Ecotin is a potent inhibitor of the contact system proteases factor XIIa and plasma kallikrein

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Received 21 March 1995; revised version received 19 April 1995

Abstract **Ecotin, a serine protease inhibitor found in the periplasm of *Escherichia coli*, has been characterized as a potent reversible tight-binding inhibitor of the human contact activation proteases factor XIIa (FXIIa) and plasma kallikrein, having K_i values of 89 pM and 163 pM, respectively. Ecotin also inhibited human leukocyte elastase (HLE) with high affinity (K_i = 55 pM). The association rate constants k_{on} for FXIIa and kallikrein were 5.3×10^5 $M^{-1} \cdot s^{-1}$ and 2.9×10^5 $M^{-1} \cdot s^{-1}$, respectively. The dissociation rate constant k_{off} for kallikrein, measured in the presence of HLE to prevent reassociation, was 6.3×10^{-5} s^{-1}; the k_{off} for ecotin with FXIIa was 4.7×10^{-5} s^{-1}. Both FXIIa and kallikrein cleaved ecotin slowly at pH 5.0, identifying Met-84 as the P_1 residue. The potent anticoagulant effect by ecotin is explained by the coincident inhibition of FXIIa, kallikrein, and FXa and suggests that it may be useful in the study of inflammatory or thrombotic disorders such as sepsis or cardiopulmonary bypass.**

Key words: Ecotin; Factor XIIa; Kallikrein; Serine protease inhibitor; Anticoagulant

1. Introduction

Contact activation is a surface mediated pathway responsible in part for the regulation of both inflammation and coagulation [1,2]. The proteins involved in this pathway include factor XII (Hageman factor), prekallikrein (Fletcher factor), high molecular weight kininogen (HMWK), factor XI, and C1 inhibitor. It has been suggested that this plasma protease system plays a significant role in a variety of clinical states including septic shock, adult respiratory distress syndrome, cardiopulmonary bypass, disseminated intravascular coagulation (DIC), and hereditary angioedema [3,4]. Inhibitors of the contact system may therefore play important roles in the regulation of inflammatory and/or thrombotic disorders.

Factor XII is the first zymogen in the intrinsic pathway of blood coagulation and is converted to its active serine protease factor XIIa (FXIIa) in plasma by exposure to negatively charged surfaces such as glass, connective tissue or collagen, endotoxin, and a wide variety of other endogenous or exogenous components. In the presence of HMWK, FXIIa can activate prekallikrein to kallikrein and factor XI to factor XIa; the kallikrein formed can activate more factor XII to FXIIa. Factor XIa generates factor IXa, which in the presence of factor VIIIa leads to the formation of factor Xa (FXa) and thrombin and ultimately results in the formation of a fibrin clot. In addition to the reciprocal feedback activation of surface bound factor XII to FXIIa, plasma kallikrein cleaves HMWK to form bradykinin, a potent endogenous vasodilator, and can activate prourokinase. Both factor XIIa and kallikrein have also been implicated in neutrophil degranulation, regulation of the complement pathway, and plasminogen activation [1,2]. A simplified scheme of the contact activation system leading to inflammation and coagulation is shown in Fig. 1.

Protein inhibitors of proteases play critical roles in the regulation of proteolytic activity in a wide variety of physiological processes. They have been extensively studied from functional, structural, and mechanistic perspectives [5,6]. Ecotin has been previously characterized as a periplasmic protein in *E. coli* and an inhibitor of the pancreatic serine proteases trypsin and chymotrypsin (bovine), porcine elastase, as well as rat mast cell chymase [7–9]. More recently, we have shown that ecotin is a very potent anticoagulant and reversible tight-binding inhibitor of human FXa with a K_i of 54 pM [10]. In this report, we show that ecotin is also a very potent reversible inhibitor of the human contact activation proteases FXIIa and plasma kallikrein as well as human leukocyte elastase (HLE).

