[UCI]I804:24011-200000267040

2019년 2월

석사학위 논문

Activation of the Blood Complement System by a Vibrio Extracellular Protease

조선대학교 대학원

생명과학과

권 소 현

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비브리오 유래 단백질분해효소에 의한 혈액 보체계 활성화 분석

2019년 2월 25일

조선대학교 대학원

생명과학과

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이 논문을 이학 석사학위 신청 논문으로 제출함.

2018년 10월

조선대학교 대학원

생명과학과

권 소 현

권소현의 석사학위논문을 인준함.

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2018년 11월

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ABSTRACT

Activation of the Blood Complement System by a Vibrio Extracellular Protease

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Vibrio vulnificus (*V. vulnificus*) is a causative agent of serious food-borne diseases in humans related to the consumption of raw seafood. It is a pathogenic bacterium that causes wound infection and septicemia. Most of fatal diseases in human are caused by septic shock, which is resulted from various virulence factors, including capsular polysaccharide and lipopolysaccharide secreted by the bacterium. In this laboratory, a broad-specificity extracellular metalloprotease (called vEP-45) has been purified and characterized from *V. vulnificus* ATCC29307. The protease can interfere with blood homeostasis through prothrombin activation and fibrinolysis. Furthermore, vEP-45 can activate the plasma contact system by cleaving key zymogen molecules, participating in the intrinsic pathway of coagulation and the kallikrein/kinin system. It has been known that the complement and coagulation systems are closely linked to maintain blood homeostasis. However, to date there are no evidences whether vEP-45 protease can activate the blood complement system. Therefore, the present study was performed to elucidate the roles of vEP-45 both on the activation of the complement system and on the population sizes of neutrophils and monocytes in mouse. The results obtained were as follows: vEP-45 could proteolytically convert

the complement precursor molecules C3, C4, and C5 to their respective active forms (C3a, C3b, C4a, C4b, C5a, and C5b) *in vitro* as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The Western blot analyses also showed that C3b and C3a could be produced from the precursor C3 by vEP-45 cleavage dose-dependently. The generations of C4b and C4a from C4 peaked at 50 and 200 ng of vEP-45 treatments, respectively, accompanied with gradual decreases at higher concentrations. The complement molecule C5a also was produced from C5 by vEP-45 in a dose-dependent manner, although C5b band could not appear clearly on the blot. These results suggest that vEP-45 can proteolytically activate the blood complement protein molecules. In this study, the ability of vEP-45 to activate the complement proteins contained in human plasma was also confirmed by Western blottings. The results showed that vEP-45 can increase the production levels of C3b and C3a by approximately 267% and 70%, respectively, in dose-dependent manners. The enzyme could increase dose-dependently the production level of C5a by approximately 266%, compared with that of non-treated control, although the C5b band could not be observed as in *in vitro* cleavage assay. These results suggest that vEP-45 can convert plasma C3 and C5 complement factors to their respective active factors, even in the plasma milieu. The effects of vEP-45 on the number of cells of immune cells such as neutrophils and monocytes were also examined *in vivo* by flow cytometry. The results showed that vEP-45 could increase the population sizes of such immune cells by an average of 32%. All these results obtained by the present study demonstrate that vEP-45 can activate the complement system to potential innate immunity.

Ⅰ. INTRODUCTION

Pathogenic bacteria produce various types of molecules including lipopolysaccharides, capsular polysaccharides, cytolysins, and metalloproteases that play important roles in bacterial virulence and pathogenesis (Hoogerwerf *et al.*, 2002; Gulig *et al.*, 2005; Kawase *et al.*, 2004; Kwon *et al.*, 2007). Among these molecules, metalloproteases may have a role in many regulatory processes, such as acquisition of nutrients, escape from host defense system, disturbance of host blood homeostasis, and induction of inflammatory response.

Vibrio vulnificus (*V. vulnificus*) is a gram-negative marine bacterium that is often found in warm coastal water (Janda *et al.*, 1988; Powell *et al.*, 2003). It is a causative agent of serious food-borne diseases in humans related to the consumption of raw seafood (Chang *et al.*, 2005). Characteristics of *V. vulnificus* infections include fever, chills, nausea, hypotensive septic shock, and the formation of secondary lesions on the extremities of patients (Jones & Oliver, 2009). *V. vulnificus* ATCC29307 secretes a 45 kDa metalloprotease, which has many biological activities associated with skin lesions and serious hemorrhagic complications (Miyoshi, 2006). The enzyme is synthesized as a zymogen consisting of a signal peptide, an N-terminal propeptide region, and catalytic domain (Fig. 1). The properties of vEP are summarized in Table 1.

The *V. vulnificus* protease has been reported to have many biological functions: proteolytic degradation of a wide variety of host proteins, such as plasma proteins involved in coagulation functions, induction of hemorrhagic tissue damage, and enhancement of vascular permeability through the generation of inflammatory mediators (Kwon *et al.*, 2007). Recently, it was reported that vEP-45 interferes with blood homeostasis through prothrombin activation and fibrinolysis (Park *et al.*, 2014). Furthermore, vEP-45 can activate the plasma contact system by cleaving key

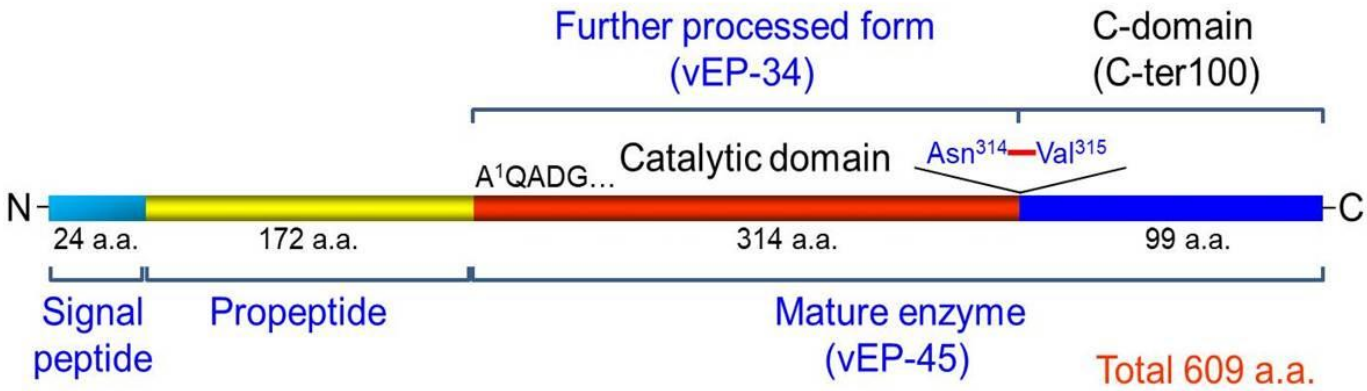
zymogen molecules, participating in the intrinsic pathway of coagulation and the kallikrein/kinin system (Park *et al.*, 2014).

On the other hands, it has been known that the blood complement and coagulation systems are closely linked each other to maintain blood homeostasis. The blood complement system plays a crucial role in the innate defense against common pathogens (Fig. 2) (Dunkelberger & Song, 2010). The system is composed of plasma proteins produced mainly by the liver or membrane proteins expressed on cell surface (Kolev *et al.*, 2014). The complement proteins collaborate in a cascade to opsonize pathogens and induce a series of inflammatory responses helping immune cells to fight infection and maintain homeostasis (Merle *et al.*, 2015). The complement cascade can be activated by three pathways: the classical, the alternative and the lectin pathways (Kolev *et al.*, 2014). Initiation of the classical pathway occurs when C1q, in complex with C1r and C1s serine proteases (the C1 complex), binds to the Fc region of complement-fixing antibodies (generally IgG1 and IgM) attached to the surface of pathogens. In turn, the autocatalytic activation of C1r and C1s cleaves C4 and C2 into large (C4b/C2a) and small (C4a/C2b) fragments. The large fragments associate on the surface of pathogens to form a C4bC2a complex, also termed C3 convertase, with the ability to cleave C3. (Dunkelberger & Song, 2010). The activation of the lectin pathway occurs in response to the recognition of mannose-binding lectins and ficolins of various carbohydrate ligands on the surface of microorganisms. This results in the activation of the mannose-binding lectin-associated serine proteases (MASP1, MASP-2 and MASP-3). MASP-2 cleaves C4 and subsequently C2, leading to the formation of the C3 convertase, as in the classical pathway (Chen *et al.*, 2010). In the alternative pathway, complement activation occurs spontaneously and continuously at a low rate (referred to as tickover), and involves the association of C3 with a water molecule to form C3(H_2O), which then recruits Factor B (FB) and Factor D (FD). FD

enzymatically cleaves FB, yielding Bb, the active serine esterase that cleaves C3 to C3a and C3b. C3b associates with Bb to form C3bBb, the C3 convertase alternative pathway. The thio-ester bond on C3 covalently reacts with various residues on cell surfaces, localizing C3 convertase formation predominantly to these sites. Properdin has dual functions, directly binding to microbial targets to provide a platform for assembly of the alternative pathway C3 convertase and increasing the stability of the C3bB/C3bBb complexes (Mathern & Heeger, 2015). All activation pathways lead to the generation of the C3 and C5 convertase enzyme complexes, which cleave C3 into the anaphylatoxin C3a and the opsonin C3b, and C5 into the anaphylatoxin C5a and C5b, respectively. Deposition of C5b onto a target initiates membrane attack complex (MAC) formation and target lysis. The opsonins promote the phagocytic uptake of pathogens by scavenger cells (Kolev *et al.*, 2014). The complement anaphylatoxins C3a and C5a contribute to inflammation and activate immune cells such as neutrophils, monocytes, and mast cells, which express the G-protein coupled anaphylatoxin receptors C3aR and C5aR. C3a is implicated in the adaptive immunity by inducing monoclonal response from B cells and up-regulation of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. In the presence of C5L2, C5a allows for C5aR internalization and subsequent induction of ERK signaling and pro-inflammatory activation of macrophages (Merle *et al.*, 2015). However, to date there are no evidences whether vEP-45 can activate the blood complement system.

This study was performed to analyze the ability of vEP-45 to cleave complement molecule to make their active forms *in vitro* and under plasma milieu. In addition, the effect of vEP-45 on the population sizes of neutrophils and monocytes in mouse was also examined. Based on these results, this study describes the effect of vEP-45 both on the activation of complement system and innate immunity.

A



B

Component		Function
Signal peptide		Targets the protein for secretion
Pro-peptide		May acts as inhibitor for the proteolytic activity
Mature enzyme	Catalytic domain	Contains the catalytic machinery including the zinc-binding motif
	C-domain (named C-ter 100)	May be important for binding to certain substrates such as collagen and elastin

Fig. 1. Overall structure and biochemical functions of vEP protease. (A) Schematic representation of the vEP domain structure. (B) Biochemical functions of the vEP protease components (Chang *et al.*, 2005).

Table 1. Brief summary of vEP properties.

