Reverse primer 2 (5'-GTGATGGTGATGGTGATTACCACTTGGCGGCGT-3'). A 1.5 kb DNA fragment was obtained and it was then used as a template for the second PCR using Forward primer 1 and Reverse primer 3 (5'-CGGGGTACCTTA ATGGTGATGGTGATGGTGATT-3'). The second PCR product was flanked by *Xho*I and *Kpn*I sites and it was purified and incubated with *Taq* DNA polymerase and dATP. The A-tailed DNA was cloned into pGEM-T-vector and the insert was

subsequently excised from the vector by cleavage with *Xho*I and *Kpn*I. This resulting DNA was then ligated into *Xho*I/*Kpn*I cut pFLAG-ATS to give the construct pvEP-ΔC100. The use of two rounds of PCR with two reverse primers was to introduce a 6 x His tag at the C-terminus of the enzyme for easy of purification.

II-4-2. Site-directed mutagenesis

The mutant G202D was made PCR with the use of megaprimers. The protocol of mutagenesis was outlined in Figure 2. Forward primer 2 (5'-AAGTCG AATGACGGTTTACGCTAC-3', underlined base indicates base change), Reverse primer 1 and pvEP were used to obtain the first PCR product which contains the mutation. This PCR product was purified and used as reverse primer in the second PCR with Forward primer 1 and pvEP to generate the entire vEP coding sequence. The DNA fragment which is flanked by *Xho*I and *Kpn*I sites was cleaved with these two restriction enzymes and then ligated into *Xho*I/*Kpn*I-cut pFLAG-ATS to yield the construct pvEP-G202D.

II-5. Enzyme expression and purification

DH5α was used as host cell for the expression of *vEP* gene. Cells were transformed with the appropriate construct by the heat shock method. A single transformed with the appropriate construct by the heat shock method. A single colony of cells harboring pvEP, pvEP-ΔC100, or pvEP-G202D was inoculated into 50 ml of LB broth containing 100 μg/ml of ampicillin and incubated at 37℃ for overnight. 500 ml of LB broth containing 100 μg/ml of ampicillin was inoculated

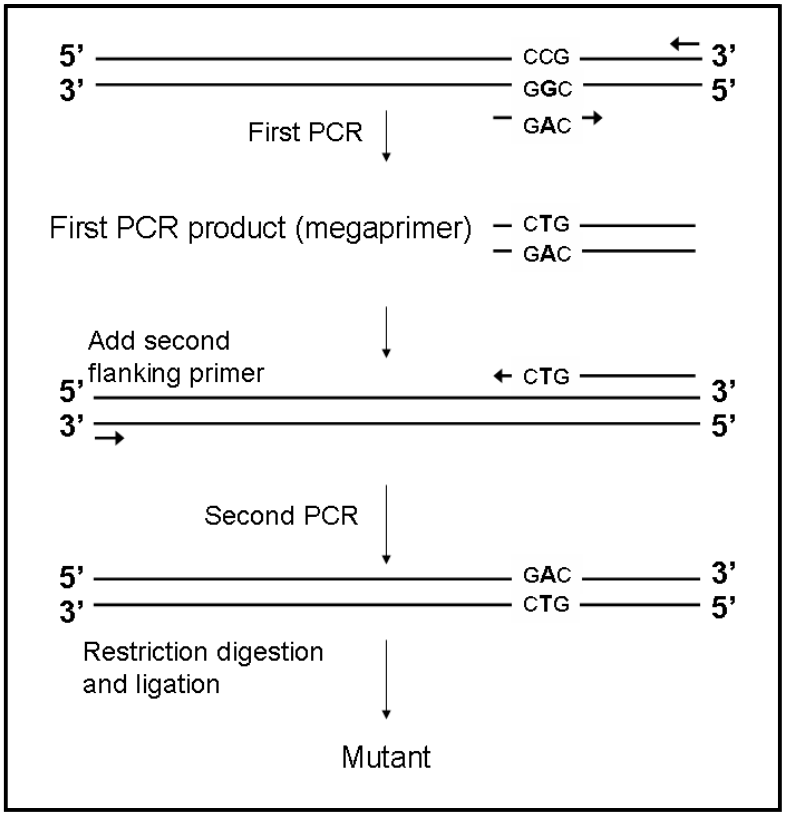

Fig. 2. Outline of mutagenesis protocol used for the construction of G202D mutant.

with 10 ml of the overnight culture. A total of four sets of 500 ml cultures were prepared for each expression. The cultures were incubated with vigorous shaking (200 rpm) at 37℃ until an A_{600} of about 0.8 was reached. IPTG was then added to the culture to a final concentration of 0.2 mM and incubated for 6h at 20℃. The cells were harvested from the cultures by centrifugation at 4,000 xg for 15 min and the pellet was resuspended in 100 ml of buffer containing 30 mM Tris-HCl (pH 8), 20% (w/v) sucrose, 1 mM EDTA, 0.3 mg/ml lysozyme and 1 mM PMSF. The cell suspension was incubated on a shaker at moderate speed for 30 min at 4℃. It was then centrifuged at 6,000 xg for 20 min at 4℃ and the supernatant was collected as a cell-free extract. Ammonium sulfate was added to the cell-free extract to give 20% saturation and the protein precipitate was removed by centrifugation at 16,000 xg for 30 min at 4℃. The supernatant was collected and the ammonium sulfate concentration was increased to 70% saturation. The resulting precipitate was collected by centrifugation at 16,000 xg for 40 min at 4℃. The pellet was dissolved in 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ followed by desalting on PD-10 columns (Amersham Biosciences) equilibrated with the same buffer. The desalted sample was applied to a HiPrep 16/10 Q FF column (Amersham Biosciences) pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes of equilibration buffer, the column was eluted with a linear gradient of NaCl from 0 to 0.4 M in the same buffer. Fractions were assayed for protease activity using azocasein as a substrate, and those fractions containing major protease activities were pooled, concentrated by ultra-filtration using an Amicon YM10 membrane (Millipore, Billerica, MA), and then further fractionated on a Source 15 Q 4.6/100 PE column (Amersham Biosciences) pre-equilibrated with the same buffer at room temperature. After washing with 10 column volumes of equilibration buffer,

the column was eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. Fractions with major protease activities were pooled, concentrated, and used as the purified enzyme and stored in small aliquots at -20℃.

II-6. Protease activity assay

Protease activity was routinely assayed with azocasein as a substrate. Reaction sample (total 200 μl) containing enzyme, 50 mM Tris-HCl (pH 7.5), and 0.25% azocasein was incubated at 37℃ for 15 min. The reaction was stopped by addition of 100 μl of 10% (w/v) trichloroacetic acid and then centrifuged at 10,000 xg for 10 min. Two hundred microliters of the supernatant was taken and the absorbance at 440 nm was measured in a 96-well plate reader (Molecular Devices).

II-7. Fluorogenic peptide assay

The design of the fluorogenic peptide {(7-methoxycoumarin-4-yl)acetyl-Gly-Arg-Thr-Ala-Thr-Ser-(ε-2,3-dinitrophenyl)Lys-amide} was based on the cleavage site of vEP obtained for the cleavage of human prothrombin (Thr^{273}-Ala^{274}). The peptide was synthesized by GenScript (USA). This peptide was designed so that the fluorescence derived from the N-terminal coumarin derivate (λ_{ex} 328 nm, λ_{em} 393 nm) was strongly quenched by the C-terminal dinitrophenyl group. Upon cleavage of the peptide by enzyme, the fluorescence increases with the release of the quencher (Knight, *et la*., 1992, Nagase, *et la*., 1994). The assay (100 μl)

contained 1 μg/ml enzyme with different concentrations of peptide (dissolved in dimethylformamide) in 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl. Enzyme and substrate were pre-incubated separately for 10 min at 37℃, and then mixed and the fluorescence was monitored for a further 10 min at the same temperature using a 96 well plate reader (Molecular Devices).

II-8. SDS-PAGE analysis

SDS-PAGE was performed according to the method of Laemmli (1970). Samples to be analyzed were mixed with an equal volume of 2 x SDS-PAGE sample buffer, heated at 100℃ for 1 min, and then loaded onto either 10% or 12% gel. After electrophoresis, protein bands were visualized by staining the gel with Coomassie blue. Molecular weight markers used consisted of β-galactosidase (*E. coli*, 116 kDa), bovine serum albumin (bovine plasma, 66 kDa), ovalbumin (chicken egg white, 45 kDa), lactate dehydrogenase (porcine muscle, 35 kDa), restriction endonuclease Bsp98I (*E. coli*, 25 kDa), β-lactoglobulin (bovine milk, 18.4 kDa), lysozyme (chicken egg white, 14.4 kDa).

II-9. Protein assay

Protein concentrations were determined with Bradford reagent (Sigma) according to the manufacturer's instructions.

