Generation of active fragments from human zymogens in the bradykinin-generating cascade by extracellular proteases from Vibrio vulnificus and V. parahaemolyticus

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ABSTRACT

Vibrio vulnificus is an opportunistic human pathogen causing septicemia, and the infection is characterized by formation of the edematous skin lesions on limbs. This pathogenic species secretes a thermolysin-like metalloprotease as a virulence determinant. The metalloprotease was confirmed to activate human factor XII-plasma kallikrein-kinin cascade that results in liberation of bradykinin, a chemical mediator enhancing the vascular permeability, from high-molecular weight kininogen. Namely, the metalloprotease showed to generate active fragments by cleavage of Arg-Ile, Arg-Val or Gly-Leu peptide bond in human zymogens (plasma prekallikrein and factor XII). In spite of induction of the sufficient vascular permeability-enhancing and edema-forming reaction in the guinea pig model, a serine protease from *V. parahaemolyticus*, a human pathogen causing primarily watery diarrhea, showed far less ability to activate and to cleave the human zymogens. These results in part may explain why only *V. vulnificus* often causes serious edematous skin damages in humans.

INTRODUCTION

Vibrio vulnificus, an opportunistic human pathogen, causes fatal septicemia characterized by formation of the edematous skin lesions on limbs (Janda et al., 1988; Park et al., 1991). This human pathogen secretes a zinc metalloprotease of the thermolysin family (Hooper, 1994; Miyoshi and Shinoda, 2000) as an important virulence determinant (Miyoshi and Shinoda, 1993). For instance, when injected into the dorsal skin of a guinea pig, *V. vulnificus* metalloprotease enhances vascular permeability through activation of the factor XII-plasma kallikrein-kinin cascade that results in liberation of bradykinin, an inflammatory mediator, from high-molecular-weight kininogen (Miyoshi et al., 1987; Molla et al., 1989). This vibrio metalloprotease may also activate the human cascade because the *in vitro* study using purified human proteins indicated conversion of both factor XII and plasma prekallikrein to active forms (Miyoshi and Shinoda, 1992). However, the mechanism by which *V. vulnificus* metalloprotease can convert human zymogens is not clarified yet. Some other human pathogenic *Vibrio* species including *V. parahaemolyticus*, an etiologic agent causing watery diarrhea, secretes serine proteases (Ishihara et al., 2002; Lee et al., 2002). Although this serine protease can digest various host proteins, its biological or pathogenic actions are not documented.

Herein, we report that *V. vulnificus* metalloprotease can generate active fragments from human zymogens, factor XII and plasma prekallikrein, through cleavage of Arg-Ile, Arg-Val or Gly-Leu peptide bond, whereas *V. parahaemolyticus* serine protease has far less activity to generate active human fragments in spite of the enough potential to activate the guinea pig factor XII-plasma kallikrein-kinin cascade.

MATERIALS AND METHODS

Substances

Bovine serum albumin, teprotide, soybean trypsin inhibitor and hexadimethrine bromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbobenzoxy-Phe-Arg-4-methyl-coumaryl-7-amide (Z-Phe-Arg-MCA) and *t*-butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-Gln-Gly-Arg-MCA) were from Peptide Institute (Minoh, Osaka, Japan). Purified human plasma prekallikrein and factor XII were purchased from Enzyme Research Laboratories Inc. (South Bend, IN, USA), and human plasma was from George-King Bio-Medical Inc. (Overland Park, KS, USA).

Preparations of vibrio proteases

The recombinant *V. vulnificus* metalloprotease (45-kDa) was isolated from the periplasmic fraction of a transformant by ammonium sulfate precipitation followed by column chromatography on a HiLoad 16/10 Phenyl Sepharose column (Amersham Biosciences, Piscataway, NJ, USA) as described (Miyoshi et al., 1997). *V. parahaemolyticus* serine protease (50-kDa) was purified according to the procedures reported by Ishihara et al. (2002). Namely, it was purified from the culture supernatant of a clinical strain by ammonium sulfate fractionation, gel filtration on a HiLoad 26/60 Sephacryl S-200 HR column, and ion-exchange chromatography on a Fractogel TMAE-650 10/2 column. Both proteases thus obtained revealed a single protein band in SDS-PAGE.