Characteristic*	Function / Sequence
Molecular weight	• SDS-PAGE: 45 kDa (mature form) and 34 kDa (autodegrading form) bands
	• Mass spectrometry (MALDI): 34,077.37 Da
	• Possible autocleavage site: The peptide bond between Asn^{314} and Val^{315}, yielding a 34,077 Da species
Optimal pH and temperature for enzyme activity	• pHs 7.5 ~ 8.0 & a temperature of 45°C
Thermal stability	• Relatively stable up to 55°C
Activator / Inhibitors	• Mn^{2+}/metal chelating agents such as $NiCl_2$ and 1,10-phenanthroline
N-terminal sequence	Vvul vEP **AQADGTGPGGNSKTGRYEFGTD** VVul_BAC22940 VWDGLNH**AQADGTGPGGNSKTGRYEFGTD**YPSFVID 225 VVul_BAC97491 VWDGLNH**AQADGTGPGGNSKTGRYEFGTD**YPSFVID 252 VPar_BAC63013 TWEGLNH**AEATGTGPGGNQKTGFYQYGTD**FPGLVIN 896 VPro_Q00971 TWDGLNH**AQADGTGPGGNTKTGRYEYGSD**FPPFVID 225 *:*****:********* *** *::*:*:*: **: (*) : Identity (:) : Strongly similar

*Adopted from Chang *et al.* (2005; 2007)

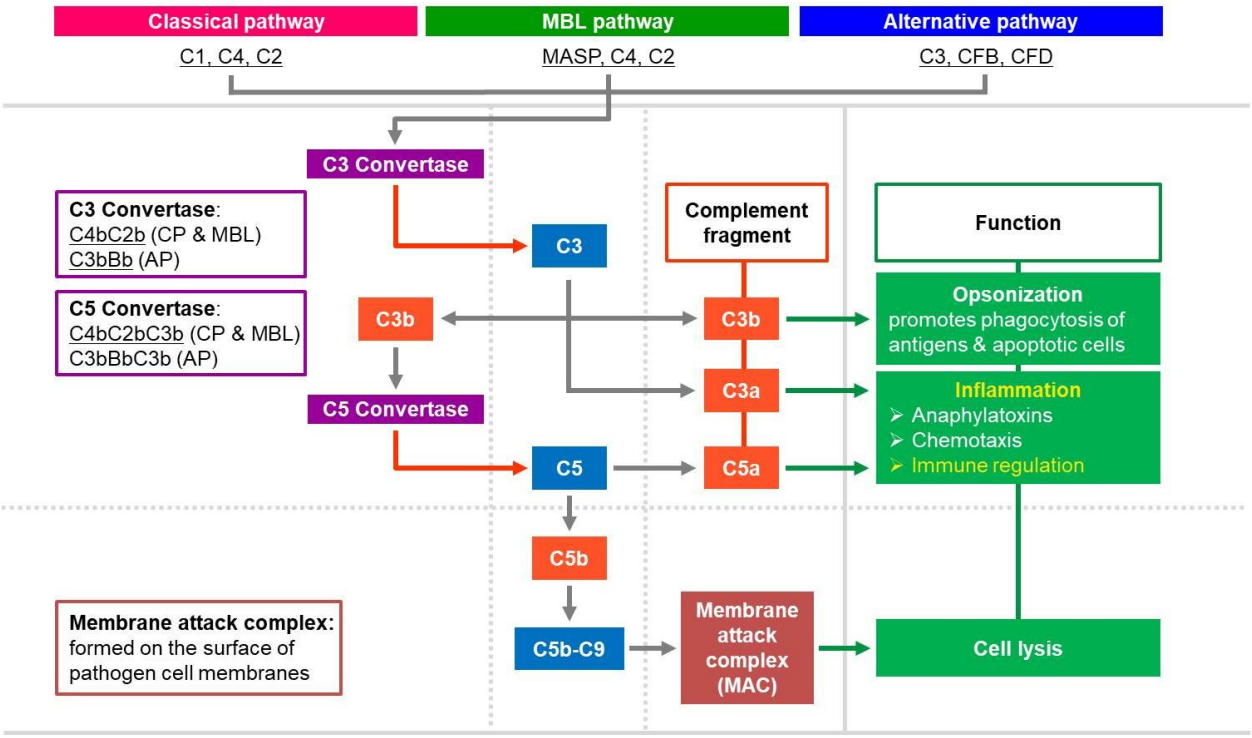


Fig. 2. Activation cascade of blood complement system. The complement cascade can be activated by three pathways: the classical, the alternative, and the lectin pathways. All three activation pathways lead to the generations of the C3 and C5 convertase enzyme complexes, which cleave C3 into the anaphylatoxin C3a and the opsonin C3b, and C5 into the anaphylatoxin C5a and C5b. Deposition of C5b onto a target initiates membrane attack complex (MAC) formation and target lysis. C3b opsonizes foreign antigens and apoptotic cells, promoting phagocytosis. The complement anaphylatoxins C3a and C5a contribute to inflammation and activate immune cells such as neutrophils, monocytes, and mast cells (Xu & Chen, 2016).

Ⅱ. MATERIALS AND METHODS

Ⅱ-1. Materials

The chromatographic columns, including HiPrep 16/10 QFF, Mono Q 4.6/100 PE, Superdex 75 10/300 GL, and PD-10 were purchased from Amersham Pharmacia Biotech Co. (Uppsala, Sweden). Yeast extract, tryptone, and bacto agar were purchased from Becton Dickinson (Baltimore, MD, USA). The plasmid pFLAG-ATS vector, 1,10-phenanthroline (1,10-PT), phenylmethylsulfonyl fluoride (PMSF), TEMED, ammonium sulfate, and other chemicals were purchased from Sigma (ST. Louis, MO, USA). The purified human complement proteins, including C3, C3a, C3b, C4, C4a, C4b, C5, C5a, and C5b and various monoclonal antibodies raised against C3, C3a, C4, C4a, C5, and C5a were purchased from Complement Technology (Tyler, TX, USA). Human plasma was prepared as follows: blood samples collected from healthy volunteers were put into a BD vacutainer tube containing 0.072 ml of 7.5% EDTA (Becton Dickinson, MD, USA) to prevent coagulation and centrifuged for 15 min at 3,000 $\times g$ to remove blood cells. The resulting plasma was stored at -70℃ until use.

Ⅱ-2. Cell culture

Escherichia coli (*E. coli*) DH5α cells were cultured in LB media containing 1% NaCl at 37℃ under aerobic conditions as described elsewhere.

Ⅱ-3. SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

performed according to the method of Laemmli (Laemmli, 1970). Protein samples were mixed with an equal volume of 6× SDS-PAGE sample buffer, boiled at 100°C for 3 min and then loaded onto 10% or 12% polyacrylamide gel. After the electrophoresis, protein bands were visualized by staining the gel with Coomassie brilliant blue. For Western blot analysis, protein samples were subjected to SDS-PAGE and then transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skim milk in TBS-T (250 mM Tris-HCl, pH 8.0, 1.5 mM NaCl, and 0.1% Tween 20) at room temperature (RT) for 2 h. The membrane was then incubated with specific primary antibodies (1:5000 in the blocking buffer) overnight at 4°C, and washed six times with TBS-T buffer. The membrane was then incubated with HRP-conjugated secondary antibodies (1:4000 in the blocking buffer) at RT for 2 h and washed five times with TBS-T buffer. The signals were detected using EZ-Western Lumi Plus system (DAEILLAB SERVICE co., Ltd., Seoul, Korea).

Ⅱ-4. Expression and purification of the recombinant vEP-45 and vEP-34 in *E. coli*

E. coli DH5α cells were used as host cell for the expression of the proteins vEP-45 and vEP-34. DH5α cells harboring recombinant plasmid pvEP-45 or pvEP-34 were inoculated into 50 ml of LB broth plus 100 μg/ml ampicillin and grown overnight at 37°C. Ten milliliters of this overnight culture were inoculated into 500 ml of fresh LB broth plus ampicillin and the cells were grown at 37°C until the OD_{600} reached 0.8. The target proteins were induced by the addition of 0.2 mM isopropyl-1-thio-β-galactopyranoside (IPTG) followed by overnight incubation at 20°C. The cells were harvested from the cultures by centrifugation for 15 min at 4,000 ×*g*

at 4°C and the resulting pellet was resuspended in 100 ml of a lysis buffer (25 mM Tris-HCl, pH 8.0, 20% sucrose, 1 mM EDTA, 1 mM PMSF, and 0.3 mg/ml lysozyme). The cell suspension was incubated on a shaker at moderate speed for 30 min at 4°C. The cell suspension was centrifuged for 20 min at 6,000 ×*g* at 4°C. The supernatant, which contained the periplasmic proteins, was subjected to precipitation with 70% saturated ammonium sulfate. The resulting protein pellet was collected by centrifugation for 40 min at 16,000 ×*g* at 4°C, dissolved in 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$, and then applied to a PD-10 column equilibrated in the same buffer to remove residual $(NH_4)_2SO_4$. The sample was then applied to a HiPrep 16/10 QFF column equilibrated with 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$. After a wash with the same buffer, the bound proteins were eluted with a linear gradient of NaCl from 0 M to 1 M in the same buffer. The active fractions were pooled, concentrated by ultrafiltration using Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore, Billerica, MA, USA), and then desalted on a PD-10 column equilibrated with 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$. The desalted proteins were loaded on a Mono Q 4.6/100 PE column equilibrated with 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and the bound proteins were eluted with a linear gradient of NaCl from 0 M to 0.7 M. Active fractions were pooled, concentrated, desalted, and loaded separately onto a Superdex 75 10/300 GL gel filtration column equilibrated with 25 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and 0.15 M NaCl. Fractions with major protease activities were pooled, concentrated, and stored in small aliquots at -20°C as the purified enzymes.

Ⅱ-5. Expression and purification of the recombinant C-ter100 in *E. coli*

E. coli DH5α cells harboring recombinant plasmid pC-ter100 were inoculated

into 50 ml of LB broth plus 100 μg/ml ampicillin and grown overnight at 37°C. Ten milliliters of this overnight culture were inoculated into 500 ml fresh LB broth plus ampicillin and the cells were grown at 37°C until the OD_{600} reached 0.8. IPTG was then added to the culture to a final concentration of 0.2 mM and incubated for overnight at 20°C. The cells were harvested from the cultures by centrifugation for 15 min at 4,000 ×*g* at 4°C. The resulting pellet was resuspended in 50 ml of a lysis buffer (30 mM Tris-HCl, pH 8.0, 20% sucrose, 1 mM EDTA, 1 mM PMSF, and 0.3 mg/ml lysozyme). The cell suspension was incubated on a shaker for 30 min at moderate speed at 4°C. It was then centrifuged for 20 min at 6,000 ×*g* at 4°C and the supernatant was collected as a cell-free extract. Ammonium sulfate was added to the cell-free extract to give 20% saturation and the protein precipitate was removed by centrifugation for 30 min at 16,000 ×*g* at 4°C. The supernatant was collected and the ammonium sulfate concentration was increased to 70% saturation. The resulting precipitate was collected by centrifugation for 40 min at 16,000 × *g* at 4°C. The pellet was dissolved in 25 mM Tris-HCl buffer (pH 7.5) followed by desalting on PD-10 columns equilibrated with the same buffer. The desalted sample was applied to an anti-FLAG M2 affinity gel pre-equilibrated with the same buffer at RT. After washing with 10 column volumes of the same buffer, the column was eluted with 0.1 M glycine-HCl buffer (pH 3.5). Fractions were neutralized with 1 M Tris-HCl buffer (pH 8.0) and concentrated by ultrafiltration using Amicon Ultra-0.5 Centrifugal Filter Unit. The purified C-ter100 protein was stored in small aliquots at -20°C.