II-10. Analysis of prothrombin activation

For the detection of prothrombin activation, 200 μl of reaction mixture containing 0.4 mg/ml prothrombin and 4 μg/ml vEP in 50 mM Tris-HCl (pH 7.5) was incubated at room temperature and 24 μl aliquots were withdrawn at different time intervals. The reaction was stopped by the addition of 1 μl of 25 mM 1,10-PT. To measure the thrombin activity, 10 μl of this sample was assayed in the presence of 0.4 mM Boc-VPR-*p*NA in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM 1,10-PT in a 100 μl reaction volume at 37℃ using a 96-well plate reader. The increase in absorbance at 405 nm due to the release of *p*NA was recorded over a period of 10 min.

II-11. Fibrinolytic activity assay

Fibrinolytic activity was assayed by measuring the decrease in turbidity of fibrin polymer in a 96-well plate using a spectrophotometric method. Ninety microliters of 1mg/ml of fibrinogen in 25 mM phosphate buffer (pH 7.5) was added to 10 μl thrombin (10 U/ml), and the fibrin polymer was allowed to form at room temperature for 1 h. Thereafter, 10 μl of vEP (0.6 μg) or plasmin (1 μg) was added to the polymer and incubated for 30 min at room temperature. The decrease in absorbance at 350 nm was then recorded with a 96-well plate reader (Molecular Devices).

II-12. Characterization of wild type and mutant vEPs

II-12-1. Effects of temperatures and pH on enzyme activity

To study the effect of assay temperatures on enzyme activity, the enzyme was assayed with azocasein as described in section II-6 but at different temperatures ranging from 37 to 75℃. The effect of pH on enzyme activity was investigated by assaying the enzyme activity with azocasein but in buffers having different pHs. The buffers used in these assays were 50 mM sodium acetate (pH 4-5.5), 50 mM sodium phosphate (pH 6-7.5), 50 mM Tris-HCl (pH 8-8.5), or 50 mM glycine-NaOH (pH 9-10.5) buffers.

II-12-2. Thermal stability of wild type and mutant vEP

To investigate the effect of temperatures on enzyme stability the purified enzyme was incubated at 55℃, 65℃ or 75℃ and samples were withdrawn at different time points (0, 1, 2, 5, 10 and 20 min) and kept on ice. The residual protease activity of the enzyme was assayed with azocasein in standard conditions. For SDS-PAGE analysis, 1,10-phenanthrolin was added to the sample to a final concentration of 2 mM and prior to electrophoresis as described in section II-8.

II-12-3. Effect of inhibitors on protease activity

The effects of cations (Ni^{2+} and Cu^{2+}), metal chelating agents (EDTA, EGTA and 1,10-phenanthrolin) and SDS on enzyme activity were studied by assaying the enzyme with azocasein in the presence of different concentrations of the additive at 37℃ for 10 min.

II-12-4. Inactivation of vEP by DEPC

The enzyme was pre-incubated with different concentrations of DEPC for 10 min at room temperature. The reaction was quenched with addition of 50 mM imidazole in 50 mM Tris-HCl (pH 7.5) and the residual enzyme activity was assayed with azocasein in standard conditions.

II-12-5. Cleavage patterns of plasma proteins

For the analysis of protein cleavage pattern, 10 μg of protein substrate was cleaved with 0.3 μg of vEP in 25 mM Tris-HCl (pH 7.5) in a total volume of 15 μl at room temperature or 37℃ for a specific time interval. Prothrombin was digested at room temperature for 5 min. Fibrinogen was digested at room temperature for 20 min. Plasminogen, γ-globulin, and BSA were digested at 37℃ for 60 min. The digestion was terminated by addition of 2 mM 1,10-phenanthrolin and an equal volume of 2 x SDS-PAGE sample buffer followed by SDS-PAGE analysis as described in section II-8.

II-12-6. Cleavage of insoluble proteins

Elastin and collagen were used as substrate for the analysis of insoluble protein cleavage by wild type and vEP mutants. Twenty micrograms of wild type vEP or vEP mutants was incubated with 1 mg of elastin in 25 mM Tris-HCl (pH 7.5) in a total volume of 500 μl at room temperature with gentle shaking for overnight. The sample was centrifuged at 10,000 xg for 10 min to separate undigested elastin from the soluble proteins/peptides released by vEP. The supernatant obtained was further precipitated with 3% TCA to precipitate vEP. After centrifugation at 10,000 xg for 10 min, the absorbance of the supernatant at 280 nm was measured against the supernatant obtained from the control (elastin only) using a spectrophotometer (Ultrospec 2000). For the cleavage of collagen type VI, the protein was first dissolved in 0.25% acetic acid, and the protein sample was then adjusted with Tris base to give a pH of about 7.5 and a protein concentration of 3 mg/ml. Twenty micrograms of this collagen was cleaved with 0.2 μg vEP in a total volume of 10 μl at 37℃ for 30 min. After cleavage, the samples were analyzed by SDS-PAGE.

III. RESULTS AND DISCUSSION

III-1. Cloning of *vEP* gene

The cloning of *vEP* gene was achieved with PCR using primers derived from the sequence for this enzyme. The PCR product was digested with *Xho*I and *Kpn*I and then ligated into *Xho*I/*Kpn*I-cut pFLAG-ATS to yield the construct shown in (Fig. 3). Wild type vEP consist of four separate domains (Fig. 1). The signal peptide enables the precursor to be targeted for secretion. The propeptide is thought to play a role in helping the enzyme to fold correctly and to inhibit the enzyme activity to prevent premature activation. The catalytic domain contains the active site of the enzyme including the metal binding site. The terminal C-domain may involve in substrate binding as well as the removal of the propeptide during activation. A mutant vEP with deletion of C-terminal 100 amino acids (ΔC100) was made to examine whether the terminal C-domain is necessary for the processing of the propeptide during the maturation of the vEP. The deletion was chosen on the basis of the putative cleavage site of vEP resulting from auto-processing. This site was determined to be at the carboxyl side of Asn^{314} based on the data obtained from mass spectrometry analysis of purified native vEP (Chang *et al*., 2005).

As vEP is highly homologous to thermolysin. In thermolysin, a residue Asp^{200} that is critical for the maintenance of thermal stability has been identified (Fig. 4). Due to the high homology between vEP and thermolysin, a residue within vEP (Gly^{202}) that corresponds to Asp^{200} in thermolysin was chosen for mutagenesis study to improve the thermal stability of vEP.

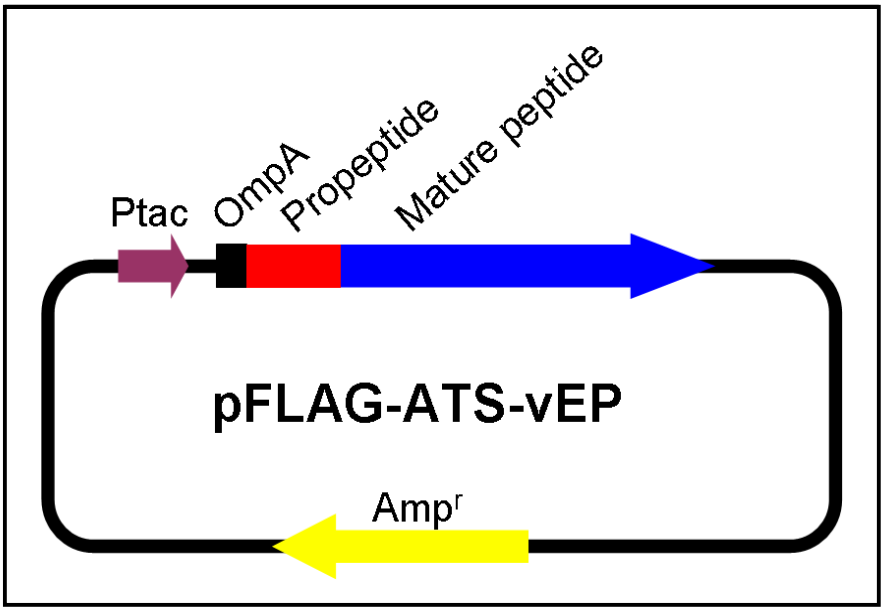

Fig. 3. Physical map of recombinant plasmid pFLAG-ATS-vEP .