2. Materials and methods

2.1. Materials

Ecotin was purified from the periplasm of cultures of *E. coli* 27C7 transformed with the ecotin expression plasmid as previously described [10]. Human FXIIa and human plasma kallikrein were obtained from Enzyme Research Labs. Inc. (South Bend, IN). Human leukocyte elastase was purchased from Elastin Products Co. Bovine trypsin, Triton X-100, and *N*-MeOSuc-Ala-Ala-Pro-Val-7-amido-4-methyl-coumarin (AAPV-MCA) were from Sigma. S2302 (H-D-Pro-Phe-Arg-pNA·2HCl) was obtained from Chromogenix and Spectrozyme P.Kal (H-D-prolyl-hexahydrotyrosyl-arginine-*p*-nitroanilide·diacetate) was from American Diagnostica. Polyacrylamide gels were purchased from Novex; molecular weight standards were from LKB (horse myoglobin peptides) and Novex (Mark 12). All other reagents were of the highest grade commercially available.

2.2. Determination of equilibrium dissociation constants

Enzyme inhibition assays were conducted in a microtiter format using equipment and analytical methods previously described [10]. Ecotin quantitation was carried out by active site titration using N^{α}-benzoyl-L-arginine-*p*-nitroanilide (Bachem) and trypsin; 4-methylumbelliferyl *p*-guanidinobenzoate (Sigma) was used to burst titrate trypsin [10].

FXIIa or kallikrein was quantitated by active site titration using ecotin as a standard and 0.5 mM S2302 as a substrate, assuming a 1:1 complex formation [7,10]; HLE was quantitated by the method of Beatty et al. [11]. There was good agreement between protein concen-

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Abbreviations: HMWK, high molecular weight kininogen; DIC, disseminated intravascular coagulation; FXIIa, factor XIIa; FXa, factor Xa; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; APTT, activated partial thromboplastin time; PT, prothrombin time.

SSDI 0014-5793(95)00466-1

trations determined by active site titrations and the manufacturer's stated concentration. The K_m for S2302 with FXIIa and kallikrein under the conditions used for each enzyme was 125 μM and 165 μM, respectively; the K_m for AAPV-MCA with HLE was 150 μM.

Apparent K_i values (K_i*) for the inhibition of FXIIa and plasma kallikrein by ecotin were measured as follows. Human FXIIa (1.6 or 12.5 nM final concentration) was incubated with quantitated ecotin (0–80 nM final concentration) in buffer A (50 mM Tris, pH 7.5, 20 mM NaCl, and 0.005% Triton X-100) containing 2 mM $CaCl_2$, in a total volume of 180 μl. After 1 h at room temperature to allow equilibration of the enzyme·inhibitor complex, 20 μl of 5 mM S2302 was added and the change in absorbance at 405 nm was monitored. The K_i* for ecotin with kallikrein was determined in a similar manner with kallikrein (1.3 nM or 4.3 nM final concentration) and 0–16 nM ecotin. The K_i* for ecotin with HLE (0.9 nM or 2.1 nM final concentration) was determined in 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl and 0.005% Triton X-100. After incubation at room temperature for 2 h, AAPV-MCA (80 μM final concentration) was added and the change in fluorescence was monitored on a Labsystems Fluoroskan II plate reader with excitation at 355 nm and emission at 460 nm. The kinetic data from each experiment were fit to Eqn. 1:

$$V_i/V_o = 1 - \frac{1 + [I_o]/[E_o] + K_i^*/[E_o] - \sqrt{(1 + [I_o]/[E_o] + K_i^*/[E_o])^2 - (4 \times [I_o]/[E_o])}}{2} \quad (1)$$

where V_i/V_o is the fractional activity (steady-state inhibited rate divided by the uninhibited rate), $[E_o]$ is the total enzyme concentration, and $[I_o]$ is the total ecotin concentration. Values for K_i* were determined by non-linear regression analysis.

