

Purification and biochemical characterization of a novel glutamyl endopeptidase secreted by a clinical isolate of *Staphylococcus aureus*

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Abstract. *Staphylococcus aureus* (*S. aureus*) is a ubiquitous Gram-positive pathogenic bacterium responsible for a majority of skin infections and toxic shock syndromes. In this study, a 34-kDa glutamate-specific serine protease (named VSPase) secreted by a clinical isolate of *S. aureus* sp. strain C-66 was purified and characterized, and VSPase-encoding gene was also cloned by PCR. VSPase enzyme purified from culture supernatant and its recombinant enzyme expressed in *E. coli* exhibited a proteolytic activity over a broad range of pH (6.0-8.5) and showed an optimal activity at 45°C. The enzyme activity was completely inhibited by DFP. The N-terminal sequence of native VSPase showed that the enzyme was produced as a form of zymogen and activated to a functional enzyme by losing its N-terminal 68 amino acid residues. VSPase specifically cleaved peptide bonds at the carboxyl sides of glutamate residues in a protein substrate such as prothrombin and exhibited its amidolytic activity towards a chromogenic substrate, Z-Phe-Leu-Glu-pNA (L-2135). The K_m , k_{cat} and k_{cat}/K_m values for VSPase were estimated to be 1.48 ± 0.156 mM,

44.4 ± 2.66 /sec and 30/mM/sec, respectively, when L-2135 was used as a substrate. It was revealed by site-directed mutagenesis that one of substitution mutations resulted in His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ residues of VSPase abolished the enzyme activity dramatically, suggesting that the three amino acid residues may compose a catalytic triad in VSPase as in typical serine proteases. Taken together, the results obtained by the present study demonstrate that VSPase is a typical glutamate-specific serine endopeptidase.

Introduction

Staphylococcus aureus (*S. aureus*) is a common gram-positive human pathogen and a major causative organism responsible for a multitude of diseases from soft tissue infections to life-threatening conditions (1). The bacterium often involves in causing toxic-shock syndrome, scarlet fever, pneumonia, sepsis and infectious endocarditis (1-4). During the course of the bacterial infection, it produces a wide spectrum of virulence factors such as exoenzymes, toxins, surface proteins and immune modulators (1,5,6). These virulence factors are expressed by a coordinated network of multiple DNA binding proteins such as Rot and SarA (7,8) and two-component systems including *agr*, *arlRS*, *saeRS*, *srrAB* and *vraSR* (9).

It is been generally believed that extracellular proteases produced by *S. aureus* also form a complex interactive network to play pleiotropic roles in the bacterial pathogenesis (10) because they can directly damage host tissues, degrade extracellular matrix proteins and induce vascular permeability (11). Therefore, the proteases can be regarded as instrumental factors both in host damage and for bacterial infection (1,10). The proteases produced by *S. aureus* can be classified into metalloproteases, cysteine and serine proteases (12). For example, aureolysin (Aur) is a typical Staphylococcal 34 kDa zinc metalloprotease that cleaves specifically the peptide bond at the carboxyl side of hydrophobic residues in protein substrates (13) and directly participates in the processing of a glutamyl endopeptidase SspA (also known as V8 protease) proenzyme (13-15). Staphopains A (ScpA) and B (SspB) are papain-like cysteine proteolytic enzymes that cleave the peptide bond between Arg-Ala residues in fibrinogen, elastin, fibronectin and kininogen (16). The typical serine proteases V8 and SPase can

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Abbreviations: 1,10-PT, 1,10-phenanthroline; BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DFP, diisopropyl fluorophosphates; DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl- β -D-thiogalactopyranoside; LB, Luria-Bertani medium; MALDI-TOF, matrix-assisted laser desorption-ionization time of flight; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLCK, tosyl-L-lysine-chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone

Key words: *Staphylococcus aureus*, glutamyl endopeptidase, serine protease, site-directed mutagenesis, PCR cloning

cleave the peptide bonds at the carboxyl termini of aspartic acid and glutamic acid residues, respectively, in their protein substrates (12,17).

Staphylococcal extracellular serine protease named SspA is synthesized as inactive proenzyme consisting of a signal peptide, an N-terminal propeptide and a mature region having catalytic function. The zymogen is activated by a staphylococcal aureolysin-dependent autocatalysis (13,18,19). The proenzyme is autoprocessed first, in which the carboxyl side of Glu⁶⁵ residue in propeptide region is cleaved, resulting in the removal of N-terminal 65 amino acid stretch. The tri-peptide (H⁶⁶AN) remained in its N-terminus is then removed by aureolysin-dependent processing to expose V⁶⁹IL in the N-terminus of active enzyme (13). Nickerson *et al.* (13) also reported that the SspA enzyme having the tripeptide (H⁶⁶AN) on its N-terminus is inactive because H⁶⁶ occludes the active site by forming hydrogen bond to a catalytic His¹¹⁹.

In this study, we purified and characterized a novel 34 kDa glutamate-specific serine endopeptidase (named VSPase) secreted by a clinical isolate of *S. aureus* sp. strain C-66. In addition, we cloned a VSPase-encoding gene to reveal the gene structure and expressed in *Escherichia coli* (*E. coli*) to obtain recombinant enzyme. We describe here the properties of this protease with emphasis on its kinetics and a catalytic triad, and show that it is a highly glutamate-specific protease that can cleave peptide bonds at the carboxyl sides of glutamates in protein substrates.

Materials and methods

Materials. Columns including HiPrep 16/10 Q FF, HiPrep 16/60 Sephacryl S-200 HR, Source 15 Q 4.6/100 PE and PD-10 were purchased from Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Synthetic chromogenic substrates, including Z-Arg-Arg-pNA (L-1225), H-Cys-pNA (L-1145), H-Gly-pNA (L-1280), H-Glu-pNA (L-1540), H-Leu-pNA (L-1305), H-Lys-pNA (L-1315) and Z-Phe-Leu-Glu-pNA (L-2135) were purchased from Bachem (Bubendorf, Switzerland). Other chromogenic substrates from Chromogenix (Milan, Italy) were as follows: Ile-Glu-(OR)-Gly-Arg-pNA (S-2222), H-D-Phe-Pip-Arg-pNA (S-2238), H-D-Val-Leu-Lys-pNA (S-2251), H-D-Ile-Pro-Arg-pNA (S-2288), Pyro-Glu-Gly-Arg-pNA (S-2444), MeO-Suc-Arg-Pro-Tyr-pNA (S-2586) and N- α -Z-D-Arg-Gly-Arg-pNA (S-2756). Two substrates, including Boc-Val-Pro-Arg-pNA and Boc-Leu-Gly-Arg-pNA were from Seikagaku (Tokyo, Japan). Protein molecular weight size markers were from Fermentas (Opelstrasse, Germany). Restriction enzymes such as *Kpn*I, *Pst*I, *Hind*III and *Dpn*I were purchased from New England BioLabs (Beverly, MA, USA). Kits for agarose gel extraction and PCR purification and *ipfu* polymerase were from iNtRON Biotechnology (Seongnam, Korea). DFP, azocasein, ammonium sulfate, BSA, trizma base, pFLAG-ATS vector and other chemicals used in this study were from Sigma (St. Louis, MO, USA).

