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Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood-clotting proteases and trypsin

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Seventy-four peptide amides of 7-amino-4-methylcoumarine (Mec) of the type Boc-Xaa-Yaa-Arg-NH-Mec were newly synthesized and tested to find specific substrates for blood-clotting proteases and trypsin. The Xaa and Yaa residues of these substrates have been replaced by 12 and 15 different amino acids, respectively. Among these peptides, the followings were found to be most sensitive substrates for individual enzymes: Boc-Asp(OBzl)-Pro-Arg-NH-Mec (k_{cat} = 160 s^{-1}, K_m = 11 μM, k_{cat}/K_m = 15000000 M^{-1} s^{-1}) for human α-thrombin, Z-<Glu-Gly-Arg-NH-Mec (k_{cat} = 19 s^{-1}, K_m = 59 μM, k_{cat}/K_m = 320000 M^{-1} s^{-1}) for bovine factor Xa, Boc-Gln-Gly-Arg-NH-Mec (k_{cat} = 5.8 s^{-1}, K_m = 140 μM, k_{cat}/K_m = 42000) for bovine factor XIIa, Boc-Asp(OBzl)-Ala-Arg-NH-Mec (k_{cat} = 9.2 s^{-1}, K_m = 120 μM, k_{cat}/K_m = 77000 M^{-1} s^{-1}) for bovine activated protein C, and Boc-Gly-Phe-Arg-NH-Mec (k_{cat} = 29 s^{-1}, K_m = 230 μM, k_{cat}/K_m = 130000 M^{-1} s^{-1}) for bovine plasma kallikrein. Moreover, Boc-Glu(OBzl)-Ala-Arg-NH-Mec (k_{cat} = 46 s^{-1}, K_m = 370 μM, k_{cat}/K_m = 120000 M^{-1} s^{-1}) was newly found as a good substrate for human factor XIa. Bovine trypsin effectively hydrolyzed peptide-NH-Mec substrates containing Ala and Pro at the P2 site. The most reactive substrate was Boc-Gln-Ala-Arg-NH-Mec (k_{cat} = 120 s^{-1}, K_m = 6.0 μM, k_{cat}/K_m = 20000000 M^{-1} s^{-1}).

Peptide amides of 7-amino-4-methylcoumarine (Mec) originally developed for the sensitive assay for α-chymotrypsin [1] and an aminopeptidase [2] have been widely used for the investigation of various proteases and peptidases including elastase [3], Xaa-prolyl dipeptidase [4], pyroglutamyl peptidase [5] and carboxypeptidases [6]. We reported previously specific and sensitive peptide-Mec substrates for α-thrombin [7], factor Xa [7], factor XIIa [8], activated protein C [9], plasma kallikrein [7], urinary kallikrein [7, 10], plasmin [11], urokinase [7], and horseshoe crab clotting factors [12]. Most of these substrates were designed based on the information from the COOH-terminal sequences of the 'activation peptides', which are liberated during the conversions of plasma serine protease zymogens to their active enzymes. The utility of these fluorogenic peptide substrates has been established for the assay of trace amounts of enzymes because of their high sensitivities. Moreover, the specific substrates for α-thrombin, factor Xa, plasma kallikrein and urokinase so far developed by our group [7] are now commercially available.

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Abbreviations. -NH-Mec, 4-methylcoumaryl-7-amide; Boc, *N-tert*-butoxycarbonyl; Z, benzyloxycarbonyl; Bzl, benzyl, <Glu, 5-oxopyrrolidone carboxylate (pyroglutamyl); -NH-Np, *p*-nitroanilide; ONp, *p*-nitrobenzyl ester.

Enzymes. Thrombin (EC 3.4.21.5); coagulation factor Xa (EC 3.4.21.6); coagulation factor IXa (EC 3.4.21.22); coagulation factor VIIa (EC 3.4.21.21); coagulation factor XIIa (EC 3.4.21.38); coagulation factor XIa (EC 3.4.21.27); plasma kallikrein (EC 3.4.21.34); trypsin (EC 3.4.21.4).

To extend the useful range of fluorogenic substrates, we have systematically synthesized a series of peptide-NH-Mec substrates, which contain different sequences with Boc-Xaa-Yaa-Arg-NH-Mec, in which the Xaa and Yaa residues of these substrates have been replaced by 12 and 15 different amino acids, respectively. Thus, the purpose of this study is to find more specific and sensitive substrates for blood-clotting proteases and trypsin, and to investigate mainly the contribution of the P2 and the P3 sites to the enzyme hydrolysis.

MATERIALS AND METHODS

Materials

Bovine pancreatic trypsin (type XI), bovine serum albumin and *p*-nitrophenyl *p'*-guanidinobenzoate · HCl and 4-methylumbelliferyl *p*-guanidinobenzoate · HCl were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fluorogenic peptide substrates were synthesized by standard chemical procedures, using Arg-NH-Mec · HCl as a starting material [2]. The products were purified further by silica gel column chromatography, partition chromatography and adsorption chromatography. The purity of these synthetic substrates was confirmed by thin-layer chromatography, high-performance liquid chromatography, elemental analysis and amino acid analysis. Factor Xa [13], factor IXaα [13], factor XIIa [14], plasma kallikrein [15], activated protein C [13] from bovine plasma, and α-thrombin [16], factor IXaβ [17] and factor XI [18] from human plasma were highly purified by the published methods. Human factor XI was converted to factor XIa by bovine factor XIIa, according to the method

of Kurachi and Davie [19]. Human factor VIIa was kindly provided by Dr Walter Kisiel (Department of Pathology, School of Medicine, University of New Mexico, Albuquerque).