2.3. Determination of association rate constants

The association rates of ecotin with FXIIa and kallikrein were determined as follows. At reference time zero, ecotin (14 nM final concentration) was added to FXIIa (10 nM final concentration) and buffer A containing 2 mM $CaCl_2$ in a total volume of 4.0 ml. Immediately thereafter, 150 μl aliquots were removed at intervals over a period of 10 min and added to microtiter wells containing 50 μl of 5 mM S2302. For kallikrein, ecotin (7 nM final concentration) was added to kallikrein (5 nM final concentration) and buffer A in a total volume of 3.0 ml at reference time zero. Immediately thereafter, 150 μl aliquots were removed at intervals over a period of 10 min and added to microtiter wells containing 50 μl of 2.5 mM Spectrozyme P.Kal. The absorbance was monitored at 405 nM and initial rates were determined for each well using the initial linear portion of each data set. The rate constants were determined by measuring free enzyme as a function of time and fitting the data to Eqn. 2:

$$[E] = [E_o] - \frac{[I_o] \times [E_o] \times (e^{t([I_o]-[E_o]) \times k_{on} \times t} - 1)}{[I_o] \times (e^{t([I_o]-[E_o]) \times k_{on} \times t} - [E_o]} \quad (2)$$

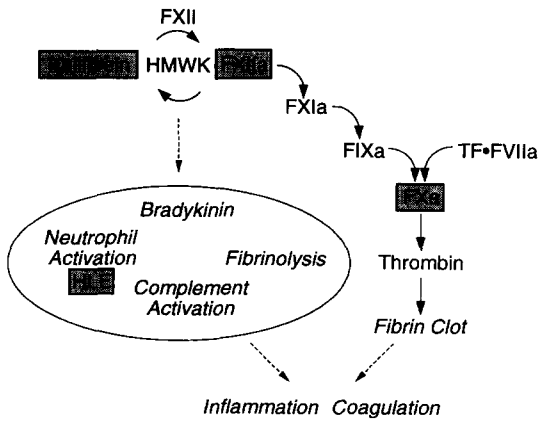

Fig. 1. Simplified scheme of the contact activation system. The serine proteases potently inhibited by ecotin are boxed and shaded. Biological consequences of contact activation are in italics.

where [E] is the concentration of free enzyme at any time t and $[E_o]$ and $[I_o]$ represent the initial concentrations of enzyme and inhibitor, respectively. Values for k_{on} were determined by non-linear regression analysis.

2.4. Determination of dissociation rate constant

The dissociation rate constant of the ecotin·kallikrein complex was measured using HLE to trap the free ecotin released from the complex. Kallikrein (2.4 μM) was incubated at room temperature for 1 h in buffer A in the presence and absence of equimolar ecotin and then at reference time zero, an aliquot was diluted 100-fold into buffer A with and without HLE (410 nM). Aliquots (150 μl) were removed at various times and added to 50 μl of 2.5 mM Spectrozyme P.Kal. Initial rates were measured at 405 nm and product curve data (free enzyme vs. time) were fit to Eqn. 3:

$$[E] = [EI]_0 \times (1 - e^{-k_{off} \times t}) \quad (3)$$

where [E] is the free [kallikrein] at any time t, $[E \times I]_0$ is the concentration of the complex at time zero, and k_{off} is the first-order dissociation rate constant. Values for k_{off} were determined by non-linear regression analysis.

2.5. Active site determination of ecotin by cleavage with FXIIa and kallikrein

In order to determine if and where ecotin is cleaved by FXIIa or kallikrein, ecotin (81.3 μM) and enzyme (3.1 μM) were co-incubated in 100 mM NaOAc pH 5.0, 50 mM $CaCl_2$. After 48 h, aliquots of the reaction mixture were removed and subjected to SDS-PAGE on 16% Tricine gels. These gels were then electroblotted onto polyvinylidene difluoride (PVDF) membranes, the membranes were stained for 30 s in 0.1% Coomassie blue R-250 in 50% methanol, destained, and the bands of interest excised and sequenced as previously described [10].