Bacterial strains and culture condition. *S. aureus* sp. strain C-66 was a kind gift from Professor Y. Lim (Chosun University School of Medicine, Gwangju, Korea) and grown in 3% tryptic soy broth (Becton-Dickinson, MD, USA) at 37°C under aerobic condition. *E. coli* DH5 α cells were routinely used for plasmid amplification and cultivated in LB medium.

SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli (20). Typically, samples were mixed with an equal volume of 2X SDS-PAGE sample buffer (120 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue and β -mercaptoethanol), heated at 100°C for 3 min, and then subjected to electrophoresis on 12% polyacrylamide gel. After electrophoresis, protein bands were visualized by staining the gel with 0.25% Coomassie Brilliant Blue.

Purification of proteases. *S. aureus* sp. strain C-66 cells were cultured until the OD₆₀₀ reached 9.0 and the culture supernatant was collected by centrifugation at 8,000 x g and 4°C for 20 min. Ammonium sulfate was added to the supernatant to reach 20% in saturation concentration and the protein precipitate was removed by centrifugation for 1 h at 12,000 x g and 4°C. The resulting supernatant was further fractionated by adding ammonium sulfate to reach 70% in saturation concentration. The proteins were collected by centrifugation for 1 h at 12,000 x g and 4°C, dissolved in 25 mM Tris-HCl buffer (pH 7.5), and desalted on PD-10 column equilibrated with the same buffer. The protein sample was applied onto a HiPrep 16/10 Q FF column equilibrated with the same buffer. The bound proteins were eluted by 0-0.5 M of NaCl linear gradient in the same buffer. Active fractions were pooled, concentrated by ultra-filtration using YM 10 membrane (Amicon), and then applied onto a HiPrep 16/60 Sephacryl S-200 HR gel filtration column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl. The active fractions were pooled and used as the purified protease. Protein concentrations were determined with Bradford reagent according to the manufacturer's instructions.

Protease activity assay. Azocasein assay was routinely used for examining a protease activity in chromatographic steps and also employed for investigating the effects of various protease inhibitors and divalent cations on enzyme activity. In this assay, a typical reaction mixture (200 μ l) was composed of 0.5% azocasein, 25 mM Tris-HCl (pH 7.5) and enzyme to be tested. The mixture was incubated for 30 min at 37°C if there was no indication. The reaction was stopped by the addition of 100 μ l of 10% TCA and centrifuged for 5 min at 10,000 x g under room temperature. The resulting supernatant was withdrawn and the absorbance at 440 nm was measured in a 96-well plate reader (Molecular Devices Corp., CA, USA). In this assay, enzyme activity was expressed as a relative value to that of no enzyme-treated control. To calculate enzyme unit and kinetics for VSPase, a synthetic chromogenic substrate Z-Phe-Leu-Glu-pNA was routinely used. The amidolytic activity of enzyme was assayed in 100 μ l of reaction mixture composed of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1 mg/ml BSA, 0.4 mM chromogenic substrate and 10% (v/v) DMF. Just after adding the chromogenic substrate, the increase in absorbance at 405 nm was monitored in a 96-well plate reader at 37°C. One unit of protease activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of pNA per min.

N-terminal amino acid sequencing and mass spectrometry. Protein samples were subjected to electrophoresis on 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membrane in 10 mM CAPS buffer (pH 11.0) containing 10% methanol. The blot was stained

Table I. Synthetic oligonucleotides used in this study.

Primer sequence (5'-3') ^a	Oligonucleotide ^b	Usage ^c
GGGGT <u>ACCC</u> CTAATTGTTCTTCGAAACTTA	fVSPase-F	Cloning of full-length VSPase-encoding gene and nucleotide sequencing
A <u>ACTGCAG</u> AACATTAATTTCTAGCTGTTTAGA	fVSPase-R	
TCAAGCTTGGGATGAAAGGTAAATTTTAAAAG	rVSPase-F	Amplification of VSPase-encoding ORF
ACGGT <u>ACCC</u> CTTATGCAGCGTCAGGGTTGT	rVSPase-R	Amplification of VSPase-encoding ORF
ACAAATAAACTTGTTCGTAGATGCTACGCAC	H119L-F	Mutagenesis for His ¹¹⁹ to Leu replacement in rVSPase
GTGCGTAGCATCTACGACA AG TTTATTTGT	H119L-R	
TATTCAGGCGAAGGT GCT TTAGCAATCGTT	D161A-F	Mutagenesis for Asp ¹⁶¹ to Ala replacement in rVSPase
AACGATTGCTAA AGC ACCTTCGCCTGAATA	D161A-R	
GGTGGTAACT TAG GTTCACCAGTATTTAAT	S237L-F	Mutagenesis for Ser ²³⁷ to Leu replacement in rVSPase
ATTAAATACTGGTGAACCTA AGT TACCACC	S237L-R	

^aThe restriction sites included in the oligonucleotides are underlined: *Kpn*I in fVSPase-F and rVSPase-R, *Pst*I in fVSPase-R and *Hind*III in rVSPase-R. The sites to be mutated are bolded. ^bF and R mean the forward and the reverse primers, respectively. ^cORF means open reading frame.

with Coomassie Brilliant Blue, followed by destaining. Target bands were excised from the blot and subjected to N-terminal sequencing using a Precise 491 HT protein sequencer (Applied Biosystems, USA). For mass spectrometric analysis, protein sample was desalted with PD-10 column, freeze-dried and subjected to MALDI-TOF. The N-terminal sequencing and the MALDI-TOF analysis were performed by Korea Basic Research Institute (Seoul, Korea).