Kinetic analysis

Enzyme activity was measured at 37°C in 50 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl, 1 mM $CaCl_2$ and 0.1 mg/ml of bovine serum albumin. Stock solutions of substrates were prepared in dimethylformamide and aliquots were diluted with the buffer before use. To a cuvette containing 1.0 ml of the buffer thermostatted at 37°C was added 20 µl of substrate solution. The initial rate of hydrolysis was measured fluorometrically with excitation at 380 nm and emission at 440 nm, after adding 20 µl enzyme solution. The substrate concentrations adjusted from 1 µM to 2 mM included the concentrations at both sides of the K_m values. Rough screenings for identification of suitable substrates were first performed at the substrate concentration of 0.2 mM and enzyme concentration of about 10 nM. Kinetic parameters were determined by direct linear plotting [20]. The concentrations of trypsin, bovine factor Xa, plasma kallikrein, activated protein C and human α-thrombin were determined by the active-site titration with *p*-nitrophenyl *p'*-guanidinobenzoate [21]. For bovine factor XIIa and human factor XIa, the concentrations were determined with 4-methylumbelliferyl *p*-guanidinobenzoate [22]. Protein concentrations of bovine and human factors IXa and human factor VIIa were estimated by assuming $A^{1\%}_{280\,nm} = 10$.

RESULTS AND DISCUSSION

For individual enzymes, we first screened the 74 peptide-NH-Mec substrates as described in Materials and Methods. Supplement Table 1 shows a comparison of the rates of hydrolysis of different peptide-NH-Mec substrates by α-thrombin, activated protein C, plasma kallikrein, and factors Xa, XIa and XIIa. Based on these results, the reactive and good substrates for individual enzymes were selected and a series of kinetic parameters were determined.

Human α-thrombin

The most reactive substrate for α-thrombin among peptide-Mec substrates shown in Supplement Table 2 was Boc-Asp(OBzl)-Pro-Arg-NH-Mec with an extremely high $k_{cat}/K_m = 15000000\ M^{-1}\ s^{-1}$. This value was comparable to that of the sensitive substrate for bovine trypsin, as described later. The k_{cat}/K_m value for this substrate was 2.7-fold higher than that for the commercially available substrate, Boc-Val-Pro-Arg-NH-Mec. α-Thrombin effectively hydrolyzed substrates containing a Pro residue at the P2 site, which was clearly demonstrated by comparing the kinetic constants for Boc-Gly-P2-Arg-NH-Mec substrates (Supplement Fig. 1 a). Apolar and non-aromatic residues such as Pro and Ala were most favorable. The contribution of a Pro residue at the P2 site to the $1/K_m$ value was especially greater than that of any other amino acid residues. On the other hand, the replacement of Pro at the P2 site by Phe particularly caused the k_{cat} to be reduced to 0.4% (Supplement Table 2 and Fig. 1 a).

The importance of apolar residues adjacent to the susceptible bond, especially a Pro residue at the P2 site [7], has also been reported by using peptide *p*-nitroanilides [23–25], peptide thioesters [24, 26], peptide chloromethanes [27, 28], fibrinopeptide analogs [29], and polypeptide substrates [30, 31]. This specificity of α-thrombin is presumed to be the result from an apolar binding site adjacent to the catalytic site in α-thrombin [32]. In contrast, the peptide-NH-Mec substrates with a Phe residue at the P2 site were less reactive. Stone and Hofsteenge [33] and Cho et al. [24] also reported that α-thrombin cleaves poorly peptide-*p*-nitroanilide substrates with a Phe residue at the P2 site. On the other hand, McRae et al. [26] have found that the P2-Phe derivative is a good substrate for α-thrombin, using peptide thioesters. This difference seems to be due to the rate-limiting step of the peptide and ester hydrolysis, that is, rate-limiting acylation for amides and rate-limiting deacylation for esters [34].

For the P3 site, aromatic and bulky groups such as Glu(OBzl), Phe and Tyr, seemed to be good, based on the results with their $1/K_m$ values (Supplement Fig. 1 b). In contrast, an acidic side chain of Glu at the P3 site appeared to reduce both k_{cat} and $1/K_m$ values. For the P4 site, the Z group was more effective than the Boc group. For example, the k_{cat} value of Z-Leu-Gly-Arg-NH-Mec is 2.8 times as high as that of Boc-Leu-Gly-Arg-NH-Mec (Supplement Table 2). Removal of the Z group from Z- < Glu-Gly-Arg-NH-Mec resulted in a 22-fold decrease in the k_{cat} value with only a twofold decrease in the $1/K_m$ value (Supplement Table 2 and Fig. 1 b). We, therefore, synthesized Z- < Glu-Pro-Arg-NH-Mec to obtain a better substrate for α-thrombin. However, the k_{cat}/K_m value for this substrate was lower than that for Boc-Asp(OBzl)-Pro-Arg-NH-Mec (Supplement Table 2).