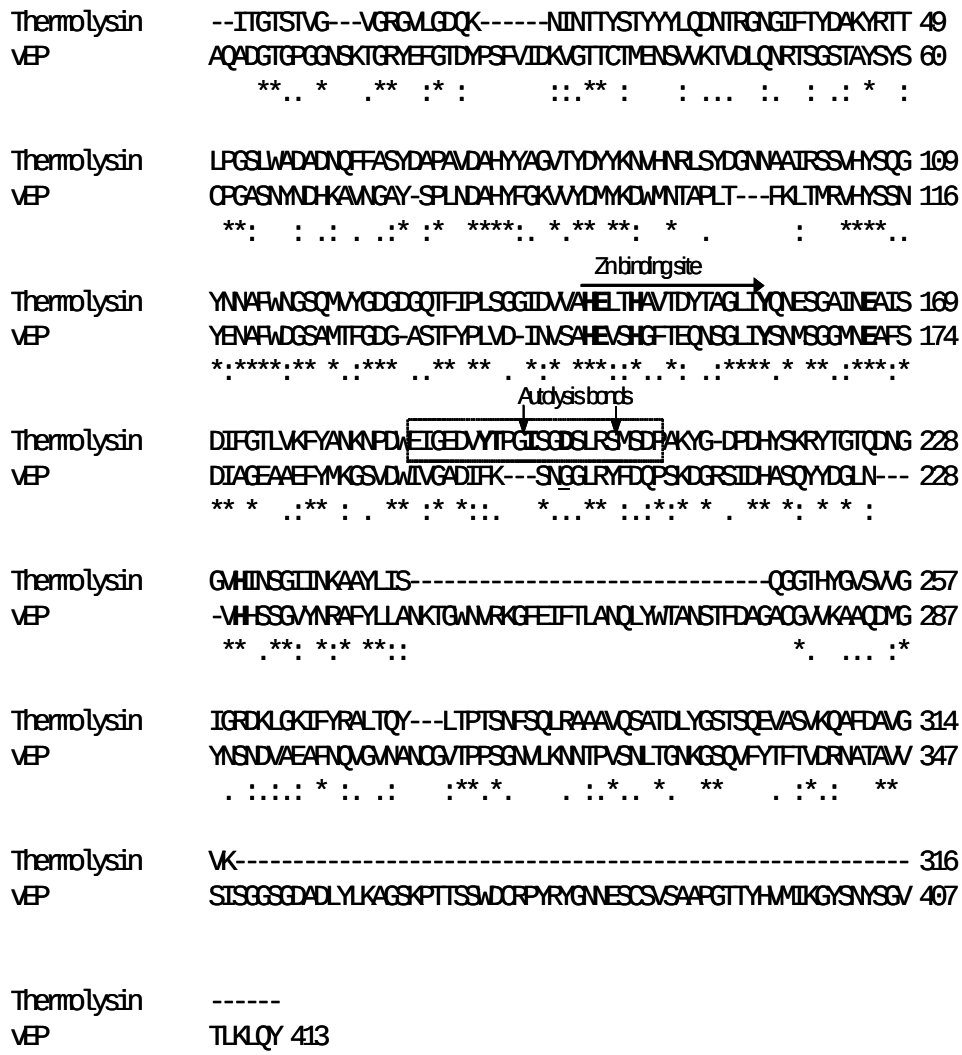

Figure 4. Alignment of thermolysin and vEP sequences. Residues in rectangular box constitutes the Ca^{2+}(4) binding site in thermolysin.

III-2. Expression and purification of vEP

vEP was expressed in *E. coli* by induction with 0.2 mM IPTG. As pFLAG-ATS is a periplasmic based expression vector, the expressed enzyme was targeted to the periplasmic region. The enzyme was expressed as a fusion protein containing the OmpA sequence fused to the whole of the structural gene of vEP. The expressed fusion protein was targeted to the periplasmic region. The enzyme was isolated from the periplasmic proteins and then subjected to two successive anion exchange chromatographic steps using HiPrep Q column and Source Q column in order. The purification of the two vEP mutants was essentially the same as for the wild type enzyme. The purification of wild type vEP, ΔC100 and G202D mutants were summarized in Tables 1, 2 and 3, respectively. The wild type enzyme had the highest specific activity. ΔC100 was less active with a specific activity of about 80% that of wild type, while G202D mutant has only 58% activity of wild type vEP. Only 15% yield was obtained for the wild type enzyme. The yields of ΔC100 and G202D were higher than wild type. SDS-PAGE analysis of the purified enzyme showed that both the wild type and G202D mutant consisted of both 45- and 35-kDa forms (Fig. 5). Wild type vEP, however, contained more 35-kDa form than that present in G202D mutant. Both enzymes also showed an intermediate product. ΔC100 which lacks the C-terminal 100 amino acids appeared only as a 35-kDa form (Fig. 5).

Table 1. Purification summary of wild type vEP protease.

Purification step	Total protein (mg)	Total Activity (U)[a]	Specific Activity (U/mg)	Yield (%)[b]
Periplasmic proteins	49.5	407,000	8,200	100
HiPrep Q	5.04	120,000	24,000	30
Source Q	2.01	607,000	30,300	15

[a] One unit (U) was defined as the amount of the protease digesting 1 μg of the azocasein in 1 min.

[b] The total activity in the periplasmic proteins was assigned the value of 100%.

Table 2. Purification summary of mutant protease ΔC100.

Purification step	Total protein (mg)	Total Activity (U)[a]	Specific Activity (U/mg)	Yield (%)[b]
Periplasmic proteins	109.7	1,005,000	9100	100
HiPrep Q	44.2	750,000	16,900	74
Source Q	29.1	700,000	24,600	69

[a] and [b], the same as described in the footnote in Table 1.

Table 3. Purification summary of mutant protease G202D.

Purification step	Total protein (mg)	Total Activity (U)[a]	Specific Activity (U/mg)	Yield (%)[b]
Periplasmic proteins	40.7	275,000	6,750	100
HiPrep Q	7.7	107,300	14,000	39
Source Q	4.4	78,400	17,800	28

[a] and [b], the same as described in the footnote in Table 1.

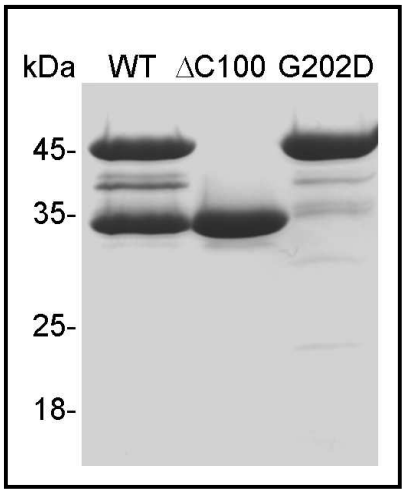

Fig. 5. SDS-PAGE analysis of purified wild type and mutant proteases ΔC100 and G202D.

III-3. Prothrombin activation by vEP

One of the important properties of vEP is its ability to activate prothrombin via proteolysis (Chang *et al.*, 2005). Thus the activation of prothrombin by wild type vEP and vEP mutants was compared over a period of 40 min at room temperature (Fig. 6). All three enzymes showed similar trends, with the activation increasing with time and reached a plateau at about 10 min. The extent of activation paralleled with the specific activity of each enzyme, with wild type showing the highest and G202D the lowest activation. Production of active thrombin was detected with the hydrolysis of Boc-VPR-*p*NA, which is specific chromogenic substrate for thrombin. These results suggest that the mutant enzymes can activate prothrombin with lowered enzyme activities. In addition, the 100 amino acids stretch at the C-terminal region of intact enzyme may play an important role in probably binding the enzyme to substrate and also in enzyme catalysis.

III-4. Fibrinolytic activity

In addition to having prothrombin activation property, vEP also possesses fibrinolytic activity being able to digest both fibrinogen and fibrin (Chang *et al.*, 2005), a property that may prove useful for the invading bacteria during infection. The fibrinolytic activity fo the recombinant enzymes were confirmed by their ability to degrade fibrin polymer, which was measured as a change of turbidity of the polymer at 350 nm (Fig. 7). Interestingly, wild type and G202D could degrade fibrin polymer equally well, whereas ΔC100 was less efficient,

owing to the absence of the C-terminal domain since fibrin is an insoluble protein. The degradation of fibrin polymer by both wild type and vEP mutants was less efficient than that shown by plasmin, the main physiological fibrinolytic enzyme. The ability of these different forms of recombinant vEP, to degrade fibrin provide further evidence to support the identity of these enzymes. However, it should be noted that the ultimate confirmation of the identity of these different of vEP could only be achieved by protein sequencing. Despite the fact that none of these enzyme has been subjected to sequencing, the properties of the enzyme examined so far and more below are sufficient to confirm that each of one of these enzymes are indeed vEP.

III-5. Effect of assay temperatures on enzyme activity

Protease activity is routinely carried out at 37℃. Figure 8. shows the effect of assay temperatures on enzyme activity for vEP. All three enzymes tested showed an optimal activity at 55℃. The increase in activity at 55℃ relative to that at 37℃ was highest for G202D, followed by ΔC100, while wild type vEP showed the lowest difference. At temperatures higher than 55℃, the activity started to drop, and at 75℃ (the highest temperature assayed), there was still significant amount of activity relative to that at 37℃. This result suggests that vEP is a relatively stable enzyme that is able to perform proteolysis at temperatures above that of physiological conditions.