PCR cloning of VSPase-encoding gene. Genomic DNA was isolated from *S. aureus* sp. strain C-66 cells using Qiagen DNeasy Tissue Kit (Hilden, Germany) and used for amplification of full-length VSPase gene by PCR. Forward (fVSPase-F) and reverse (fVSPase-R) primers that were designed and synthesized on the basis of *S. aureus* serine protease operon sequences (18) were used for the PCR (Table I). PCR program used for amplifying full-length VSPase gene was as follows: hold for 5 min at 95°C; 30 cycles for 1 min at 94°C, 50°C for 40 sec and 72°C for 2 min; followed by a hold for 7 min at 72°C to ensure full extension. For this PCR, a Perkin-Elmer Thermal cycler 9700 was used. After purification, the amplified DNA was cloned into pGEM-T Easy vector (Promega, Madison, USA) and the resulting recombinant plasmid was named pGEM-VSPase. The nucleotide sequence of insert DNA contained in pGEM-VSPase was analyzed using M13 universal primers as described elsewhere. On the other hand, the entire coding region for VSPase was also obtained by PCR from the pGEM-VSPase plasmid using the primers listed in Table I. PCR was performed for 1 min at 94°C, 30 sec at 50°C and 1 min 30 sec at 72°C for 30 cycles using the Eppendorf Mastercycler gradient. The resulting PCR product was digested both with *Hind*III and *Kpn*I, and ligated to *Hind*III/*Kpn*I-cut pFLAG-ATS vector to yield the pFLAG-ATS-VSPase construct.

Nucleotide sequencing and data analysis. Nucleotide sequence analysis was performed by Bioneer (Daejeon, Korea). Multiple sequence alignments were performed using European Molecular

Biology Laboratory Tool ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2>) (21).

Construction of VSPase mutant genes using site-directed mutagenesis. Various VSPase mutants were constructed by *Dpn*I-mediated site-directed mutagenesis method as described previously (22). In this mutagenesis, double-stranded pFLAG-ATS-VSPase vector DNA and synthetic oligonucleotide primers containing desired mutations (Table I) were used. Briefly, the annealed primers were extended by temperature cycling (30 sec at 94°C, 1 min at 55°C and 6 min 30 sec at 68°C for 12 cycles) with *ipfu* DNA polymerase, treated with *Dpn*I, and then transformed into *E. coli* strain DH5α. Positive clones were selected and the sequence identities of mutant genes constructed were confirmed by nucleotide sequencings.

Expression and purification of recombinant VSPase (rVSPase) and its mutant enzymes in *E. coli*. *E. coli* DH5α cells harboring pFLAG-ATS-VSPase or mutant clones were inoculated into 50 ml of LB broth containing 100 µg/ml ampicillin and cultured overnight at 37°C. The cell aliquot (10 ml) was then inoculated in 500 ml of a fresh LB broth containing 100 µg/ml of ampicillin and grown at 37°C until OD₆₀₀ reached 0.8. The target proteins were induced by the addition of 0.2 mM of IPTG, followed by overnight incubation at 20°C. The cells were harvested, resuspended in 100 ml of a buffer (30 mM Tris-HCl, pH 8.0, 20% sucrose, 1 mM EDTA and 0.3 mg/ml lysozyme), and then incubated at 4°C for 30 min. The cell suspension was centrifuged for 20 min at 18,000 x g and 4°C and the resulting supernatant was subjected to ammonium sulfate precipitation at 70% saturation. The protein pellet was collected by centrifugation for 30 min at 35,000 x g and 4°C, dissolved in 25 mM Tris-HCl buffer (pH 7.5), and then applied to PD-10 column equilibrated in the same buffer to remove a residual ammonium sulfate. The sample was applied to a HiPrep 16/10 Q FF column equilibrated with 25 mM Tris-HCl (pH 7.5). After washing with the same buffer, the bound proteins were eluted

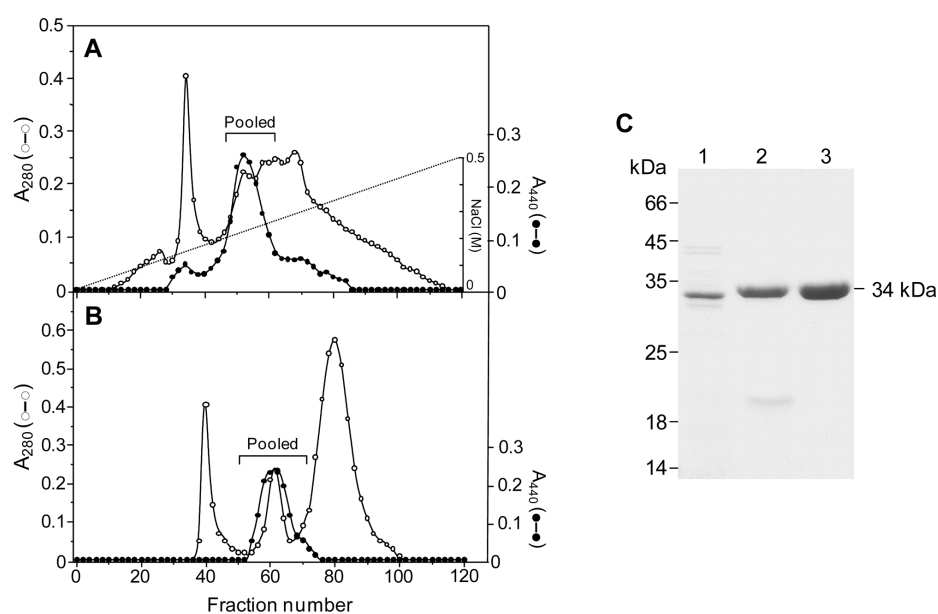


Figure 1. Purification of VSPase from culture supernatant of *S. aureus* sp. strain C-66 cells. (A) Ion exchange chromatography on HiPrep 16/10 Q FF column. The proteins precipitated with 20-70% ammonium sulfate were loaded on the column and eluted with a linear gradient of 0-0.5 M NaCl. Proteolytic activity contained in each fraction was assayed with azocasein assay as described in Materials and methods and active fractions from fraction numbers 47-62 were pooled as indicated and used for further purification. (B) Size-exclusion chromatography on HiPrep 16/60 Sephacryl S-200 HR. The proteins pooled from the HiPrep 16/10 Q FF column were loaded onto the column and fractionated at a flow rate of 0.5 ml/min. Active fractions were pooled from fraction numbers 55-72 and used as purified enzyme. In panels (A) and (B), the protein concentration (○-○) was determined by measuring the absorbance at 280 nm and the protease activity (●-●) using azocasein as a substrate was assayed by measuring the absorbance at 440 nm. (C) SDS-PAGE of active fractions from various purification steps. Proteins obtained from each purification step were electrophoresed on 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue to visualize. Lane 1, proteins from 20-70% ammonium sulfate cut-off; lane 2, from HiPrep 16/10 Q FF column chromatography; lane 3, from HiPrep 16/60 Sephacryl S-200 HR size exclusion column chromatography. Approximately 34 kDa purified enzyme in size is indicated.

