2009年 2月

碩士學位論文

A serine protease secreted by *Staphylococcus aureus* evokes a vascular permeability accompanied with production of pro-inflammatory cytokines

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2009年 2月 25 日

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指導敎授 李 正 燮

이 論文을 理學 碩士學位申請 論文으로 提出함

2008 年 10月

朝 鮮 大 學 校 大 學 院

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A serine protease secreted by *Staphylococcus aureus* evokes a vascular permeability accompanied with production of pro-inflammatory cytokines

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Staphylococcus aureus (*S. aureus*) is a ubiquitous Gram-positive bacterium responsible for a majority of skin infections as well as causing toxic shock syndrome. In this study, a glutamate-specific serine endopeptidase (named VSPase) secreted by *S. aureus* sp. strain C-66 was purified and characterlized in terms of its involvement in the induction of inflammatory response and vascular permeability. VSPase clearly increased the expression levels of the genes for pro-inflammatory cytokines such as TNF-α and IL-1β, and also up-regulated an inflammatory regulator cyclooxygenase-2 in their levels of transcription, as determined by RT-PCR,

ELISA, and Western blot analysis. VSPase could induce the degradation of IκB resulting in the translocation of NF-κB proteins into nucleus, as judged by Western blot analysis and super shift assay with anti-p65 antibody, respectively. These results suggest that VSPase can activate NF-κB signaling pathway through the degradation of IκB proteins, leading the production of pro-inflammatory cytokines and an inflammatory regulator. Interestingly, wild type VSPase could cause an increased vascular permeability on guinea pig system in a dose-dependent manner, whereas its mutant enzyme S237L that was totally deficient in proteolytic activity could not, as examined by Miles assay. These results suggest that the vascular permeability caused by VSPase may be related to its proteolytic activity. Taken together, all results obtained by this study demonstrate that VSPase plays an important role in the expression of pro-inflammatory cytokines and inflammatory regulator through the activation of NF-κB and also can enhance a vascular permeability during the Staphylococcal infection.

I. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a ubiquitous bacterium and produces numerous toxins including super antigens that cause unique disease entities such as toxic-shock syndrome, scarlet fever and has acquired resistance to practically all antibiotics (Cai *et al.*, 2007). Its main habitats are the nasal membranes and skin of warm-blooded animals. The bacteria cause a range of infections from mild, such as skin infectious and food poisoning (Becker *et al.*, 2003), to life-threatening, such as pneumonia, sepsis and infectious endocarditis (Ferry *et al.*, 2005). Moreover, *S. aureus* has an impressive capacity to adjust to the environment since it has developed a multitude of invasive and evasive mechanisms to cope with host defence (Calander *et al.*, 2004, Calander *et al.*, 2006).

During the bacterium-host cell interaction, bacterial surface proteins play a key role in the pathogenesis of disease. These proteins were expressed in various size and shape on the bacterial surface sequentially engage with complimentary receptors on target cells. These early events paly a primary role in the bacterial adherence, colonization and internalization. Once the initial niche has been established, bacteria may use their extracellular armamentarium to gain access to the tissue either by subverting host defense mechanisms, therapeutic regimes, host cellular signaling systems, by employing molecular mimicry, or by using a brutal force of their ability to digest the target tissue. The surface of Staphylococci are decorated with an array of variety of proteins. In addition, Staphylococci secrete a variety of proteins many of them are in fact enzymes of a variety of nature.

Many studies have been performed mapping *S. aureus* virulence determinants with the aim of improving our ability to combat *S. aureus* infections. So far, little is known concerning the impact of *S. aureus* extracellular proteases on virulence. The major proteolytic enzymes secreted by *S. aureus* consist of metalloprotease (Drapeau *et al.*, 1972), serine glutamate-specific endopeptidases including V8 protease (Houmard *et al.*, 1972), staphylocoagulase (SC) (Zolli *et al.*, 1963), SPase (Yoshikawa *et al.*, 1992), and two related cysteine proteases referred to as staphopain (ScpA) and cysteine protease (SspB) (Shaw *et al.*, 2004). Among they V8 protease is thought to combat host defenses and facilitate tissue invasion and nutrient acquisition.

Alteration in the immune response after infection, multiple trauma, and posttraumatic sepsis are recognized as physiological reactions of the organism to restore homeostasis. The level of these immunological changes correlates with the degree of tissue damage as well as with the severity of haemorrhage and ischaemia. Cytokines are known to be integral components of this immune response. The local release of pro-inflammatory and anti-inflammatory cytokines after severe to infection indicates their potential to induce systemic immunological alterations. It appears that the balance or imbalance of these different cytokines partly controls the clinical course in these host.

Overproduction of either pro-inflammatory cytokines or anti-inflammatory mediators may result in organ dysfunction (Hildebrand *et al.*, 2005). The major pro-inflammatory cytokines involved in the response to bacterial infection and trauma include tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These cytokines, which are predominantly

produced by monocytes and macrophages, mediate a variety of frequently overlapping effects, and their actions can be additive. The concept is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. For example, genes that are pro-inflammatory are type II phospholipase (PL) A2, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). These genes code for enzymes that increase the synthesis of platelet-activating factor (PAF), leukotrienes, prostanoids, and nitric oxide (NO) (Dinarello, 2000). Another class of genes that are pro-inflammatory chemokines includes interleukin-8 (IL-8), monocyte chemotactic protein type 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and macrophage inflammatory protein-2 (MIP-2).

Many of the transcriptional effects of inflammatory cytokines, including TNF-α, are mediated by activation of the transcription factor, nuclear factor-kappaB (NF-κB) (Rothwarf *et al.*, 1999). The NF-κB has attracted wide spread attention among researchers in many fields based on the following: its unusual and rapid regulation, the wide range of genes that it controls, its central role in immunological processes, the complexity of its subunits, and its apparent involvement in several diseases (Tak *et al.*, 2001). A primary level of control for NF-κB is through interactions with an inhibitor protein called IκB. Recent evidence confirms the existence of multiple forms of IκB that appear to regulate NF-κB by distinct mechanisms (Hayden *et al.*, 2004).

NF-κB can be activated by exposure of cells to lipopolysarcchariде (LPS) or inflammatory cytokines, such as TNF-α, IL-1β, viral infection or expression of certain viral gene products, UV irradiation and B or T cell activation

(Crinelli *et al.*, 2000). Activation of NF-κB to move into the nucleus is controlled by the targeted phosphorylation and subsequent degradation of IκB (Fig. 1). In the nucleus, NF-κB dimers bind to target DNA elements and activate transcription of genes encoding proteins involved with immune or inflammation responses and with cell growth control (Baldwin, 1996).

S. aureus is the most frequently isolated pathogen in gram-positive sepsis, which suggest that some factors from this bacterium is associated with septic shock induction (Mattsson *et al.*, 2001). It is well known that Staphylococcal components can induce the production or release of many basoactive mediators, including PAF, prostaglandin (PG) (Okiji *et al.*, 1989), and bradykinin (BK) as well as cytokines, in particular, of interleukins and TNF-α. These mediators, expecially those like BK and PAF, can potently increase of vascular permeability. Three microvascular events characterize acute inflammation; arteriolar vasodilatation, neutrophil recruitment, and vascular permeability increase (Lotufo *et al.*, 2006).

Vascular permeability increase occurs at the very beginning of the inflammatory response and is initially triggered by agents released by mast cells, which activate endothelial receptors promoting endothelial retraction and junction disorganization, leading to gap formation between endothelial cells in venules and capillaries (Lotufo *et al.*, 2006). A substantial increase in capillary permeability is also a feature of acute inflammation in bacterial infections (Miles *et al.*, 1952).

Recent studies show that *S. aureus* produces cysteine proteases that are Staphopains A and B (so called ScpA and SspB). These proteases

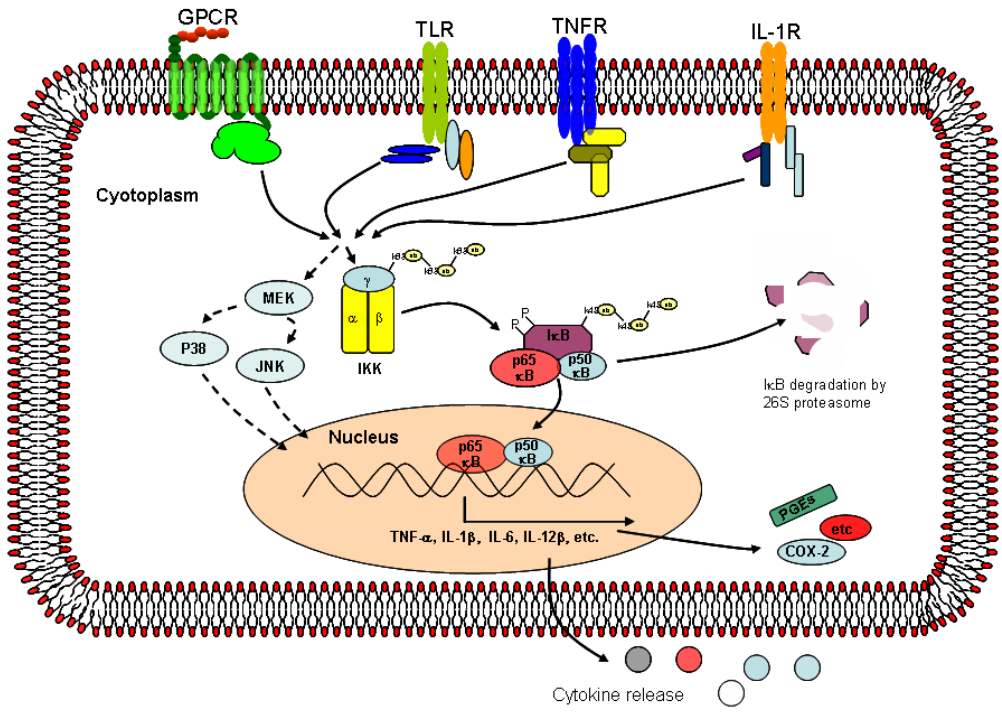


Fig. 1. Signaling pathway in macrophage. During infection, host cells sense bacterial-derived products (LPS, protease, PGN, and DNA, *etc.*) via specific receptors (TLR, GPCR, IL-1R, and TNFR, *etc.*) (Janssens *et al.*, 2003). The activation of macrophage results in the production of numerous mediators involved in innate immunity and inflammation. This event triggers phosphorylation of downstream signal transducers including inhibitor of κB (IκB) kinase (IKK), which in turn would induce the formation of an IKK-activating signalsome that activates IKK through a mechanism that involves Lys63-linked IKKγ ubiquitination. NF-κB proteins activated by phosphorylated NF-κB subunits (for example, p50 and p65) liberated from IκB-NF-κB complex would be subsequently translocalized into nucleus for DNA binding to modulate inflammatory effector gene expression (Annane *et al.*, 2005).

induce the release of bradykinin that is a final product of plasma kallikrein/kinin system activation which causes vascular leakage and leads to hypotension. However, aureolysin (Sabat *et al.*, 2000) and V8 protease do not increase vascular permeability (Imamura *et al.*, 2005).

This study describes an extracellular protease named VSPase from *S. aureus.* The protease has an identical N-terminal sequence to those of SPase and V8 proteases. This study also demonstrates that VSPase can induce and/or increase inflammatory gene expression in Raw 264.7 cells and show that the enzyme activity of VSPase is essential for cytokine production and the induction of vascular permeability.