Ⅱ-6. Cleavages of C3, C4, and C5 by vEP-45 *in vitro*

Reaction mixtures consisting of human complement proteins C3 (5 μg), C4 (5 μg), or C5 (5 μg) and different concentrations of vEP-45 (0.5, 2, 10, or 50 ng) in

phosphate buffered saline (PBS, pH 7.5) were incubated for 3 min at 37°C. When the Western blottings were performed, reaction mixtures consisting of human complement proteins C3 (1 μg), C4 (0.5 μg), or C5 (0.5 μg) and vEP-45 (0, 25, 50, 100, 200, or 300 ng) in PBS (pH 7.5) were incubated for 3 min at 37°C. Thereafter, the reactions were terminated by the addition of 1 mM of 1,10-PT (Park *et al.*, 2014). The cleaved products were separated by 10% or 15% SDS-PAGE and visualized by Coomassie blue staining or Western blottings.

Ⅱ-7. Cleavages of C3 and C5 by vEP-45 in plasma milieu

Human plasma was diluted with PBS (pH 7.5) to a final concentration of 10%. Reaction mixtures consisting of 10 μl of 10% human plasma and vEP-45 (0.5, 1, or 2 μg) diluted in PBS (pH 7.5) were incubated for 3 min at 37°C. Thereafter, the reactions were terminated by the addition of 1 mM of 1,10-PT (Park *et al.*, 2014). The cleaved products were separated by SDS-PAGE and detected by Western blottings.

Ⅱ-8. Flow cytometry

BALB/c mice at 7 weeks of age were injected with 10 mg/kg each of lipopolysaccharide (LPS), vEP-45, vEP-34, and C-ter100 via the tail vein. Whole blood was collected 3 h later by retro-orbital bleeding via k2-ethylenediaminetetra-acetic acid-coated (K2-EDTA-coated) tubes (BD Vacutainer, Becton Dickinson, NJ, USA). Thereafter, the reactions were terminated by the addition of 1 mM of 1,10-PT (Park *et al.*, 2014). One hundred microliters of whole blood were aliquoted in 5 ml polystyrene round bottom test tubes (Falcon, BD Labware, NJ, USA) and blocked

with 20 μl of human Fc receptor binding inhibitor (eBioscience, CA, USA) and incubated on ice for 15 min to block FcRs. Antibodies raised against CD11b-APC, Ly-6C-PerCP, and Ly-6G-PE (eBioscience, CA, USA) in a total volume of 50 μl were then added at the concentrations suggested by the manufacturer. Cells were stained in the dark for 25 min at RT, and then 2 ml of 1× RBC lysing solution (eBioscience, CA, USA) was added, mixed, and incubated with the cells in the dark for 20 min at RT to lysis red blood cells. The cell solution was then centrifuged for 5 min at 500 ×*g* at RT, and the supernatant was discarded (Lewis *et al.*, 2013). The cells were then washed twice with 2 ml of Flow Cytometry Staining Buffer (eBioscience, CA, USA), resuspended in 200 μl of the same buffer. FACS analysis was performed on a FACS-Calibur (Becton Dickinson, USA), and data were analysed using the WinMDI 2.8 software. Red blood cells are excluded by gating based on forward and side scatter characteristics. Cell types were identified by size and positive expression of surface markers; Ly-6G for neutrophils, Ly-6C and CD11b for monocytes.

Ⅲ. RESULTS AND DISCUSSION

III-1. Expression and purification of the recombinant vEP-45, vEP-34, and C-ter100

Using an expression vector pFLAG-ATS, the active recombinant vEP-45 and vEP-34 enzymes were purified from the periplasmic proteins of the *E. coli* transformants, in which 70% ammonium sulfate fractionation, HiPrep 16/10 QFF, Mono Q 4.6/100 PE, and Superdex 75 10/300 GL gel filtration columns were employed as described previously (Chang *et. al.,* 2005) (Fig. 3B). The C-ter100 peptide was purified from the periplasmic proteins of the *E. coli* transformants, using anti-FLAG M2 affinity column chromatography as described in Materials and Methods (Fig. 3C). Overall schematic structures of vEP-45, vEP-34, and C-ter100 used in this study are represented in Fig. 3A.

III-2. Cleavage of C3 by vEP-45 *in vitro*

The blood complement system can be activated by three pathways (the classical, the lectin, and the alternative pathways), which results in cleavage of inactive C3 protein into the functional fragments C3a and C3b (Merle *et al.*, 2015). As illustrated in Fig. 4A, the complement C3 precursor is composed of two chains, in which α chain (110 kDa) and β chain (75 kDa) are linked by an intrastrand disulfide bond (Lambris, 1988). The cleavage of C3 at the α chain leads to the generation of the C3b which comprises two polypeptide chains (a 101 kDa α′ chain and a 75 kDa β chain) and 9 kDa C3a (Lambris, 1988). In this study, the ability of vEP-45 to cleave the human complement protein C3 into C3a and C3b was

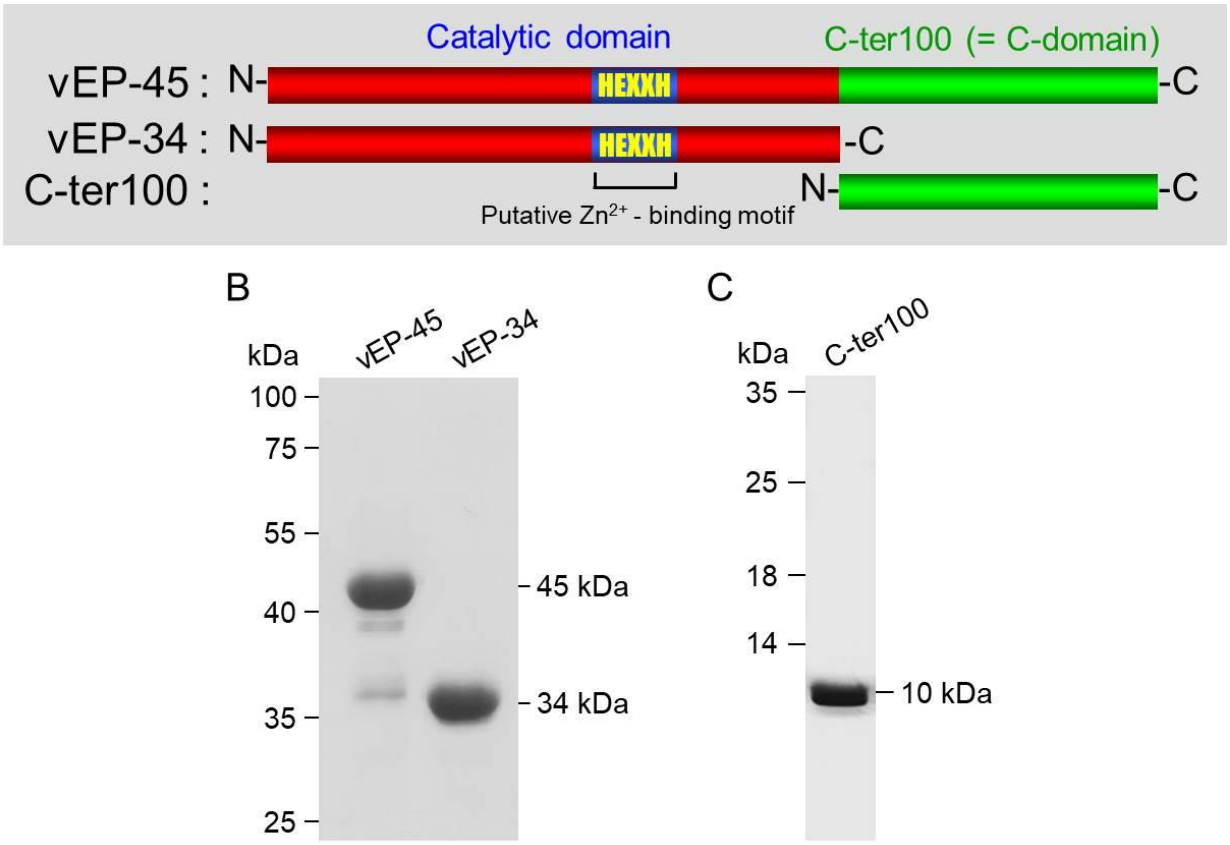


Fig. 3. Purification of recombinant vEP-45, vEP-34, and C-ter100 from *E. coli*. (A) Overall schematic structures of vEP-45, vEP-34, and C-ter100. (B) SDS-PAGE of purified vEP-45 and vEP-34. The purified vEP-45 and vEP-34 enzymes resolved by a Superdex 75 10/300 GL were subjected to 12% SDS-polyacrylamide gel under reducing conditions. (C) SDS-PAGE of purified C-ter100. The purified C-ter100 peptide was subjected to 15% SDS-polyacrylamide gel under denaturing conditions.

examined *in vitro* (Figs. 4 and 5). When human complement protein C3 (5 μg) and vEP-45 (0.5, 2, 10, or 50 ng) were incubated for 10 min at 37°C and subjected to SDS-PAGE, bands of 101 kDa and 9 kDa were produced from α′ chain of C3b and C3a, respectively (Fig. 4B). The generations of C3b and C3a from human complement protein C3 peaked at 0.5 ng of vEP-45 treatment, accompanied with gradual decreases at higher concentrations (Figs. 4C and 4D; Table 2). To determine whether a similar *in vitro* cleavage pattern was detectable by Western blot analysis, human C3 (1 μg) and vEP-45 (0, 25, 50, 100, 200, or 300 ng) were incubated for 3 min at 37°C and subjected to Western blot analysis. As shown in Fig. 5, C3b and C3a were dose-dependently produced from the precursor C3 by vEP-45 cleavage. These results suggest that vEP-45 can proteolytically cleave C3 into C3a and C3b.

III-3. Cleavage of C4 by vEP-45 *in vitro*

Both classical and lectin pathways cleave C4 into active fragment C4b and a small fragment C4a (Avirutnan *et al.*, 2011; Merle *et al.*, 2015). As illustrated in Fig. 6A, the complement C4 precursor is composed of three chains, in which α chain (97 kDa), β chain (75 kDa), and γ chain (33 kDa) are linked by intrastrand disulfide bonds (Inal & Schifferli, 2002). The cleavage of C4 at α chain releases C4b which comprises three polypeptide chains (a 88 kDa α′ chain, a 75 kDa β chain, and a 33 kDa γ chain) and 9 kDa C4a (Inal & Schifferli, 2002). In this study, the ability of vEP-45 to cleave the human complement protein C4 into C4a and C4b was examined *in vitro* (Figs. 6 and 7). When human complement protein C4 (5 μg) and vEP-45 (0.5, 2, 10, or 50 ng) were incubated for 10 min at 37°C and subjected to SDS-PAGE, C4b α′ chain and C4a appeared as two bands of 88 kDa