Permeability-enhancing activity

The vascular permeability-enhancing activity was measured as described (Miyoshi et al., 1987). The protease sample diluted with 0.9 % NaCl were injected into the dorsal skin of a male Hartley guinea pig (300-400 g), which was previously injected intravenously with 5 % Evans blue (1.0 ml/kg of body weight). At 30-min postinjection, the dorsal skin was flayed, and the area of the blueing spot was measured. In order to test effect of teprotide or soybean

trypsin inhibitor, each substance (5 nmol) was mixed with the sample and administrated intradermally. The animals used were anesthetized with sodium pentobarbital (20 mg/kg of body weight).

Generation of plasma kallikrein in mammalian plasma

In order to remove α-macroglobulin (α M), a broad-spectrum protease inhibitor in mammalian plasma (Sottrup-Jensen, 1989), human or guinea pig plasma was pretreated with the IgG antibody against each α M at 4 ^{o}C for 20 min. Thereafter, the antibody-treated plasma was diluted to 10-fold with the assay buffer (50 mM Tris-HCl buffer supplemented with 0.9 % NaCl, 0.02 % NaN$_3$, 0.01 % bovine serum albumin and 0.05 % hexadimethrine bromide; pH 8.0). This diluted plasma (0.5 ml) was incubated with an appropriate amount of the sample at 30 ^{o}C in the presence of 0.2 mM Z-Phe-Arg-MCA, and the increase of absorbance at 370 nm due to liberation of 7-amino-4-metylcoumarin (AMC) from the substrate was measured periodically. Thereafter, the amount of AMC liberated was calculated.

The experiments were repeated two or three times; however, only the representative results from one series of experiments were shown in "RESULTS".

Activation of human zymogens

To test activation by *V. vulnificus* metalloprotease, purified human plasma prekallikrein (36 nM) or factor XII (68 nM), of which concentration is about one-fifth in human plasma, was mixed with the metalloprotease (10 nM) and the suitable substrate (0.2 mM) in a total of 0.5 ml of the assay buffer. The reaction mixture was incubated at 30 ^{o}C, the increase of absorbance at 370 nm was measured periodically, and the amount of AMC liberated was calculated. In case of *V. parahaemoyticus* serine protease, the serine protease (50 nM) was allowed to act on plasma prekallikrein (36 nM) in the presence or absence of factor XII (68

nM).

The experiments were repeated two or three times; however, only the representative results from one series of experiments were shown in "RESULTS".

Fragmentation of human zymogens

In the absence of bovine serum albumin, *V. vulnificus* metalloprotease (0.1 μM) was allowed to act on plasma prekallikrein (1.4 μM) or factor XII (2.5 μM) at 30 ^{o}C for 1 min. In case of *V. parahaemoyticus* serine protease, the serine protease (0.8 μM) was allowed to act on human factor XII (3.0 μM) for 1 or 5 min. Thereafter, the reaction was terminated by the addition of an equal volume of 10 % trichloroacetic acid. The precipitate was rinsed two times with 100 % ethanol and mixed with 0.5 M Tris-HCl containing 2 % SDS, 5 % 2-mercaptoethanol, 0.05 % bromophenol blue and 70 % glycerol (pH 6.8). The sample thus prepared was treated at 100 ^{o}C for 5 min for SDS-PAGE. SDS-PAGE using a Phast System (Amersham Biosciences) was carried out with a PhastGel^{TM} Gradient 10-15 Gel according to the instructions provided by the manufacturer.

Determination of N-terminal amino acid sequences

The proteins separated by SDS-PAGE were transferred to a PVDF membrane using a Phast System and stained with Coomassie brilliant blue. Thereafter, the membrane was rinsed several times with distilled water, and each protein band was cut off. The N-terminal amino acid sequence was determined by automated Edman degradation with a Model 491 protein sequencer (Applied Biosystems, Foster City, CA, USA) equipped with an online Model 120A PTH-amino acid analyzer.

RESULTS AND DISCUSSION

Activation and fragmentation of human zymogens by V. vulnificus *metalloprotease*

The recombinant *V. vulnificus* metalloprotease was used in the present experiments because of convenience of its purification (Miyoshi et al., 1997). The metalloprotease gene transformed into *Escherichia coli* is expressed by its own promoter, and the enzyme precursor produced is processed normally through autocatalytic removal of the N-terminal propeptide. Therefore, the recombinant enzyme has an equal proteolytic activity to the native enzyme isolated from the culture supernatant of the vibrio. In order to reconfirm the correct maturation of the recombinant *V. vulnificus* metalloprotease, it was allowed to act on human plasma prekallikren or factor XII in the presence of the suitable substrate, Z-Phe-Arg-MCA (for plasma kallikrein) or Boc-Gln-Gly-Arg-MCA (for activated factor XII). Although the recombinant enzyme itself showed no amidolytic activity, steady generation of the amidolytic activity toward each of the substrates was observed in a time-dependent manner (Fig. 1A), indicating activation of either human zymogens by the recombinant enzyme which was maturated correctly.