II. MATERIALS AND METHODS

II-1. Materials

HiPrep 16/10 Q FF column, Source 15 Q 4.6/100 PE column, Superdex 75 10/300 GL column and PD-10 column were purchased from Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from BioWhittaker (Walkersville, MD, USA). TNF-α enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN). Polyvinylidene fluoride (PVDF) membrane was obtained from Bio-Rad (Richmond, Calif). The synthetic chromogenic substrate, Z-Phe-Leu-Glu-*p*NA (L-2135) was obtained from Bachem (Bubendorf, Switzerland). The following antibodies for the antigens were from Santa Cruz Biotechnology (Santa Cruz, CA): TNF-α, IL-6, IκBα, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Anti-rabbit, anti-goat, and anti-mouse immunoglobulin Gs coupled to peroxidases were also from the same company. Rabbit polyclonal antibody raised against phospho-NF-κB p65 was obtained from Cell Signaling Technology (Beverly, MA, USA). Protein molecular weight markers were obtained from Fermentas (Darmstadt, Germany). Restriction enzymes *Kpn* I, *Hin*d III and *Dpn* I were purchased from New England BioLabs (Beverly, MA, USA). An agarose gel extraction kit, PCR purification kit and i*pfu* polymerase obtained from iNtRON Biotechnology (Seonnam, Korea). The plasmid pFLAG-ATS vector, azocasein, ammonium sulfate, bovine serum albumin

(BSA), trizma base, and other chemicals used were obtained from Sigma. (St. Louis, MO, USA).

II-2. Cultivation of bacterial strains and Raw 264.7 cells

S. aureus sp. strain C-66 was a kind gift from Prof. Y. Lim (Chosun University School of Medicine, Gwangju, Korea) and grown in 3% tryptic soy broth (Bacton Dickinson, MD, USA) at 37℃ under aerobic conditions. *Escherichia coli* (*E. coli*) DH5α cells were cultivated in Luria Bertani (LB) medium. Raw 264.7 cells that were normally used for examining the induction of inflammatory response by VSPase were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37℃ under 5% CO_2 condition.

II-3. Purification of native VSPase protease

The culture supernatant of *S. aureus* sp. strain C-66 cells were collected by centrifugation at 8,000 x*g* and 4℃ for 20 min and ammonium sulfate was added to 70% saturation. The resulting precipitate was collected by centrifugation at 12,000 x*g* and 4℃ for 30 min, dissolved in 25 mM Tris-HCl (pH 7.5), and desalted on PD-10 column equilibrated with the same buffer. The sample was applied to a HiPrep 16/10 Q FF equilibrated with the same buffer. The VSPase was eluted with a linear gradient of NaCl from 0 to 500 mM in the same buffer. Active fractions were pooled, concentrated by ultra-filtration using Amicon YM 10 membrane (Millipore, MA, USA), and applied to a Superdex 75 10/300 GL gel filtration column equilibrated with 50

mM Tris-HCl (pH 7.5) containing 150 mM NaCl. The active fractions were pooled and used as the purified protease. Protein concentrations were determined with Bradford reagent (Sigma, USA) according to the manufacturer's instructions.

II-4. Cloning of *VSPase* gene

The entire coding region of *VSPase* gene was amplified by PCR from the chromosomal DNA of *S. aureus* sp. strain C-66 using the following oligonucleotides: 5'-TCAAGCTTGGGATGAAAGGTAAATTTTTAAAAG-3' and 5'-ACGGTACCCCTTATGCAGCGTCAGGGTTGT-3' as the forward and reverse primers, respectively (The underlined bases in the primers represent the introduced restriction sites, *Hin*d III in forward primer and *Kpn* I in reverse primer). PCR was performed for 1 min at 94℃, 30 sec at 50℃, and 1 min 30 sec at 72℃ for 30 cycles using the Eppendorf Mastercycler gradient. After purification, the PCR product was digested with *Hin*d III and *Kpn* I, further purified and then ligated to *Hin*d III/*Kpn* I-cut pFLAG-ATS vector to yield the pFLAG-ATS-*VSPase* construct.

II-5. Construction of *VSPase* mutants with site-directed mutagenesis method

VSPase mutants were made by *Dpn* I-mediated site-directed mutagenesis as shown in Fig. 2. In this mutagenesis, the pFLAG-ATS-*VSPase* double-stranded DNA vector and two synthetic

oligonucleotide primers containing the desired mutations were used. Table 1 shows the oligonucleotide primers used. As shown in Fig. 2, the annealed primers were extended by temperature cycling with i*pfu* DNA polymerase as described by the manufacturer. After the thermal cycling (30 sec at 94℃, 1 min at 55℃ and 6 min 30 sec at 68℃ for 12 cycles), the PCR products and the parental DNA templates were treated with *Dpn* I endonuclease its (target sequence is 5'-GmA↓TC-3' in which an arrow indicates the cleavage site), which is specific for methylated and hemi-methylated DNA to select for mutation containing synthesized DNA. The recombinant plasmid DNA was then transformed into *E. coli* strain DH5α (Fig. 2). Positive clones were selected and their DNA sequences were determined to confirm the expected mutation.

II-6. Expression and purification of VSPase and its mutant enzymes

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℃. Ten milliliters of this overnight culture was used to inoculate 500 ml of fresh LB broth containing ampicillin and cells were grown at 37℃ until the A_{600} reached 0.8. Four sets of this culture were prepared. The target protein was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by overnight incubation at 20℃. The cells were harvested, resuspended in 100 ml of 30 mM

Table 1. Oligonucelotides used in site-directed mutagenesis.

Gene modification	Oligonucleotide[a]	Primer sequence (5' to 3')[b]
Point mutation for Ser^{237} to Leu	S237L-F	GGTGGTAACTTAGGTTCACCAGTATTTAAT
	S237L-R	ATTAAATACTGGTGAACCTAAGTTACCACC
Point mutation for His^{119} to Leu	H119L-F	ACAAATAAACTTGTCGTAGATGCTACGCAC
	H119L-R	GTGCGTAGCATCTACGACAAGTTTATTTGT
Point mutation for Asp^{161} to Ala	D161A-F	TATTCAGGCGAAGGTGCTTTAGCAATCGTT
	D161A-R	AACGATTGCTAAAGCACCTTCGCCTGAATA

[a]F and R mean the forward and the reverse primers, respectively.
[b]Sites for mutations are underlined.

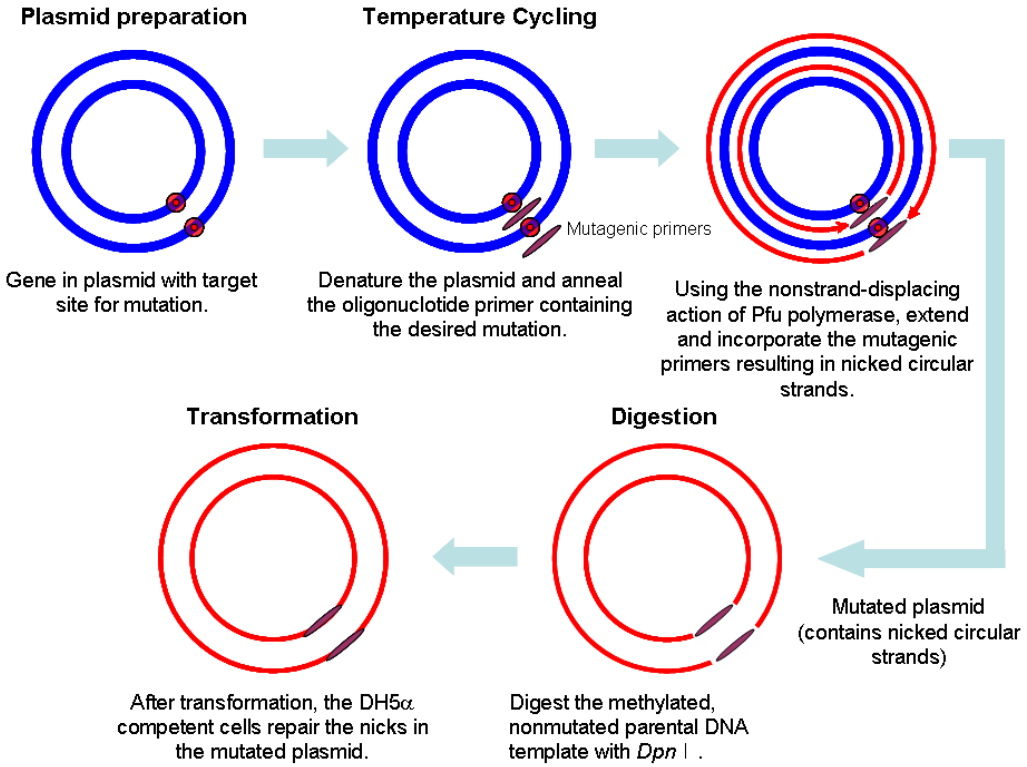


Fig. 2. Outline of mutagenesis protocol used for the construction of VSPase mutants.

Tris-HCl (pH 8.0)/ 20% sucrose/ 1 mM EDTA/ 0.3 mg/ml lysozyme, and then incubated at 4℃ for 30 min. The cell suspension was centrifuged at 18,000 x*g* for 20 min at 4℃. The supernatant, which contained the periplasmic protein, was subjected to ammonium sulfate precipitation at 70% saturation. The resulting protein pellet was collected by centrifugation at 35,000 x*g* for 30 min at 4℃, dissolved in 25 mM Tris-HCl (pH 7.5), and then applied to PD-10 column equilibrated in the same buffer to remove residual $(NH_4)_2SO_4$. 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 with a linear gradient of NaCl from 0 to 500 mM in the same buffer. Fractions with 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 300 mM in the same buffer. Fractions with major protease activity or size were pooled and purified enzymes were stored in small aliquots at -20℃ until used.

II-7. Protease activity assay

Protease activity was routinely assayed with azocasein as a substrate. Reaction sample (total 200 μl) containing enzyme, 25 mM Tris-HCl (pH 7.5), and 0.5% azocasein was incubated at 37℃ for 20 min. The reaction was stopped by addition of 100 μl of 10% (w/v) trichloroacetic acid and then centrifuged at 10,000 x*g* 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 Corporation, California, USA).

To calculated enzyme unit and kinetics for VSPase, synthetic chromogenic substrate, Z-Phe-Leu-Glu-*p*NA (L-2135) was routinely used. The amidolytic activity of enzyme was assayed in 100 μl of reaction mixture composed of 50 mM Tris-HCl (pH 7.5), 0.9% NaCl, 0.1 mg/ml BSA, enzyme, and 0.4 mM of the chromogenic substrate. After the addition of the chromogenic substrate, the increase in absorbance at 405 nm was monitored in a 96-well plate reader at 37℃. One unit of protease activity was defined as the amount of enzyme that catalyzes of 1 μmol *p*NA per min under the conditions described.

II-8. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

After treatment with VSPase, S237L or LPS, cells were lysed and total RNAs were isolated using easy-spin total RNA extraction kit, (iNtRON Biotechnology, Korea) according to the manufacturer's instruction. cDNAs were made from 1 μg of RNA using oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase (Bioneer, Korea). PCR amplification using 0.5 μg of complementary DNA (cDNA) aliquots were performed by adding 2.5 mM dNTPs, 2.5 units *Taq* DNA polymerase, and 10 pmol each of forward and reverse primers (Table 2) in a PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3). Reactions were processed in a DNA thermal cycler (Eppendorf, NY, USA) through 45 cycles of 30 sec of denaturation at 94℃, 30 sec of annealing at different temperatures (Table 2) and followed by

Table 2. Sequences of the primers used for RT-PCR.