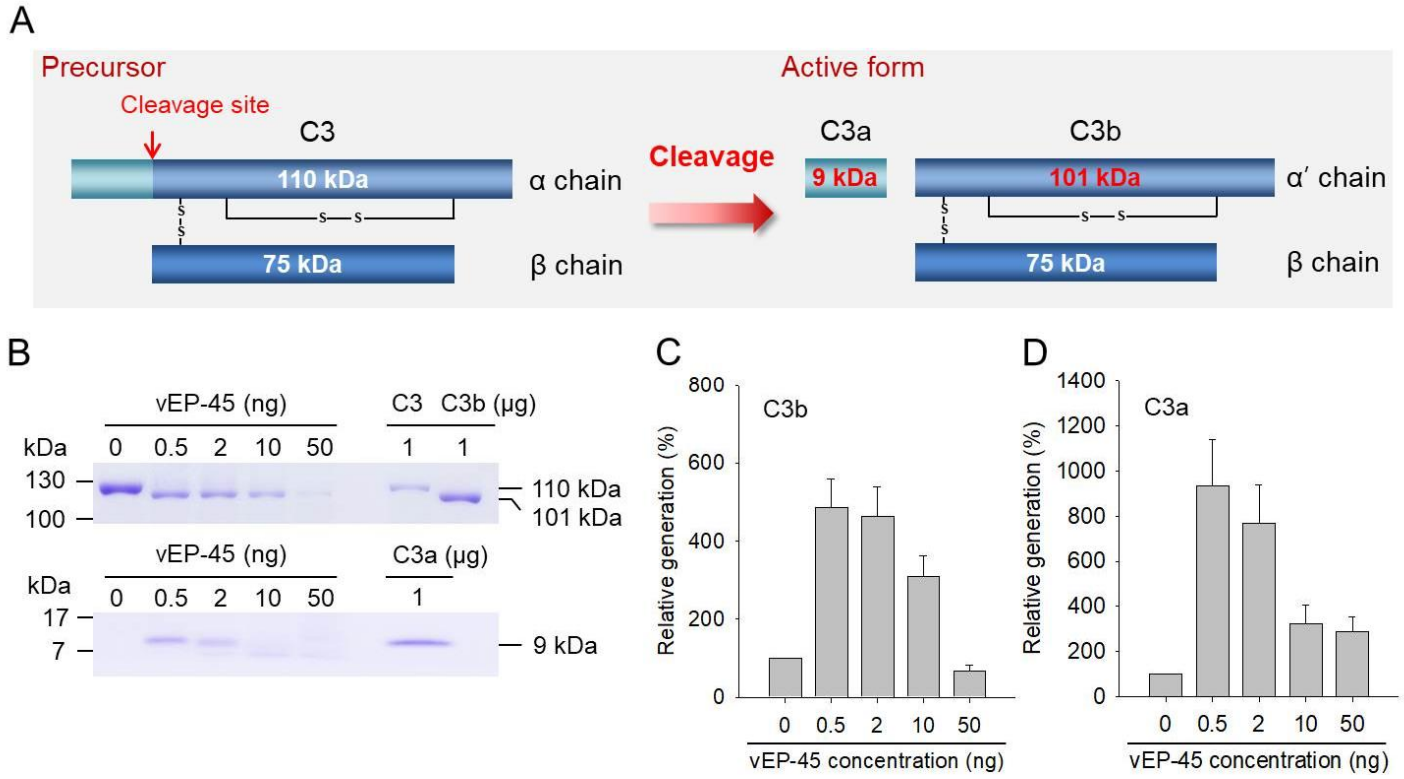


Fig. 4. Cleavage of human complement protein C3 by vEP-45 as shown by SDS-PAGE. (A) Schematic structures of C3, C3a, and C3b. (B) Cleavage of C3 by vEP-45. Human complement protein C3 (5 μg) was incubated with vEP-45 (0.5, 2, 10, or 50 ng) for 10 min at 37°C. Proteins from each sample were separated by SDS-PAGE and stained with Coomassie brilliant blue. (C and D) Histograms showing the productions of C3b α′ chain (C) and C3a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

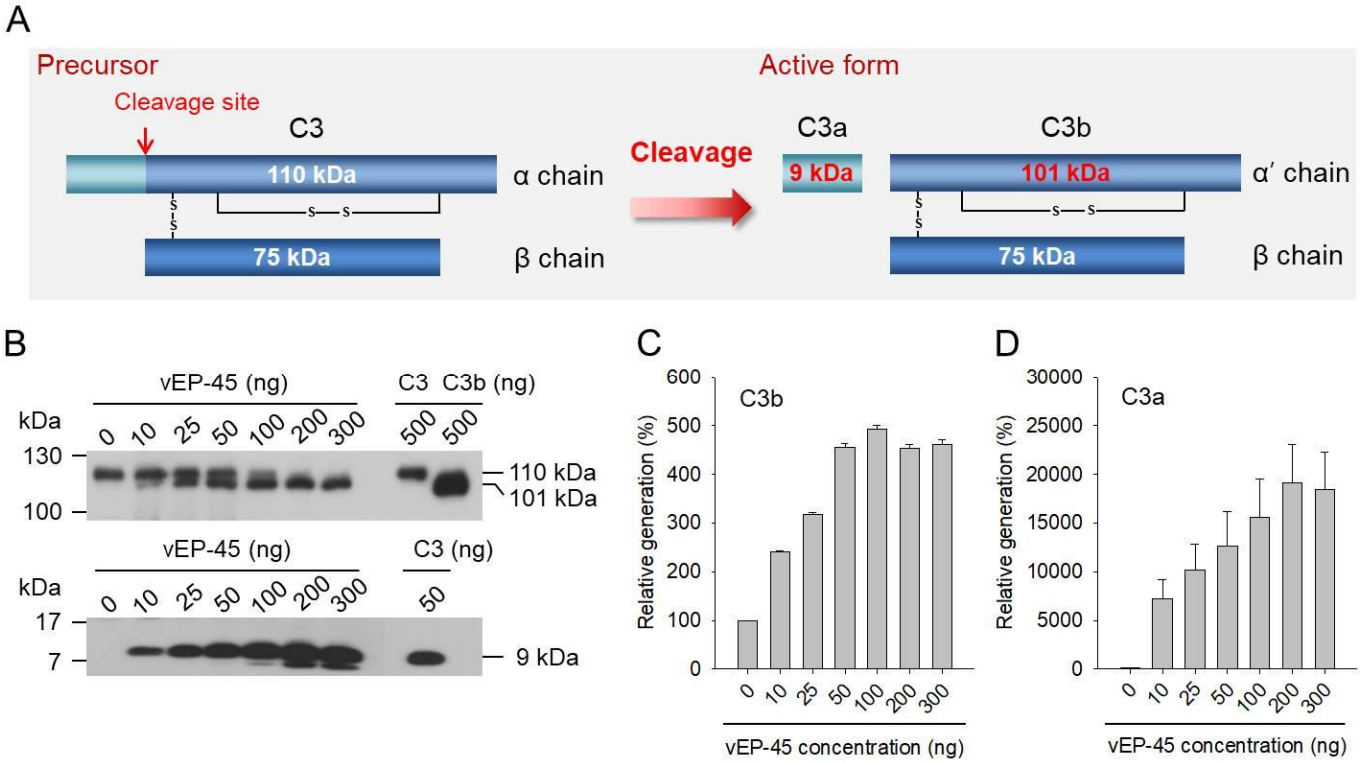


Fig. 5. Cleavage of human complement protein C3 by vEP-45 as shown by Western blottings. (A) Schematic structures of C3, C3a, and C3b. (B) Cleavage of C3 by vEP-45. Human complement protein C3 (1 μg) was incubated with vEP-45 (10, 25, 50, 100, 200, or 300 ng) for 3 min at 37°C. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with anti-C3 antibody (upper panel) or anti-C3a antibody (lower panel). (C and D) Histograms showing the productions of C3b α′ chain (C) and C3a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

Table 2. Relative generations of C3b and C3a from C3 by vEP-45.

Active form	SDS-PAGE		Western blottings	
	vEP-45 concentration (ng)	Relative generation (%)[a]	vEP-45 concentration (ng)	Relative generation (%)[a]
C3b	0	100	0	100
	0.5	486	10	240
	2	465	25	317
	10	310	50	455
	50	69	100	493
			200	454
			300	462
C3a	0	100	0	100
	0.5	935	10	7222
	2	768	25	10186
	10	322	50	12652
	50	288	100	15579
			200	19116
			300	18406

[a] The relative generations were quantified by densitometric analysis after SDS-PAGE or Western blottings, in which the value obtained from non-treated control group was regarded as 100%.

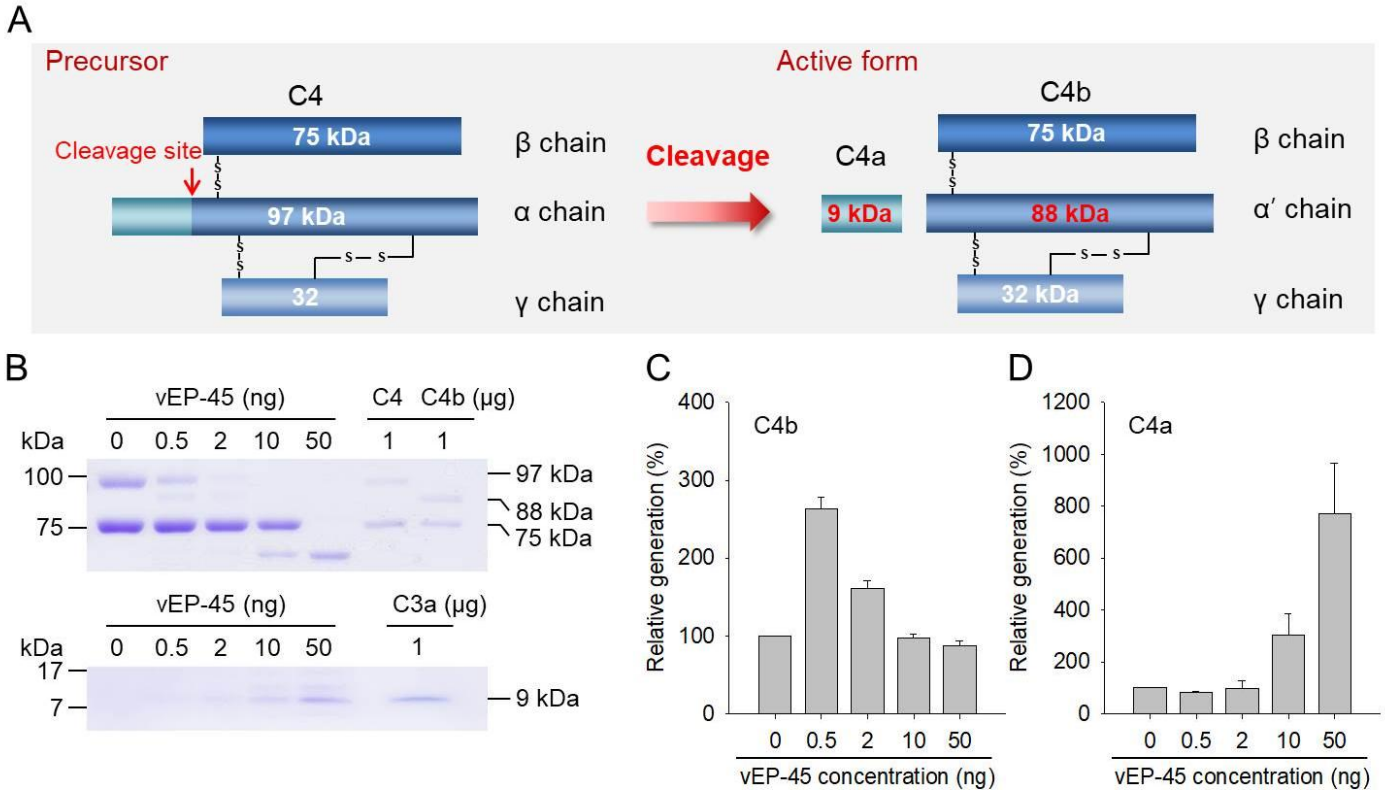


Fig. 6. Cleavage of human complement protein C4 by vEP-45 as shown by SDS-PAGE. (A) Schematic structures of C4, C4a, C4b. (B) Cleavage of C4 by vEP-45. Human complement protein C4 (5 μg) was incubated with vEP-45 (0.5, 2, 10, or 50 ng) for 10 min at 37°C. Proteins from each sample were separated by SDS-PAGE and stained with Coomassie brilliant blue. (C and D) Histograms showing the productions of C4b α′ chain (C) and C4a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