Table II. Purification summary of VSPase enzyme from the culture supernatant of *S. aureus* sp. strain C-66.

Fraction	Total protein (mg)	Total activity (U) ^a	Specific activity (U/mg)	Yield (%) ^b
Culture supernatant	158.9	44.62	0.28	100
(NH ₄) ₂ SO ₄	116.4	37.48	0.32	84
HiPrep 16/10 Q FF	3.4	19.20	5.64	43
HiPrep 16/60 Sephacryl S-200 HR	1.1	12.57	11.43	28

^aOne unit (U) was defined as the amount of protease that catalyzes the release of 1 μ mol of *p*NA/min. ^bThe total activity in culture filtrate was assigned the value of 100%.

with a linear gradient of NaCl ranging from 0 to 0.5 M in the same buffer. Fractions having major protease activity or size were pooled and further chromatographed on a Source 15 Q 4.6/100 PE column equilibrated in 25 mM Tris-HCl (pH 7.5). After washing with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 0 to 0.3 M. Active fractions were pooled and the purified enzymes were stored in small aliquots at -20°C until used.

Results

Purification and characterization of VSPase. An active extracellular protease named VSPase was purified from the culture supernatant of *S. aureus* sp. strain C-66 cells by employing ammonium sulfate precipitation, anion exchange

chromatography and size-exclusion chromatography in order (Fig. 1). Maximal protease activity could be observed in culture supernatant when the cells were cultured at 37°C for 10 h until OD₆₀₀ value reached 9.0 (data not shown). To purify VSPase enzyme, the culture supernatant obtained from this culture condition was subjected to 20-70% ammonium sulfate fractionation and the resulting proteins were loaded onto HiPrep 16/10 Q FF column. The bound proteins were eluted by a linear gradient (0-0.5 M) of NaCl and the proteolytic activity contained in each fraction was assayed with azocasein as a substrate (Fig. 1A). As shown in Fig. 1A, most of protease activities appeared from fraction numbers 47-62, which were eluted by 200-300 mM concentration range of NaCl. The pooled active fractions were applied onto a HiPrep 16/60 Sephacryl S-200 HR column and the proteins

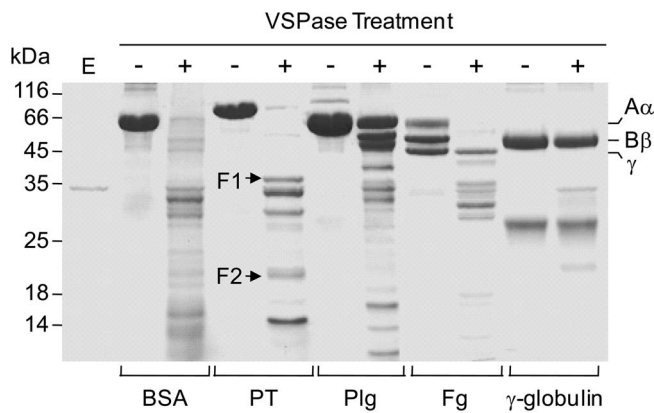


Figure 2. Proteolytic cleavage of VSPase to various plasma proteins. Ten microgram each of corresponding proteins were digested with 0.5 μ g of purified VSPase for 30 min at 37°C, electrophoresed on 12% SDS-polyacrylamide gel, and then stained with Coomassie Brilliant Blue to visualize. Symbols + and - mean the addition and the omission of VSPase enzyme in the reaction, respectively. E, VSPase only; BSA, bovine serum albumin; PT, prothrombin; Plg, plasminogen; Fg, fibrinogen. F1 and F2 indicate the peptide fragments subjected to N-terminal sequencing to analyze the cleavage site of VSPase enzyme.

were eluted at a flow rate of 0.5 ml/min (Fig. 1B). Active fractions were pooled from fractions 55 to 72 and used as a purified enzyme for further study (Fig. 1B). The purification of VSPase enzyme is summarized in Table II. The purified

enzyme showed a specific activity of 11.43 U/mg protein, which represented approximately 41-fold increase over the culture supernatant. About 1.1 mg of enzyme could be obtained from 1,000 ml of culture supernatant (Table II). The purified enzyme appeared as a single band on SDS-PAGE, having an apparent molecular mass of 34 kDa (Fig. 1C). However, MALDI-TOF analysis gave a molecular mass of 29,688 Da. The N-terminal amino acid sequencing result showed that the N-terminus of purified enzyme was composed of N-VILPNNDRHQITDITNGHYA-C (Fig. 3).

Enzymatic properties of VSPase. The purified VSPase enzyme exhibited a proteolytic activity over a broad range of pH (6.0-8.5) and showed an optimal activity at 45°C when azocasein was used as a substrate (data not shown). Of 16 chromogenic substrates tested (e.g., L-1225, L-1145, L-1280, L-1540, L-1305, L-1315, L-2135, S-2222, S-2238, S-2251, S-2288, S-2444, S-2586, S-2756, Boc-Leu-Gly-Arg-pNA and Boc-Val-Pro-Arg-pNA), only L-2135 (Z-Phe-Leu-Glu-pNA) could be hydrolyzed by VSPase. These results suggest that VSPase is a typical glutamyl endopeptidase. Kinetic data obtained with L-2135 showed that K_m , k_{cat} , and k_{cat}/K_m values for VSPase were 1.48 ± 0.156 mM, 44.4 ± 2.66 /sec and 30/mM/sec, respectively. In addition, VSPase was capable of cleaving various plasma proteins, such as BSA, prothrombin, plasminogen, fibrinogen and γ -globulin, among which prothrombin and fibrinogen were the most efficient substrates for VSPase (Fig. 2). In spite of the efficient cleavage of prothrombin by