Gene		Primer sequence (5' to 3')	Expected PCR products in size (bp)	Annealing temperature (℃)	GeneBank Accession No.
TNF-α	Forward	TTTCCTCCCAATACCCCTTC	197	48	Y00467
	Reverse	AGTGCAAAGGCTCCAAAGAA			
IL-1β	Forward	TGTGAAATGCCACCTTTTGA	205	50	AK168047
	Reverse	GTAGCTGCCACAGCTTCTCC			
IL-12β	Forward	GGGACATCATCAAACCAGAC	226	53	NM_008352
	Reverse	ACTTCGGTAGATGTCTTCTC			
COX-2[a]	Forward	CAGCAAATCCTTGCTGTTCC	658	50	BC052900
	Reverse	CCATCCTTGAAAAGGCGCAG			
PGEs[b]	Forward	ATGCCTTCCCCGGGCCTG	462	48	AK150013
	Reverse	TCACAGATGGTGGGCCAC			
MIP-2[c]	Forward	AACAAAGGCAAGGCTAACTGA	203	53	X53798
	Reverse	AACATAACAACATCTGGGCAAT			
GAPDH	Forward	TCAGCAATGCATCCTGCACCAC	252	55	BC082592
	Reverse	TGCCAGTGAGCTTCCCGTTCAG			

[a]Cyclooxygenase-2; [b]Prostaglandin E synthase; [c]Macrophage-inflammatory protein-2.

40 sec of elongation at 72℃. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide (EtBr), and visualized using a UV transilluminator. The band densities were quantified using NIH Scion Image software (Scion Corp., Frederick, MD).

Real time quantitative polymerase chain reaction (real time PCR) was performed using a Rotor-Gene 3000 (Corbett Research, Australia). Each sample was subjected to multiplex real time PCR for 40 cycles (denaturation for 20 sec at 94℃, annealing for 20 sec at 50℃, extension for 30 sec at 72℃) using SYBR Green system (Qiagen, Valencia, CA).

II-9. Enzyme-linked immunosorbent assay (ELISA)

The 5 x 10^5 cells were plated on 48-well plates the day before VSPase stimulation. Cells were treated with LPS (1 μg/ml), VSPase (3 μg/ml) or S237L (3 μg/ml) for 3 h at 37℃. The TNF-α protein levels were determined using ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

II-10. SDS-PAGE and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Typically, samples were mixed with an equal volume of 2x SDS-PAGE sample buffer, heated at 100℃ for 3 min, and then subjected to electrophoresis. After electrophoresis, protein bands were visualized by staining the gel with 0.25% Coomassie brilliant blue. For Western blot

analysis, samples were subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked at room temperature for 2 h with blocking solution (TBS containing 5% skim milk and 0.1% Tween 20) and incubated with various antibodies diluted in the blocking solution at 4℃ for overnight. The membrane was washed three times in TBS plus 0.1% Tween 20 and incubated with a diluted peroxidase-conjugated secondary antibody for 2 h. The blot was washed three times in TBS plus 0.1% Tween 20, once in TBS only, and treated with WestZol Western Blotting Detection Reagents (iNtRON Biotechnology, Korea). To detect signals, the blot was exposed to X-ray film and developed according to manufacturer's instructions.

II-11. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from Raw 264.7 cells before and after stimulation with VSPase for various time periods. Cell pellets were suspended in 100 μl of hypotonic buffer containing 10 mM HEPES (pH 7.9), 0.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM phenylmethylsulphonyl fluoride (PMSF) for 5 min on ice and centrifuged at 15,000 x*g* for 5 min. The resulting nuclear pellet was then resuspended in a low-salt buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 0.2 mM PMSF for 20 min at 4℃. Samples were then centrifuged (15,000 x*g*) at 4℃ for 20 min and nuclear extracts were aliquoted. Protein concentrations were measured with Bradford protein assay

reagent (Sigma, USA) and equal amounts were loaded for EMSA. A double-stranded synthetic oligonucleotide containing κB element (^{32}P-AGCTTGGGGACTTTCC-3') was prepared and end-labeled with 5 units of T_4 polynucleotide kinase in the presence of 50 μCi (3,000 Ci/mmol) of [γ-^{32}p]ATP (Amersham Phamacia Biotech., Uppsala, Sweden). Binding reactions were performed in a total 20 μg of reaction mixture composed of 10 μg of nuclear extracts and 0.5 pmol of the radioactively labeled oligonucleotide in a binding buffer (20 mM HEPES, pH 7.9, 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 250 ng of poly (dI-dC)). Samples were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel with 0.25x TBE buffer (22.2 mM Tris, 22.2 mM boric acid, 0.5 mM EDTA) and then by autoradiography on X-ray film described elsewhere (Crinelli *et al.*, 2000). Super shift assay were carried out with anti-p65 antibody as described previously (Velasco *et al.*, 1997).

II-12. Vascular permeability assay

The guinea pig (body weight, 250 to 350 g, male) was lightly anesthetized with diethyl ether (JUNSEI). Sixty five milligram per kilogram body weight of Evans blue was administered intravenously, followed by an intradermal injection of 50 μl of test sample (dissolved in 10 mM PBS) into the clipped flank of the guinea pig. A solution of Evans blue (5% solution in 0.6% saline) was filtered through a 0.2 μm micropore filter before use. After 10 min, the guinea pig was sacrified by bleeding. The blueing tissues were cut out and incubated in 3 ml of formamide at 60℃ for 48 h. The dye

exclusion was determined by measuring the absorbance at 620 nm and expressed as a measure in μg of Evans blue dye efflux.

III. RESULTS AND DISCUSSION

III-1. A Glu-specific extracellular serine protease named VSPase secreted by *S. aureus* sp. strain C-66

III-1-1. Purification of native VSPase

An active extracellular protease designated to as VSPase was purified from the culture supernatant of *S. aureus* sp. strain C-66 by employing three purification steps consisting of ammonium sulfate precipitation, weak anion exchange, and size exclusion chromatographies in order. Only one activity peak was obtained from HiPrep 16/10 Q FF and Superdex 75 10/300 GL column chromatographies (data not shown). The purification of wild type VSPase is summarized in Table 3. The purification yield was 1.44%. The amount of purified protease obtained was 1.5 mg with a total activity of 43.27 units/mg. The purified enzyme appeared as a single band with apparent molecular weight of approximately 34 kDa, as judged by SDS-PAGE (Fig. 3). There was no difference in migration between reducing and non-reducing conditions (data not shown). Mass spectrometry analysis using MALDI-TOF showed that the mass of VSPase was found to be 29,688 Da. N-terminal amino acid sequencing revealed the sequence NH_2-VILPNNDRHQITDTTNGHYA-COOH, which had 100% identity with those of SPase and V8 proteases.

Table 3. Purification summary of native VSPase.

Purification step	Total protein (mg)	Total activity (U)[a]	Specific Activity (U/mg)	Yield (%)[b]
Culture supernatant	104.2	ND[c]	ND[c]	100
70% $(NH_4)_2SO_4$	94	202.5	2.15	90.2
HiPrep 16/10 Q FF	7.5	146	19.47	7.19
Superdex 75 10/300 GL	1.5	63.4	43.27	1.44

[a] One unit (U) was defined as a substrate the amount of protease that catalyzes the release of 1 mmol of *p*NA/min when the chromogenic substrate (Z-Phe-Leu-Glu-*p*NA) was used.

[b] The total proteins in the culture supernatant were assigned the value of 100%.

[c] ND, not detectable.

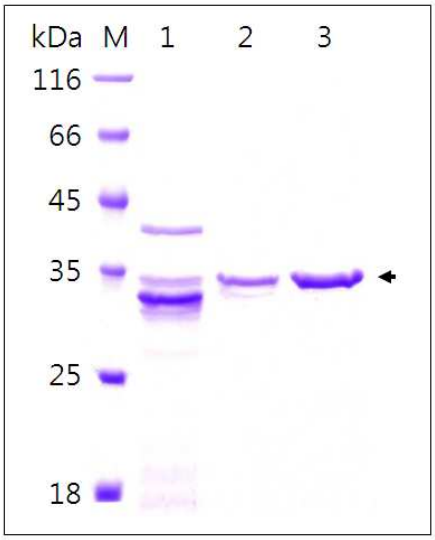


Fig. 3. SDS-PAGE of proteins obtained from various purification steps. The proteins were electrophoresed on 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Lanes M, Protein molecular weight markers; 1, ammonium sulfate fraction (0 to 70%); 2, HiPrep Q column chromatography; 3, size exclusion chromatography. An arrow indicates the purified native VSPase enzyme.

III-1-2. Cloning of *VSPase* and its mutant genes

The cloning of *VSPase* gene was achieved with PCR using primers derived from the sequence for V8 protease as described in Materials and Methods. The PCR products were digested with *Hin*d III and *Kpn* I and then ligated into *Hin*d III/*Kpn* I-cut pFLAG-ATS vector to make the construct shown in Fig. 4. The resulting 6.4 kb recombinant plasmid named pFLAG-ATS-*VSPas*e harboured V*SPase* gene (Fig. 4). As shown in Fig. 4, three other mutant *VSPase*-encoding genes were also constructed in the same vector. The nucleotide sequence of cloned *VSPase* gene was determined from which amino acid sequence was also deduced (Fig. 5). As shown in Fig. 5, *VSPase* gene had one open reading frame composed of 1,020 nucleotides that could produce a protein composed of 336 amino acids. When the deduced amino acid sequence of VSPase was compared with those of other Staphylococcal proteases, there were a high sequence homology between VSPase, SPase, and V8 proteases (Fig. 6). Identity between VSPase and SPase was found to be 98% and that between VSPase and V8 protease was 97%. The sequence comparison also showed that there was a putative active-site serine and histidine residues within concensus motives, (T-x(2)-[GC]-[NQ]-S-G-S-x-[LIVM]-[FY]) and ([ST]-G-[LIVMFYW](3)-[GN]-x(2)-T- [LIVM]-x-T-x(2)-H) that are frequently found in Glu-specific proteases (Dancer *et al.*, 1990). Typically V8 superfamily shares a catalytic triad formed by Ser, His and Asp (Reed *et al.*, 2001). On the basis of this observation, PCR-based site-directed mutagenesis was performed to reveal the amino acids composed of catalytic triad probably contained in VSPase.

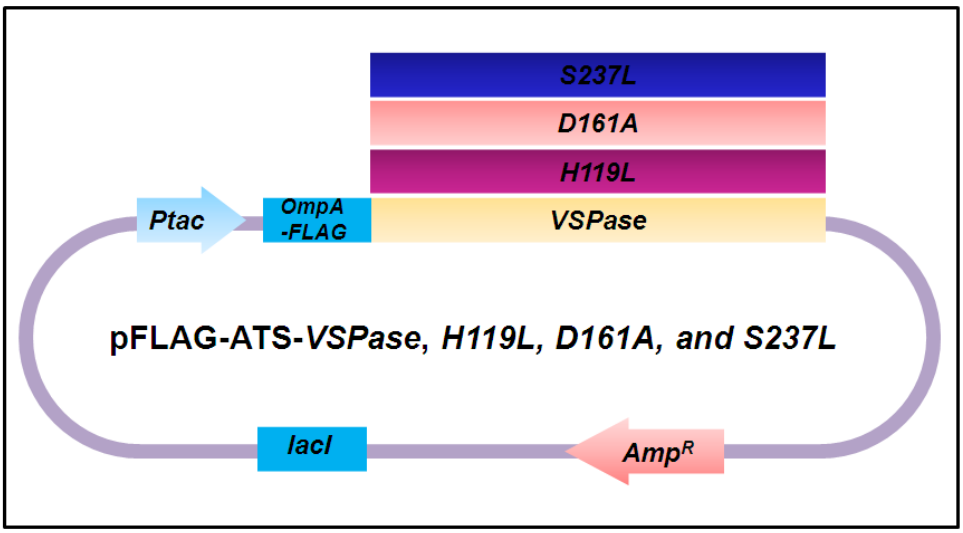


Fig. 4. Physical maps of recombinant plasmids of pFLAG-ATS-*VSPase* and its derivatives.