3. Results and discussion

3.1. Determination of equilibrium dissociation constants

Apparent equilibrium dissociation values (K_i*) were determined using methods derived for tight-binding inhibitors since there was substantial inhibition at concentrations of ecotin comparable to that of the protease [12,13]. The inhibition of FXIIa, kallikrein and HLE by ecotin under equilibrium conditions is shown in Fig. 2. If the inhibitor and substrate compete for the same active site which is almost certainly true since ecotin is cleaved by the enzyme (see below), the true K_i value is generally related to K_i* by the expression $K_i = K_i^*/(1 + [S]/K_m)$, where K_m is the Michaelis constant for substrate [14]. However, since ecotin·enzyme complex dissociation is negligible during the assay (see below), the K_i* for ecotin is equal to the true K_i. In this manner, K_i values of 89 pM and 163 pM were calculated for ecotin binding to FXIIa and kallikrein, respectively (Table 1). The K_i for ecotin with kallikrein of 163 pM measured under equilibrium conditions is in very good agreement with the K_i measured kinetically where the ratio of k_{off}/k_{on} is 217 pM (see below). The K_i value for ecotin with HLE was 55 pM (Table 1); this is about 24-fold higher affinity than has been reported for ecotin with porcine pancreatic elastase [9].

Based on active site titrations with ecotin concentrations $> 100 \times K_i$, the interaction with FXIIa, kallikrein and HLE was stoichiometric, i.e. a 1:1 molar ratio of enzyme-to-ecotin was found for the E·I complexes formed. We previously demonstrated that ecotin exists primarily as a dimer at concentrations above ~390 nM and that the ecotin·FXa complex consists of one ecotin dimer and two FXa molecules [10]. In addition, the trypsin·ecotin complex has also been shown by biochemical methods [7] and by X-ray crystallography [15] to consist of one ecotin dimer and two trypsin molecules. By analogy, we postu-

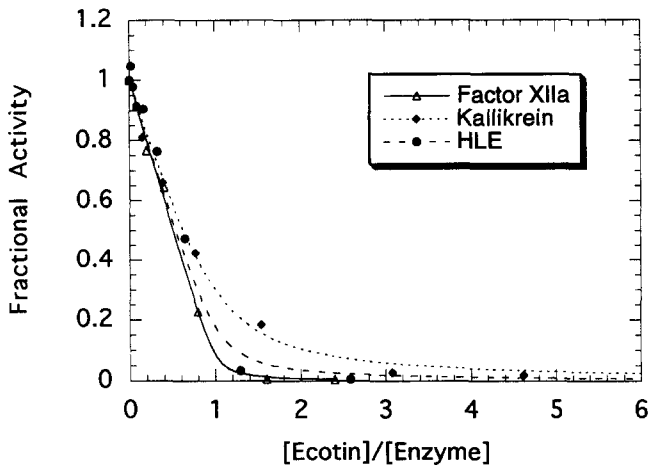

Fig. 2. Determination of the apparent equilibrium dissociation constants of ecotin with factor XIIa, kallikrein, and HLE. The K^*_i values were determined by non-linear regression analysis of the data using Eqn. 1; the curves represent the fractional activity determined from equation 1 using the calculated K^*_i values. The calculated K^*_i values for factor XIIa (△), kallikrein (◆), and HLE (●) were 101 pM, 168 pM, and 80 pM, respectively; the enzyme concentrations were 12.5 nM, 1.3 nM, and 2.1 nM, respectively.

late that inhibition by ecotin at high concentrations involves one ecotin dimer with two protease molecules. The equations used to calculate the various kinetic constants assume that the complex contains one molecule each of enzyme and inhibitor. These equations are appropriate since the equilibrium and kinetic measurements were conducted at a concentration range well below the K_d for ecotin dimerization.

3.2. Determination of association and dissociation rate constants

The rate of association of ecotin with FXIIa or kallikrein was measured during the initial phase of the reaction, where dissociation of the E·I complex is negligible, and the rate constants were calculated from the integrated form of the second-order rate equation [10]. The association of ecotin with FXIIa and kallikrein is rapid with association rate constants (k_{on}) of 5.3×10^5 $M^{-1} \cdot s^{-1}$ and 2.9×10^5 $M^{-1} \cdot s^{-1}$, respectively (Fig. 3; Table 1).