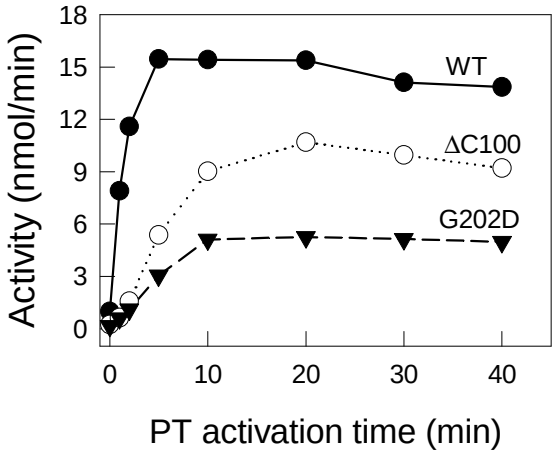

Fig. 6. Activation of prothrombin by vEP, ΔC100, and G202D proteases. Thrombin activity was measured with thrombin-specific chromogenic substrate (Boc-VPR-*p*NA). Prothrombin (PT; 0.4 mg/ml) was activated by vEP (4 μg/ml) at room temperature, aliquots were withdrawn at different time intervals, and the reaction was stopped by the addition of 1 mM 1,10-PT to. The cleavage products were assayed for thrombin activity in the presence of 1 mM 1,10-PT and 0.4 mM of Boc-VPR-*p*NA at 37℃.

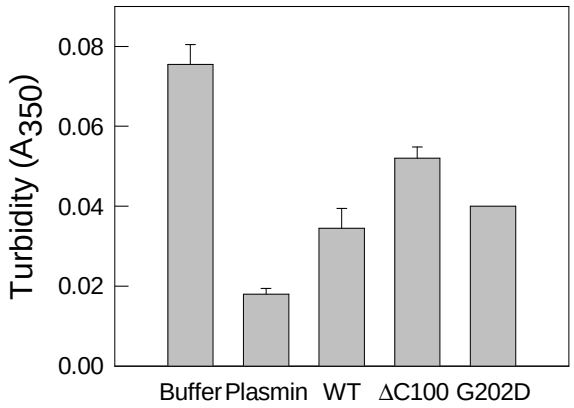

Fig. 7. Fibrinolytic activity of wild type vEP and mutant proteases. Fibrinolytic activity was measured by a decrease in the turbidity of fibrin polymer. Enzymes were applied as spots onto the fibrin polymer (catalyzed by thrombin with fibrinogen as substrate) and allowed to incubate at room temperature for 30 min. The decrease in turbidity was then measured at 350 nm. The data are the mean values from two separate experiments performed in triplicate.

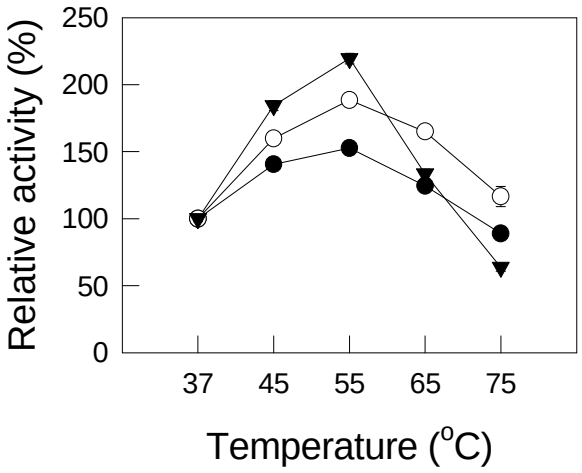

Fig. 8. Effect of assay temperatures on wild type (WT) and vEP mutants. Protease activity was assayed with azocasein at different temperatures as described in Materials and Methods. Symbols: ●, WT; ○, ΔC100; ▼, G202D. The data are the mean values from two separate experiments performed in triplicate.

III-6. pH dependence

The activity verse pH profiles were similar for both wild type and vEP mutants in that all enzymes exhibited maximal activity at around pH 8. Removing the C-terminal 100 amino acids or changing Gly^{202} to Asp from vEP did not appear to alter the pH-dependence of the enzyme with respect to protease activity.

III-7. Thermal stability of proteases

Thermolysin is a very thermal stable enzyme. Since there is a high degree of homology between thermolysin and vEP, the thermal stability of vEP was investigated and compared with that of thermolysin. The enzymes were first heated at three different temperatures (55℃, 65℃ or 75℃), and the residual activity of the enzyme was assayed with azocasein as a substrate at 37℃. At 55℃, there was no degradation of wild type, ΔC100 or thermolysin (Fig. 10A). Although there was no loss of protein for G202D due to degradation at this temperature, there was significant conversion of the 45-kDa form to 35-kDa beyond 5 min of incubation. This appears to suggest that changing Gly^{202} to Asp decreases the thermal stability of the enzyme instead of increasing as anticipated. The loss of 45-kDa form of G202D also correlated with the loss of enzyme activity (Fig. 10B). At 65℃, significant loss of protein was seen with both wild type and G202D, and although the degradation of protein occurred mainly for the 45-kDa, loss of the 35-kDa was also noticeable, especially for G202D at 20 min incubation (Fig. 11A). For the wild type, the loss of the

45-kDa form resulted in the accumulation of the 35-kDa form from 1 to 5 min of incubation. ΔC100 also showed a steadily loss of protein with incubation time, but the trench of protein loss was less severe compared with that of the 45-kDa seen with the wild type and G202D. The loss of protein simultaneously led to a loss of enzyme activity (Fig. 11B). Thermolysin showed no significant loss of protein or activity at 65℃. At 75℃, complete loss of protein and activity were seen with both wild type and mutant vEP after 5 min of incubation (Fig. 12). Thermolysin also showed some degradation but the majority of the protein was still intact, even after 20 min of incubation. This results shows that thermolysin is a much more thermal stable enzyme than vEP. The degradation of vEP also suggests that it is a biphasic event with initial specific cleavage leading to the transient accumulation of the 35-kDa form, followed by more non-specific cleavage leading to complete loss of the enzyme. Changing Gly^{202} to Asp also has an effect on the enzyme thermal stability, but instead of increasing it relative to the wild type, it actually decreased the thermal stability of the enzyme.

III-8. Substrate specificity

Removing the C-terminal 100 amino acids from vEP protease reduces the enzyme specific activity by about 80%. Since no residue that constitutes the active site of vEP is located in the region comprised by the terminal 100 amino acids, the effect on enzyme activity may due to a decrease in substrate binding ability. Comparison of the cleavage profile of wild type vEP and the two mutants for various plasma proteins showed wild type and G202D had similar patterns while the pattern of ΔC100 was noticeably different, both in the degree of

cleavage and in the band pattern. The most significant difference was seen with the cleavage of γ-globulin (Fig. 13). Wild type vEP could readily cleave γ-globulin whereas only about 50% of the protein was cleaved by G202D and about 90% of the protein still remained intact in the case of ΔC100. These results suggest that the C-terminal domain is essential for the cleavage of γ-globulin by vEP, and that it enable the enzyme to better cleave γ-globulin by enabling the vEP to better bind to γ-globulin. This is a significant feature since γ–globulin is involved in the defense mechanism of both human and animal.

III-9. Cleavage of insoluble proteins

The cleavage of insoluble proteins by vEP was also examined using elastin and collagen as substrates. The elastin used in the digest could not be dissolved in a suitable aqueous solvent that is permissive to vEP activity. Thus, the protein was mixed with a buffer containing 20 μg/ml enzyme and incubated for overnight at room temperature. The undigested elastin was precipitated with TCA and the soluble peptides derived from cleavage of elastin by vEP were detected as in increase in absorbance at 280 nm. As shown in Figure 14A, wild type vEP produced the most soluble peptides compared to either mutant. But significantly less soluble peptides were released by ΔC100, about 30% that of wild type enzyme, and therefore confirming the important role played by the C-terminal domain in the cleavage of insoluble protein. Collagen is also an insoluble protein, but it could still be dissolved in acidic condition followed by adjustment of the pH to around 7.5 used in the cleavage reaction. However,

unlike the cleavage of elastin, the extent and pattern of collagen cleavage was similar between wild type and ΔC100. In contrast, lesser cleavage of collagen was observed for G202D, and the major difference in pattern being the absence of a band at around 45 and 27 kDa, which could be seen in the cleavage by with wild type and ΔC100 proteases. The C-terminal domain appears to have little or no effect in the case of collagen cleavage, and the lesser cleavage afforded by G202D could relate to its lower enzyme activity compared with either wild type or ΔC100 proteases. Thus it appears that as long as a protein can be dissolved in solution, its cleavage by vEP is less likely to be influenced by the absence of the C-terminal domain. However, in the case of γ-globulin cleavage, the absence of the C-terminal domain significantly reduced the cleavage of the protein although γ-globulin is a soluble protein. The effect of the C-terminal domain on the binding and subsequent cleavage of a protein substrate therefore appears to be different for different substrate proteins, a feature that requires further investigation.