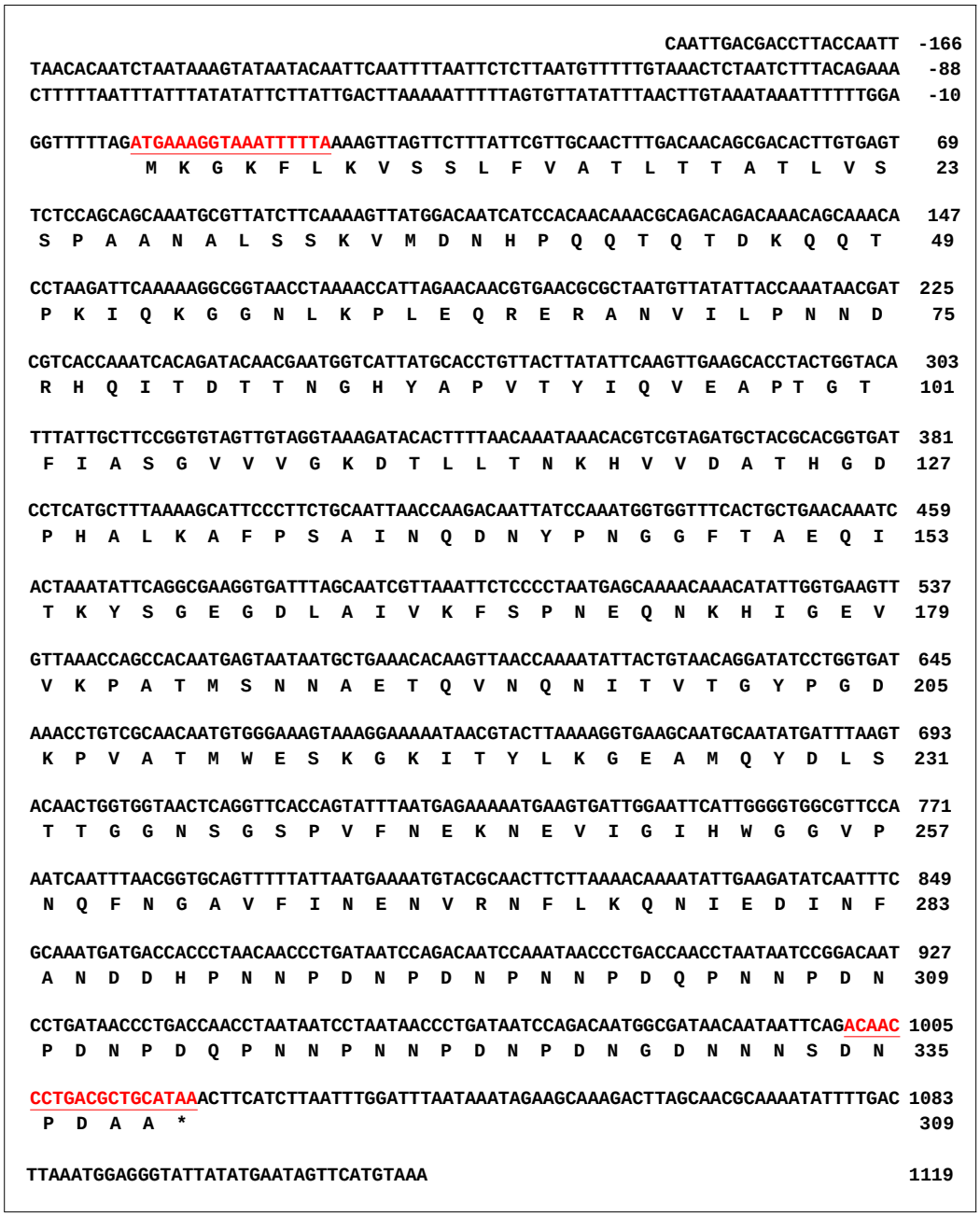


Fig. 5. The nucleotide and the deduced amino acid sequences of *VSPase* gene. The underlined nucleotide sequences indicate the forward and the reverse primer binding sites used for cloning of *VSPase* gene.

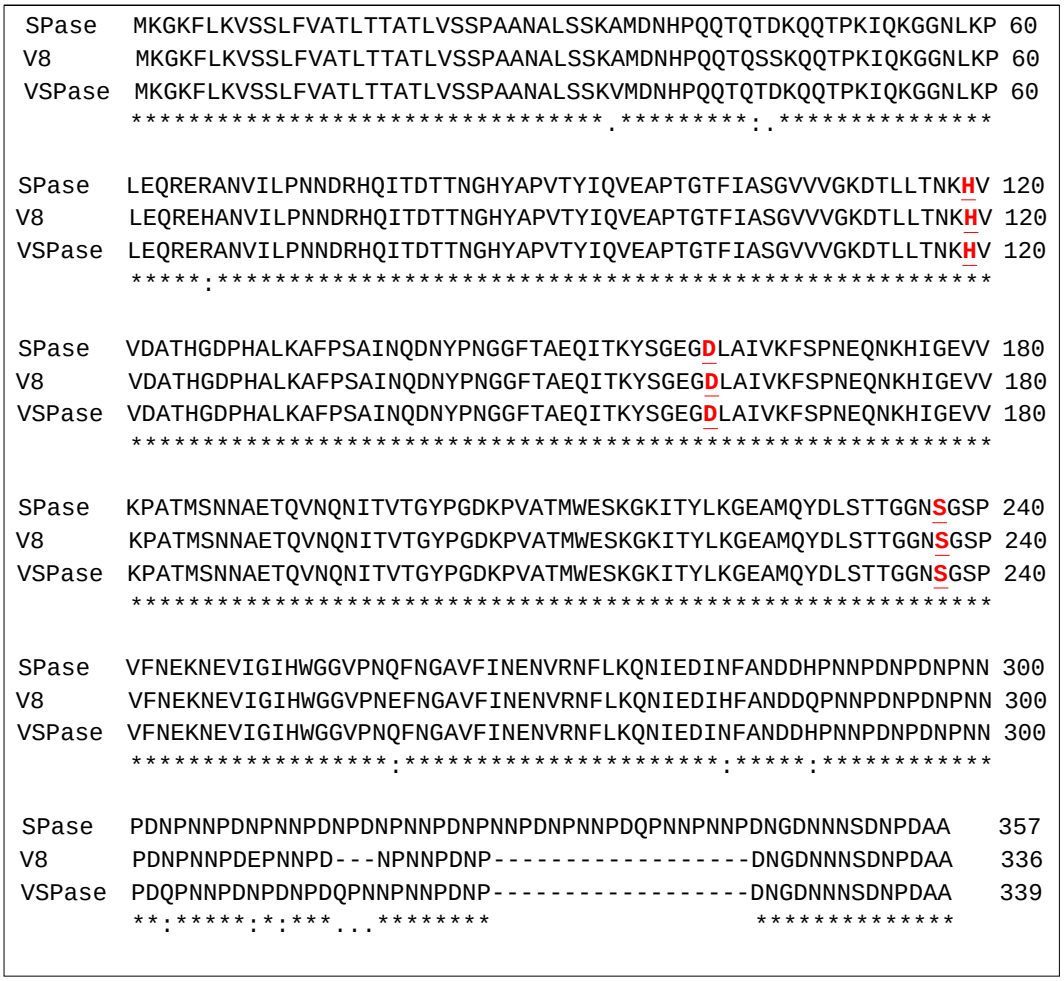


Fig. 6. Alignment of amino acid sequences between VSPase, SPase, and V8 proteases. Identical residues in all three sequences are asterisked. Three amino acid residues shown by underline indicate the members of a putative catalytic triad in the active enzyme, which are typically found in serine proteases.

The resulting mutant enzymes named H119L, D161A, and S237L were expressed and purified. Three amino acid residues (His^{119}, Asp^{161}, and Ser^{237}) of putative catalytic triad within VSPase was changed to Leu^{119}, Ala^{161}, and Leu^{237}, respectively (Fig. 4).

III-1-3. Expression and purification of recombinant VSPase and its mutants

Recombinant VSPase was expressed in *E. coli* as an N-terminal fusion protein with FLAG peptide using pFLAG-ATS vector as shown previously (Fig. 4). In this vector system, a signal sequence that is known as OmpA (outer membrane protein A) and a responsible for the secretion of FLAG fusion protein crosses the inner, cytoplasmic membrane into the periplasmic space is located (Oka *et al.*,1985).

Therefore, the expressed VSPase enzyme could be isolated from the periplasmic proteins using two successive anion exchange chromatographic steps including HiPrep 16/10 Q FF column and Source 15 Q 4.6/100 PE column in order (Fig. 7). The purification of recombinant VSPase (wild type) and mutant enzymes is summarized in Tables 4 and 5, respectively. The final yields of the mutant enzymes were more than twice that of wild type VSPase. Fig. 8 shows the SDS-PAGE results. Wild type VSPase, S237L, and D161A appeared to be homogeneous, as they migrated as a single band with an estimated molecular weight of approximately 34 kDa on the SDS-polyacrylamide gel. However, the H119L was found to be 37 kDa form that probably contains propeptide region.

Table 4. Purification summary of recombinant VSPase.

Purification step	Total protein (mg)	Total activity (U)[a]	Specific Activity (U/mg)	Yield (%)[b]
Periplasmic proteins	64	ND[c]	ND[c]	100
HiPrep 16/10 Q FF	35	297.5	8.5	54.7
Source 15 Q 4.6/100 PE	4.6	166.1	36.1	7.2

[a] One unit (U) was defined as a substrate the amount of protease that catalyzes the release of 1 mmol of *p*NA/min when the chromogenic substrate (Z-Phe-Leu-Glu-*p*NA) was used.

[b] The total proteins in the culture supernatant proteins were assigned the value of 100%.

[c] ND, not detectable.

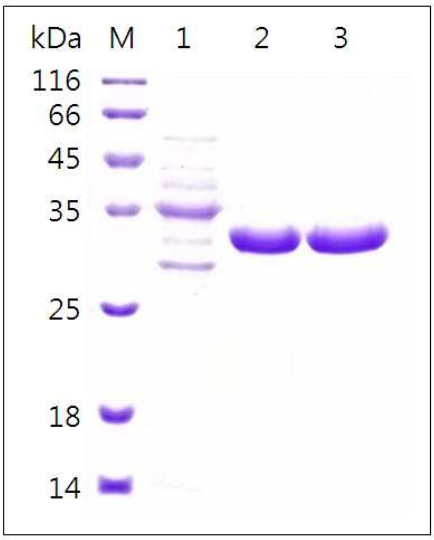


Fig. 7. SDS-PAGE of proteins obtained from various purification steps of recombinant VSPase. The proteins obtained from each purification steps were electrophoresed on 12% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Lanes M, Protein molecular weight markers; 1, periplasmic fraction; 2, HiPrep Q column chromatography; 3, Source Q column chromatography.