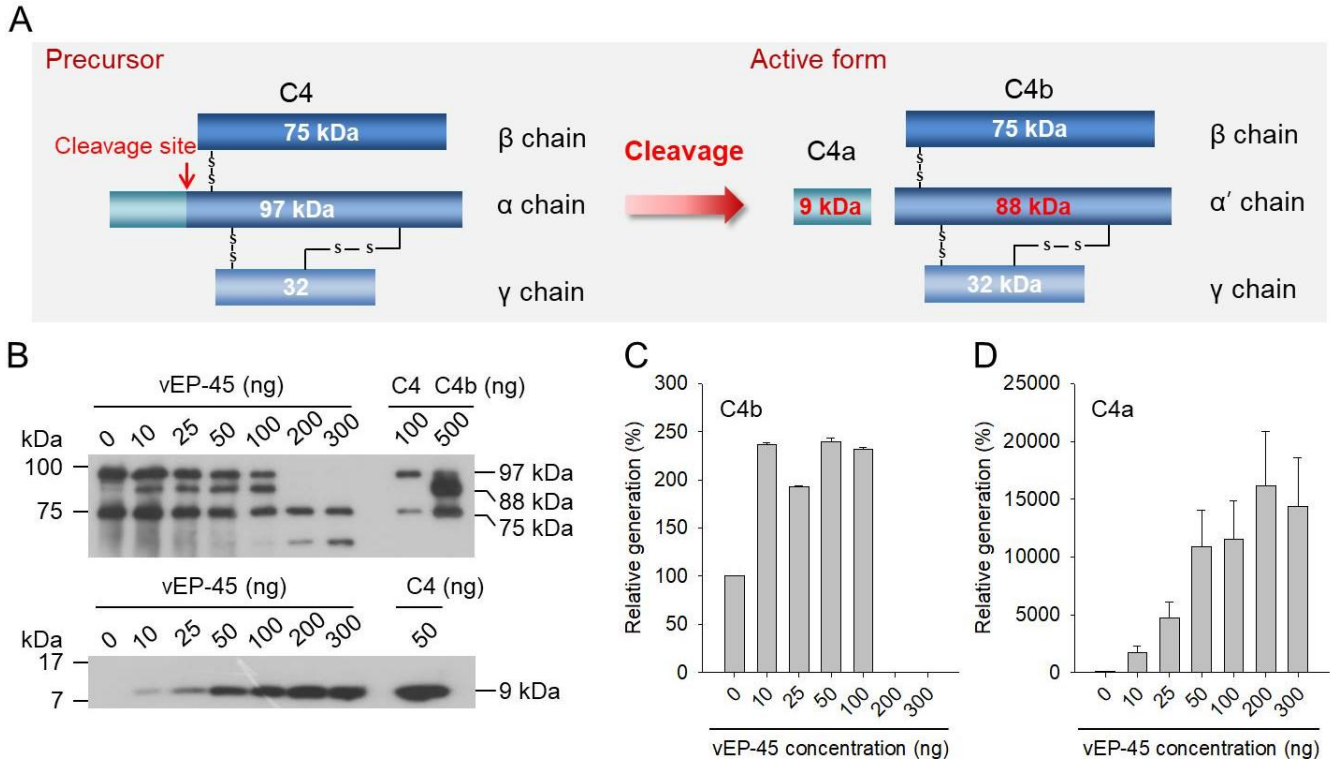


Fig. 7. Cleavage of human complement protein C4 by vEP-45 as shown by Western blottings. (A) Schematic structures of C4, C4a, and C4b. (B) Cleavage of C4 by vEP-45. Human complement protein C4 (0.5 μg) was incubated with vEP-45 (10, 25, 50, 100, 200, or 300 ng) for 3 min at 37°C. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with anti-C4 antibody (upper panel) or anti-C4a antibody (lower panel). (C and D) Histograms showing the productions of C4b α′ chain (C) and C4a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

and 9 kDa, respectively (Fig. 6B). Similarly to C3 cleavage, maximal C4b production was obtained with 0.5 ng of vEP-45 and decreased at higher concentrations but, in this case, C4a production peaked at 50 ng of vEP-45 in a dose-dependent manner (Figs. 6C and 6D; Table 3). To determine whether a similar *in vitro* cleavage pattern was detectable by Western blot analysis, human C4 (0.5 μg) and vEP-45 (0, 25, 50, 100, 200, or 300 ng) were incubated for 3 min at 37°C and subjected to Western blot analysis. As shown in Fig. 7, the generations of C4b and C4a from C4 peaked at 50 and 200 ng of vEP-45 treatments, respectively, accompanied with gradual decreases at higher concentrations (Fig. 7; Table 3). These results suggest that vEP-45 can proteolytically cleave C4 into C4a and C4b.

III-4. Cleavage of C5 by vEP-45 *in vitro*

The active complement C3b binds to the C3 convertase to form a new enzymatic complex, that is, C5 convertase which cleaves C5 into anaphylatoxins C5a and C5b (Dunkelberger & Song, 2010; Merle *et al.*, 2015). As illustrated in Fig. 8A, the complement C5 precursor is composed of two chains, in which α chain (115 kDa) and β chain (75 kDa) are linked by an intrastrand disulfide bond (Krisinger *et al.*, 2012). The cleavage of C5 at α chain releases the C5b which comprises two polypeptide chains (a 104 kDa α′ chain and a 75 kDa β chain) and 11 kDa C5a (Krisinger *et al.*, 2012). In this study, the ability of vEP-45 to cleave the human complement protein C5 into C5a and C5b was examined *in vitro* (Figs. 8 and 9). When human complement protein C5 (5 μg) and vEP-45 (0.5, 2, 10, or 50 ng) were incubated for 10 min at 37°C and subjected to SDS-PAGE analysis, C5a was found at 11 kDa and the band of α′ chain of C5b was barely visible (Fig. 8B). The generation of C5a was increased by vEP-45 in a dose-dependent manner (Fig. 8D; Table 4). To determine whether a similar *in vitro* cleavage pattern was

Table 3. Relative generations of C4b and C4a from C4 by vEP-45.

Active form	SDS-PAGE		Western blottings	
	vEP-45 concentration (ng)	Relative generation (%)[a]	vEP-45 concentration (ng)	Relative generation (%)[a]
C4b	0	100	0	100
	0.5	263	10	237
	2	161	25	192
	10	98	50	240
	50	88	100	232
			200	0
			300	0
C4a	0	100	0	100
	0.5	82	10	1752
	2	97	25	4752
	10	303	50	10866
	50	771	100	11525
			200	16122
			300	14371

[a] The relative generations were quantified by densitometric analysis after SDS-PAGE or Western blottings, in which the value obtained from non-treated control group was regarded as 100%.

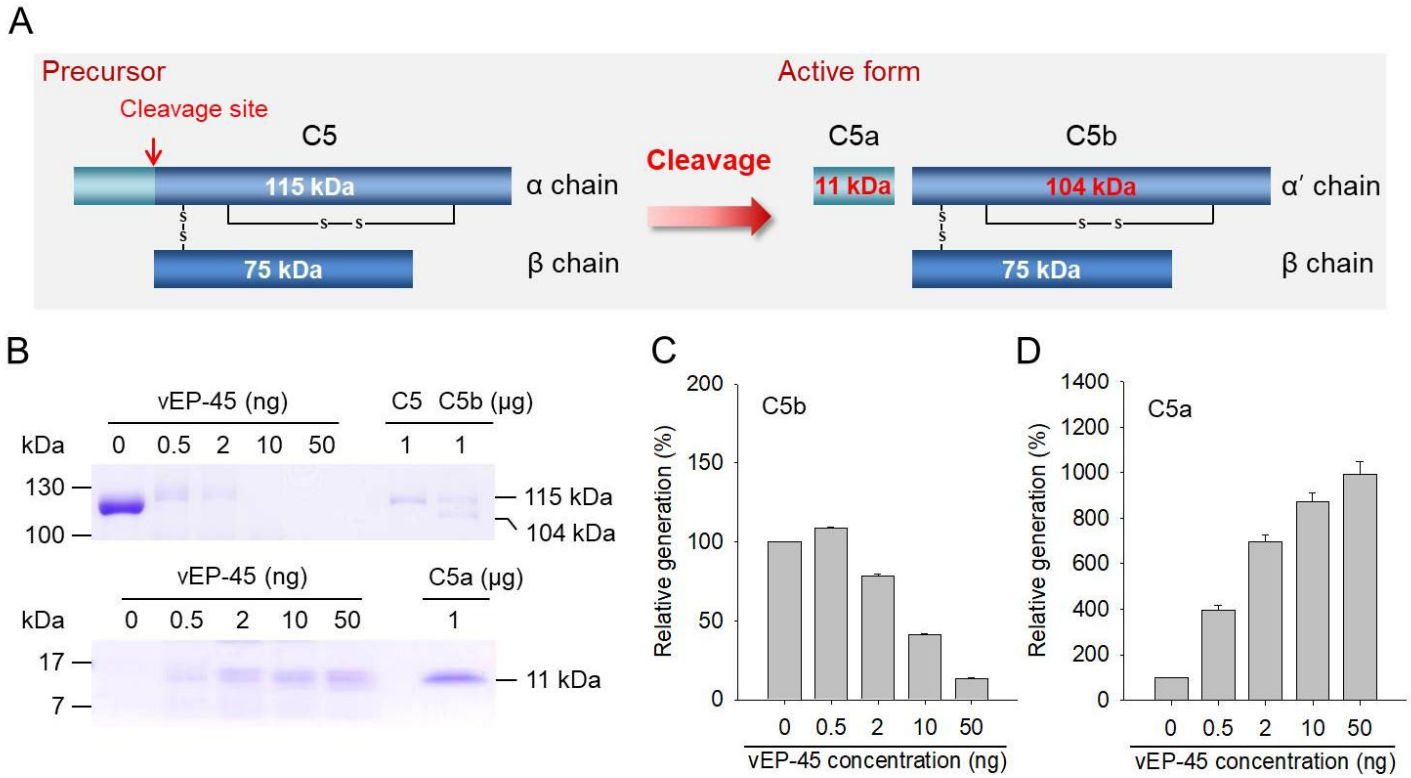


Fig. 8. Cleavage of human complement protein C5 by vEP-45 as shown by SDS-PAGE. (A) Schematic structures of C5, C5a, and C5b. (B) Cleavage of C5 by vEP-45. Human complement protein C5 (5 μg) was incubated with vEP-45 (0.5, 2, 10, or 50 ng) for 10 min at 37°C. Proteins from each sample were separated by SDS-PAGE and stained with Coomassie brilliant blue. (C and D) Histograms showing the productions of C5b α′ chain (C) and C5a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