Various endogenous serine proteases have been documented to achieve activation of the human zymogens through digestion of the N-terminal polypeptides, which results in generation of the active C-terminal fragments (Tanaka et al., 1992; Hovinga et al., 1994). *V. vulnificus* metalloprotease was tested to see if it also causes fragmentation of the human zymogens. When incubated at 30 $^{\circ}$C for 1 min, the metalloprotease could generate the 36-kDa fragment from plasma prekallikrein (Fig. 2A). Analysis of the N-terminal amino acid sequence of the fragment revealed that *V. vulnificus* metalloprotease cleaved Arg^{371}-Ile^{372} peptide bond, of which the cleavage site is identical with those of endogenous serine proteases including activated factor XII (Tanaka et al., 1992). Likewise, the 30-kDa fragment was generated via cleavage of the peptide bond, Arg^{353}-Val^{354} or Gly^{357}-Leu^{358}, when factor XII was incubated with the metalloprotease for 1 min (Fig. 2A). It should be noted that one of

these cleavage sites is identical with that of plasma kallikrein (Hovinga et al., 1994).

Fig. 3 shows the proposed mechanism in the activation of the human factor XII-plasma kallikrein-kinin cascade by *V. vulnificus* metalloprotease. The human cascade may be activated via generation of active C-terminal fragments through hydrolysis of the amino group side of hydrophobic amino acid residues.

On the studies using synthetic oligopeptide substrates, as well as other bacterial metalloproteases of the thermolysin family (Morihara and Tsuzuki, 1971), *V. vulnificus* metalloprotease have been believed to hydrolyze specifically the peptide bond at the amino group side of the P_1' amino acid residue, usually a hydrophobic amino acid residue (Miyoshi and Shinoda, 2000). The results shown in the present study using the native protein substrates are apparently consistent with this conclusion. Namely, *V. vulnificus* metalloprotease was found to cleave Arg-Ile/Val or Gly-Leu peptide bond of human plasma prekallikrein or factor XII.

Activation and fragmentation of human zymogens by V. parahaemolyticus *serine protease*

The serine protease from *V. parahaemolyticus*, a human pathogen causing gastrointestinal diseases, was also found to evoke enough permeability-enhancing reaction in the *in vivo* guinea pig model (data not shown). As well as that by *V. vulnificus* metalloprotease (Miyoshi et al., 1987), the permeability enhancement caused by the serine protease was augmented about 3-fold by simultaneous administration of teprotide, a kininase inhibitor, whereas inhibited almost completely by soybean trypsin inhibitor, a well-known inhibitor of plasma kallikrein (data not shown). Since each inhibitor showed no effect on the proteolytic activity, it might be concluded that *V. parahaemolyticus* serine protease also enhanced vascular permeability through activation of the factor XII-plasma kallikrein-kinin cascade.

To verify activation of the guinea pig cascade, the serine protease was allowed to act on

guinea pig plasma, and generation of plasma kallikrein was traced periodically by measuring liberation of AMC from the substrate. As well as *V. vulnificus* metalloprotease (Miyoshi and Shinoda, 1992; Miyoshi et al., 1994), *V. parahaemolyticus* serine protease could not generate significant amounts of plasma kallikrein because of quickly inactivation by α M (data not shown). However, as shown in Fig. 4, the serine protease could elicit time-dependent generation of plasma kallikrein after a short lag period when allowed to act on α M-free plasma. In case of α M-free human plasma, whereas, far less generation of plasma kallikrein was observed after as long as an 8-min lag period (Fig. 4). It should be emphasized that, in both cases, *V. vulnificus* metalloprotease showed much more efficient generation of plasma kallikrein without the lag period (Fig. 4). These findings suggest indirect generation of kallikrein by *V. parahaemolyticus* serine protease. In order to study the indirect kallikrein generation, the serine protease was allowed to act on purified human plasma prekallikrein in the presence or absence of factor XII. Only in the presence of factor XII, generation of plasma kallikrein was observed after an 8-min lag period (Fig. 1B) indicating that *V. parahaemolyticus* serine protease may slowly generate the active fragment from human factor XII, which in turn converts plasma prekallikrein to kallikrein.