Table 5. Purification summary of VSPase mutant enzymes.

	Total protein (mg)	Specific Activity (U/mg)	Yield[a] (%)
H119L	6.6	ND[b]	13.50
D161A	11.6	ND[b]	15.45
S237L	9.9	ND[b]	16.90

[a] The total proteins in the culture supernatant proteins were assigned the value of 100%.
[b] ND, not detectable.

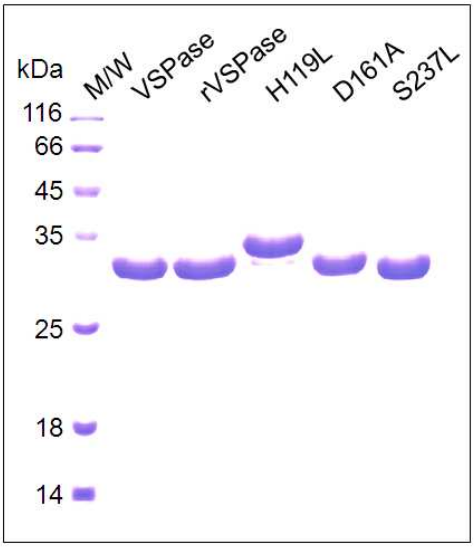


Fig. 8. SDS-PAGE of purified enzymes. The same amount (5 μg) of each enzyme was separated on 12% polyacrylamide gel and stained with Coomassie brilliant blue. M/W, protein molecular weight size markers.

Extracellular proteases are generally synthesized as inactive proenzymes consisting of a signal peptide, an N-terminal propeptide, and a mature region having catalytic activity. The propeptide is thought to act as a molecular chaperone by assisting with the proper folding of the protease during maturation (Tang *et al.*, 2002). As a result, the mutation of the active site histidine in VSPase may result in a loss of the autoprocessing activity, leading to maintain the propeptide region on the H119L enzyme.

III-1-4. Enzymatic properties of VSPase

VSPase exhibited an optimal activity around pH 6.0~8.5 and at 45℃, when azocasein was used as a substrate. Among the various chromogenic substrates tested, only Z-Phe-Leu-Glu-*p*NA (L-2135) was an efficient substrate for VSPase (Table 6). The N-terminal sequences two fragments derived from the cleavage of prothrombin by VSPase were found to be Gly^{313}-Arg-Thr-Ala-Thr^{317} and Gly^{367}-Ser-Asp-Ala-$Glu^{371.}$ As shown in Fig. 9B, Gly^{313} and Gly^{367} residues in prothrombin are located all behind at Glu^{312} and Glu^{366}, respectively. Which the amidolytic activity of VSPase against a chromogenic substrate L-2135 (Table 6), these results indicate that VSPase cleaves specifically the carboxy-termini of glutamic acids in peptides and therefore in a glutamate-specific endopeptidase. The effect of divalent cations on VSPase activity was studied. Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+} had no significant effect on VSPase activity. EDTA and 1,10-phenanthroline, which are inhibitors of metalloproteases also showed no effect. VSPase was not sensitive to other serine protease inhibitors such as aprotinin, leupeptin (also cysteine protease inhibitor), TPCK, TLCK, and PMSF (Table 7). However, addition of 10 mM diisopropylfluorophosphate (DFP) almost completely

abolished the VSPase activity. These results suggest that VSPase is a serine protease.

Enzyme kinetics of recombinant VSPase and its mutant enzymes were determined with the chromogenic substrate described in Materials and Methods. The K_M value of VSPase was found to be 0.910 ± 0.07 mM (Table 8). Kinetic data obtained with Z-Phe-Leu-Glu-*p*NA showed that VSPase was less efficient than V8 protease but more active than the *S. aureus* cysteine protease toward this peptide substrate. Both native VSPase and recombinant VSPase had similar amidolytic activity for the peptide substrate, whereas those for H119L, D161A, and S237L were dramatically diminished. Furthermore, amidolytic activity of H119L is not detectable (Fig. 10). These results clearly show that the three residues, His^{119}, Asp^{161}, and Ser^{237} are composed of a catalytic triad in VSPase.

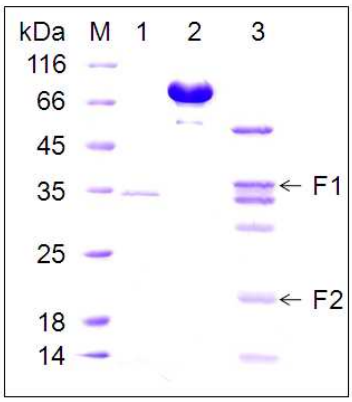
As shown in the schematic diagram of Fig. 11, it was strongly assumed that VSPase should be composed of the following three regions: (1) a signal peptide region (29 a.a.) that enables the precursor protein to be targeted for secretion; (2) a propeptide region (39 a.a.) that may play a role in helping the enzyme to fold correctly and to inhibit the enzyme activity; and (3) a mature enzyme region (271 a.a.) that contains a catalytic triad composed of histidine 119, aspartate 161, and serine 237 residues, which is typically found in all serine proteases (Rawlings *et al.*, 1994).

Table 6. Substrate specificity for VSPase.

Chromogenic substrate	Availability*
Z-Phe-Leu-Glu-*p*NA	**Yes**
Boc-Leu-Gly-Arg-*p*NA	No
Boc-Val-Pro-Arg-*p*NA	No
H-D-Phe-Pip-Arg-*p*NA	No
H-D-Val-Leu-Lys-*p*NA	No
H-D-Ile-Pro-Arg-*p*NA	No
Meo-Suc-Arg-Pro-Tyr-*p*NA	No
H-Arg-Arg-*p*NA	No
H-Cys-*p*NA	No
H-Gly-*p*NA	No
H-Lys-*p*NA	No
H-Glu-*p*NA	No

*The amidolytic activity by VSPase was measured as described in Materials and Methods. Enzyme activity was expressed as a Yes or No compared to that of detection availability.

A



B

F1: Ile^{311}-Glu^{312}-↓Gly^{313}-Arg^{314}-Thr^{315}-Ala^{316}-Thr^{317}

F2: Val^{365}-Glu^{366}-↓Gly^{367}-Ser^{368}-Asp^{369}-Ala^{370}-Glu^{371}

Fig 9. Cleavage of prothrombin by VSPase. Prothrombin (10 μg) was digested with 0.5 μg of VSPase in a total reaction volume of 10 ul at room temperature for 30 min. (A) Samples were subjected to SDS-PAGE on 12% gel. Lanes M, Protein molecular weight markers; 1, VSPase only; 2, prothrombin only; 3, prothrombin plus VSPase. F1 and F2 indicate the fragments that were subjected to N-terminal sequencing (B). The underlined amino acid sequences show the results of N-terminal sequencings. The arrow indicates the cleavage site for VSPase.

Table 7. Effects of metal ions and protease inhibitors on VSPase activity.

Additive[a]	Activity (%)[b]
None	100
$CaCl_2$ (1 mM)	116.2
$CuCl_2$ (1 mM)	94.4
$MnCl_2$ (1 mM)	114.4
$MgCl_2$ (1 mM)	116.5
$NiCl_2$ (1 mM)	113.5
$ZnCl_2$ (1 mM)	116.9
Aprotinin (0.5 mM)	106.7
Leupeptin (1 mM)	107
EDTA (10 mM)	104.48
TPCK (1 mM)	102.5
TLCK (1 mM)	102.8
1,10-PT (10 mM)	102
DFP (10 mM)	6.8
PMSF (10 mM)	97

[a]The final concentrations used are in parenthesis. VSPase activity was assayed with azocasein as a substrate with or without corresponding additive at 37 ℃ for 15 min. DFP, diisopropyl fluorophosphate; EDTA ethylenediaminetetraacetic acid; PMSF, phenylmethanesulphonylfluoride; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone.

[b]Enzyme activity was expressed as a relative value compared to that of non-treated control.

Table 8. Comparison of kinetic parameters for VSPase, V8, and cysteine proteases.

Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
VSPase[a]	0.910±0.07	6.06±0.21	6,698±734
V8 protease[b]	0.286	6.6	23,300
Cysteine protease[b]	0.5	0.16	320

[a]K_M and k_{cat} value of the enzyme were obtained using Z-Phe-Leu-Glu-*p*NA as a substrate.
[b]From Potempa *et al.*(1988).

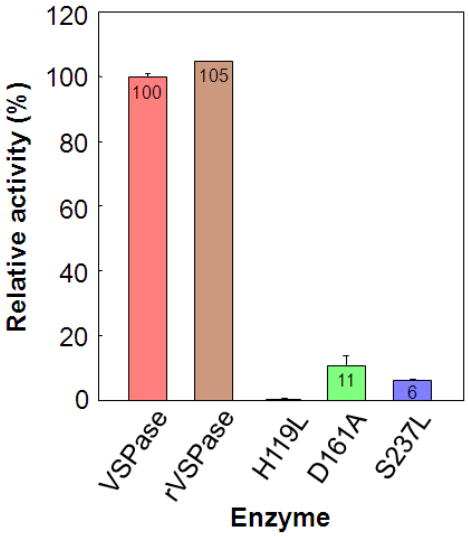


Fig. 10. Amidolytic activity of the purified enzymes. Amidolytic activity was determined as described in Materials and Methods. In this assay, 10 µg/ml of each enzyme was used.

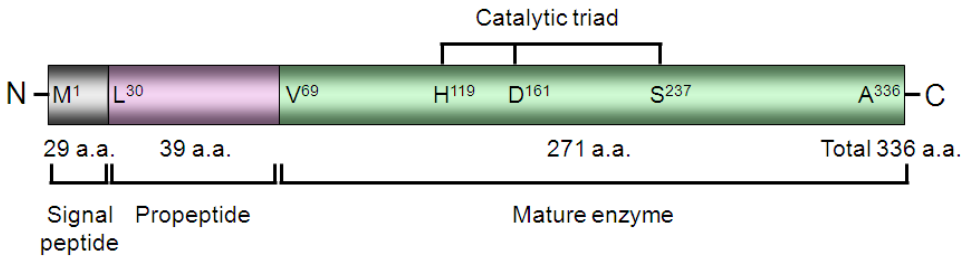


Fig. 11. Schematic representation of VSPase structure.

III-2. Inflammatory response caused by VSPase

III-2-1. Effect of VSPase on pro-inflammatory cytokines and chemokine gene expression in cultured Raw 264.7 cells

To examine whether VSPase can induce an inflammatory response, mRNA levels of pro-inflammatory cytokine genes for TNF-α and IL-1β were measured in Raw 264.7 cells. The transcription levels of TNF-α and IL-1β genes were increased by treatment with VSPase, as determined by real time PCR (Fig. 12). When 3 μg/ml of VSPase was treated, the transcription levels of TNF-α and IL-1β genes were increased greately for 3 h and reached at maximum level for 9 h (Fig. 12). ELISA also showed that VSPase in fact could increase TNF-α production, up to 657 pg/ml in a dose-dependent manner (Fig. 13). However, a VSPase mutant enzyme S237L that has a loss of enzyme activity could not at all doses treated (Fig. 13). These results suggest that the enzyme activity of VSPase is required for the production of inflammatory cytokines such as TNF-α and IL-1β.

The effect of VSPase on the induction of another pro-inflammatory cytokine gene including IL-12β was also examined using RT-PCR (Fig. 14). As shown in Fig. 14, the transcription level of a gene for IL-12β was also increased when 3 μg/ml of VSPase was treated.