Bovine factor Xa

The most sensitive substrate for factor Xa was found to be Z- < Glu-Gly-Arg-NH-Mec (Supplement Table 3). The k_{cat}/K_m value for this substrate was 5.6-fold higher than that for the commercially available substrate, Boc-Ile-Glu-Gly-Arg-NH-Mec.

The contributions of the P2 site were investigated (Supplement Fig. 2 a). The P2-Gly was most effective as previously reported [7, 24, 26, 35, 36], resulting in the increase of the k_{cat} value. In addition, the Gly and Ala residues contributed to increase the $1/K_m$ value. The Phe residue was obviously effective with regard to the k_{cat} value.

Supplement Fig. 2 b demonstrates the contribution of the P3 and the P4 sites. The k_{cat} value for Z- < Glu-Gly-Arg-NH-Mec was strikingly high. The contribution of < Glu at the P3 site and of the Z group at the P4 site to the k_{cat} value was also observed for human α-thrombin (Supplement Fig. 1 b). The substitution of Z for Boc in Boc-Leu-Gly-Arg-NH-Mec caused a 2.5-fold increase in the k_{cat}/K_m value (Supplement Table 3 and Fig. 2 b). Substrates with bulky apolar residues at the P3 site such as Glu(OBzl) and Leu affected on increasing the $1/K_m$ value. However, those with charged groups such as Glu and Lys at the P3 site resulted in poorer substrates, suggesting the existence of an apolar binding site near the catalytic center of bovine factor Xa.

Bovine activated protein C

Boc-Asp(OBzl)-Ala-Arg-NH-Mec was found to be the most sensitive substrate, which showed a 3.3-fold higher k_{cat}/K_m value than that of the commercially available substrate, Boc-Leu-Ser-Thr-Arg-NH-Mec (Supplement Table 4).

The substrate specificity of activated protein C towards its natural substrates remains unknown, although one of the

cleavage sites for the inactivation of factor VIIIa has been reported by Eaton et al. [37]. The specificity of this enzyme has been examined by using peptide-NH-Mec substrates [9], peptide *p*-nitroanilides [33], thioesters [24], and peptide-chloromethane inhibitors [33]. The sensitive substrates so far known were as follows: Boc-Leu-Ser-Thr-Arg-NH-Mec with k_{cat}/K_m = 25000 M^{-1} s^{-1} [9], Z-Ser-Phe-Arg-NH-Np with k_{cat}/K_m = 65000 M^{-1} s^{-1} [24], and D-Ile-Pro-Arg-NH-Np with k_{cat}/K_m = 330000 (for human activated protein C) [33]. The enzyme has been demonstrated to select an apolar residue such as Phe at the P2 site [24] and an apolar D-amino acid residue at the P3 site [33]. Moreover, Ohno et al. [9] and Cho et al. [24] have indicated the importance of Ser at the P3 site for effective hydrolysis. Unlike factor Xa, activated protein C accommodates a bulky residue such as Val or Asp(OBzl) at the P2 and P3 sites (Supplement Fig. 3a, b and c). These results suggest the existence of a considerably hydrophobic region near the catalytic site of bovine activated protein C.

Unfortunately, Boc-Asp(OBzl)-Ala-Arg-NH-Mec is also a sensitive substrate for α-thrombin (Supplement Table 2). Therefore, for measurement of the activity of protein C in plasma, α-thrombin has to be inactivated with antithrombin III plus heparin or hirudin prior to the assay using this substrate.

Bovine factor XIIa

Factor XIIa preferentially hydrolyzed substrates containing Gly, Pro and Ala residues at the P2 site (Supplement Table 1). The sensitive substrate was found to be Boc-Gln-Gly-Arg-NH-Mec (Supplement Table 5), and a Gly residue at the P2 site appeared to contribute to the k_{cat} value (Supplement Fig. 4a).

Supplement Fig. 4b shows the effect of the P3 site. The Gln and the Lys residues at the P3 site were favorable, which play an important role in the k_{cat} value. On the other hand, the P3-Glu resulted in a decrease in the k_{cat} value. Cho et al. [24] have also observed that human factor XIIa is quite reactive towards the *p*-nitroanilide substrates containing a Lys residue at the P3 site. In fact, the cleavage site of human factor XI hydrolyzed by human factor XIIa contains a Lys residue at the P3 site [18].

We also synthesized a pentapeptide-NH-Mec, Boc-Ala-Pro-Arg-Pro-Arg-NH-Mec, the sequence of which originates from that located in the reactive site of popcorn factor XIIa inhibitor [38]. However, the k_{cat}/K_m value of factor XIIa for this substrate was 2.8-fold lower than that for the Boc-Gln-Gly-Arg-NH-Mec substrate (Supplement Table 5).

Human factor XIa

Cho et al. [24] reported a synthetic substrate for bovine factor XIa, Z-Glu-Gly-Arg-NH-Np with k_{cat}/K_m = 4100 M^{-1} s^{-1}. We found a new sensitive substrate for human factor XIa, Boc-Glu(OBzl)-Ala-Arg-NH-Mec, with k_{cat}/K_m = 120000 M^{-1} s^{-1}. Human factor XIa exhibited the specificity towards Ala and Pro residues at the P2 site (Supplement Table 1). The contributions of the P3 site were investigated by using Boc-P3-Ala-Arg-NH-Mec substrates (Supplement Fig. 5). Facor XIa accommodated bulky apolar residues such as Glu(OBzl) and Ser(Bzl) at the P3 site. The P3-Gln was very effective with regard to the k_{cat} value, whereas it was unfavorable in respect to the $1/K_m$ value.