As shown in Fig. 2B, *V. parahaemolyticus* serine protease also generated the 30-kDa fragment from factor XII when the mixture of the concentrated proteins were allowed to incubate at 30 $^{\circ}$C. The analysis of the N-terminal amino acid sequence of the fragment revealed that the Thr^{352}-Arg^{353} peptide bond was cleaved by the serine protease. This cleavage site is only 1 residue toward the N-terminal from that of *V. vulnificus* metalloprotease. Taken together, although *V. parahaemolyticus* serine protease is also a potent activator of the human factor XII-plasma kallikrein-kinin cascade, its action is operated after a long lag-period, and its potential is far less than *V. vulnificus* metalloprotease.

In spite of the powerful ability to generate plasma kallikrein *in vitro* (Fig. 4), in guinea

pig model, *V. vulnificus* metalloprotease elicited slightly weaker permeability-enhancing reaction *in vivo* (data not shown). Thermolysin and related metalloproteases have been documented to have the kininase activity causing digestion of bradykinin (Zevaco and Desmazeaud, 1980; Wolz and Bond, 1990; Carvalho et al., 1992). Therefore, due to its own kininase activity, *V. vulnificus* metalloprotease may attenuate the vascular permeability-enhancing reaction evoked by bradykinin generated *in situ*. In deed, when *V. vulnificus* metalloprotease was allowed to act on bradykinin, the steady hydrolysis and inactivation of bradykinin was observed in a dose-dependent manner. Additionally, carboxypeptidase B, a kininase-like pancreatic enzyme known to reduce the permeability-enhancing reaction induced by guinea pig plasma kallikrein (Imamura et al., 1984), showed negligible effect on the permeability enhancement elicited by *V. vulnificus* metalloprotease.

Production of the metalloprotease by *V. vulnificus* is positively regulated by the quorum-sensing or cell density-dependent system, in which a small extracellular substance called the autoinducer operates as a bacterial pheromone (Kim et al., 2003). Namely, expression of the metalloprotease gene is augmented at stationary growth phase because of sufficient bacterial multiplication followed by accumulation of the autoinducer. Watanabe et al. (2004) recently reported that *V. vulnificus* has prominent potentials to grow and to produce the metalloprotease in human serum, which are thought to be a crucial cause of serious systemic diseases by this species. In addition to them, the present study evidently showed that *V. vulnificus* metalloprotease had sufficient ability to activate the human bradykinin-generating cascade. This finding may explain, at least partially, why only *V. vulnificus* often causes fatal infections in humans.

The guinea pig model has been used for evaluation of abilities of various microbial proteases to activate the factor XII-plasma kallikrein-kinin cascade (Molla et al., 1989;

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Kaminishi et al., 1990; Maruo et al., 1993). However, the present data demonstrated that this model might sometimes cause overestimation of the pathological ability of the protease tested. On guinea pig factor XII and plasma prekallikrein, the amino acid sequences around the cleavage sites are different from those of human zymogens (Semba et al., 1993; Shibuya et al., 1999). These replacements of amino acids may cause significant increase in activation efficiency of the guinea pig zymogens by microbial proteases. Therefore, as suggested by Shibuya et al. (1999), the ability to hydrolyze the synthetic peptides mimicking the cleavage sites of human zymogens may be more credible to evaluate the pathological potentials of the proteases.

In summary, even though it does not show the excellent activity in guinea pig models, *V. vulnificus* metalloprotease may be a suitable enzyme to activate the bradykinin-generating cascade in humans.

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FIGURE LEGENDS

Fig. 1. Activation of human zymogens by vibrio proteases. (A) *V. vulnificus* metalloprotease (10 nM) was mixed with plasma prekallikrein (36 nM) and Z-Phe-Arg-MCA (0.2 mM), or factor XII (68 nM) and Boc-Gln-Gly-Arg-MCA (0.2 mM) in a total of 0.5 ml of the assay buffer. The reaction mixture was incubated at 30 $^{\circ}$C, and liberation of AMC from the substrate was measured periodically. (B) *V. parahaemolyticus* serine protease (50 nM) was allowed to act on plasma prekallikrein (36 nM) in the presence or absence of factor XII (68 nM) at 30 $^{\circ}$C, and liberation of AMC from Z-Phe-Arg-MCA was measured periodically.