Because the affinity of ecotin for kallikrein is so high, the rate of dissociation of the ecotin·kallikrein complex was measured after a 100-fold dilution into buffer containing a 17-fold molar excess of HLE. Ecotin binds with high affinity to HLE (Table 1), which was used to trap free ecotin and prevent its reassociation with kallikrein. In the absence of HLE, reassociation of ecotin with kallikrein was so rapid that free kallikrein was barely detectable. Progress of dissociation was monitored as the increase in hydrolysis rate of Spectrozyme P.Kal, which is not hydrolyzed by HLE, resulting from the increase in free enzyme with time (Fig. 4). A control lacking ecotin showed that the specific activity of kallikrein (74 OD_{405} $min^{-1} \cdot nmol^{-1}$) was unaffected by the presence of HLE over the time course of the experiment. The time dependent dissociation of the ecotin·kallikrein complex was fit to Eqn. 3; the value for k_{off} is 6.3×10^{-5} s^{-1} (Table 1).

We were unable to measure a dissociation constant for ecotin with FXIIa since reassociation of ecotin with FXIIa was so rapid that free FXIIa was barely detectable. HLE inactivated FXIIa and could therefore not be used to measure the dissociation rate by trapping free ecotin. The dissociation rate constant of ecotin with FXIIa as calculated from $k_{on} \times K_i$ is 4.7×10^{-5} s^{-1} (Table 1).

Based on activated partial thromboplastin time (APTT) clotting assays, approximately 300 nM ecotin is required to prolong the clotting time 10-fold [10]; at this concentration, the interaction of ecotin with FXIIa and kallikrein is essentially irreversible since the K_i is much less than the ecotin concentration [16]. The slow dissociation rates predict a half-life for the E·I complexes of ca. 3 h. Furthermore, the association rates are relatively rapid, suggesting that ecotin could play a significant role in vivo. However, it is difficult to predict the relative degree of inhibition of each enzyme where many other factors may contribute to these interactions.

3.3. Reactive site determination of ecotin with factor XIIa and plasma kallikrein

The interaction of ecotin with FXIIa and kallikrein results in a slow cleavage of the inhibitor by the enzyme under certain in vitro conditions. Fig. 5 illustrates the amount of cleavage of ecotin by FXIIa and kallikrein at pH 5.0 in 48 hours. The N-terminal sequences obtained (Fig. 5) revealed that both FXIIa and kallikrein slowly cleave ecotin between Met-84 and Met-85. These results define Met-84 as the P_1 residue [17] which agrees with studies using trypsin, chymotrypsin, and porcine

Table 1
Equilibrium dissociation and kinetic constants for ecotin with factor XIIa, plasma kallikrein, HLE and factor Xa[a]

Enzyme	K_i (pM)	$k_{on} \times 10^{-5}$ ($M^{-1} \cdot s^{-1}$)	$k_{off} \times 10^5$ (s^{-1})
Factor XIIa	89 ± 51 (n = 5)	5.3 ± 0.3 (n = 3)	4.7[b]
Plasma kallikrein	163 ± 24 (n = 6)	2.9 ± 0.1 (n = 4)	6.3; 4.7[b]
HLE	55 ± 23 (n = 3)	n.d.[c]	n.d.[c]
FXa[d]	54	13.5	6.5

[a] The number of independent determinations is indicated in brackets. [b] Values in parenthesis are calculated from $(K_i) \times (k_{on})$. [c] n.d. = not determined. [d] Taken from Seymour et al. [10].

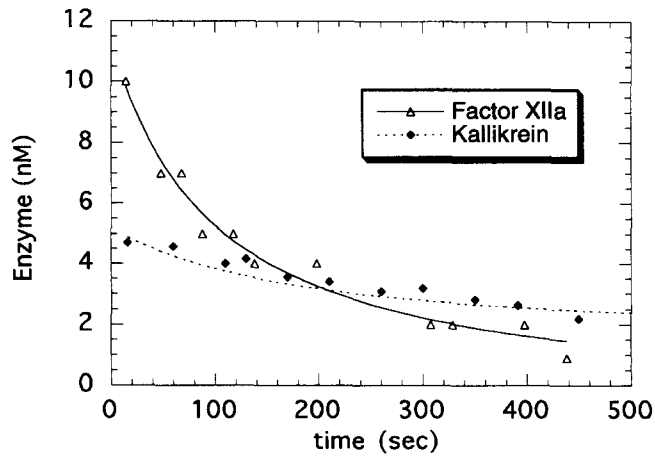

Fig. 3. Determination of ecotin association rates with factor XIIa and kallikrein. The association rate constants for ecotin were determined by nonlinear regression analysis of the data using Eqn. 2; the curves represent the free enzyme concentration determined from Eqn. 2 using the calculated k_{on} values. The data shown yielded k_{on} values of 5.6×10^5 $M^{-1} \cdot s^{-1}$ for FXIIa (△) and 2.9×10^5 $M^{-1} \cdot s^{-1}$ for kallikrein (◆).

