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# Pleiotropic effects of a vibrio extracellular protease on the activation of contact system

Jung Eun Park [a], Jong Woo Park [a], Weontae Lee [b], Jung Sup Lee [a,*]

[a] Department of Biomedical Science, BK21-plus Research Team for Bioactive Control Technology, National Research Center for Dementia, College of Natural Sciences, Chosun University, Gwangju 501-759, Republic of Korea
[b] Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea

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ABSTRACT

Many proteases secreted by pathogenic bacteria can affect seriously on hemostatic system. We have reported that an extracellular zinc metalloprotease (named vEP-45) from *Vibrio vulnificus* ATCC29307 activates prothrombin to active thrombin, leading the formation of fibrin clot. In this study, the effects of vEP-45 on the intrinsic pathway of coagulation and the kallikrein/kinin system were examined. The protease could activate proteolytically clotting factor zymogens, including FXII, FXI, FX, and prothrombin, to their functional enzymes *in vitro* and plasma milieu. In addition, it could cleave plasma prekallikrein (PPK) to form an active kallikrein as well as actively digest high-molecular weight kininogen (HK), probably producing bradykinin. In fact, vEP-45 could induce a vascular permeability in a dose-dependent manner *in vivo*. Taken together, the results demonstrate that vEP-45 can activate plasma contact system by cleaving key zymogen molecules, participating in the intrinsic pathway of coagulation and the kallikrein/kinin system.



## 1. Introduction

Plasma contact system, integral part of innate immunity, is composed of the intrinsic pathway of coagulation and the kallikrein/kinin system [1–3]. Four major factors control this system in a cascade manner [2]. These include three serine protease zymogens [factor XII (FXII), factor XI (FXI) and plasma prekallikrein (PPK)] and a non-enzymatic protein, high-molecular weight kininogen (HK). Of these factors, FXII acts as an initiator to activate the contact system cascade. At the initial stage of activation, FXII zymogen is auto-activated to active FXII (FXIIa) through a conformational change when it contacts to negatively charged surfaces such as kaolin, heparin, and dextran sulfate [1], when the endothelium that lines the blood vessel is changed to pro-coagulant state [4], and/or when the platelets are stimulated to release polyphosphates (also called Poly P) [2]. The activated FXIIa cleaves then PPK to produce active kallikrein (Kall), which in turn digest HK to release proinflammatory nonapeptide bradykinin (BK), causing the expansion of blood vessels and also inducing vascular permeability [5–7]. At the same time, FXIIa also triggers the activation of the intrinsic pathway of coagulation by converting FXI to active FXI (FXIa) that activates proteolytically FX zymogen to active FX (FXa). The FXa generated cleaves prothrombin to make thrombin that cleaves fibrinogen, leading to the formation of cross-linked fibrin [3]. The proteolytic activities of FXIIa, FXIa, and Kall are controlled by an endogenous typical serpin, C1 esterase inhibitor (also known as C1-INH), to be inhibited [3,8].

Bacteria secrets a variety of proteases involved in activating the kallikrein/kinin system and the intrinsic pathway of coagulation [3]. In fact, many bacterial proteases act as activators for plasma zymogens, which are participating in the activation of the contact system [3,8,9]. In previous studies, we have reported that an opportunistic pathogenic marine bacterium *Vibrio vulnificus* (*V. vulnificus*) ATCC29307 secretes a zinc metalloprotease (named vEP-45) that interferes with blood homeostasis through prothrombin activation and fibrinolysis [10,11]. vEP-45 is a broad-specificity protease and cleaves actively various blood clotting-associated plasma proteins such as prothrombin and fibrinogen [10]. Furthermore, a peptide derived from vEP-45-cleaved prothrombin shows an actual thrombin activity capable of producing functional

*Abbreviations:* 1,10-PT, 1,10-phenanthroline; AFC, 7-amido-4-trifluormethylcoumarin; BK, bradykinin; FX, factor X; FXI, factor XI; FXII, factor XII; HK, high-molecular weight kininogen; Kall, kallikrein; PBS, phosphate buffered saline; PPK, plasma prekallikrein; *p*NA, *para*-nitroaniline; PT, prothrombin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; serpin, serine proteinase inhibitor; TH, thrombin; vEP, vibrio extracellular protease.

* Corresponding author. Address: Department of Biomedical Science, College of Natural Sciences, Chosun University, 309 Pilmun-daero, Dong-gu, Gwangju 501-759, Republic of Korea. Fax: +82 62 230 6768.
*E-mail address:* jsplee@chosun.ac.kr (J.S. Lee).

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fibrin monomers, which are spontaneously polymerized and cross-linked in the presence of FXIIIa [10,11].

These previous results led us to examine what influence vEP-45 might have on the activation of contact system *in vitro* and plasma milieu. We describe here the pleiotropic effects of vEP-45 protease on the activation of kallikrein/kinin system and the intrinsic pathway of coagulation *in vitro* and under plasma milieu, together with its involvement in the induction of vascular permeability in guinea pig system.

## 2. Materials and methods

### 2.1. Materials

Various human zymogens and their active enzymes, including FXII, FXIIa, FXI, FXIa, PPK, Kall, and HK were purchased from Enzyme Research Laboratories (IN, USA). Protein size markers were from Fermentas (Darmstadt, Germany). Bradford protein assay kit, 1,10-PT, Evans blue dye, and SDS were obtained from Sigma (St. Louis, MO, USA). Diethyl ether was from Junsei (Tokyo, Japan). Synthetic chromogenic substrates, including H-D-Pro-Phe-Arg-*p*NA (S-2302) and N-α-Z-D-Arg-Gly-Arg-*p*NA (S-2765), were purchased from Chromogenix (Milan, Italy). Boc-Val-Pro-Arg-*p*NA was from Seikagaku (Tokyo, Japan). A fluorogenic peptide substrate (H-D-Val-Leu-Arg-AFC) was from Calbiochem (Darmstadt, Germany).