III-10. Cleavage of synthetic peptide substrate

The kinetic properties of wild type vEP and the two mutants were investigated by examing the cleavage of the fluorogenic peptide substrate by the enzymes. The results are shown in Table 4. Both wild type vEP and ΔC100 had similar K_m values for the peptide substrate, whereas the K_m value for G202D was approximately 3 folds lower. Removal of the C-terminal domain appears to have no significant impact on substrate binding. On the other hand, substituting Gly for Asp at residue 202 seems to enhance the binding of substrate. However,

this substitution had a drastic effect on the k_{cat}, which was about 22 folds lower compared with the wild type. The deletion of the C-terminal domain caused only a moderate decrease in k_{cat}. The specificity constant (k_{cat}/K_m) is an indication of the catalytic efficiency of the enzyme for a given substrate. As shown in Table 4, wild type vEP appears to be the most efficient enzyme among the three, followed by ΔC100. G202D is the least efficient enzyme. The extent of impact that a single amino acid change has on the structure and activity of an enzyme is often difficult to predict. Gly^{202} is not a residue that is constitutes the active site. The impact that this residue was expected to have on the enzyme is a change in thermal stability. From the result, it is clear that changing it to Asp significantly decreased the catalysis of the enzyme although, substrate binding was somewhat enhanced.

It should be noted that the k_{cat} obtained with the peptide substrate shows a different trench to the specific activity obtained with azocasein (Tables 1, 2 and 3), whereby, the decrease in specific activity for G202D relative to that of the wild type enzyme is less than two folds. This may due to the difference in substrate between azocasein (which is a whole protein) and the peptide substrate. Thus the change in substrate binding and catalysis can vary depending on whether the substrate is a whole protein or a peptide. Another cause of difference between the two results could be due to the methodologies of the assay. In the case of azocasein, the activity of the enzyme was obtained from endpoint reading whereas in the case of the peptide substrate, the activity was determined from the initial rate of the reaction. In conclusion, the deleting the C-terminal domain of vEP did not significantly affect enzyme activity, whereas changing Gly^{202} to Asp mainly affected the catalysis of the enzyme and at the same time, improved the binding of peptide substrate.

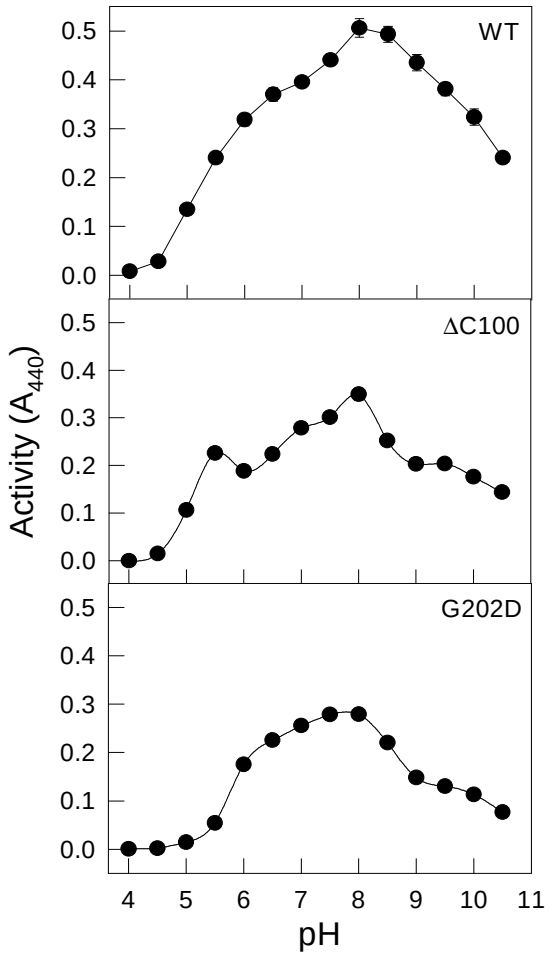

Fig. 9. pH-dependence of wild type (WT) and vEP mutants. Protease activity was assayed with azocasein as described previously at various pHs using the following buffer systems: 50 mM sodium acetate (pH 4.0~5.5); 50 mM sodium phosphate (pH 6.0~7.5); 50 mM Tris-HCl (pH 8.0~-8.5); 50 mM glycine-NaOH (pH 9.0~10.5). The data are the mean values from two separate experiments performed in triplicate.

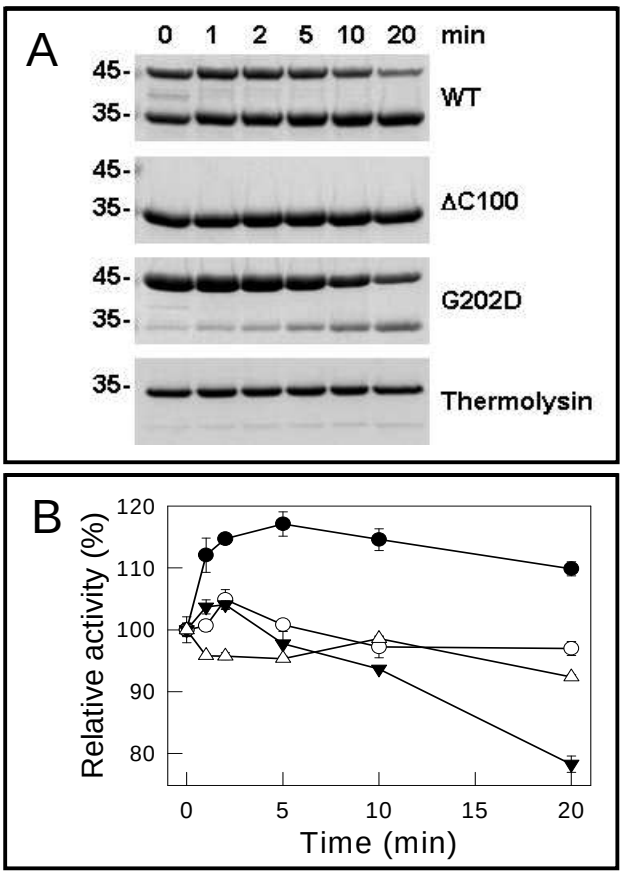

Fig. 10. Thermal stability of wild type (WT) and vEP mutants at 55℃. (A) SDS-PAGE of enzymes incubated at 55℃ for various time intervals. (B) Residual activity of enzymes as assayed with azocasein at 37℃ following incubation of enzymes at 55℃. Symbols: ●, WT; ○, ΔC100; ▼, G202D; ▽, thermolysin. The data are the mean values from two separate experiments performed in triplicate.

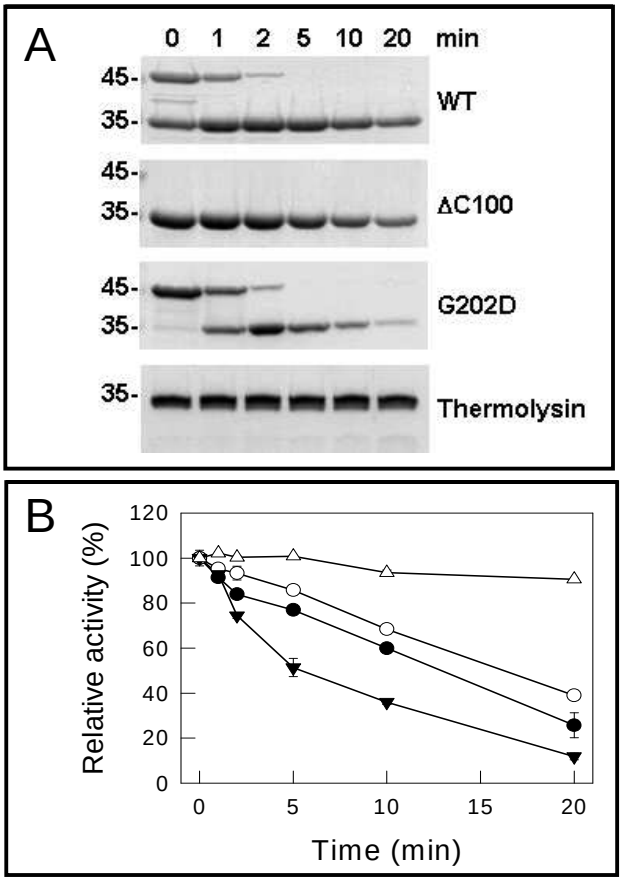

Fig. 11. Thermal stability of wild type (WT) and vEP mutants at 65℃. (A) SDS-PAGE of enzymes incubated at 65℃ for various time intervals. (B) Residual activity of enzymes as assayed with azocasein at 37℃ following incubation of enzymes at 65℃. Symbols: ●, WT; ○, ΔC100; ▼, G202D; ▽, thermolysin. The data are the mean values from two separate experiments performed in triplicate.