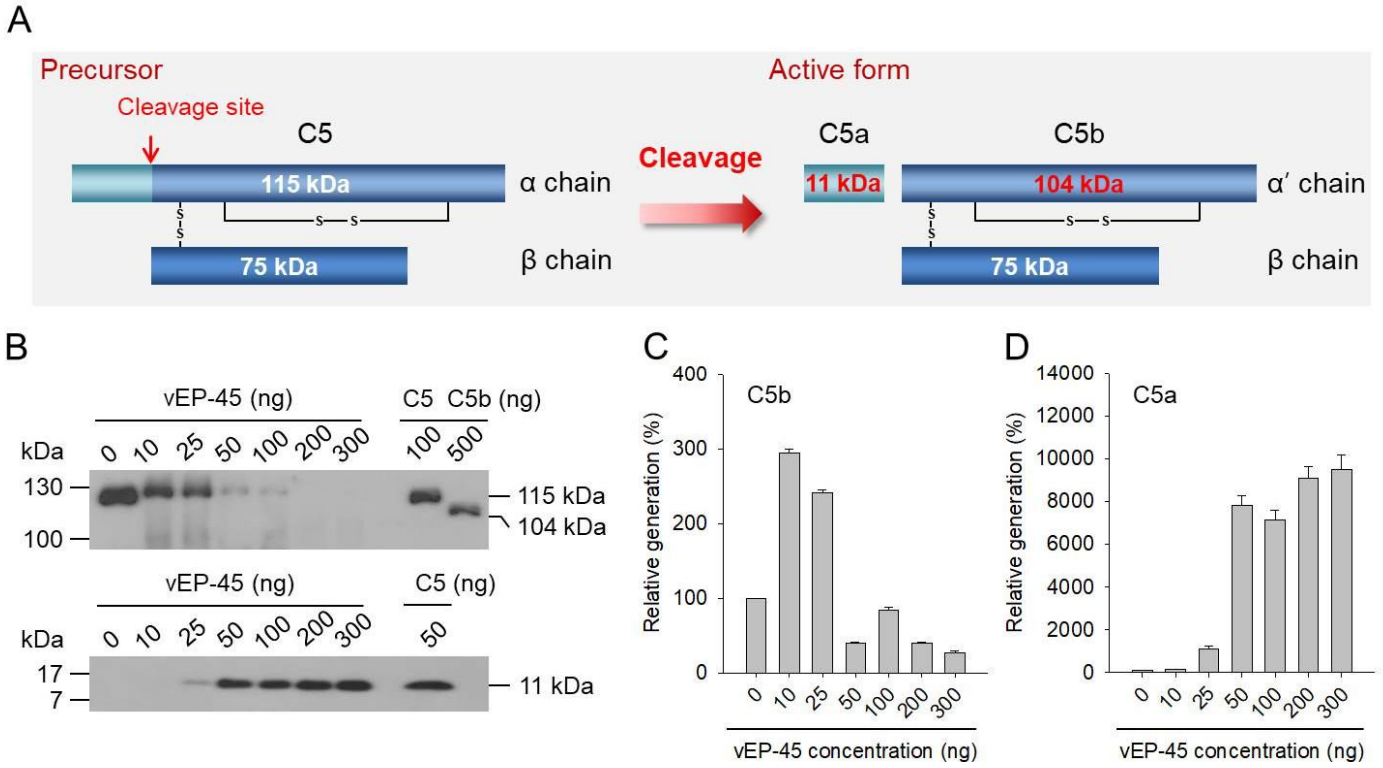


Fig. 9. Cleavage of human complement protein C5 by vEP-45 as shown by Western blottings. (A) Schematic structures of C5, C5a, and C5b. (B) Cleavage of C5 by vEP-45. Human complement protein C5 (0.5 μg) was incubated with vEP-45 (10, 25, 50, 100, 200, or 300 ng) for 3 min at 37°C. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with anti-C5 antibody (upper panel) or anti-C5a antibody (lower panel). (C and D) Histograms showing the productions of C5b α′ chain (C) and C5a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

Table 4. Relative generations of C5b and C5a from C5 by vEP-45.

Active form	SDS-PAGE		Western blottings	
	vEP-45 concentration (ng)	Relative generation (%)[a]	vEP-45 concentration (ng)	Relative generation (%)[a]
C5b	0	100	0	100
	0.5	109	10	295
	2	78	25	241
	10	41	50	40
	50	13	100	85
			200	40
			300	28
C5a	0	100	0	100
	0.5	398	10	138
	2	697	25	1122
	10	871	50	7808
	50	993	100	7157
			200	9079
			300	9509

[a] The relative generations were quantified by densitometric analysis after SDS-PAGE or Western blottings, in which the value obtained from non-treated control group was regarded as 100%.

detectable by Western blot analysis, human C5 (0.5 μg) and vEP-45 (0, 25, 50, 100, 200, or 300 ng) were incubated for 3 min at 37°C and then subjected to Western blot analysis. As shown in Fig. 9, the complement molecule C5a was produced from C5 by vEP-45 in a dose-dependent manner, although C5b α′ chain band was not detected on the blot (Fig. 9; Table 4). These results suggest that vEP-45 can proteolytically cleave C5 into C5a.

III-5. Cleavages of C3 and C5 by vEP-45 in plasma milieu

The complement system is composed of plasma proteins produced mainly by the liver or membrane proteins expressed on cell surface and operates in plasma (Kolev *et al.*, 2014; Merle *et al.*, 2015). The presented results obtained from *in vitro* experiments showed that vEP-45 could convert the complement proteins to their active forms (Figs. 4 ~ 9). However, further evaluation was necessary to confirm the vEP-45 ability of activating those complements present in plasma milieu. To examine the ability, 10% of human plasma was treated with vEP-45 (0.5, 1, or 2 μg) for 3 min at 37°C and subjected to Western blot analysis. As shown in Fig. 10, the production levels of C3b and C3a were increased by vEP-45 to approximately 267% and 70%, respectively, in dose-dependent manners (Fig. 10; Table 5). Consistent with the *in vitro* findings, the C5b α′ chain band was barely visible, whereas the production level of C5a was increased by vEP-45 to approximately 266% in a dose-dependent manner, compared with that of non-treated control (Fig. 11; Table 6). These results suggest that vEP-45 actually can convert plasma C3 and C5 to their respective active factors, even in the plasma milieu.

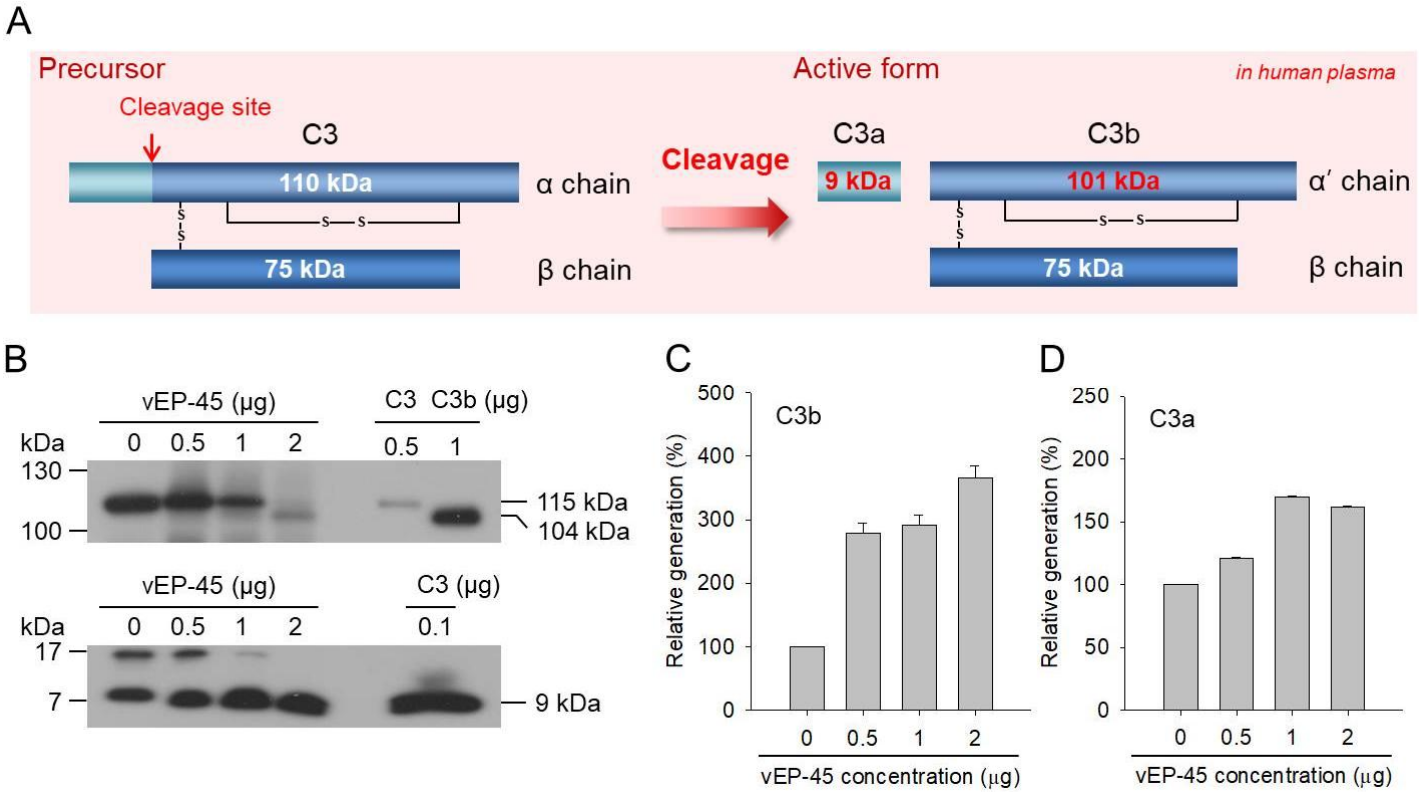


Fig. 10. Cleavage of C3 by vEP-45 in plasma milieu. (A) Schematic structures of C3, C3a, and C3b. (B) Cleavage of C3 by vEP-45 in plasma milieu. Samples of human blood plasma (10%) were incubated with vEP-45 (0.5, 1, or 2 μg) for 3 min at 37°C. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with anti-C3 antibody (upper panel) or anti-C3a antibody (lower panel). (C and D) Histograms showing the productions of C3b α′ chain (C) and 35a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

Table 5. Relative generations of C3b and C3a from human plasma by vEP-45.

Active form	vEP-45 concentration (μg)	Relative generation (%)[a]
C3b	0	100
	0.5	279
	1	291
	2	366
C3a	0	100
	0.5	121
	1	170
	2	161

[a] The relative generations were quantified by densitometric analysis after Western blottings, in which the value obtained from non-treated control group was regarded as 100%.