The specificity of facor XIa towards peptide-NH-Mec substrates is quite different from that towards a natural substrate. The amino acid sequence of the cleavage site required for the activation of factor IX by factor XIa has been identified as -Leu-Thr-Arg-Ala- [39]. However, the k_{cat}/K_m value for the substrate of Boc-Leu-Thr-Arg-NH-Mec synthesized here was 10-fold lower than that for the best substrate (Supplement Table 6).

Boc-Glu(OBzl)-Ala-Arg-NH-Mec is also one of the sensitive substrates for trypsin (Supplement Table 8). Thus, the substrate specificity of human factor XIa towards peptide-NH-Mec is very similar to that of trypsin.

Bovine plasma kallikrein

The substrate specificity of plasma kallikrein has been studied with peptide-*p*-nitroanilides [26] and peptide-NH-Mec [7]. Plasma kallikrein exhibits a very strict specificity towards a Phe residue at the P2 site [7, 26]. In the present study, Boc-Gly-Phe-Arg-NH-Mec was found to be the most selective substrate (Supplement Table 7). However, its k_{cat}/K_m value (130000 M^{-1} s^{-1}) was only 1.3 times as high as that of the commercially available substrate, Z-Phe-Arg-NH-Mec. The present data also indicate that extension of the peptide chain of Z-Phe-Arg-NH-Mec has little effect on the reactivity. The P3-Gly of the sensitive substrate may contribute to facilitate the Phe at the P2 site to be orientated more susceptibly to the active site of plasma kallikrein, because of its large conformational space in the Ramachandran map.

Plasma kallikrein also exhibit the specificity towards a Thr residue at the P2 site, like activated protein C. Bovine plasma kallikrein shows a 1.8-fold higher k_{cat}/K_m value for Boc-Met-Thr-Arg-NH-Mec than that of activated protein C (Supplement Tables 4 and 6). In fact, the amino acid sequence of the cleavage site required for the activation of human factor XII by plasma kallikrein is -Met-Thr-Arg-Val- [40].

Factor IXa and factor VIIa

McRae et al. [26] have reported that bovine factor IXa is reactive to the peptide thioesters and amides containing Phe or Trp residues at the P2 site, such as Z-Trp-Arg-SBzl with k_{cat}/K_m = 92000 M^{-1} s^{-1} and Z-Trp-Arg-NH-Mec with k_{cat}/K_m = 73 M^{-1} s^{-1}. On the other hand, Zur and Nemerson [41] previously found that bovine factor VIIa efficiently hydrolyzes an *N*-acylated arginine ester, Z-Arg-ONp with k_{cat}/K_m = 9000 M^{-1} s^{-1} [41].

In the present study, we could not find any good substrates for factors IXa and VIIa. The sequences located close to the cleavage sites required for the activations of factors X [42–44] and IX [39, 45] were taken as a guideline for synthesizing substrates, that is, Boc-Val-Val-Arg-NH-Mec, Boc-Leu-Thr-Arg-NH-Mec, and Boc-Phe-Ser-Arg-NH-Mec. However, these substrates were essentially unsusceptible to factors IXa and VIIa. These results indicate that factors IXa and VIIa may have a very strict specificity and recognize both of the NH_2-terminal (P1, P2 and P3) and the COOH-terminal (P1′, P2′ and P3′) sides for hydrolysis of the peptide bond, like renin [46].

Bovine trypsin

Trypsin hydrolyzed all the peptide-NH-Mec substrates quite well, and the k_{cat}/K_m values for tripeptide-NH-Mec substrates ranged over $1.6-200\times10^5$ M^{-1} s^{-1} (Supplement Table 8). The most reactive substrate was found to be Boc-Gln-Ala-Arg-NH-Mec.

Table 1. *Relative activities of blood clotting proteases and trypsin*
The k_{cat}/K_m value for the hydrolysis of Boc-Gly-Phe-Arg-NH-Mec by human α-thrombin was taken as 1.0. A dash (——) indicates not determined. The value for the most sensitive substrate for individual proteases is underlined

Substrates	Human α-thrombin	Bovine Xa	Bovine activated protein C	Bovine XIIa	Human XIa	Bovine plasma kallikrein	Trypsin
P4 P3 P2 P1							
Boc-Asp(OBzl)-Pro-Arg-NH-Mec	<u>2542</u>	1.1	4.9	2.7	—	—	2203
Z- <Glu-Gly-Arg-NH-Mec	20	<u>54</u>	—	—	—	0.4	237
Boc-Asp(OBzl)-Ala-Arg-NH-Mec	203	—	<u>13</u>	—	—	—	814
Boc- Gln -Gly-Arg-NH-Mec	7.6	3.2	—	<u>7.1</u>	—	0.4	830
Boc-Glu(OBzl)-Ala-Arg-NH-Mec	119	—	3.1	—	<u>20</u>	—	2881
Boc- Gly -Phe-Arg-NH-Mec	1.0	—	1.9	—	—	<u>22</u>	107
Boc- Gln -Ala-Arg-NH-Mec	105	—	—	2.9	—	1.4	<u>3390</u>