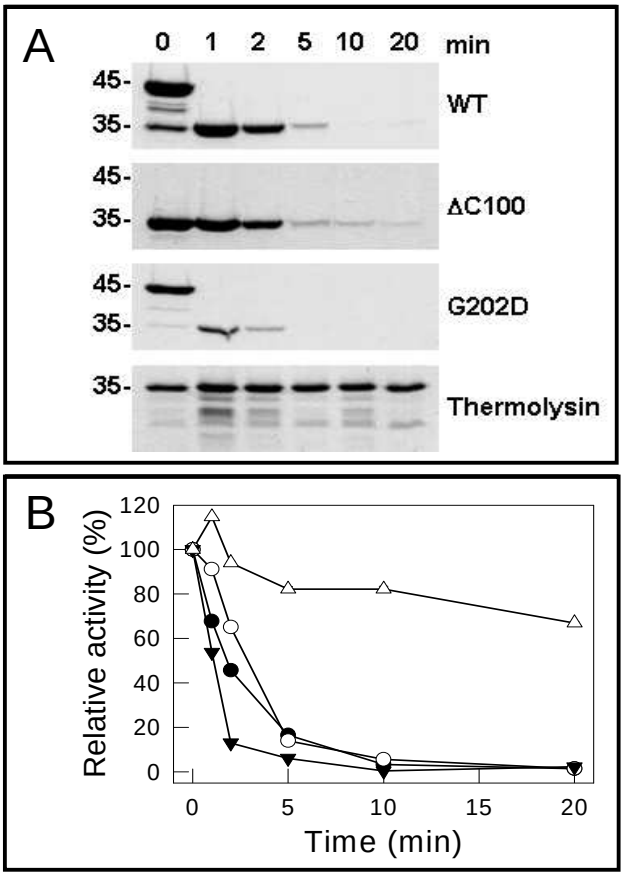

Fig. 12. Thermal stability of wild type (WT) and vEP mutants at 75℃. (A) SDS-PAGE of enzymes incubated at 75℃ for various time intervals. (B) Residual activity of enzymes as assayed with azocasein at 37℃ following incubation of enzymes at 75℃. Symbols: ●, WT; ○, ΔC100; ▼, G202D; ▽, thermolysin. The data are the mean values from two separate experiments performed in triplicate.

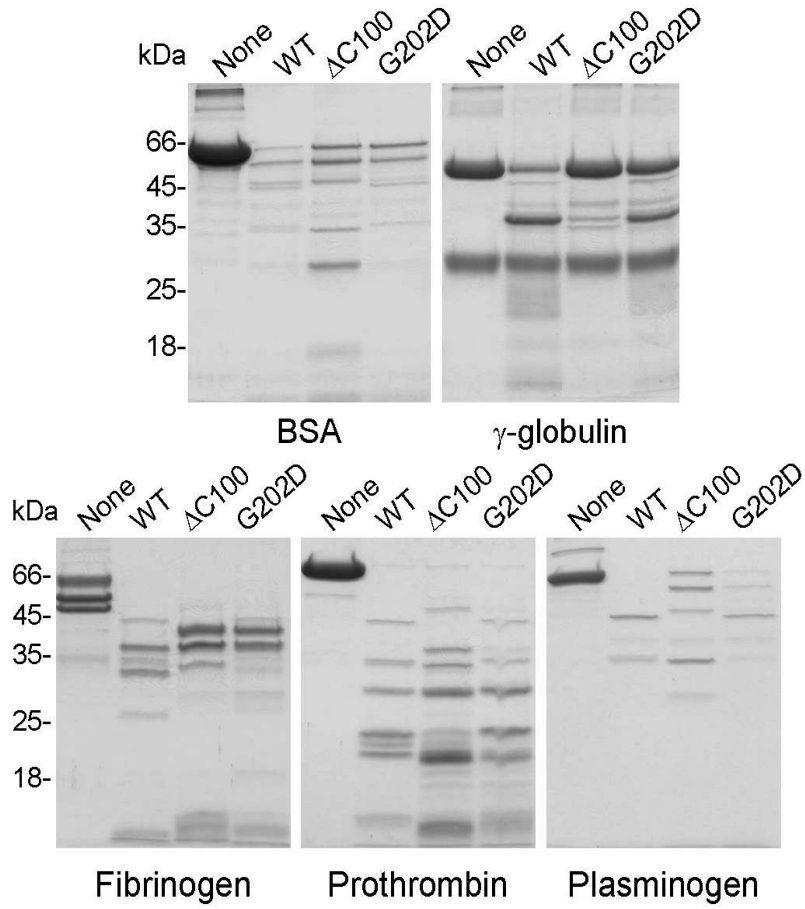

Fig. 13. Substrate specificity of wild type (WT) and vEP mutants. Ten micrograms of each protein as indicated was incubated with 0.3 μg of purified enzyme and then separated on 12% polyacrylamide gel. Fibrinogen was digested for 20 min at room temperature. Prothrombin was digested for 5 min at room temperature. Plasminogen, BSA and γ-globulin were digested for 60 min at 37℃.

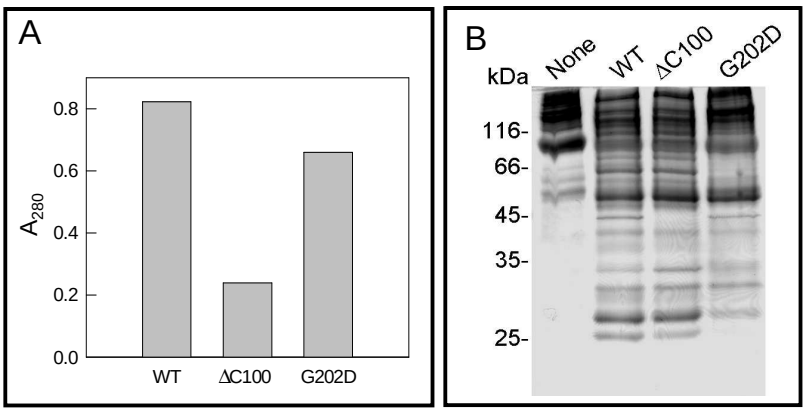

Fig. 14. Cleavage of insoluble protein. (A) Twenty micrograms of enzyme was incubated with 1 mg of elastin in 25 mM Tris-HCl (pH 7.5) in a total volume of 500 μl at room temperature for overnight. The sample was centrifuged at 10,000 xg for 10 min to collect the supernatant. Two hundred microliters of the soluble protein was taken and the absorbance at 280 nm was measured. (B) Twenty micrograms of collagen was incubated with 0.2 μg of purified enzyme and then separated on 12% polyacrylamide gel.

Table 4. Kinetic parameters for the cleavage of peptide substrate by vEP and mutant enzymes.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type vEP	0.117	4.2	35.6
∆C100	0.124	3.3	26.9
G202D	0.039	0.2	4.9

III-11. Effect of inhibitors on enzyme activity

The activity of native vEP was previously reported to be inhibited by metal chelating agents such as EDTA, EGTA and 1,10-phenanthrolin as well as certain cations such as Ni^{2+} and Cu^{2+} (Chang *et al*., 2005). The effects of these inhibitors on the recombinant enzyme were therefore investigated. EDTA, EGTA and 1,10-PT were all inhibitory to wild type and mutant enzymes (Figs. 15, 16 and 17) with 1,10-PT being the most and EDTA being the least potent inhibitor. Cu^{2+} was more inhibitory than Ni^{2+}. The apparent K_i for Ni^{2+} is similar between wild type vEP and G202D, whereas the apparent K_i for Cu^{2+} is lower for G202D, suggesting that G202D may be more sensitive than the wild type to inhibition by Cu^{2+}. ΔC100 was less sensitive to Ni^{2+} compared with wild type, but the sensitivity toward Cu^{2+} is similar for both enzyme as shown by similar apparent K_i values (Table 5). SDS is an anionic detergent that can bind proteins through electrostatic interaction. Only about 30% of the enzyme activity remained for wild type and G202D in the presence of 0.1% SDS (Fig. 18). ΔC100 showed a slightly higher resistance to SDS having about 40% of activity remaining at 0.1% SDS. Higher concentration of SDS was not tested because it also caused the loss of azo group from azocasein and therefore interferes with the enzyme activity assay.