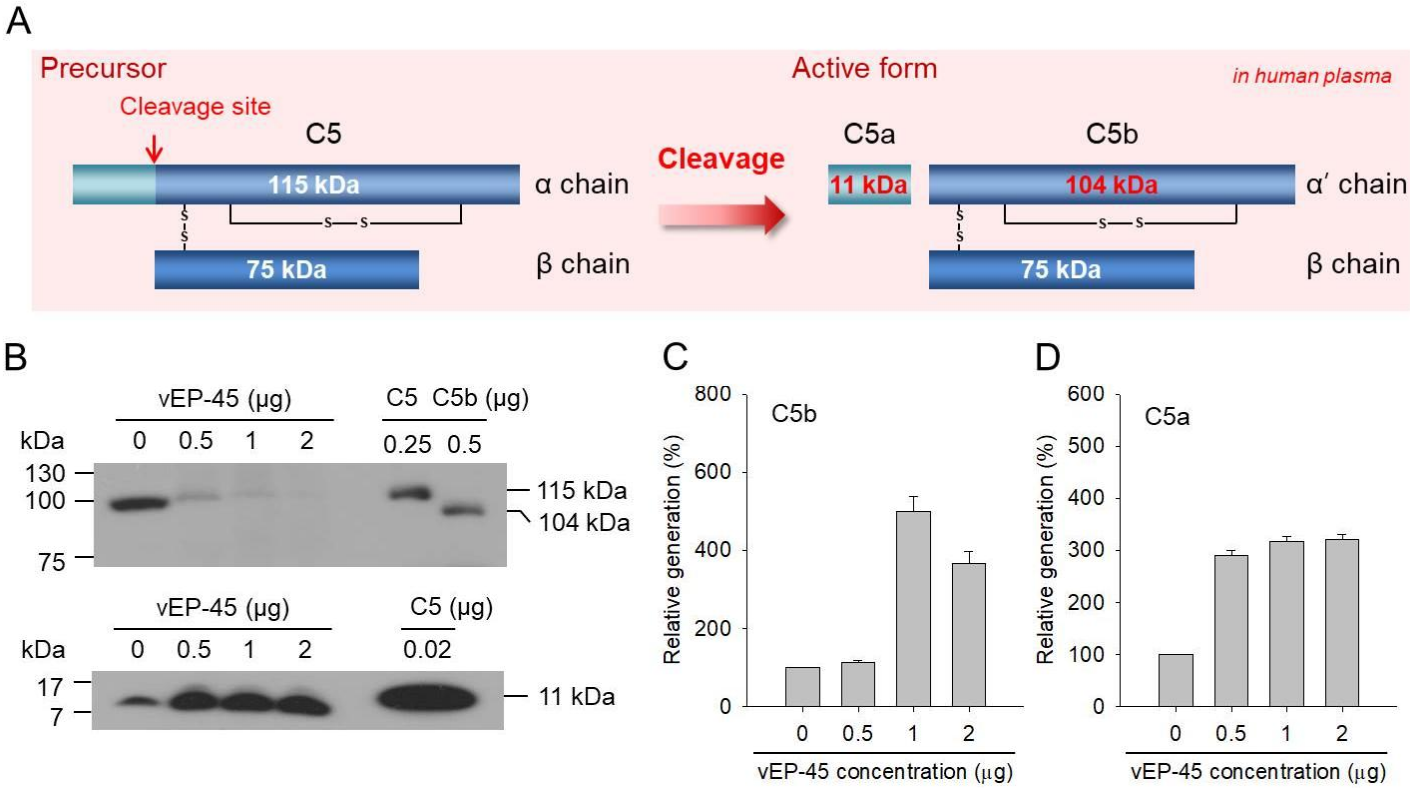


Fig. 11. Cleavage of C5 by vEP-45 in plasma milieu. (A) Schematic structures of C5, C5a, and C5b. (B) Cleavage of C5 by vEP-45. Samples of human blood plasma (10%) were incubated with vEP-45 (0.5, 1, or 2 μg) for 3 min at 37°C. Proteins from each sample were separated by SDS-PAGE and Western blottings were performed with anti-C5 antibody (upper panel) or anti-C5a antibody (lower panel). (C and D) Histograms showing the productions of C5b α′ chain (C) and C5a (D) upon treatments with different concentrations of vEP-45, in which the value obtained from non-treated control was regarded as 100%.

Table 6. Relative generations of C5b and C5a from human plasma by vEP-45.

Active form	vEP-45 concentration (μg)	Relative generation (%)[a]
C5b	0	100
	0.5	113
	1	500
	2	366
C5a	0	100
	0.5	289
	1	317
	2	322

[a] The relative generations were quantified by densitometric analysis after Western blottings, in which the value obtained from non-treated control group was regarded as 100%.

III-6. Effects of vEP-45 on the population sizes of neutrophils and monocytes *in vivo*

The complement anaphylatoxins C3a and C5a contribute to inflammation by activating immune cells such as neutrophils, monocytes, and mast cells, which express the G-protein coupled anaphylatoxin receptors C3aR and C5aR (Merle *et al.*, 2015). It has been reported that vEP-45 can activate plasma contact system by cleaving plasma prekallikrein (PPK) to form an active kallikrein as well as actively digest high-molecular weight kininogen (HK), probably producing bradykinin which induces inflammation with implications for host defense and innate immunity (Park *et al.*, 2014; Weidmann *et al.*, 2017). Furthermore, C-domain of vEP-45 can induce inflammatory response by up-regulating various pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), inflammatory regulators (Cox-2, iNOS, and PGE), and MIP-2 in their transcription levels (data not shown). Therefore, it could be expected that vEP-45 can induce innate immunity. On the basis of this background, the effects of vEP-45 on the population sizes of immune cells such as neutrophils and monocytes *in vivo* were examined by flow cytometry (Figs. 12 and 13). For the experiments, mice were injected with 10 mg/kg LPS, vEP-45, vEP-34, or C-ter100 via the tail vein, and whole bloods were collected 3 h later. The flow cytometric analyses with the bloods collected showed that LPS resulted in increasing approximately 2-fold the numbers of neutrophils ($Ly\text{-}6G^+$) (Fig. 12; Table 7) and monocytes ($Ly\text{-}6C^+CD11b^+$) (Fig. 13; Table 7), compared to those of non-treated control mice as expected (Alexander & Rietschel, 2001). When the mice were injected with vEP-45, vEP-34, and C-ter100 under the same experimental conditions, the numbers of neutrophils increased to 5.63-, 2.83-, and 4.75-folds, respectively, compared to those of non-treated control mice (Fig. 12; Table 7). In addition, the numbers of monocytes increased to 4.47-, 2.41-, and 3.77-folds by vEP-45, vEP-34, and C-ter100,

respectively, under the same experimental conditions (Fig. 13; Table 7). Interestingly, the elevated levels of the numbers of such immune cells by vEP-34 was relatively lower than by vEP-45 and C-ter100 (Fig. 12C and 13C). All these results suggest that vEP-45 can potentiate the innate immunity by increasing such immune cells through by its C-domain.

Taken together, all the results obtained by this study showed that 1) vEP-45 can cleave the blood complement factors, including C3, C4, and C5, to activate their active forms *in vitro*; 2) vEP-45 can cleave C3 and C5 to produce their active forms in plasma milieu; and finally 3) vEP-45 can up-regulate the population sizes of neutrophils and monocytes *in vivo*. Therefore, the results demonstrate that vEP-45 can activate the complement system to potential innate immunity (Fig. 14).

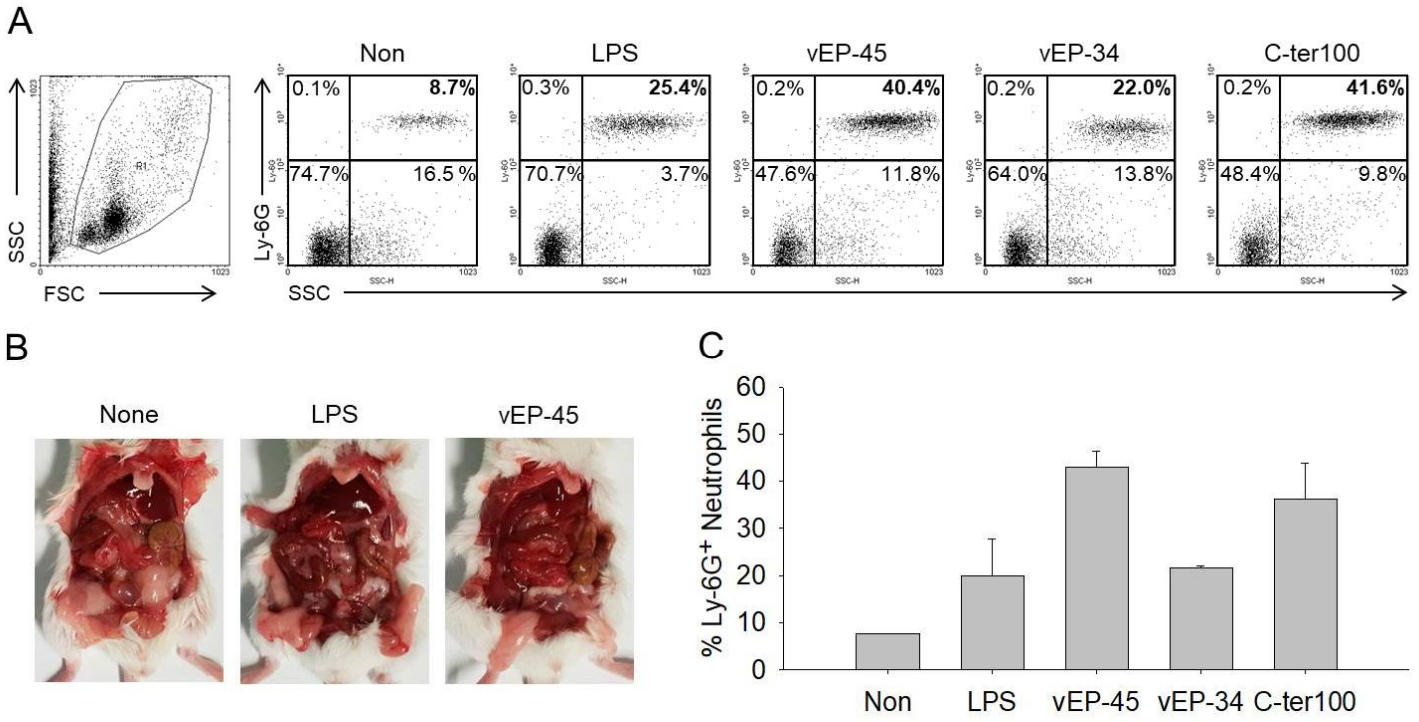


Fig. 12. Effects of vEP−45 on population size of neutrophils *in vivo*. The mice were injected with 10 mg/kg each of LPS, vEP−45, vEP−34, and C−ter100 via the tail veins. The numbers of neutrophils were analyzed 3 h later by FACS. (A) Representative FACS profiles of blood cells from mice. Neutrophils were gated based on forward and side scatter characteristics and then examined for expression of Ly−6G. Upper right quadrants show Ly−6G^{+} neutrophils. (B) Anatomical observations of mice injected with none, LPS, and vEP−45 as indicated. The photographs were taken 3 h later the injections. (C) The histogram showing the percentages of Ly−6G^{+} neutrophils. Data were obtained from two independent experiments.