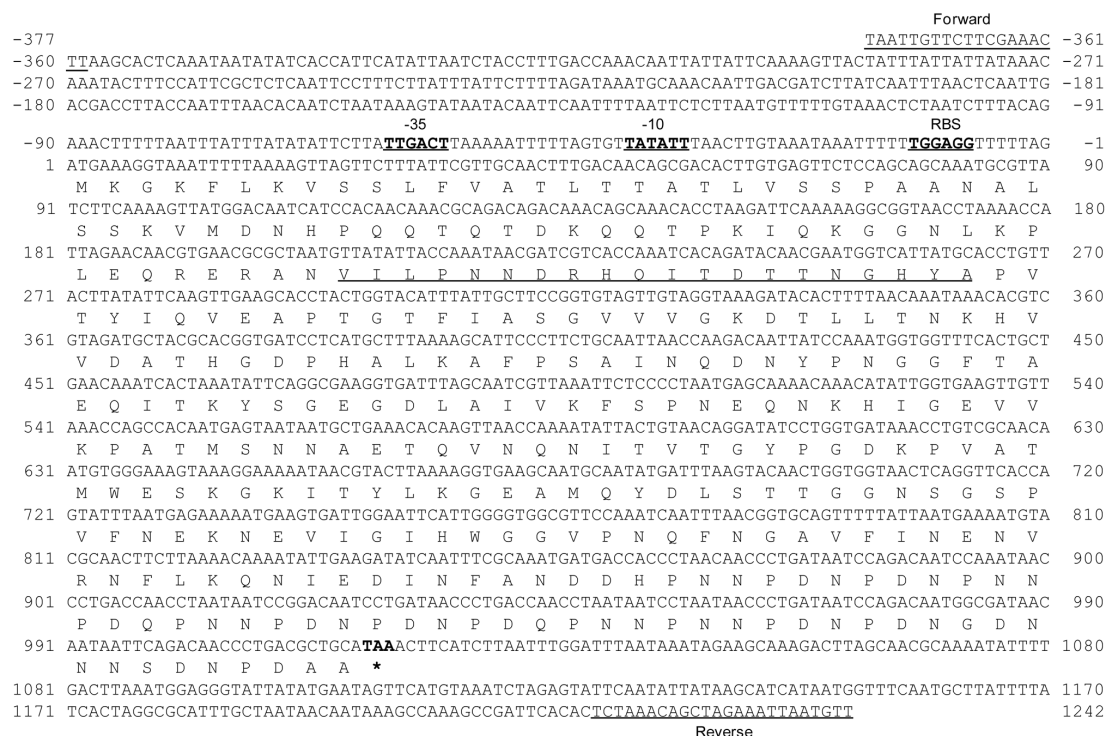


Figure 3. The nucleotide and the deduced amino acid sequences of cloned VSPase-encoding gene. The gene was amplified from the genomic DNA of *S. aureus* sp. strain C-66 cells by PCR as described in Materials and methods. The nucleotide sequence of cloned gene was analyzed by dideoxycytidine termination method, from which amino acid sequence was deduced. The conserved -35, -10 boxes and RBS (ribosome binding site) are underlined with bold characters. The N-terminal sequence determined by peptide sequencing of purified VSPase enzyme is also bolded and underlined in the deduced amino acid sequence. The open reading frame of cloned gene is composed of 1,020 nucleotides that can encode 339 amino acids. The start and the stop codons in the cloned gene are found to be ATG and TAA (indicated by an asterisk), respectively. Forward and reverse primer binding sites for nucleotide sequencing are underlined as indicated by forward and reverse, respectively. GeneBank accession no. for the cloned gene is EF457973.

VSPase, there was no detectable thrombin activity when a typical chromogenic substrate for thrombin (Boc-Val-Pro-Arg-pNA) was used (data not shown). This result suggests that VSPase does not have an ability to activate prothrombin unlike vEP protease from *Vibrio vulnificus* (23,24). The A α chain of fibrinogen was totally degraded by VSPase whereas the B β and γ chains were relatively resistant to cleavage (Fig. 2). As in prothrombin activation by VSPase, the spontaneous polymerization of fibrin monomer also did not occur when fibrinogen was incubated with the enzyme, as judged by turbidity assay (data not shown). On the other hand, various divalent cations and protease inhibitors were tested for their effects on VSPase activity. As shown in Table III, divalent cations including Ca²⁺, Cu²⁺, Mn²⁺, Mg²⁺, Ni²⁺ and Zn²⁺ showed no significant effects on VSPase activity. Furthermore, the enzyme activity was not inhibited by the treatments with PMSF, TPCK, TLCK, leupeptin and aprotinin (Table III). These non-inhibitory effects of inhibitors were also common in serine proteases such as GluSE and V8 (25). The metalloprotease inhibitors such as EDTA and 1,10-PT also had no effect (Table III). However, the addition of 10 mM DFP completely abolished the enzyme activity (Table III). These results suggest that VSPase is an enzyme included in serine protease family, not a metalloprotease.

Determination of VSPase cleavage site. The cleavage site for VSPase was revealed by N-terminal sequencings with two peptide fragments (F1 and F2 in Fig. 2) resulted from prothrombin cleavage by the enzyme. The sequencing results showed that the amino-terminal sequences of F1 and F2 were Gly³¹³-Arg-Thr-Ala-Thr and Gly³⁶⁷-Ser-Asp-Ala-Glu, respectively. In prothrombin, Glu³¹² and Glu³⁶⁶ are ahead by Gly³¹³ and Gly³⁶⁷, respectively (26). Therefore, the results clearly indicate that VSPase cleaves the peptide bonds between glutamate and glycine in the protein substrate, specifically at the carboxyl sides of glutamates. This conclusion was also supported by the fact that VSPase exerted its amidolytic activity toward Z-Phe-Leu-Glu-pNA as described above.

Cloning and nucleotide sequence analysis of VSPase gene. VSPase-encoding gene was cloned by PCR using a pair of synthetic oligonucleotide primers (Table I) as described in Materials and methods. A DNA fragment of about 1,600 bp in size could be amplified by the PCR from the genomic DNA of *S. aureus* sp. strain C-66 cells (data not shown), and the nucleotide sequence of the PCR product was analyzed (Fig. 3). The nucleotide sequence analysis showed that the amplified gene contained an open reading frame (ORF) composed of 1,020 nucleotides, starting from an initiation codon ATG and ending at a stop codon TAA, which could produce a polypeptide of 339 amino acid residues (Fig. 3). In addition, the sequence VILPNDRHQITDITNGHYA was found from the deduced amino acid sequence, which is the same N-terminal sequence of purified VSPase (Fig. 3). This result suggests that VSPase is produced as a form of zymogen and can be activated to a functional enzyme by losing 68 amino acids from its N-terminus. The nucleotide sequence analysis also showed that highly conserved three kinds of *cis*-acting elements that can control the transcription of the gene were located on the upstream region of the gene (Fig. 3). They

Table III. Effects of various metal ions and protease inhibitors on VSPase activity.