Trypsin cleaved substrates with Ala and Pro residues at the P2 site effectively (Supplement Fig. 7a). Basic side chains at the P2 site were also effective with regard to the $1/K_m$ value. On the other hand, the substitution of Phe for Ala at the P2 site in Boc-Gly-Phe-Arg-NH-Mec resulted in a decrease in both k_{cat} and $1/K_m$ values. As shown in Supplement Fig. 7b, a Gln residue at the P3 site exhibited an increase in the k_{cat} value. The replacement of the P3-Gln by Glu and Glu(OBzl) caused a slight decrease in the k_{cat}/K_m value. The importance of the L-isomer in the P3 site was found by comparing the kinetic parameters between Boc-Gln-Ala-Arg-NH-Mec and Boc-D-Gln-Ala-Arg-NH-Mec (Supplement Table 8). The L-isomer was 2.3-fold more active than the D-isomer, which was due to an increase in the $1/K_m$ value. Substrates with aromatic residues or apolar residues at the P3 site resulted in poorer substrates (Supplement Fig. 7b).

In comparison with the contribution of the P2 and the P3 sites, we found that the experimentally measured k_{cat}/K_m values agree well with calculated ones, using the following equation: calculated k_{cat}/K_m value for Boc-P3-P2-Arg-NH-Mec $= A \times B/C$, where A, B, and C are experimentally determined by the k_{cat}/K_m values for Boc-P3-Gly-Arg-NH-Mec, Boc-Gly-P2-Arg-NH-Mec, and Boc-Gly-Gly-Arg-NH-Mec. These parameters for 14 peptide substrates are shown in Supplement Table 8. By comparing the measured and calculated parameters, the correlation coefficient, $r = 0.94$, was obtained (Supplement Fig. 8). This result indicates that the P2 and the P3 sites independently contribute to the catalytic efficiency for bovine trypsin.

In conclusion, we have developed more sensitive peptide-NH-Mec substrates than commercially available ones for α-thrombin, factor Xa, factor XIIa, activated protein C, plasma kallikrein, and trypsin. For human factor XIa, we discovered the sensitive substrate which enables a rapid and accurate assay at the amount of 10 ng protein. In general, blood-clotting proteases except for plasma kallikrein exhibit the relatively strict specificity towards peptide-NH-Mec substrates containing apolar (Ala and Pro) or Gly residue at the P2 site and bulkier residues at the P3 site, although they show the substantial difference in the catalytic efficiency towards the substrates. The relative k_{cat}/K_m values for clotting proteases and trypsin are summarized in Table 1. It is clear that Boc-Asp(OBzl)-Pro-Arg-NH-Mec, Z-<Glu-Gly-Arg-NH-Mec and Boc-Gly-Phe-Arg-NH-Mec could be used, respectively, as highly specific substrates for α-thrombin, factor Xa and plasma kallikrein. For factor XIIa and factor XIa, Boc-Gln-Gly-Arg-NH-Mec and Boc-Glu(OBzl)-Ala-Arg-NH-Mec would be also useful in the presence of a specific inhibitor for α-thrombin. For the assay of activated protein C, Boc-Asp(OBzl)-Ala-Arg-NH-Mec is most reliable to use, but the α-thrombin activity in the test sample has to be inactivated prior to the assay.