### 2.2. Expression and purification of vEP-45 protease and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

*Escherichia coli* (*E. coli*) DH5α cells were cultured in Luria–Bertani (LB) medium as described elsewhere. vEP-45 protease was expressed and purified from *E. coli* DH5α cells harboring a recombinant plasmid pvEP-45 as described previously [10]. SDS–PAGE was performed according to the method of Laemmli [12]. Typically, protein samples were mixed with an equal volume of 2× SDS–PAGE sample buffer, heated at 100 °C for 3 min, and then subjected to electrophoresis on 12% polyacrylamide gel. After electrophoresis, protein bands were visualized by staining the gel with 0.25% Coomassie brilliant blue.

### 2.3. vEP-45-mediated cleavage of FXII, FXI, PPK, and HK

Reaction mixture consisted of 10 μg each of proteins to be digested (FXII, FXI, PPK, or HK) and 0.2 μg of vEP-45 in a reaction buffer (50 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1 mg/ml BSA) was incubated for 1 or 5 min at 37 °C. Thereafter, the reaction was terminated by the addition of 1 mM of 1,10-PT and the resulting products were electrophoresed on 12% SDS–polyacrylamide gel, followed by staining with 0.25% Coomassie brilliant blue to visualize.

### 2.4. vEP-45-induced activation of FXII, FXI, FX, and PPK

Reaction mixture consisted of 5 μg each of zymogens (FXII, FXI, FX, or PPK) and 0.2 μg of vEP-45 in the same reaction buffer described in Section 2.3 was incubated for 1 or 5 min at 37 °C and the reaction was terminated by the addition of 1 mM of 1,10-PT. Thereafter, the activated enzyme activities were observed as follows: FXIIa and the FXIa activities were examined with the chromogenic substrate S-2302, in which the increases in absorbance at 405 nm were monitored every 30 s for 10 min at 37 °C in a 96-well plate reader (Molecular Devices). Kall activity was assayed with 0.4 mM of H-D-Val-Leu-Arg-AFC by measuring $\lambda_{em}$ = 505 nm and $\lambda_{ex}$ = 400 nm every 30 s at 37 °C for 10 min in a micro spectrofluorometer (Molecular Devices), from which the activity resulted was expressed as the relative fluorescence unit (RFU).

### 2.5. vEP-45-induced activation of the components of contact system in plasma milieu

Human plasma was prepared as described previously [13] and diluted with phosphate buffered saline (PBS) to a final concentration of 10%. To examine the activation of contact system components, 90 μl of 10% plasma and 10 μl of vEP-45 (1 μg) were mixed in the presence or absence of 1 mM of 1,10-PT and then 0.4 mM each of various synthetic peptide substrates (S-2302 for FXIIa and FXIa, H-D-Val-Leu-Arg-AFC for kallikrein, S-2765 for FXa, and Val-Pro-Arg-*p*NA for thrombin) was added. The activity produced was then monitored every 2 min for 30 min at 37 °C as described in Section 2.4.

### 2.6. vEP-45-induced vascular permeability in guinea pig

Evans blue dye was dissolved in PBS to a final concentration of 5% and filtered through a sterilized membrane (0.2 μm in pore size) before use. A guinea pig (300 g in body weight, male) was lightly anesthetized with diethyl ether and then the dye prepared was administered intravenously (65 mg per kilogram body weight), followed by the intradermal injection of 50 μl of vEP-45 (1 or 3 μg). After 10 min, the guinea pig was sacrificed and photographed to visualize the induced vascular permeability. For the quantification of the dye leakage, the skin around injection point (approximately one square centimeter) was cut out, soaked in 3 ml of formamide, and incubated for 48 h at 60 °C to allow the dye release. The amount of dye exclusion was determined by measuring the absorbance at 620 nm and expressed as a measure in μg of Evans blue dye efflux [6].

## 3. Results and discussion

### 3.1. vEP-45 can cleave and activate zymogens involved in contact system

The possible ability of vEP-45 to activate the zymogens involved in the intrinsic pathway of coagulation and the kallikrein/kinin system was first examined *in vitro* (Fig. 1). To see that polypeptide fragments capable of consisting active enzymes can be generated by vEP-45 cleavage, 10 μg each of zymogens (FXII, FXI, FX, and PPK) was digested with 0.2 μg of vEP-45 at 37 °C for 1 or 5 min and the resulting products were analyzed on 12% SDS–polyacrylamide gel (Fig. 1). As shown in Fig. 1A–D, polypeptide fragments comparable sizes to the chains comprising native enzymes seemed to be produced by vEP-45 cleavage from the corresponding zymogens, as indicated at the right sides of panels. These results suggest that vEP-45 may activate the zymogens through proteolysis as in the case of prothrombin activation [10]. Based on these results, the actual zymogen activation ability of vEP-45 was examined *in vitro* using chromogenic or fluorogenic peptide substrates specific for the activated enzymes (Fig. 1E–H). As shown in Fig. 1E, there was a clear increase in absorbance at 405 nm when 5 μg of FXII was digested with 0.2 μg of vEP-45 at 37 °C for 1 or 5 min and then 0.4 mM of S-2302 was added as a substrate, with no increase with zymogen only or vEP-45 alone. The relative FXIIa activity derived from vEP-45-cleaved FXII increased to an average of 9.07-folds, compared to that of non-cleaved zymogen (Fig. 1I). In this case, the adjusted unit of FXIIa activity was equivalent to approximately 0.96, where 1 unit was defined as an average absorbance value obtained with one μg of native enzyme. These results clearly show that vEP-45 can proteolytically activate FXII to active