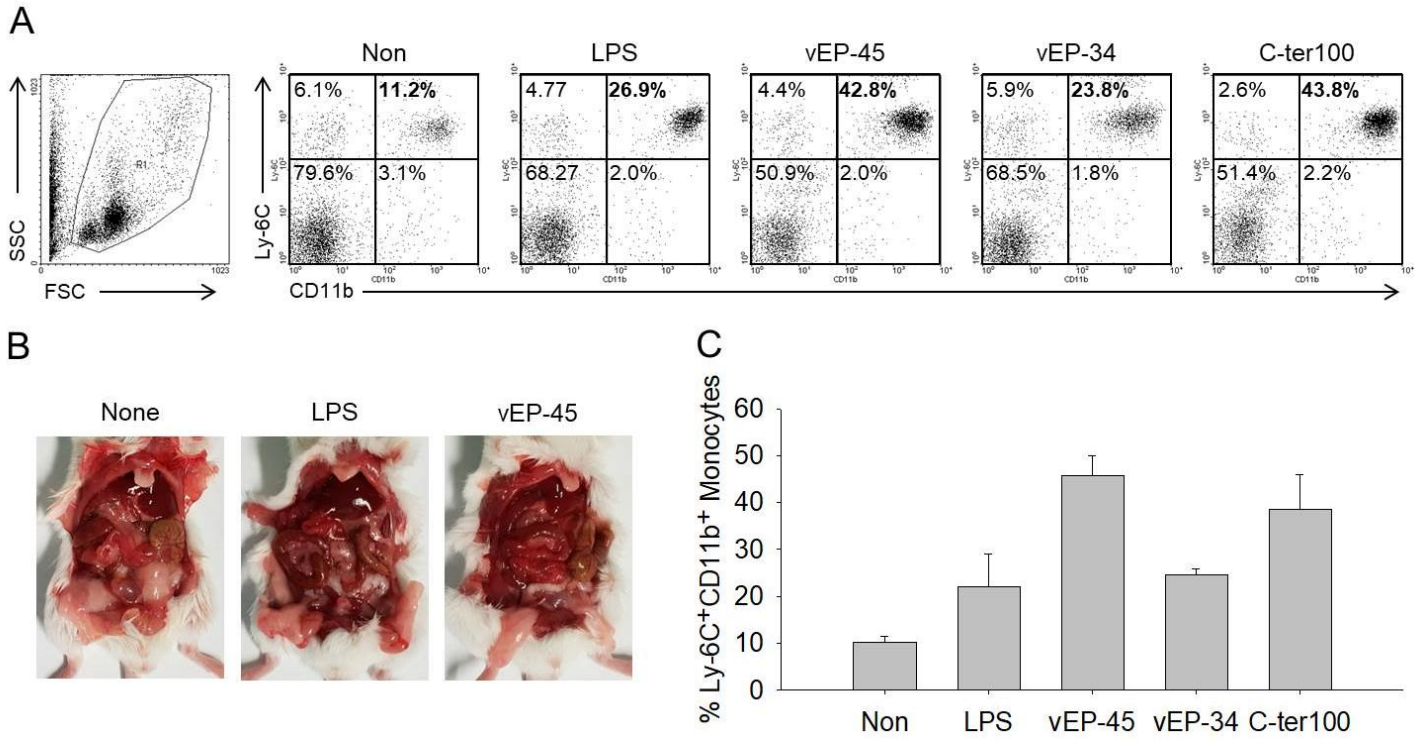


Fig. 13. Effects of vEP−45 on population size of monocytes *in vivo*. The mice were injected with 10 mg/kg each of LPS, vEP−45, vEP−34, and C−ter100 via the tail veins. The numbers of monocytes were analyzed 3 h later by FACS. (A) Representative FACS profiles of blood cells from mice. Monocytes were gated based on forward and side scatter characteristics and then examined for expression of Ly−6C and CD11b. Upper right quadrants show $Ly-6C^{+}CD11b^{+}$ monocytes. (B) Anatomical observations of mice injected with none, LPS, and vEP−45 as indicated. The photographs were taken 3 h later the injections. (C) The histogram showing the percentages of $Ly-6C^{+}CD11b^{+}$ monocytes. Data were obtained from two independent experiments.

Table 7. Effects of vEP-45 on the population sizes of immune cells *in vivo*.

Treatment[a]	Neutrophils		Monocytes	
	(%)	Increase in fold	(%)	Increase in fold
Non	7.6	1	10.2	1
LPS	19.9	2.61	22.1	2.16
vEP-45	42.9	5.63	45.7	4.47
vEP-34	21.6	2.83	24.7	2.41
C-ter100	36.2	4.75	38.6	3.77

[a] The mice were injected with 10 mg/kg of LPS, vEP-45, vEP-34, and C-ter100 and the numbers of cells were counted by flow cytometry as described in Materials and Methods.

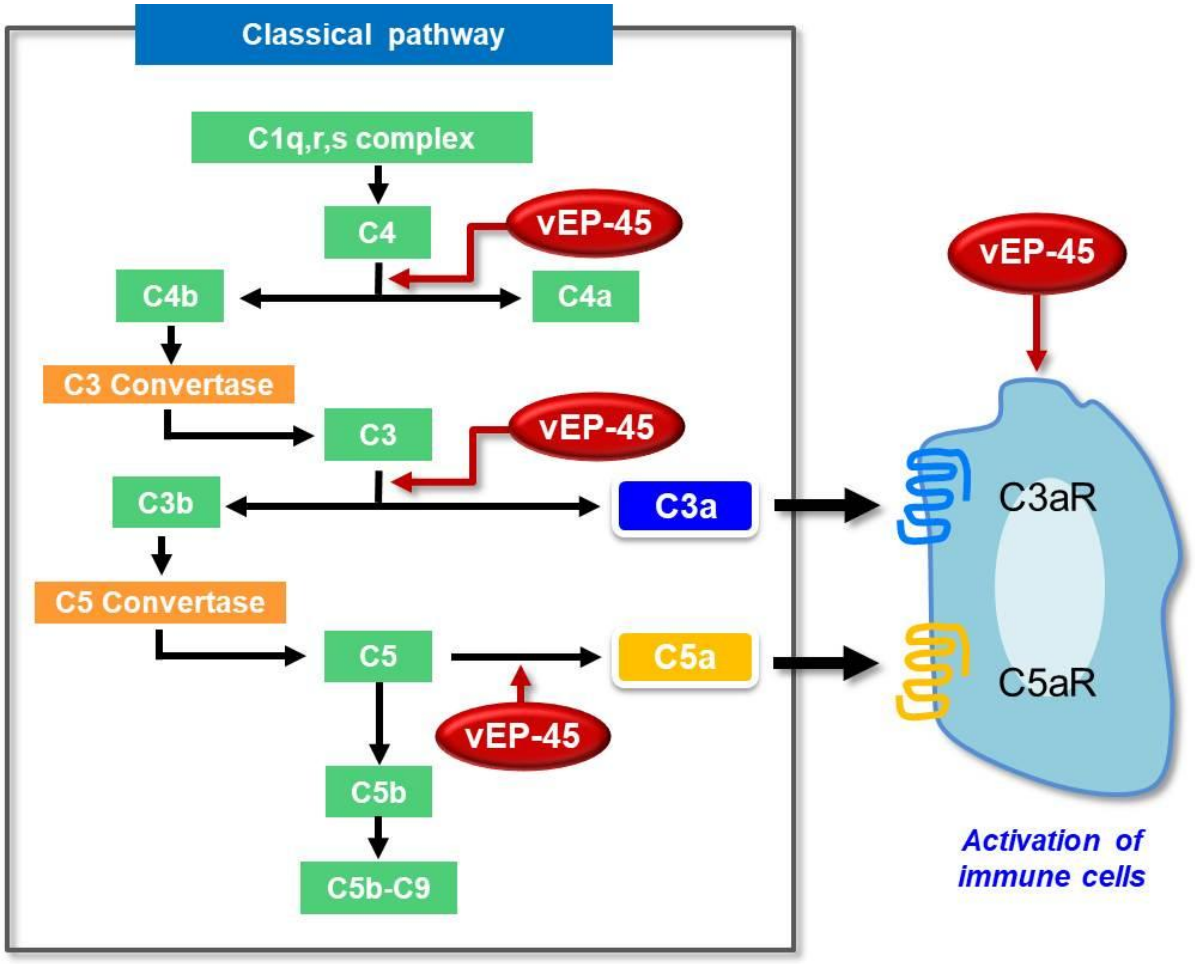


Fig. 14. vEP-45 can induce the activation of complement system and increase the numbers of immune cells. vEP-45 can proteolytically activate the blood complement protein molecules. Furthermore, vEP-45 can increase the population sizes of the immune cells.

Ⅳ. 초록

비브리오 유래 단백질분해효소에 의한 혈액 보체계 활성화 분석

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Vibrio vulnificus(*V. vulnificus*)균은 해산물을 날 것으로 섭취했을 때 유발되는 비브리오 패혈증의 원인균이다. 일반적으로 비브리오와 같은 병원성 세균들은 금속-함유 단백질 분해효소(metalloprotease)를 비롯한 capsular polysaccharide, lipopolysaccharaide 등을 분비한다. 본 연구실은 *V. vulnificus* ATCC29307 균주로부터 분비되는 단백질분해효소(vEP-45로 명명됨)를 암호화하는 유전자를 클로닝하여 염기서열을 분석하였으며, 이 유전자를 대장균에서 과발현시킨 후 순수분리하여 그 생화학적 특성을 규명한 바 있다. vEP-45는 프로트롬빈(prothrombin)을 트롬빈(thrombin)으로 활성화시킬 뿐만 아니라 교차연결된 피브린(cross-linked fibrin)을 분해하여 혈액의 항상성을 교란시킨다. 또한 이 효소는 혈액 응고의 내인성 경로(intrinsic pathway)에 작용하는 FXII 등 다양한 zymogen들을 절단함으로써 혈장 접촉계(plasma contact system)를 활성화시킬 수 있다. 혈액 보체계(blood complement system)와 응고계(blood coagulation system)는 서로 밀접하게 연관되어 있으며, 이들의 활성화는 혈액의 항상성 유지에 매우 중요하다. 따라서 혈액 응고계와 접촉계를 활성화시키는 vEP-45에 의한 혈액 보체계 활성화 여부 및 선천성 면역(innate immunity)에 미치는 영향을 규명하는 연구는 병원성 세균이 분비하는 단백질분해효소의 기능을 근본적으로 이해하는데 있어 매우

중요하다. 본 연구에서는 비브리오 감염 시 분비되는 vEP-45에 의한 보체계와 면역세포의 활성화 여부를 분석함으로써 이 단백질분해효소에 의한 선천성 면역기능의 변화를 규명하고자 하였다. 본 연구를 통해 얻은 주요결과는 다음과 같다. 시험관 내에서 인간 보체계 전구분자들(C3, C4 및 C5 등)을 vEP-45로 절단한 결과, 이들의 활성화된 형태인 C3a/C3b, C4a/C4b 및 C5a/C5b가 생성됨을 sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE)로 확인하였다. 이러한 결과는 Western blot 분석으로도 재확인하였다. Western blotting 결과, C3a/C3b의 생성은 vEP-45 농도 의존적으로 증가하였으며, C4b 및 C4a는 각각 50 및 200 ng의 vEP-45를 처리했을 때 가장 높게 생성됨을 확인하였다. Western blot 상에서 C5b band는 관찰되지 않았으나, C5a의 생성은 vEP-45 농도-의존적임을 확인하였다. 이러한 vEP-45에 의한 보체계 인자들의 활성화가 혈장 내에 존재하는 보체들로 부터도 일어나는지를 Western blotting으로 확인 하였다. 그 결과, vEP-45 처리농도에 비례하여 C3a와 C3b가 267% 및 70%로 각각 증가하며, C5a는 약 266% 증가함을 확인하였다. 그러나 시험관 내 실험결과와 동일하게 이 경우에도 C5b band는 확인할 수 없었다. 이러한 결과들은 vEP-45가 혈장 내에 존재하는 C3와 C5를 잘라 C3a/C3b 및 C5a/C5b로 각각 활성화시킬 수 있음을 시사한다. 이러한 vEP-45에 의한 보체계 활성화가 선천성 면역반응에 어떤 영향을 미치는지를 분석하기 위해 vEP-45를 주사한 생쥐에서 호중구 및 단핵구 세포 수를 형광-활성 세포 선별기(fluorescence-activated cell sorter, FACS)를 이용한 flow cytometry로 분석하였다. 그 결과, vEP-45는 호중구와 단핵구 세포를 평균 약 32% 증가시킴을 확인하였다. 이상의 결과들은 vEP-45가 비브리오 감염 시 혈액 보체계와 선천성 면역반응을 활성화 시킬 수 있음을 시사하는 것이다.

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