Fig. 2. Fragmentation of human zymogens by vibrio proteases. (A) Human plasma prekallikrein (1.4 µM) or factor XII (2.5 µM) was mixed with the assay buffer (-) or 0.1 µM of *V. vulnificus* metalloprotease (+). Thereafter, the reaction mixture was incubated at 30 $^{\circ}$C for 1 min, and subjected to SDS-PAGE using a PhastGel$^{\text{TM}}$ Gradient 10-15 Gel. (B) Human factor XII (3.0 µM) was mixed with the assay buffer (-) or 0.8 µM of *V. parahaemolyticus* serine protease (+). Each mixture was incubated at 30 $^{\circ}$C for 1 or 5 min, and subjected to SDS-PAGE using a PhastGel$^{\text{TM}}$ Gradient 10-15 Gel.

Fig. 3. Schematic representation of activation mechanism of the human factor XII-plasma kallikrein-kinin cascade by *V. vulnificus* metalloprotease.

Fig. 4. Generation of plasma kallikrein in α M-free mammalian plasma by vibrio proteases. At 30 $^{\circ}$C, *V. vulnificus* metalloprotease (20 nM) or *V. parahaemolyticus* serine protease (50 nM) was allowed to act on 0.5 ml of the 10-fold diluted guinea pig or human plasma pretreated with the antibody against α M, and liberation of AMC from Z-Phe-Arg-MCA was measured periodically.
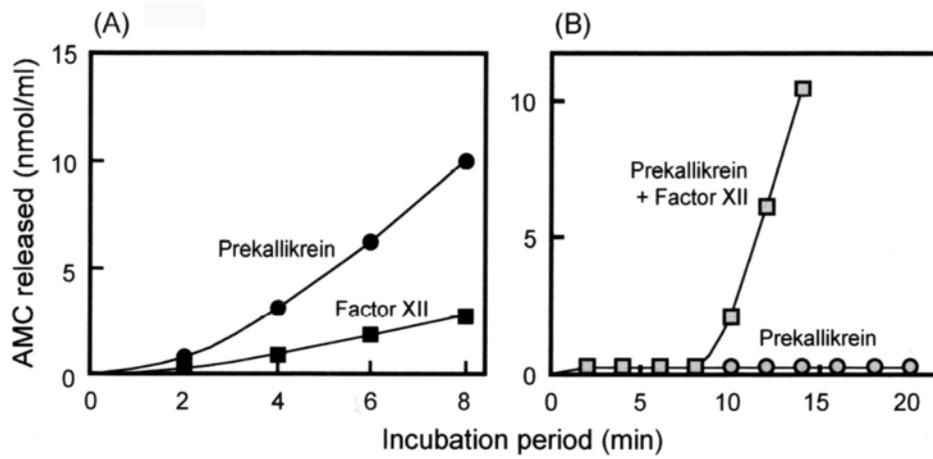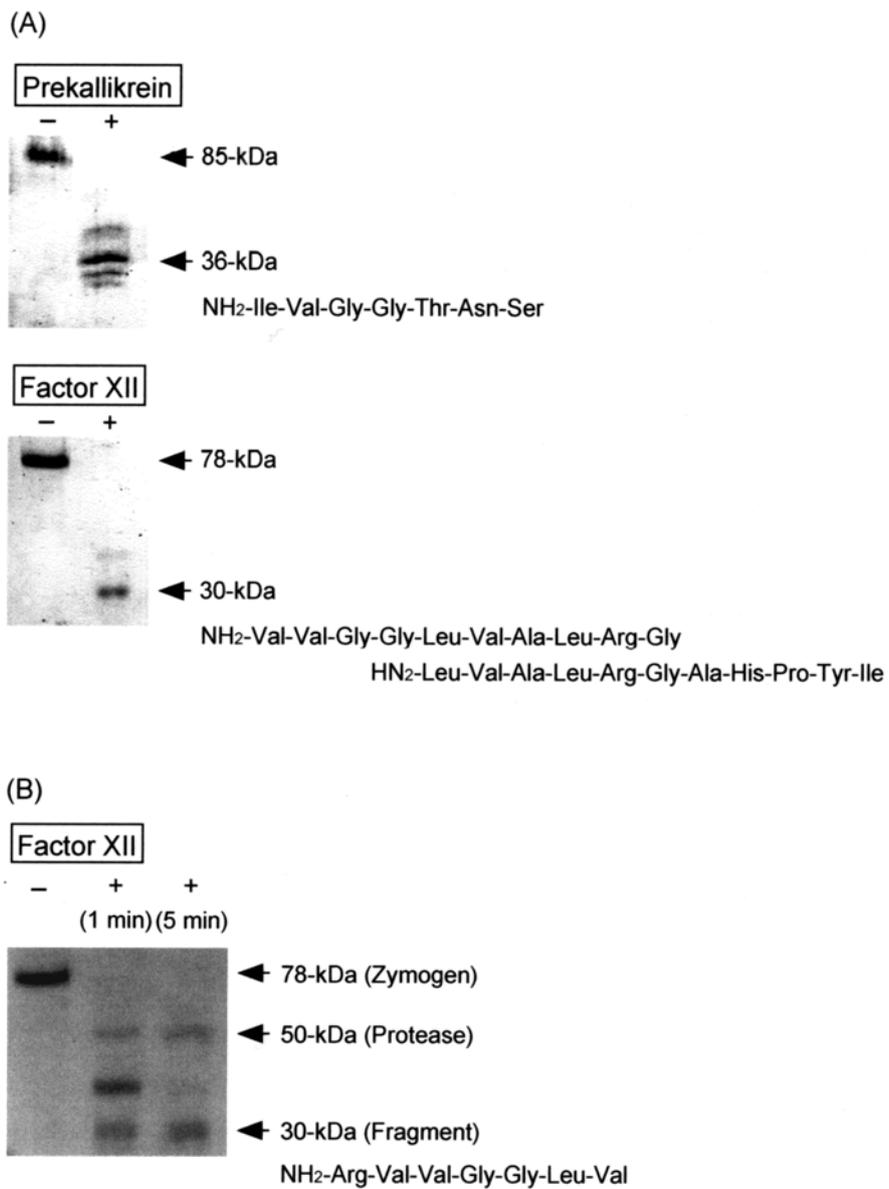
15