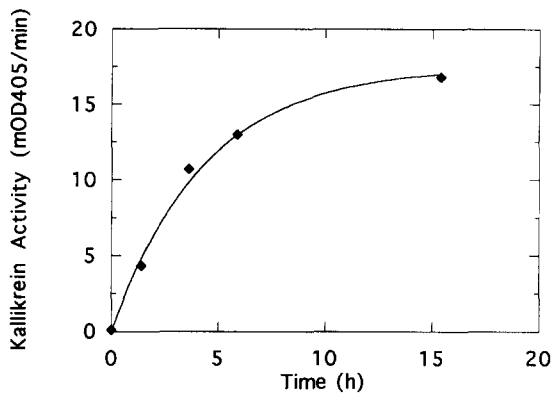

Fig. 4. Determination of ecotin dissociation rate with kallikrein. The dissociation of ecotin from kallikrein in the presence of HLE to prevent reassociation is shown (◆). The curve results from non-linear regression analysis of the data fit to equation 3 using the calculated k_{off} value of 6.3×10^{-5} s^{-1}, where the measured velocity is proportional to free enzyme.

pancreatic elastase [8] and FXa [10]. In studies with these proteases, the replacement of the P_1 methionine residue with various sidechains led to mutants that were still potent inhibitors [9,18], suggesting that residues other than P_1 significantly contributed to binding. The X-ray crystal structure of the ecotin·trypsin complex has shown that a second discrete and distal binding site exists [15].

3.4. Anticoagulant properties

We previously demonstrated that ecotin is a potent anticoagulant as assessed by both APTT and prothrombin time (PT) assays; a 10-fold prolongation of clotting times was found at concentrations of ca. 0.3 μM and ca. 2 μM, respectively [10]. The prolonged clotting times are consistent with inhibition of FXa; however, the greater potency observed in the APTT assay, which measures intrinsic coagulation pathway activation, likely reflects the additional inhibition of the contact activation proteases FXIIa and plasma kallikrein. Thus, the coincident inhibition of FXa, FXIIa, and kallikrein by ecotin leads to a more potent anticoagulant effect than one due to the inhibition of FXa alone.

3.5. Other inhibitors of contact activation proteases

The contact system is primarily regulated by C1 inhibitor, a naturally occurring human protein inhibitor belonging to the serpin family of protease inhibitors. C1 inhibitor binds irreversibly to FXIIa and kallikrein and is the primary physiological inhibitor of both of these proteases; α_2-macroglobulin is another significant inhibitor of kallikrein [1,2]. In the presence of heparin, antithrombin-III can inhibit FXIIa and kallikrein; however, it inhibits thrombin and factor Xa (FXa) more effectively. Kallikrein and factor XIIf, a 30 kDa fragment containing the serine protease domain of FXII, are more potently inhibited by a mutant form of α_1-proteinase inhibitor containing an arginine in the P_1 position than by C1 inhibitor [19,20].

A number of other exogenous protein inhibitors of FXIIa and kallikrein have been previously identified, although none bind with the very high affinity of ecotin. *Curcurbita maxima* trypsin inhibitor III (CMTI III) is a 29 amino acid protease inhibitor containing 3 disulfide bonds isolated from squash seeds which inhibits FXIIa with a K_i of 3 nM [21]. This inhibitor (also known as pumpkin seed Hageman factor inhibitor) does not inhibit kallikrein or thrombin, but weakly inhibits plasmin and FXa [22]. In addition, corn contains a 112 residue inhibitor known to inhibit only trypsin and FXIIa [23]. Plasma kallikrein is also reversibly inhibited by bovine basic pancreatic trypsin inhibitor (BPTI, aprotinin), which also inhibits plasmin and a number of other serine proteases and has been used to treat patients with acute pancreatitis and during cardiopulmonary bypass [24,25]. Replacement of the P_1 lysine residue with arginine resulted in a mutant BPTI having a K_i of 15 nM for kallikrein, which was 20-fold higher affinity than with BPTI itself [26]. It is interesting to note that the active site binding loop of BPTI from P_2 to P'_1 is reported to be isostructural with the binding loop of ecotin [15].