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REFERENCES

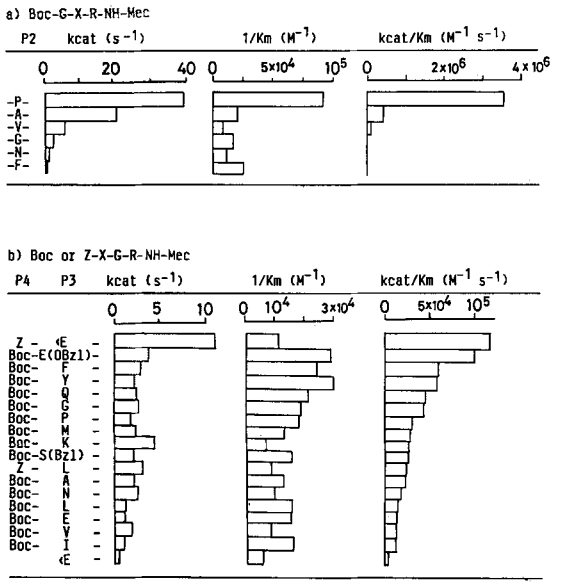
1. Zimmerman, M., Yurewicz, E. C. & Patel, G. (1976) *Anal. Biochem. 70*, 258–262.
2. Kanaoka, Y., Takahashi, T. & Nakayama, H. (1977) *Chem. Pharm. Bull. 25*, 3126–3128.
3. Zimmerman, M., Ashe, B., Yurewicz, E. C. & Patel, G. (1977) *Anal. Biochem. 78*, 41–51.
4. Kato, T., Hama, T., Kojima, K., Nagatsu, T. & Sakakibara, S. (1978) *Clin. Chem. 24*, 1163–1166.
5. Fujiwara, K. & Tsuru, D. (1978) *J. Biochem. (Tokyo) 83*, 1145–1149.
6. Kunugi, S., Fukuda, M. & Hayashi, R. (1985) *Eur. J. Biochem. 153*, 37–40.
7. Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T. & Sakakibara, S. (1977) *J. Biochem. (Tokyo) 82*, 1495–1498.
8. Sugo, T., Kato, H., Iwanaga, S., Takada, K. & Sakakibara, S. (1985) *Eur. J. Biochem. 146*, 43–50.
9. Ohno, Y., Kato, H., Morita, T., Iwanaga, S., Takada, K., Sakakibara, S. & Stenflo, J. (1981) *J. Biochem. (Tokyo) 90*, 1387–1395.
10. Kato, H., Adachi, N., Iwanaga, S., Abe, K., Takada, K., Kimura, T. & Sakakibara, S. (1980) *J. Biochem. (Tokyo) 87*, 1127–1132.
11. Kato, H., Adachi, N., Ohno, Y., Iwanaga, S., Takada, K. & Sakakibara, S. (1980) *J. Biochem. (Tokyo) 88*, 183–190.
12. Nakamura, T., Morita, T. & Iwanaga, S. (1986) *Eur. J. Biochem. 154*, 511–521.
13. Hashimoto, N., Morita, T. & Iwanaga, S. (1985) *J. Biochem. (Tokyo) 97*, 1347–1355.
14. Fujikawa, K., Kurachi, K. & Davie, E. W. (1977) *Biochemistry 16*, 4182–4194.
15. Fujikawa, K., Walsh, K. A. & Davie, E. W. (1977) *Biochemistry 16*, 2270–2277.
16. Kawabata, S., Morita, T., Iwanaga, S. & Igarashi, H. (1985) *J. Biochem. (Tokyo) 97*, 1073–1078.
17. Theodorsson, B., Hedner, U., Nilsson, I. M. & Kisiel, W. (1983) *Blood 61*, 973–981.

18. Fujikawa, K., Chung, D. W., Hendrickson, L. E. & Davie, E. W. (1986) *Biochemistry 25*, 2417–2424.
19. Kurachi, K. & Davie, E. W. (1977) *Biochemistry 16*, 5831–5839.
20. Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.
21. Chase, T. & Shaw, E. (1970) *Methods Enzymol. 19*, 20–27.
22. Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A. & Elmore, D. T. (1973) *Biochem. J. 131*, 107–117.
23. Lottenberg, R., Hall, J. A., Blinder, M., Binder, E. P. & Jackson, C. M. (1983) *Biochim. Biophys. Acta 742*, 539–557.
24. Cho, K., Tanaka, T., Cook, R. R., Kisiel, W., Fujikawa, K., Kurachi, K. & Powers, J. C. (1984) *Biochemistry 23*, 644–650.
25. Pozsgay, M., Szabó, G. Cs., Bajusz, S., Simonsson, R., Gáspár, R. & Elödi, P. (1981) *Eur. J. Biochem. 115*, 491–495.
26. McRae, B. J., Kurachi, K., Heimark, R. L., Fujikawa, K., Davie, E. W. & Powers, J. C. (1981) *Biochemistry 20*, 7196–7206.
27. Lijnen, H. R., Uytterhoeven, M. & Collen, D. (1984) *Thrombos. Res. 34*, 431–437.
28. Walker, B., Wikstrom, P. & Shaw, E. (1985) *Biochem. J. 230*, 645–650.
29. Lobo, A. P., Wos, J. D., Yu, S. M. & Lawson, W. B. (1976) *Arch. Biochem. Biophys. 177*, 235–244.
30. Chang, J.-Y. (1985) *Eur. J. Biochem. 151*, 217–224.
31. Chang, J.-Y., Alkan, S. S., Hilschmann, N. & Braun, D. G. (1985) *Eur. J. Biochem. 151*, 225–230.
32. Sonder, S. A. & Fenton, J. W., II (1984) *Biochemistry 23*, 1818–1823.
33. Stone, S. R. & Hofsteenge, J. (1985) *Biochem. J. 230*, 497–502.
34. Hirohara, H., Phillip, M. & Bender, M. L. (1977) *Biochemistry 16*, 1573–1580.
35. Aurell, L., Friberger, P., Karlsson, G. & Claeson, G. (1977) *Thrombos. Res. 11*, 595–609.
36. Lonsdale-Eccles, J. D., Hogg, D. H. & Elmore, D. T. (1980) *Biochim. Biophys. Acta 612*, 395–400.
37. Eaton, D., Rodriguez, H. & Vehar, G. A. (1986) *Biochemistry 25*, 505–512.
38. Mahoney, W. C., Hermodson, M. A., Jones, B., Powers, D. D., Corfman, R. S. & Reeck, G. R. (1984) *J. Biol. Chem. 259*, 8412–8416.
39. Kurachi, K. & Davie, E. W. (1982) *Proc. Natl Acad. Sci. USA 79*, 6461–6464.
40. McMullen, B. A. & Fujikawa, K. (1985) *J. Biol. Chem. 260*, 5328–5341.
41. Zur, M. & Nemerson, Y. (1978) *J. Biol. Chem. 253*, 2203–2209.
42. Titani, K., Fujikawa, K., Enfield, D. L., Ericsson, L. H., Walsh, K. A. & Neurath, H. (1975) *Proc. Natl Acad. Sci. USA 72*, 3082–3086.
43. Leytus, S. P., Chung, D. W., Kisiel, W., Kurachi, K. & Davie, E. W. (1984) *Proc. Natl Acad. Sci. USA 81*, 3699–3702.
44. Fung, M. R., Hay, C. W. & MacGillivray, R. T. A. (1985) *Proc. Natl Acad. Sci. USA 82*, 3591–3595.
45. Katayama, K., Ericsson, L. H., Enfield, D. L., Walsh, K. A., Neurath, H., Davie, E. W. & Titani, K. (1979) *Proc. Natl Acad. Sci. USA 76*, 4990–4994.
46. Murakami, K., Ohsawa, T., Hirose, S., Takada, K. & Sakakibara, S. (1981) *Anal. Biochem. 110*, 232–239.