Fig. 1

(A)

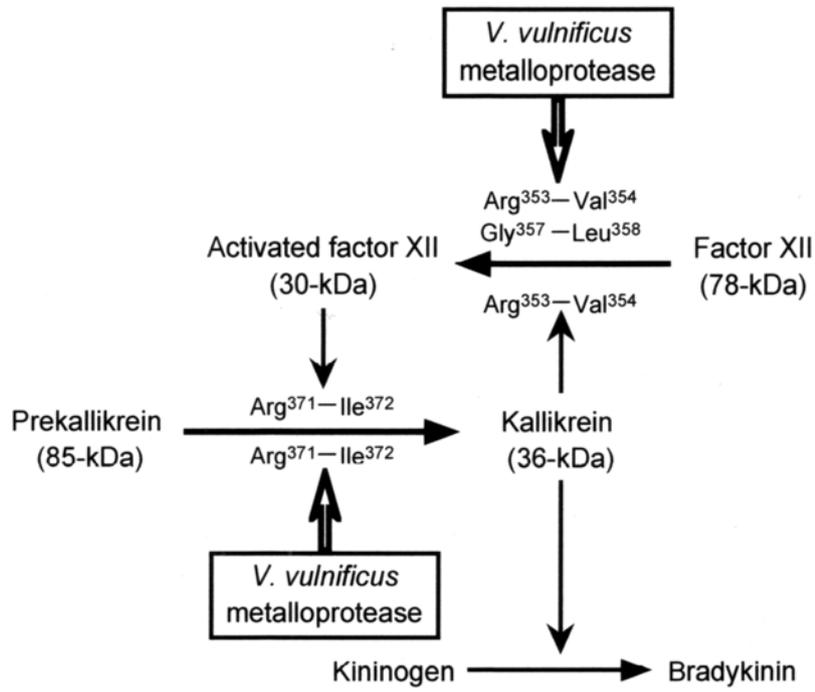AMC released (nmol/ml)

Prekallikrein

Factor XII

(B)

Prekallikrein
+ Factor XII

Prekallikrein

Incubation period (min)

Fig. 2

(A)

Prekallikrein
− +

◄ 85-kDa

◄ 36-kDa

NH2-Ile-Val-Gly-Gly-Thr-Asn-Ser

Factor XII
− +

◄ 78-kDa

◄ 30-kDa

NH2-Val-Val-Gly-Gly-Leu-Val-Ala-Leu-Arg-Gly

HN2-Leu-Val-Ala-Leu-Arg-Gly-Ala-His-Pro-Tyr-Ile

(B)

Factor XII
−   +   +
  (1 min)(5 min)

◄ 78-kDa (Zymogen)

◄ 50-kDa (Protease)

◄ 30-kDa (Fragment)

NH2-Arg-Val-Val-Gly-Gly-Leu-Val

Fig. 3

Fig. 4