The transcription levels of TNF-α, IL-1β, and IL-12β were increase to

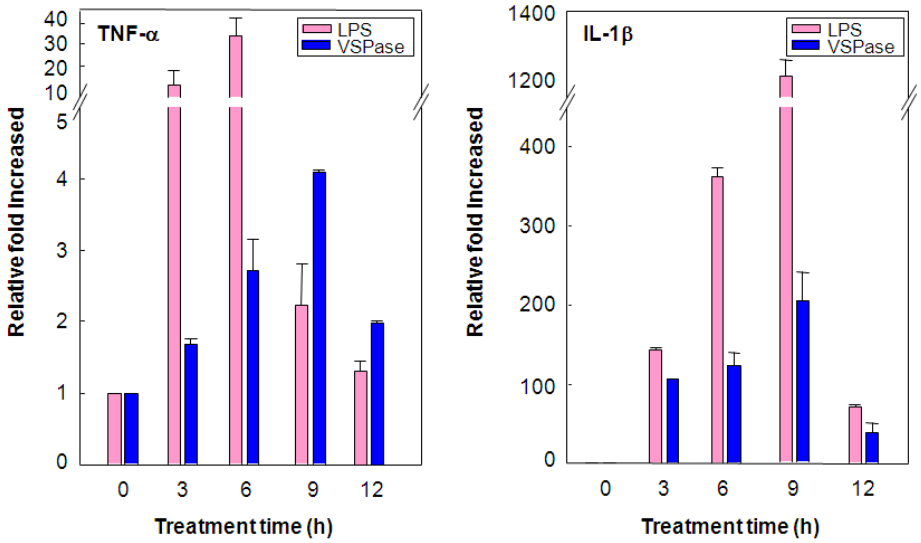


Fig. 12. Transcription levels of genes for TNF-α and IL-1β after treatment with VSPase. Raw 264.7 cells were treated with LPS (1 μg/ml) or VSPase (3 μg/ml) for various time periods and total RNAs were isolated. Real time PCR was performed using TNF-α, IL-1β, and GAPDH primer sets. The mRNA expression levels were expressed as a fold increase compared to that of GAPDH as an internal control.

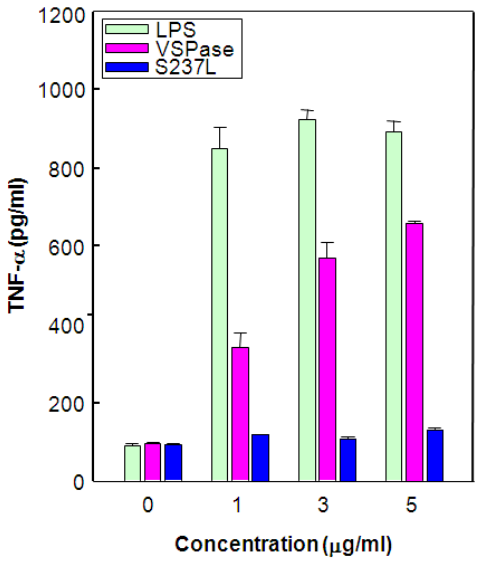


Fig. 13. Production of TNF-α in Raw 264.7 cells by VSPase. Raw 264.7 cells ($1x10^5$) were treated with different concentrations of LPS, wild type VSPase or mutant enzyme S237L for 3 h. TNF-α concentration in the culture supernatant was measured using an ELISA kit for TNF-α.

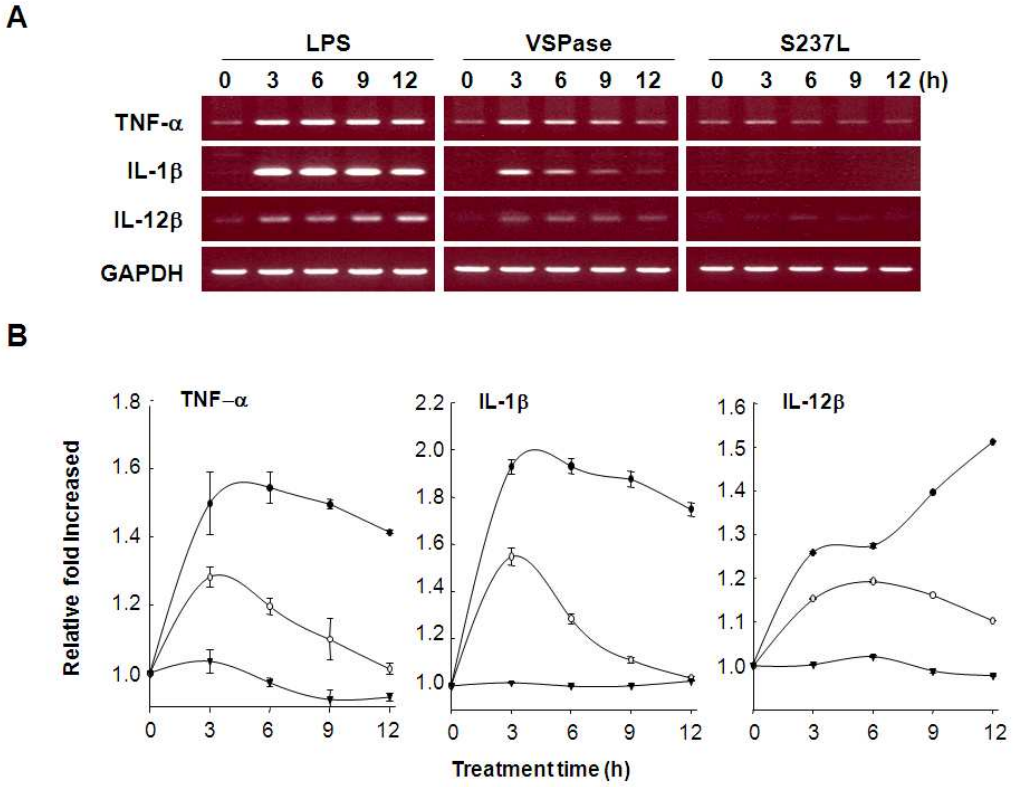


Fig. 14. Effects of LPS and VSPase on the pro-inflammatory cytokine mRNA levels in Raw 264.7 cells. Cells were treated with LPS (1 μg/ml), VSPase (3 μg/ml) or S237L (3 μg/ml) and total RNAs were extracted at 0, 3, 6, 9, and 12 h. The expression levels of TNF-α, IL-1β, IL-12β, and GAPDH mRNAs were determined by RT-PCR. (A) The RT-PCR products were separated on 2% agarose gel and stained with ethidium bromide to visualize. (B) Relative cytokine gene expressions were quantified using Scion Image software. Symbols ●, LPS; ○, VSPase; ▼, S237L.

1.3-, 1.5-, and 1.2-folds, respectively, compared with that of non-treated control. In addition, the MIP-2 mRNA level also clearly increased by VSPase treatment with the same concentration. The increased level was estimated to be 1.4-fold, compared with that of non-treated control at 3 h (Fig. 15).

Among the chemokines, the MIP-2 gene is inducible in multiple, different cell types and response to a variety of pro-inflammatory stimuli (Armstrong *et al.*, 2004). Furthermore, the expression of MIP-2 contributes to the significantly neutrophils recruitment to amplify the inflammatory process. Therefore, these results suggest that VSPase function as an immunostimulant and the proteolytic activity of VSPase is closely related to the induction of pro-inflammatory response in Raw 264.7 cells.

III-2-2. Effect of VSPase on PGEs and COX-2 mRNA expression in Raw 264.7 cells

In this study, the involvement of VSPase in induction of PGEs and COX-2 in their transcription levels was also investigated. Prostaglandin is an important autacoids, which modulate inflammatory reaction. COX-2 is an enzyme, which is primarily responsible for the induced production of prostaglandins. Enhanced expression of COX-2 has been found at inflammatory sites in animals with inflammatory diseases. As shown in Fig. 16, mRNA transcripts for COX-2 and PGEs genes increased in Raw 264.7 cells after treatment with VSPase, in which the transcription levels of those genes were increase to 1.2-folds, compared with that of non-treated control.

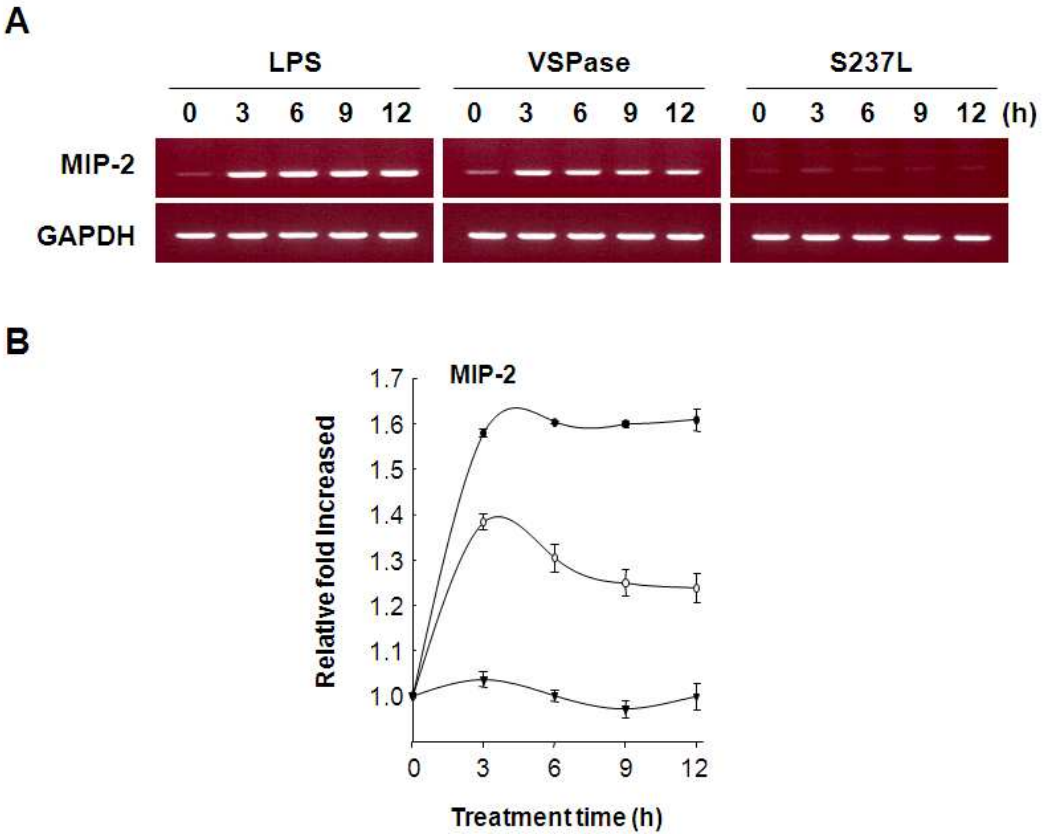


Fig. 15. MIP-2 mRNA expression by VSPase in cultured Raw 264.7 cells. Raw 264.7 cells were plated in 100 mm culture dishes and treated with LPS (1 μg/ml), VSPase (3 μg/ml) or S237L (3 μg/ml) for 0 to 12 h. After preparation of total RNAs from the cells, the mRNA levels were analysed by RT-PCR with specific primers for MIP-2 or GAPDH as indicated. (A) The RT-PCR products were separated on 2% agarose gel and stained with ethidium bromide to visualize. (B) Relative cytokine gene expressions were quantified using Scion Image software. Symbols ●, LPS; ○, VSPase; ▼, S237L.