Additive	Concentration (mM)	Activity (%) ^a
Control	-	100
Ca ²⁺	1	116.2
Cu ²⁺	1	94.4
Mn ²⁺	1	114.4
Mg ²⁺	1	116.5
Ni ²⁺	1	113.5
Zn ²⁺	1	116.9
TPCK	1	102.5
TLCK	1	102.8
Leupeptin	0.5	107
Aprotinin	10	106.7
EDTA	10	104.8
1,10-PT	10	102
DFP	10	6.8
PMSF	10	97

^aVSPase activity was assayed with azocasein as a substrate with or without the corresponding additive at 37°C for 15 min as described in Materials and methods.

included two -35 (TTGACT) and -10 (TATATT) boxes that are often found in the promoters of extracellular protease genes of *S. aureus* and a putative ribosome binding site (TGGAGG) (19). VSPase had a significant sequence similarity to other Staphylococcal glutamyl endopeptidases in the level of amino acid sequence (Fig. 4). The sequence comparison analysis revealed that VSPase had ~98, 97, 71 and 51% sequence identities with those of SPase from *S. aureus* ATCC 12600, V8 from *S. aureus* subsp. MW2, PROM from *S. warneri* and GluSE proteases from *S. epidermidis*, respectively, with an average of 79.3% (Fig. 4). Comparing the deduced amino acid sequence of VSPase with those of four Staphylococcal proteases, it seemed to be clear that VSPase zymogen had a propeptide region composed of 68 amino acids (Fig. 4), as the N-terminus of purified enzyme started with the sequence VILPNDRHQITDITNGHYA. The sequence comparison also showed that VSPase had highly conserved His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ residues, which might compose a catalytic triad as in typical serine proteases (Fig. 4). In addition, VSPase had a typical Pro-Asx-Asp (Glx) repeat at its C-terminus as in other proteases being compared, except in GluSE protease (Fig. 4).

Expression of recombinant VSPase and construction of VSPase mutant enzymes. Recombinant VSPase (named rVSPase) was expressed in *E. coli* DH5 α cells harboring a plasmid pFLAG-ATS-VSPase as described in Materials and methods and purified by employing two successive chromatographic steps of HiPrep 16/10 Q FF and Source 15 Q 4.6/100 PE columns (data not shown). Approximately 3.7 mg of rVSPase could be obtained from 64 mg of the *E. coli* periplasmic proteins. The purified rVSPase showed a similar

	1	30	60	90					
VSPase	<u>MKGKFLKVSS</u>	<u>LFVATLTTAT</u>	<u>LVSSPAANAL</u>	<u>SSKVMNDHPO</u>	<u>OTOTDKOOTP</u>	<u>KIQKGGNLKP</u>	<u>LEQERERANVI</u>	<u>LPNNDRHQIT</u>	<u>DTTNGHYAPV</u>
SPase	MKGKFLKVSS	LFVATLTTAT	LVSSPAANAL	SSKAMDNDHPQ	QTQTDKQOTP	KIQKGGNLKP	LEQERERANVI	LPNNDRHQIT	DTTNGHYAPV
V8	MKGKFLKVSS	LFVATLTTAT	LVSSPAANAL	SSKAMDNDHPQ	QSQSSKQOTP	KIQKGGNLKP	LEQREHANVI	LPNNDRHQIT	DTTNGHYAPV
PROM	MKVKFFTASS	LLIATLTST	LINP--AAHE	TTSSTDNDHQ	TTQSQQQKTP	KIDKGNNVKP	VEKKERANVI	LPNNDRHQIN	DTTLGHYAPV
GluSE	MKKRFLSICT	MTIAALATT	MVNT--SYAK	TDTESHNHSS	LGTEKNVLD	INSSSHNIK	SQNKSYPSVI	LPNNNRHQIF	NTTQGHYDAV
	::	*.***:***:	***:***:.*	:...:***:	:...:*.~:	:...:*.~*	*.~:~:~:~*	*****~.	***.***:~*
	91	120	150	180					
VSPase	<u>TYIQVEAPT</u>	<u>TFIASGVVVG</u>	<u>KDTLLTNKHV</u>	<u>VDATHGDPHA</u>	<u>LKAFPSAINQ</u>	<u>DNYPNGGFTA</u>	<u>EQITKYSSEG</u>	<u>DLAIVKFSFN</u>	<u>EQNKHIGEVV</u>
SPase	TYIQVEAPT	TFIASGVVVG	KDTLLTNKHV	VDATHGDPHA	LKAFPSAINQ	DNYPNGGFTA	EQITKYSSEG	DLAIVKFSFN	EQNKHIGEVV
V8	TYIQVEAPT	TFIASGVVVG	KDTLLTNKHV	VDATHGDPHA	LKAFPSAINQ	DNYPNGGFTA	EQITKYSSEG	DLAIVKFSFN	EQNKHIGEVV
PROM	TFVQVQSN	TFIASGVVVG	KDKLLTNKHV	VDATHGNPRA	LKAFPSAVNQ	NNYPNGGFTG	EQITKYPGNA	DLAIVKFSFN	NQNKHIGEVV
GluSE	SFIYIPIDGG	YMSGSGVVVG	ENEILTNKHV	VNGAKGNPRN	ISVHPSAKNE	NDYPNGKFVG	QEIIYPYGN	DLAILRVSPN	EHNQHIGQVV
	::	:::~*****	*.:~*****	***:~***:	:::~***.~*	*****~*~:	***.***.~*	*****~***	*.:~***~*
	181	210	240	270					
VSPase	<u>KPATMSNNAE</u>	<u>TQVNQNITVT</u>	<u>GYPGDKPVAT</u>	<u>MWESKGKITY</u>	<u>LKGEAMQYDL</u>	<u>STTGGNSGSP</u>	<u>VFNEKNEVIG</u>	<u>IHWGGVNPQF</u>	<u>NGAVFINENV</u>
SPase	KPATMSNNAE	TQVNQNITVT	GYPGDKPVAT	MWESKGKITY	LKGEAMQYDL	STTGGNSGSP	VFNEKNEVIG	IHWGGVNPQF	NGAVFINENV
V8	KPATMSNNAE	TQVNQNITVT	GYPGDKPVAT	MWESKGKITY	LKGEAMQYDL	STTGGNSGSP	VFNEKNEVIG	IHWGGVNEF	NGAVFINENV
PROM	TPATLSDNAD	TSQNQPITVT	GYPGDKPLAT	MWESRGKITQ	IQGEDMHYDL	STTGGNSGSP	VFNSRNEVIG	IHWGGAANSY	NGAVFINNNV
GluSE	KPATISSNTD	TRINENITVT	GYPGDKPLAT	MWESVGKVVY	IGGEELRYDL	STVGGNSGSP	VFNGKNQVIG	IHYGGVDNKY	NSSVYINDFV
	..~*	*.~***~***	*****~***:	***.~***:	:~*~*.~***	***:~*****	***.~*****	***.~*~*.~*	*.:~***~*~*
	271	300	Pro-Asx-Asp (Glx) repeat				339		
VSPase	<u>RNFLKQNI</u>	<u>INFANDDHPN</u>	<u>NPDNPDNPNP</u>	<u>PDQPNNDPNP</u>	<u>DNPDQPNPNP</u>	<u>NPDNP----</u>	<u>-----</u>	<u>---DNGDNNN</u>	<u>SDNPDA</u>
SPase	RNFLKQNI	INFANDDHPN	NPDNPDNPNP	PDNPNNDPNP	NNPDNDPNP	NPDNPNNDPN	PNNPDQPNNP	NNPDNDGN	SDNPDA
V8	RNFLKQNI	INFANDDHPN	NPDNPDNPNP	PDNPNNDPNP	DNPDNDGN-N	NSDNPDA--	-----	-----	-----
PROM	QNFLKQNI	IFHNSD--N	NDNDNDNNGT	DDNNNNNDND	DN--NYDNDP	AA-----	-----	-----	-----
GluSE	QQFLRNIPD	INIQ-----	-----	-----	-----	-----	-----	-----	-----
	:~~*	*.:~***:~:	***:~***:~:	:~***:~:	:~***:~:	:~***:~:	:~***:~:	~***:~***:	~***:~***:

Figure 4. Alignment of the amino acid sequence of VSPase with those of typical glutamate-specific endopeptidases from bacteria. The first 68 amino acids that presumably act as a signal peptide and propeptide region in VSPase zymogen are underlined. The N-terminal amino acid sequence of VSPase is shown with bold characters. The highly conserved three amino acid residues (His¹¹⁹, Asp¹⁶¹ and Ser²³⁷) that may compose of a catalytic triad and the conserved Pro-Asx-Asp (Glx) repeats located in each enzyme are represented by bold characters. The alignment was performed with ClustalW2 software. Asterisk (*) means that the residues in the column are identical in all aligned sequences. Colon (:) indicates that conserved substitutions are located. Dot (.) means that semi-conserved substitutions are located. VSPase, protease from *S. aureus* sp. strain C-66; SPase, from *S. aureus* ATCC 12600; V8, from *S. aureus* subsp. *aureus* MW2; PROM, from *S. warneri*; GluSE, from *S. epidermidis*.

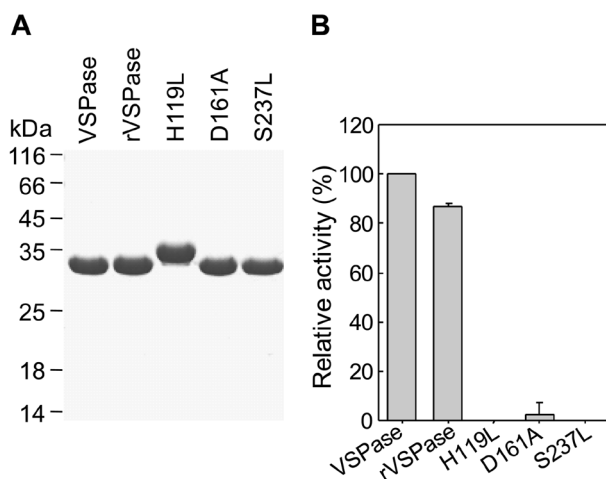


Figure 5. Purification and enzyme activity assay of VSPase mutant enzymes. (A) SDS-PAGE of purified enzymes. VSPase was purified from the culture supernatant of *S. aureus* sp. strain C-66. rVSPase was from *E. coli* cells harboring pFLAG-ATS-VSPase as described in Materials and methods. Genes for H119L, D161A and S237L mutant enzymes were constructed using *DpnI*-mediated site-directed mutagenesis, expressed in *E. coli*, and purified as described in Materials and methods. In the mutagenesis, three amino acid residues, His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ in VSPase were replaced with Leu, Ala and Leu, respectively, making H119L, D161A and S237L mutant enzymes. The purified enzymes (5 µg each) were electrophoresed onto 12% polyacrylamide gel and stained with Coomassie Brilliant Blue to visualize. (B) Amidolytic activities of the purified enzymes. The enzyme activities were assayed with a synthetic peptide substrate (Z-Phe-Leu-Glu-pNA) as described in Materials and methods, in which 500 ng each of corresponding enzyme was incubated with the chromogenic substrate for 20 min at 37°C and the increases in absorbance at 405 nm were monitored in a 96-well plate reader at 37°C. Enzyme activity was expressed as a percentage relative to that of VSPase.

specific activity (12.73 U/mg) to that of secreted native VSPase enzyme (Table II). In addition, the purified rVSPase enzyme exhibited the nearly same enzymatic properties as the native enzyme in terms of substrate specificity, cleavage site and inhibitory response to DFP (data not shown). As mentioned in previous section, VSPase seemed to have a catalytic triad that might be composed of His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ as shown by the amino acid sequence comparison (Fig. 4). To investigate the roles of these amino acids in catalysis, three kinds of mutant genes that can incorporate each of Leu¹¹⁹, Ala¹⁶¹ and Leu²³⁷ instead of His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ in rVSPase were constructed using *DpnI*-mediated site-directed mutagenesis. In the mutagenesis, three pairs of synthetic oligonucleotides that contained corresponding codons for the amino acids (Table I) and double-stranded pFLAG-ATS-VSPase vector DNA as a template to be mutated were used as described in Materials and methods. The identities of mutant genes constructed were confirmed by nucleotide sequencings (data not shown). The mutant genes were transformed into *E. coli* and the enzymes expressed were purified homogeneously using two purification steps including HiPrep 16/10 Q FF and Source 15 Q 4.6/100 PE column chromatographies in order (Fig. 5A). The mutant enzymes each containing Leu¹¹⁹, Ala¹⁶¹ and Leu²³⁷ residues instead of His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ in rVSPase were named H119L, D161A and S237L, respectively. As shown in Fig. 5B, H119L and S237L did not show any detectable activity toward a chromogenic substrate L-2135. A mutant enzyme D161A also showed a background level of activity. These results suggest that the three amino acid residues, His¹¹⁹, Asp¹⁶¹ and Ser²³⁷ in VSPase play critical roles in the enzymatic action, which probably consist a catalytic triad as in other serine proteases.