3.6. Implications

The physiological role of ecotin remains unknown. However, since ecotin does not inhibit any known *E. coli* proteases, its location in the periplasm has suggested a role in protecting the bacteria from proteases found in the mammalian gut [7]. The inhibition of the pancreatic enzymes which are present in the mammalian gastrointestinal tract supports this hypothesis. The potent inhibition of FXIIa, plasma kallikrein, and FXa by ecotin was unexpected and is somewhat more difficult to rationalize. In addition, ecotin potently inhibited HLE, a serine pro-

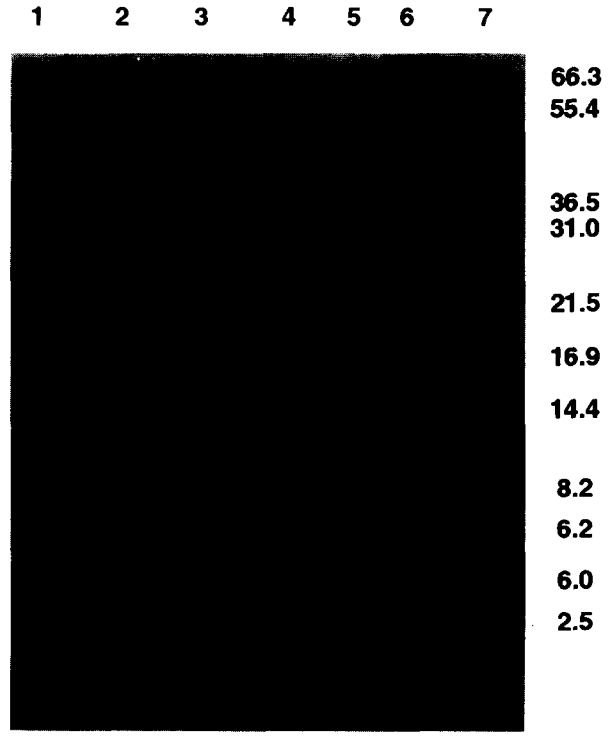

Fig. 5. Cleavage of ecotin by FXIIa and kallikrein and reactive site determination. Samples were incubated as described in the text for 48 h at room temperature, reduced, run on 16% Tricine gels and blotted onto PVDF. Lane 1 = ecotin; lane 2 = ecotin with FXIIa; lane 3 = ecotin with kallikrein; lane 4 = kallikrein; lane 5 = FXIIa; and lanes 6 and 7 = molecular weight markers from Novex and LKB, respectively, with the associated M_r values $\times 10^{-3}$. Arrows indicate the bands which were later excised from the blot and sequenced. The amino-terminal sequence of the lower bands in lanes 2 and 3 (~6.2 kDa) were MA-P; the amino-terminal sequence of the upper bands in lanes 2 and 3 (~8.2 kDa) was AESVQ and corresponded to the amino-terminus of ecotin.

tease found in azurophilic granules of neutrophils, which is released upon activation and has been implicated in the proteolytic destruction of connective tissue proteins and the pathogenesis of a variety of inflammatory disorders [27]. A variety of serine proteases including FXa, FXIIa, kallikrein, and HLE become activated or released during septic shock [3,4]. Inhibition of FXa blocks DIC, however it does not prevent shock or organ damage [28]. Conversely, inhibition of FXIIa can prevent hypotension, but does not block DIC [29]. Since ecotin potently inhibits FXa, FXIIa, and kallikrein as well as HLE, it is tempting to speculate that ecotin, a protein derived from *E. coli*, may provide an effective tool for studying and potentially modulating the lethal effects of Gram-negative bacteria induced septicemia.

Acknowledgements: The authors would like to thank Bill Henzel for the sequencing data.

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