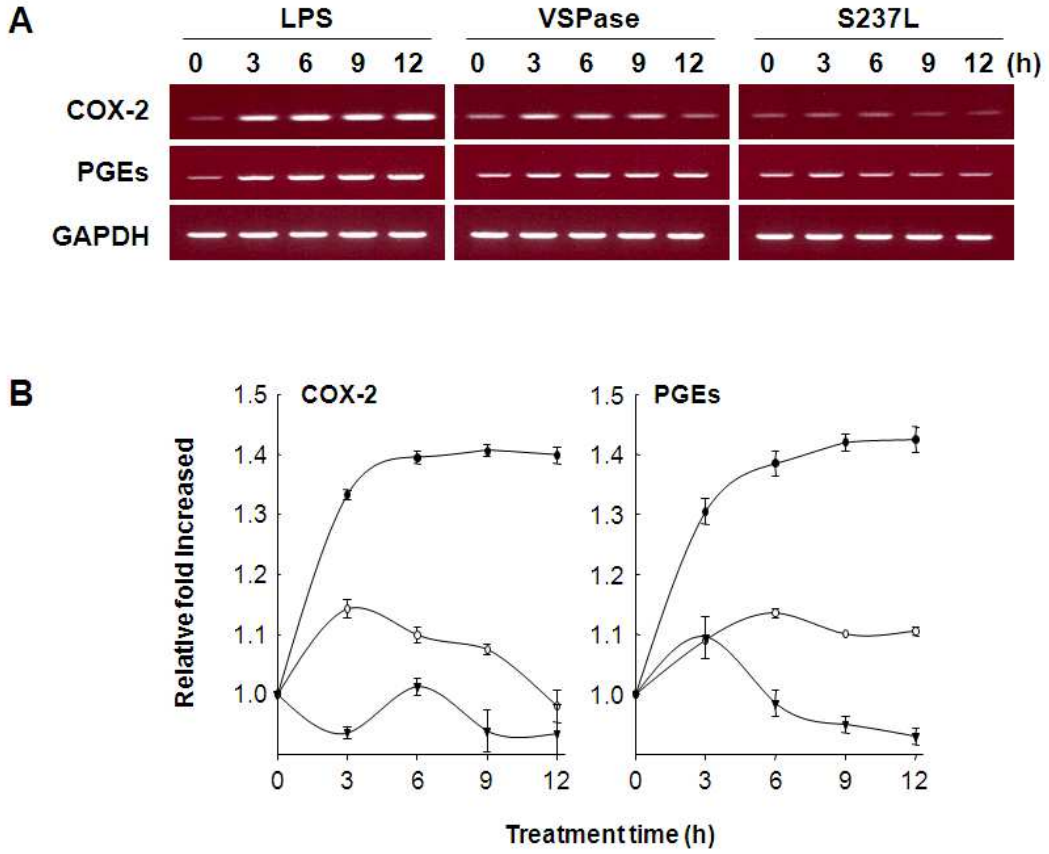


Fig. 16. Up-regulation of prostaglandin E2 synthase (PGEs) and cyclooxygenase-2 (COX-2) mRNA expression by VSPase. For PGEs and COX-2, Raw 264.7 cells were treated with 1 μg/ml LPS or 3 μg/ml of VSPase and S237L for various time periods. Total RNAs were isolated and RT-PCR were performed using PGEs, COX-2 and GAPDH primer sets. (A) The RT-PCR products were separated on 2% agarose gel and stained with ethidium bromide to visualize. (B) Relative cytokine gene expressions were quantified using Scion Image software. Symbols ●, LPS; ○, VSPase; ▼, S237L.

COX-2 expression markedly enhanced after 3 h, decreasing again after 6 h. PGEs expression also weakly increased after 3 h. However, S237L has no effect on COX-2 and PGEs mRNA expression. The increased expression of COX-2 and PGEs are commonly associated with inflammation. Therefore, the increased mRNA expression of PGEs and COX-2 further supports a role for VSPase as an inflammatory initiator.

III-2-3. Activation of NF-κB by VSPase

Activation of NF-κB constitutes an important step in the course of several immune and inflammatory responses. Two main regulatory mechanisms of NF-κB activity have been reported (Baldwin, 1996). One is the precise nucleotide sequence of the κB motif to which NF-κB binds, in which the transcriptional activity depends on variations in the consensus sites and in the flanking regions (Bours *et al.*, 1993, Wulczyn *et al.*, 1992). The other involves the association of NF-κB with inhibitory subunits such as the various forms of IκB proteins and the formation of an inactive complex in the cytosol (Thanos *et al.*, 1995). It is also well documented that LPS can activate NF-κB in cultured macrophage cells (Crinelli *et al.*, 2000).

These observations demonstrate a possibility that VSPase can activate genes for cytokines and pro-inflammatory mediators through by the activation of NF-κB signaling pathway. Immunoblot analysis of cytosolic extracts showed that IκBα was quickly degraded in the VSPase-stimulated cells at 30 min like in LPS (Fig. 17). This result suggest that IκB degradation induced by VSPase can give a free the NF-κB proteins from the inactivated complex that is composed of IκB and NF-κB in cytoplasm (Scheinman *et al.*, 1995, Auphan *et al.*, 1995). Therefore, the IκBα degradation induced by VSPase

would result in NF-κB activation, leading to traslocation of the proteins into nucleus.

In this study, it was confirmed that the NF-κB proteins activated by VSPase could bind to their target genes in nucleus, as judged by EMSA and super shift assay (Figs. 17 and 18). EMSA experiment showed that there was a clear band shift when a concensus NF-κB binding probe was incubated with a nuclear of extracts prepared from VSPase-treated cells (Fig. 18A). This result suggest that VSPase can induce NF-κB activation through IκB degradation and the activated NF-κB proteins are translocated into nucleus. The NF-κB activation and the translocation into nucleus were confirmed once again using super shift assay with anti-p65 antibody (Fig. 18B). As shown in Fig. 18B, the nuclear extracts prepared from Raw 264.7 cells treated with VSPase were sufficient to make super-shifted band. All these results conclude that VSPase can induce IκB degradation, now the freed NF-κB proteins translocated into nucleus, bind to the upstream concensus NF-κB binding sequences of target genes, and finally increase the transcription levels of the genes.

III-2-4. Induction of vascular leakage by VSPase

Vascular leakage activity of VSPase was studied with Miles assay (Miles *et al.*, 1952). As shown in Fig. 19, VSPase could induce a vascular leakage in a dose-dependent manner. However, a mutant enzyme S237L had no effect on vascular leakage activity. These results suggest that the proteolytic activity of the enzyme maybe to the induction of vascular permeability. Together with that, it cannot be ruled out a possibility that the mast cells activated by various cytokines that had been induced by VSPase

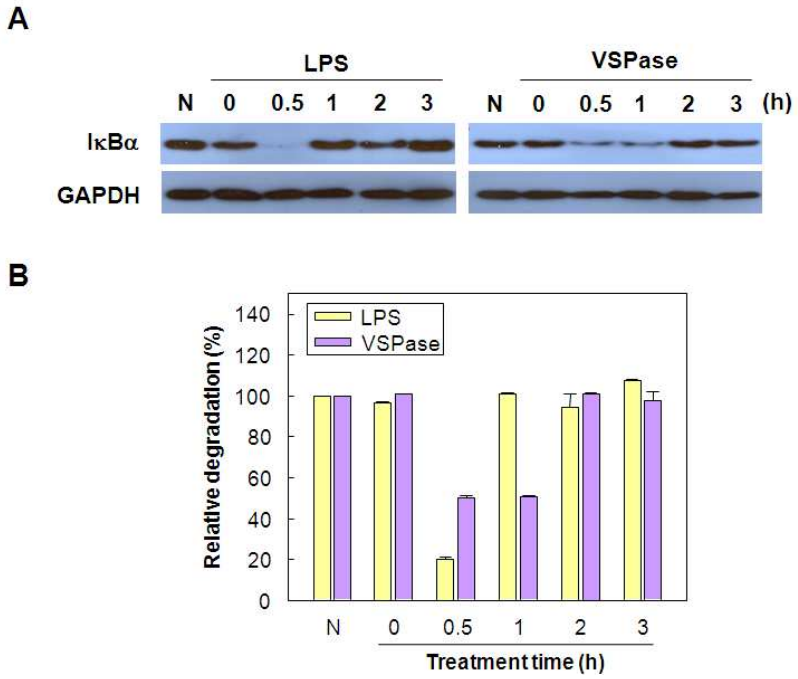


Fig. 17. Degradation of IκBα in Raw 264.7 cells treated with VSPase. Cells were incubated with LPS (1 μg/ml) or VSPase (3 μg/ml) for various time periods as indicated and cytosolic extracts (80 μg) were subjected into 12% SDS-PAGE. (A) Western blotting was performed with anti-IκBα or anti-GAPDH antibodies. (B) Relative degradation of IκBα was obtained by analyzing the signal intensity of autoradiogram bands of the panel A and expressed as percentage change, compared to untreated control. N, no treatment.

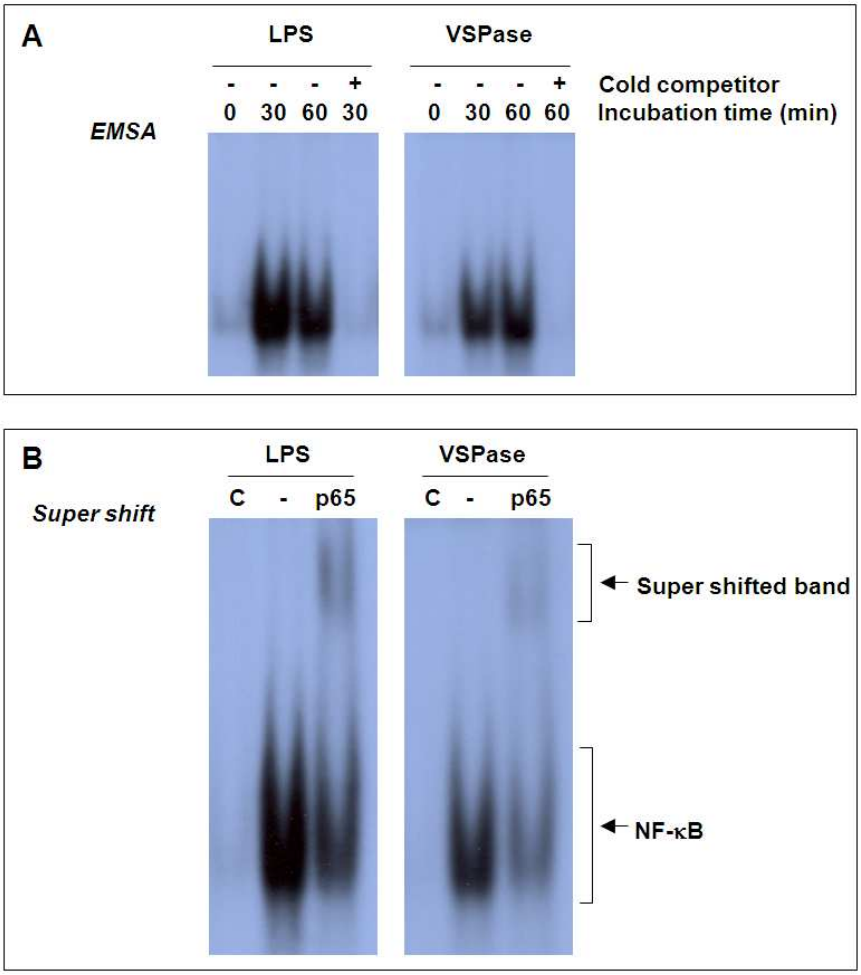


Fig. 18. Activation of NF-κB by VSPase. Raw 264.7 cells were treated with LPS (1 μg/ml) and VSPase (3 μg/ml) as indicated and the nuclear extracts were prepared for EMSA, in which [γ-^{32}P]-labeled concensus NF-κB binding element was used as a probe. (A) Time course of NF-κB activation by LPS and VSPase stimulation. (B) Super shift assay with anti-p65 antibody. C, cold competitor only.