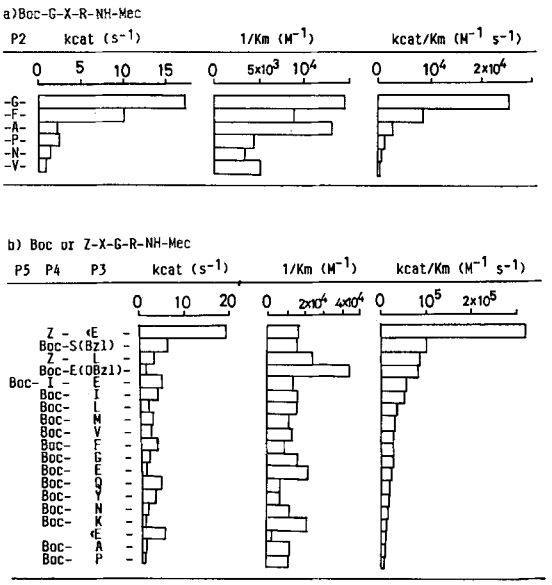
Supplementary Material to

Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood clotting proteases and trypsin

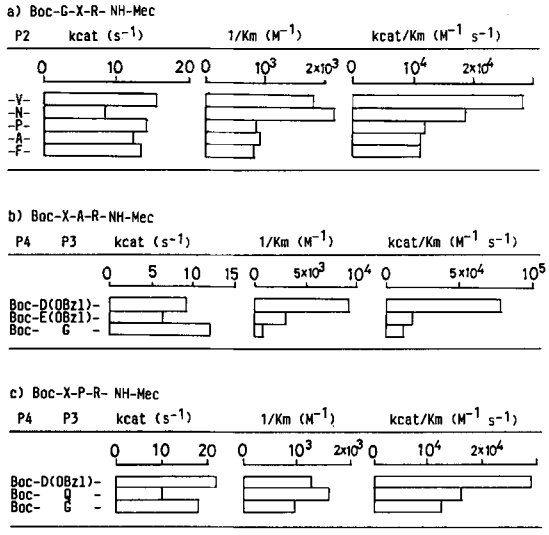
Shun-ichiro Kawabata, Takako Miura, Takashi Morita, Hisao Kato, Kazuo Fujikawa, Sadaaki Iwanaga, Katsumi Takada, Terutoshi Kimura and Shumpei Sakakibara



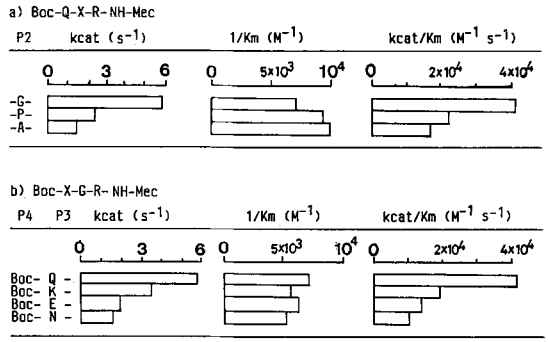
Supplement Fig. 1. Substrate specificity of human α-thrombin toward Boc-Gly-P2-Arg-NH-Mec (a) and Boc-P3-Gly-Arg-NH-Mec (b) substrates. The standard IUPAC one-letter code for amino acid residues was used. Data are from Table 2.



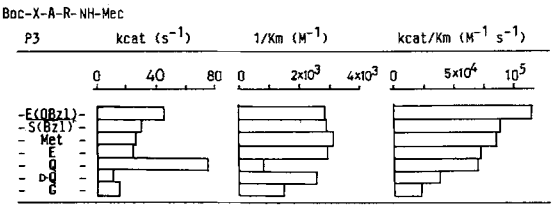
Supplement Fig. 2. Substrate specificity of bovine factor Xa toward Boc-Gly-P2-Arg-NH-Mec (a) and Boc-P3-Gly-Arg-NH-Mec or Z-P3-Gly-Arg-NH-Mec (b) substrates. Data are from Table 3.