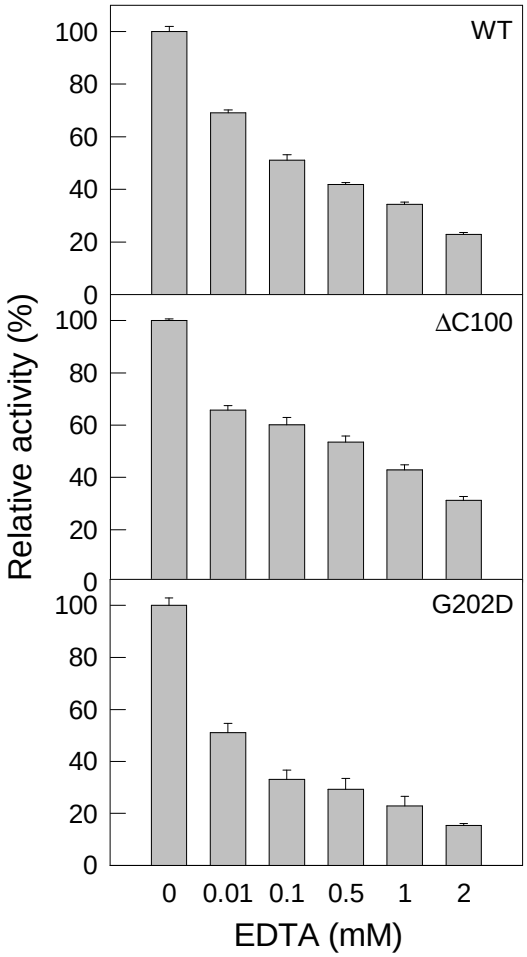

Fig. 15. Inhibition of vEP activity by EDTA. The enzyme activity was assayed with azocasein as a substrate in the presence of different concentrations of EDTA. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

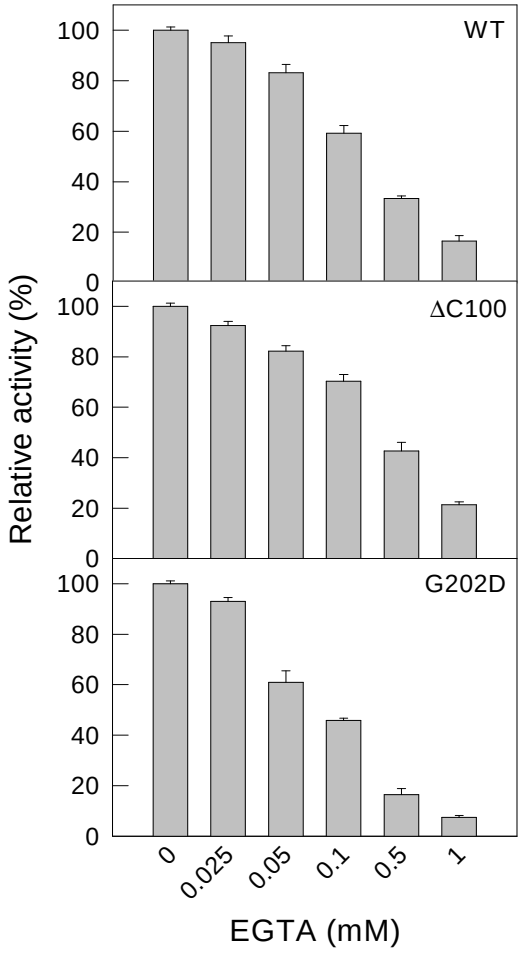

Fig. 16. Inhibition of vEP activity by EGTA. The enzyme activity was assayed with azocasein as a substrate n the presence of different concentrations of EGTA. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

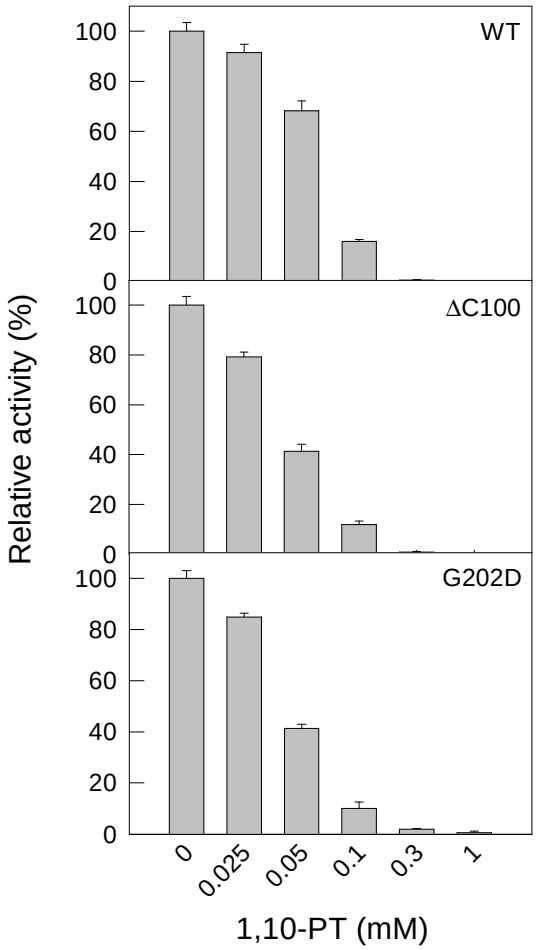

Fig. 17. Inhibition of vEP activity by 1,10-phenanthrolin (1,10-PT). The enzyme activity was assayed with azocasein as a substrate in the presence of different concentrations of 1,10-PT. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

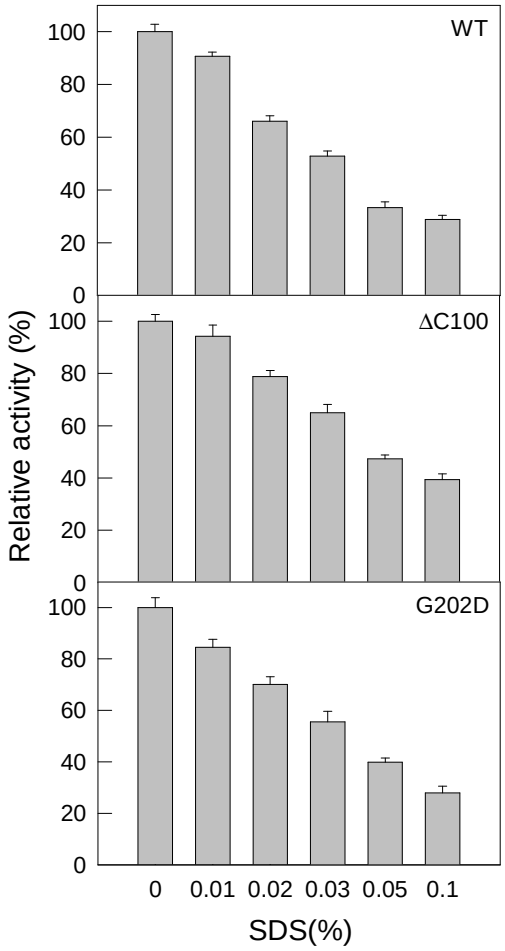

Fig. 18. Inhibition of vEP activity by SDS. The enzyme activity was assayed with azocasein as a substrate in the presence of various concentrations of SDS. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

Table 5. Inhibition of vEP enzymes by Ni^{2+} and Cu^{2+} ions.

Enzyme	K_{iapp} (μM)	
	$NiCl_2$	$CuCl_2$
Wild type vEP	159.7 ± 31.6	62.8 ± 10.1
ΔC100	289.9 ± 41.4	75.7 ± 15.8
G202D	166.4 ± 25.9	35.9 ± 8.90

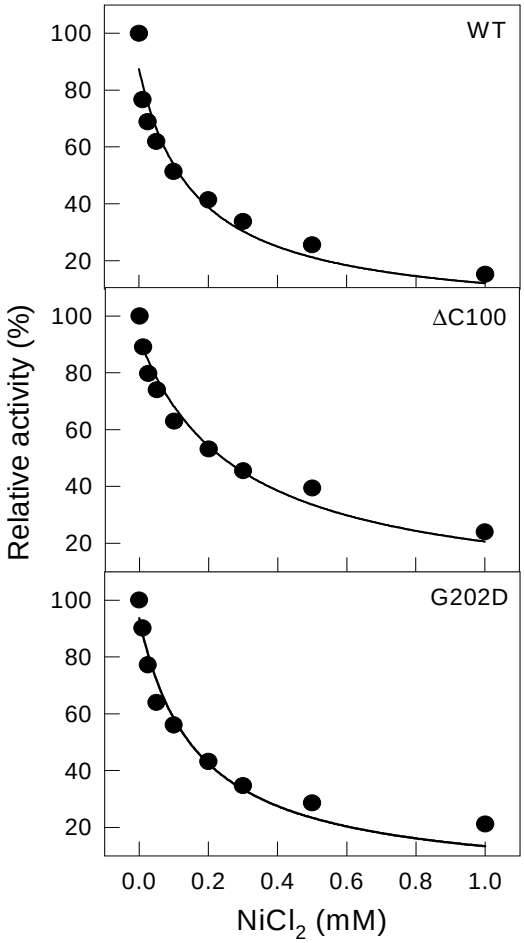

Fig. 19. Inhibition of vEP activity by Ni^{2+}. The enzyme activity was assayed with azocasein as a substrate in the presence of various concentration of Ni^{2+}. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