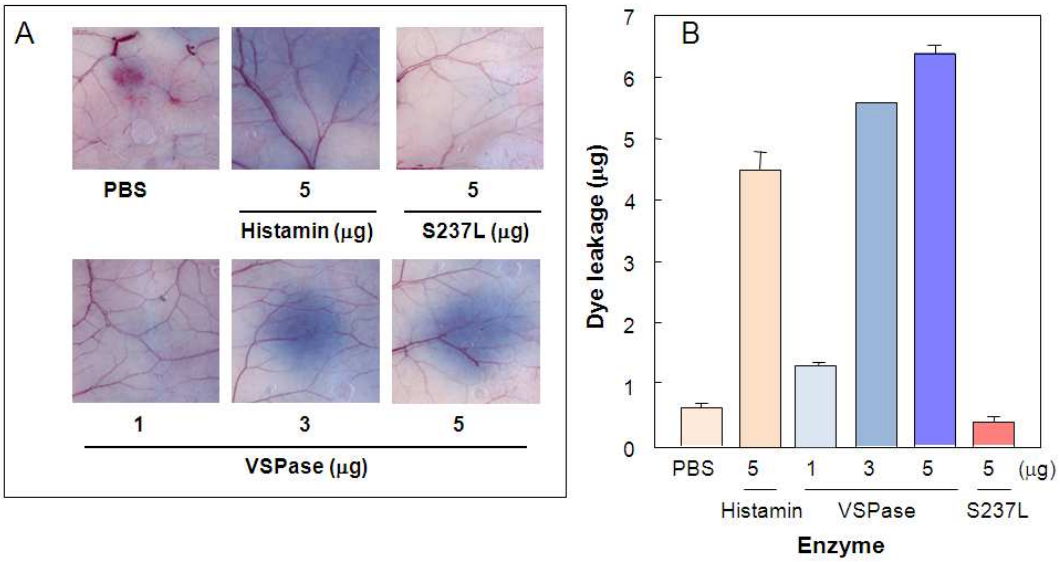


Fig. 19. Induction of vascular permeability by VSPase. A guinea pig (body weight 250 g, male) was lightly anesthetized with diethyl ether and 65 mg of Evans blue per kg body weight was administered intravenously. (A) Fifty microliters of histamin (5 μg), VSPase (1, 3, and 5 μg) or S237L (5 μg) were injected intradermally. After 10 min, dye leakage was detected by the presence of blue spots at the injection sites. (B) Dose response effect of VSPase on vascular permeability. The amount of dye extracted from the skin by soaking in formamide solution was quantitated by spectrophotometry at 620 nm.

treatment can increase the vascular permeability, regardless of VSPase's proteolytic activity. It has been reported that Stapophain A (ScpA) from *Staphylococcus aureus*, streptopain (SpeB) from *Streptococcus pyogenes*, 56 K-protease from *Serraia marcescens* (Maruo *et al.,* 1993), ASP from *Aeromonas sobria*, subtilisin from *Bacillus subtillis*, gingipains-R produced by *Porphyromons gingivalis,* and a metalloprotease from *Vibrio vulnificus* (Miyoshi *et al.*, 1987) are capable of inducing vascular permeability. In addition, there is only one report that a bacterial serine protease called ASP can act as vascular permeability enhancer (Imamura *et al.*, 2006).

In conclusion, all results obtained by the present study suggest that VSPase plays an important role in the increase expressions of pro-inflammatory cytokines and inflammatory regulator through the activation of NF-κB and also can enhance a vascular permeability.

IV. 적요

포도상 구균의 세린계열 단백질 분해효소에 의한 혈관투과성 증가와 염증유발 사이토카인의 발현촉진에 관한 연구

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포도상 구균은 그람 양성균으로서 피부에 감염하여 여드름, 종기, 농가진 등을 유발할 뿐만 아니라 심하게는 폐렴, 패혈증, 골수염 및 독성쇼크증후군 등을 유발한다. 이러한 증상들은 포도상 구균의 감염 후 생성되는 여러 종류의 독소 및 효소에 의하여 발생하며, 숙주 체내에서 균의 증식 및 감염증 확산에 중요한 역할을 한다. 본 연구에서는 새롭게 발견된 포도상 구균인 *Staphylococcus aureus* C-66 균주의 배양액으로부터 글루탐산을 특이적으로 인지하여 절단하는 세린계열의 단백질분해효소(VSPase라 칭함)를 황산암모늄 침전법과 HiPrep Q 그리고 gel filtration을 이용한 두 단계의 크로마토그래피를 통해 분리 및 정제하여 그 특성을 규명하였다. 정제한 단백질의 아미노말단 서열분석 결과, VSPase는 포도상 구균의 단백질 분해효소인 V8과 유사하다는 것을 확인하였으며, V8 효소의 염기서열을 바탕으로 합성한 한 쌍의 프라이머와 중합효소 연쇄반응을 이용하여 VSPase 유전자를 증폭한 후, pFLAG-ATS 발현벡터에 클로닝한 후, 대장균에서 재조합 VSPase를 발현시켜 순수 분리하였다. 포도상 구균의 배양액에서 분리한

VSPase와 대장균에서 생산된 재조합 VSPase는 동일한 효소활성을 가지고 있었다. VSPase는 다양한 혈장 단백질을 분해하는 활성을 지니고 있었으며, 발색기질(Z-Phe-Leu-Glu-*p*NA)을 이용한 실험 결과, 단백질에서 글루탐산의 카르복시 말단의 펩티드결합을 가수분해하는 단백질 내부 가수분해효소(endopeptidase)임을 확인하였다. 이 효소의 K_M 값은 0.910±0.07 mM 이였으며, k_{cat} 값은 6.06±0.21 s^{-1}였다. 또한 pH 6.0~8.5까지 넓은 범위에 거쳐 효소활성을 보였다. VSPase는 활성부위에 "세린-히스티딘-아스파트산"으로 이루어진 촉매삼인조(catalytic triad)를 지닌 세린계열의 단백질분해효소임을 부위이적 돌연변이 유도실험을 통하여 확인하였다. 119번째 히스티딘을 류신으로 치환시킨 H119L, 161번째 아스파트산을 알라닌으로 치환시킨 D161A, 237번째 세린을 류신으로 치환시킨 S237L 돌연변이 효소는 효소활성을 전혀 지니지 못하였다. VSPase를 생쥐 대식 세포주(Raw 264.7 세포)에 처리하였을 때, 염증유발 사이토카인인 TNF-α가 다량 생성되었으며, IL-1β, IL-12β 등의 발현도 촉진되었다. 또한 염증유발 케모카인인 MIP-2 및 염증 조절인자들인 cyclooxygenase-2(COX-2)와 prostaglandin E2 synthase(PGEs) 등의 발현을 촉진시켰다. 이러한 사이토카인의 생성은 VSPase에 의해 활성화된 전사인자 NF-κB에 의해 이루어짐을 확인하였다. 항-IκB 항체를 이용한 면역블롯 결과, VSPase는 처리 후 30분에서 IκB의 분해를 유도하였다. IκB 분해에 의해 활성화된 NF-κB의 핵 내로의 이동은 NF-κB 결합부위를 지닌 올리고 뉴클레오티드 탐침을 이용한 전기영동 이동성-변화분석(Electrophoretic Mobility Shift Assays; EMSA)와 항-p65 항체를 이용한 super shift assay로 확인하였다. 기니피그를 이용한 실험동물 모델에서 VSPase는 1 μg의 소량에서도 투과성을 증가시키는 반면, S237L은 5 μg에서도 혈관투과성을 증가시키지 못 하였다. 이러한 결과는 VSPase의 단백질분해효소 활성과 혈관투과성 증가가 관련이 있음을 시사하는 것이다. 본 연구에서 얻은 연구결과들은 포도상 구균에서 분비되는 단백질분해효소가 이 균의 생체 감염과정에서 여러 가지 염증유발 사이토카인, 케모카인 및 염증조절자들을 유도생성시킬 수 있으며, 혈관투과성을 증가시킬 수 있음을 보여주는 것이다

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저작물 이용 허락서

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논문제목	한글 **포도상 구균으로부터 분비되는 세린계열 단백질 분해효소에 의한 혈관투과성 증가와 염증유발 사이토카인의 발현촉진에 관한 연구**				
	영문 **A serine protease secreted by *Staphylococcus aureus* evokes a vascular permeability accompanied with production of pro-inflammatory cytokines**				

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

- 다 음 -

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

동의여부 : 동의(0) 반대( )

2009 년 2 월 25 일

저작자: 박 종 우 (인)

조선대학교 총장 귀하

감사의 글

어느덧 실험실 생활을 시작한지 4년이라는 시간이 지났습니다. 정신없이 지나가버린 학사 2년과 아쉬움이 남는 2년간의 석사 학위 기간이었지만 많은 사람들의 도움을 통해 학위논문을 완성하게 되어 감사의 뜻을 전하고자 합니다.

우선 분자세포생물학 실험실의 정신적 지주이시며 언제나 열과 성을 다하여 지도해주시는 우리 이정섭 교수님 정말 감사합니다. 그리고 그동안 지도해주신 생명공학과교수님들과 함경수 소장님을 비롯한 생물신소재학과 교수님들, 바쁘신 와중에도 기꺼이 심사위원이 되어주신 유호진 교수님과 정혜광 교수님께 감사드립니다.

지난 4년 동안 실험도 가르쳐주시고 많이 싸워(?)주신 우리 실험실 최고 선배 정은누나 너무 감사해요.. 그리고 석사 동기 은희, 으리, 막내 아닌 막내 재영이도 모두 고맙단다. 우리 실험실 식구는 아니지만 많은 도움을 주신 홍석이형이랑 민영이에게도 감사의 뜻을 전하고 싶습니다.

바쁘다고 연락도 잘하지 않는 나와 상대해주는 친구들, 특히 창호 그리고 언제나 응원해주는 현지 모두 다 고맙단다. 다른 친구들도 논문을 전해 주지는 못 하겠지만 고마워..

감사의 글에서 빠져서는 안 될 우리 가족들.. 힘들게 일 하시면서도 막내아들 공부를 위해 지원을 아끼시지 않는 아빠, 엄마.. 그리고 나이 먹은 동생 용돈 챙겨 주고 걱정해주느라 힘든 형, 누나.. 사랑하는 우리 가족들 모두에게 죄송하고.. 너무 너무 감사해요~

마지막으로 논문을 쓰면서 제 자신에게서 많은 부족함을 느끼고 반성할 수 있는 계기가 되었습니다. 박사학위를 시작하기에 앞서 석사학위동안 아쉬웠던 점들을 마음속 깊이 세기고 이를 바탕으로 더 나은 모습으로 성장하기 위해 노력해 보고자 합니다. 그동안 지켜봐주신 모든 분들께 다시 한 번 감사의 뜻을 전하며, 앞으로도 많은 관심 부탁드립니다.