Discussion

It is been reported that *S. aureus* secretes several kinds of proteases in culture medium. The enzymes include serine proteases (15,27), cysteine proteases (28) and metalloprotease (12). In this study, we have purified a glutamate-specific serine protease named VSPase from the culture filtrate of a clinical isolate of *S. aureus* sp. strain C-66 and characterized in terms of substrate specificity and enzyme kinetics. The purified VSPase is thermally stable up to 75°C. At preincubation temperature 75°C, approximately 65.4% of the enzyme activity retain, compared to that of at 25°C (data not shown). Interestingly, VSPase undergoes auto-degradation when it is incubated at a temperature of 55°C or higher, but the extent of degradation is the most severe at 55°C, resulting in almost complete loss of protein within 10 min of incubation (data not shown). However, at 65 and 75°C, the degradation was less severe (data not shown). The auto-degradation phenomenon seems to be correlated with the loss of enzyme activity, as assayed with azocasein as a substrate. Furthermore, time-dependent incubation of VSPase at 55°C shows that more than half of the enzyme can be degraded within 2 min (data not shown).

In terms of kinetics, VSPase has a similar catalytic efficiency with V8 protease ($k_{cat}/K_m = 27.7/\text{mM}/\text{sec}$), as it has k_{cat}/K_m value of $30.0/\text{mM}/\text{sec}$ (29). However, VSPase differs from V8 protease in several aspects of substrate specificity. V8 protease shows an amidolytic activity towards all both Z-Phe-Leu-Glu-pNA and Z-Glu-pNA (29), however, VSPase does not exhibit the activity towards H-Glu-pNA, meaning that VSPase has a little bit greater substrate specificity than V8 protease. In other words, V8 protease cleaves peptide bonds at the carboxyl side of any glutamate residue in peptide and protein substrates whereas VSPase requires at least two hydrophobic residues ahead of glutamate for the cleavage. In practice, VSPase cleaves the carboxyl sides of glutamates in the two sequences, Ala³¹⁰-Ile³¹¹-Glu³¹²-Gly³¹³ and Ile³⁶⁴-Val³⁶⁵-Glu³⁶⁶-Gly³⁶⁷ of prothrombin. These results demonstrate that for the cleavage of VSPase, not only the presence of glutamate is essential, but the sequence surrounding the N-terminal side of glutamate is also important, together with the nature of the amino acids such as the presence of at least two hydrophobic residues.

The nucleotide sequence analysis of cloned VSPase-encoding gene shows that the gene can produce a polypeptide of 339 amino acid residues (Fig. 3). As shown in Fig. 3, the deduced amino acids from 69th to 88th completely coincide with the N-terminal sequence of the purified VSPase. This result suggests that the N-terminal region consisted of 68 amino acids is processed from VSPase zymogen, resulting in producing a mature enzyme. Therefore, it is expected that the mature form of VSPase is composed of 271 amino acids, having a calculated molecular mass of 29,351 Da, which is well matched with the mass (29,688 Da) determined by MALDI-TOF. The result also shows that VSPase zymogen has a propeptide region that might occlude the active site and a catalytic domain containing a catalytic machinery.

It has been known that V8 proenzyme cannot be properly processed to a functional enzyme in *E. coli* (15,30-32). Unlike V8 protease, rVSPase expressed in *E. coli* appears a functionally active processed form (Fig. 5). rVSPase migrates the same distance as native enzyme on SDS-polyacrylamide gel (Fig. 5A),

and its N-terminus starts with VILPNN (data not shown) as in native enzyme. Even the mutant enzymes D161A and S237L that only have background levels of enzyme activity have the same molecular weights of 34 kDa as native VSPase (Fig. 5). However, a mutant enzyme H119L seems to be larger, as it has approximately 37 kDa, than that of rVSPase on SDS-polyacrylamide gel (Fig. 5A), which is corresponding to the size of premature form of rVSPase containing the propeptide region. However, H119L enzyme can be processed to 34 kDa when the enzyme is incubated with rVSPase for a short period of time (data not shown). These results demonstrate that VSPase zymogen can be autoprocessed by itself to active enzyme by losing the propeptide region. However, the processing of rVSPase in *E. coli* is still unclear; therefore it should be clarified in a further study.

Staphylococcal glutamyl endopeptidases have a high sequence homology in amino acid sequence level (Fig. 4). When the amino acid sequences from VSPase, SPase, V8 protease, PROM and GluSE enzymes were compared, the sequence identity is found to be approximately 79.25% (Fig. 4). However, the homology between C-terminal parts of the same enzymes is about 51.75%. This distinct difference between the C-terminal regions mainly dues to the absence or the length of Pro-Asx-Asp (Glx) repeats (Fig. 4). It is postulated that Pro-Asx-Asp (Glx) repeat may be responsible for substrate binding of the enzymes, however, Yabuta *et al* reported that the C-terminal repeat sequence is not needed for the enzymatic activity (31). Our recent data performed with the truncated VSPase mutant enzyme in the repeat also showed that the enzyme activity was as much as wild-type VSPase (unpublished data).

With the overall sequence homology, serine protease superfamily shares a typical catalytic triad formed by serine, histidine and aspartate residues (25,33). The result of amino acid sequence alignment (Fig. 4) shows that VSPase may also have a highly conserved catalytic triad. The three amino acid residues (His¹¹⁹, Asp¹⁶¹ and Ser²³⁷) that may compose a putative catalytic triad in VSPase can be changed to Leu¹¹⁹, Ala¹⁶¹ and Leu²³⁷, respectively, using site-directed mutagenesis. As expected, substitution mutations in one of the three amino acids of VSPase result in abolishing dramatically enzyme activity (Fig. 5B). These results clearly show that the three residues can make a catalytic triad in VSPase enzyme as in other typical serine proteases (34-36).

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