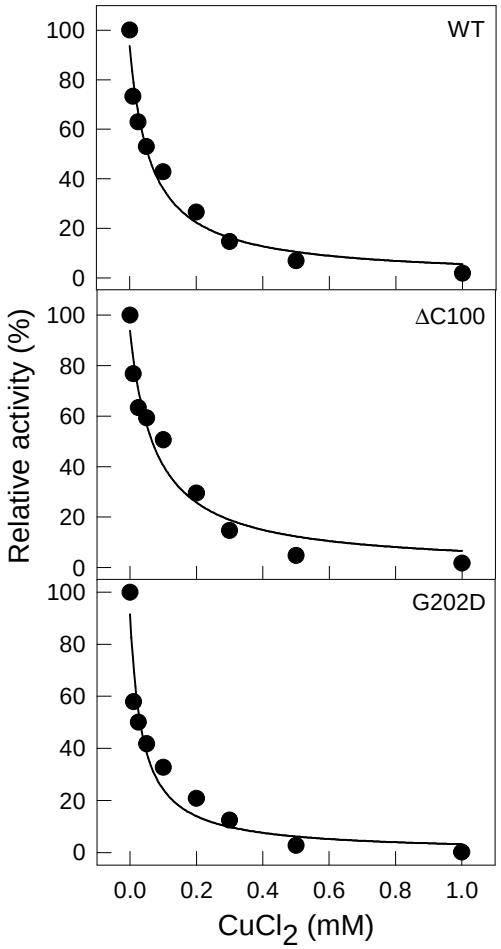

Fig. 20. Inhibition of vEP activity by Cu^{2+}. The enzyme activity was assayed with azocasein as a substrate in the presence of various concentration of Cu^{2+}. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

III-12. Effect of DEPC

DEPC is a His modification that has been routinely used to inactivate enzyme with active site containing His residues. Since vEP active site also contains His residues the effect of DEPC on the activity of vEP was also investigated. The does-dependent inactivation of vEP by DEPC is shown in Figure 21. The loss of enzyme activity confirms the importance of His residue in the active site of vEP. However, even at 2 mM DEPC, there was still about 20% of enzyme activity remaining. This suggests that vEP might be a closely packed enzyme with the active site being less accessible to DEPC than other enzymes in which such DEPC concentration would result in total loss of enzyme activity.

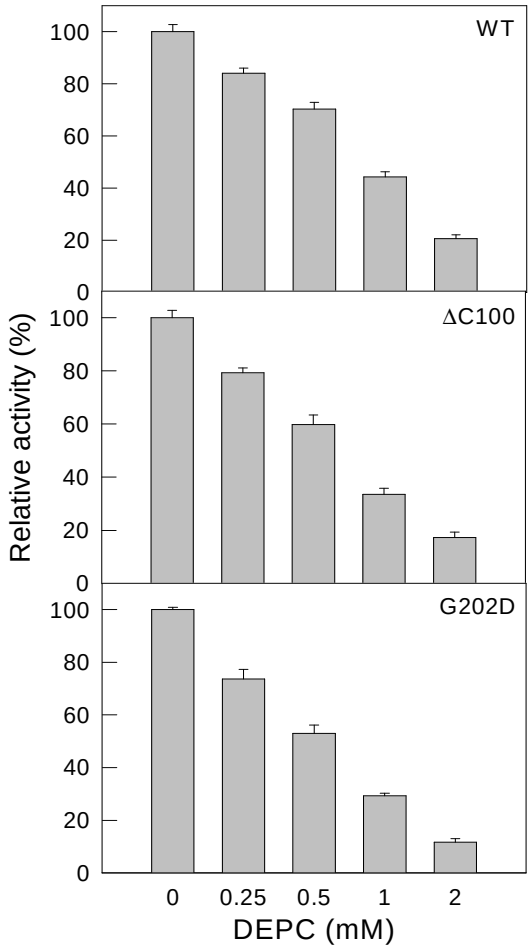

Fig. 21. Inactivation of vEP activity by DEPC. The enzyme was incubated with different concentration of DEPC for 20 min and the reaction was terminated with access imidazloe. The residual enzyme activity of the DEPC-inactivated sample was assayed with azocasein as a substrate. The data are the mean values from two separate experiments performed in triplicate. WT indicates wild type vEP enzyme.

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V. 적요

*Vibrio vulnificus*에서 분비되는 단백질분해효소 유전자의 클로닝 및 위치 지정 돌연변이 유도

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세균으로부터 분비되는 단백질 분해효소들는 세균의 생활주기에서 생리학적으로 중요한 역할을 할 뿐만 아니라 숙주세포에 독성을 발휘하여 여러 가지 질병을 유발하는 것으로 알려져 있다. 본 연구에서 사용한 *Vibrio vulnificus*(*V. vulnificus*) ATCC 29307 균주에서 분비되는 metalloprotease(vEP라 명명됨)는 Zn^{2+} 이온을 함유하고 있으며, 혈액응고에 관여하는 다양한 혈장 단백질을 분해하여 세균이 혈관에 침투하는 것을 용이하게 하는 등, 많은 생물학적 기능을 가지고 있다. vEP 단백질 분해효소는 *V. vulnificus* ATCC 29307 균주의 배양액으로부터 분리 되었으며, 프로트롬빈 활성화능과 피브린분해능을 갖고 있다. 따라서 본 연구에서는 이미 알려진 *vEP* 유전자의 염기서열을 기초로 중합효소 연쇄반응을 통해 이 유전자를 증폭시키고 pFLAG-ATS 발현벡터에 클로닝한 후, 대장균에서 발현시켰다. 재조합 vEP(rvEP로 명명함)는 발현벡터의 주변 세포질 공간(periplasmic space)으로 분비되어 온전한 활성을 갖는 효소로 발현되었다. 발현된 효소는 두 가지 종류의 이온교환 크로마토그래피(HiPrep Q column과 Source Q column)를 이용하여 분리하였고, 45 kDa과 35 kDa의 두 가지 크기로 존재함을 확인하였다. 본 연구에서는 rvEP의 온도, pH, 기질에 대한 반응성, 그리고 억제제에 대한 반응성 등을 중심으

로 그 특성을 규명하였고, 위치-지정 돌연변이유도(site-directed mutagenesis) 방법을 이용, 두 개의 돌연변이체 vEP 단백질들(ΔC100과 G202D)을 제조하여 그 특성을 야생형 vEP 효소와 비교, 분석 하였다. ΔC100으로 명명한 돌연변이체 단백질은 vEP의 C-말단을 구성하고 있는 100개의 아미노산들을 제거한 돌연변이체이며, G202D는 vEP 단백질의 202번째 아미노산인 글리신을 아스파트산으로 치환한 치환형 돌연변이체 효소이다. ΔC100 돌연변이체 효소는 rvEP와 비교할 때 20% 정도 낮은 효소활성을 갖고 있었으며, G202D는 약 42% 더 낮은 활성을 보였다. vEP는 프로트롬빈을 잘라 기능성 트롬빈(functional thrombin)을 생성할 뿐만 아니라 교차연결된 피브린을 분해하는 것으로 알려져 있다. 본 연구에서는 ΔC100과 G202D 돌연변이체 효소들의 프로트롬빈 활성화능과 피브린 분해능을 야생형 효소의 활성능과 비교, 분석 하였다. 실험결과, ΔC100과 G202D 효소들은 야생형 vEP 효소능에 비해 각각 58%와 33.1%의 프로트롬빈 활성능을 갖고 있었으며, 피브린 분해능에 있어서도 더 낮은 분해능을 보였다. G202D 효소는 rvEP와 ΔC100에 비해 고온에 비교적 더 불안정하였으며, ΔC100 효소는 rvEP와 기질 특이성 등에서 큰 차이를 보였다. 특히 rvEP에 비해 ΔC100에 의한 감마글로블린, 피브리노겐, 프로트롬빈 등의 절단이 현저하게 감소하는 것을 확인 하였으며, 금속이온에 대해서도 서로 다른 민감도를 가지고 있다는 사실을 규명하였다. ΔC100과 G202D 효소의 활성에 미치는 Ni^{2+}과 Cu^{2+}이온의 영향을 분석한 결과, rvEP는 Ni^{2+} 이온에 의해 약 75%의 효소활성이 감소한데 비해 ΔC100은 60%의 효소활성이 감소하는 것으로 보아 이 돌연변이체 효소는 rvEP보다 Ni^{2+}이온에 더 큰 민감성을 보인다는 사실을 알 수 있었다. G202D효소는 Cu^{2+}이온에 의해 효소활성이 43% 감소한 반면, rvEP는 27%의 효소활성이 감소한 것으로 보아 G202D가 Cu^{2+}이온에 의해 rvEP보다 더 민감하다는 것을 알 수 있었다. 따라서 이러한 결과를 종합하여 볼 때, vEP의 C-말단 부분은 기질과의 결합반응에서 중요한 역할을 하는 것으로 생각되며, 202번째 글리신 아미노산이 효소의 온도에 대한 안정성에 매우 중요한 역할을 하는 것으로 판단된다.