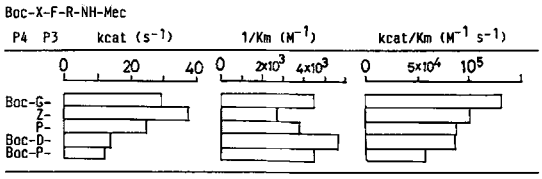
Supplement Fig. 3. Substsrate specificity of bovine activated protein C toward Boc-Gly-P2-Arg-NH-Mec (a), Boc-P3-Ala-Arg-NH-Mec (b), and Boc-P3-Pro-Arg-NH-Mec (c) substrates. Data are from Table 4.



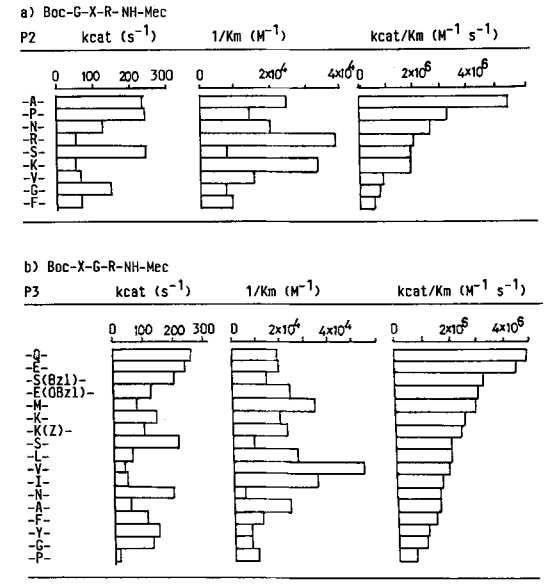
Supplement Fig. 4. Substrate specificity of bovine factor XIIa toward Boc-Gln-P2-Arg-NH-Mec (a) and Boc-P3-Gly-Arg-NH-Mec (b) substrates. Data are from Table 5.



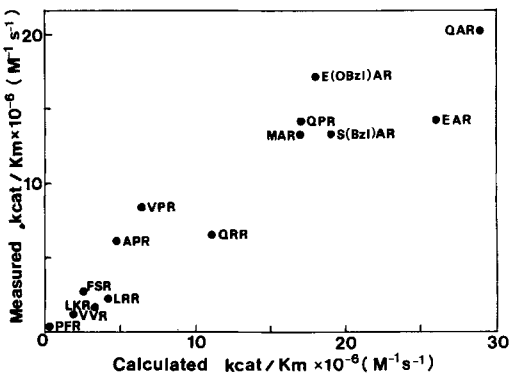
Supplement Fig. 5. Substrate specificity of human factor XIa toward Boc-P3-Ala-Arg-NH-Mec substrates. Data are from Table 6.



Supplement Fig. 6. Substrate specificity of bovine plasma kallikrein toward Boc-P3-Phe-Arg-NH-Mec substrates. Data are from Table 7.



Supplement Fig. 7. Substrate specificity of bovine trypsin toward Boc-Gly-P2-Arg-NH-Mec (a) and Boc-P3-Gly-Arg-NH-Mec (b) substrates. Data are from Table 8.



Supplement Fig. 8. Comparison between measured and calculated kcat/Km values. Data are from Table 8.

Supplement Table 1. Specific activities of clotting enzymes and plasma kallikrein toward peptide-NH-Mec substrates[a)]

No.	Substrates					Human α-thrombin	Bovine Xa	Bovine activated protein C	Bovine XIIa	Human XIa	Bovine plasma kallikrein
	P6	P5	P4	P3	P2 P1						
1			Boc-	Gly	-Gly-Arg-NH-Mec	65	74	31	1.1	86	9
2			Boc-	Ala	-Gly-Arg-NH-Mec	73	25	16	1.1	23	11
3			Boc-	Val	-Gly-Arg-NH-Mec	50	97	16	1.0	26	19
4			Boc-	Leu	-Gly-Arg-NH-Mec	35	101	17	0.6	55	24
5			Boc-	Ile	-Gly-Arg-NH-Mec	33	>142	17	0.9	23	16
6			Boc-	Pro	-Gly-Arg-NH-Mec	105	13	12	0.1	-	11
7			Boc-	Met	-Gly-Arg-NH-Mec	123	92	23	1.8	61	22
8			Boc-	Glu	-Gly-Arg-NH-Mec	68	38	22	1.9	134	17
9			Boc-	Asn	-Gly-Arg-NH-Mec	144	55	25	1.4	129	18
10			Boc-	Gln	-Gly-Arg-NH-Mec	169	61	32	>4.7	246	41
11			Boc-	Lys	-Gly-Arg-NH-Mec	113	21	22	2.2	67	15
12			Boc-	Phe	-Gly-Arg-NH-Mec	160	108	20	0.7	62	12
13			Boc-	Tyr	-Gly-Arg-NH-Mec	140	121	17	0.8	68	10
14				<Glu	-Gly-Arg-NH-Mec	24	98	82	1.6	199	17
15				Z	-Gly-Arg-NH-Mec	31	15	15	0.2	25	13
16				Glutaryl	-Gly-Arg-NH-Mec	-[b)]	14	-	0.2	2	-
17			Z -	Gly	-Gly-Arg-NH-Mec	72	71	28	1.0	26	36
18			Z -	Leu	-Gly-Arg-NH-Mec	151	125	18	0.6	133	62
19			Z -	<Glu	-Gly-Arg-NH-Mec	597	>142	85	0.7	16	21
20			Boc-Glu(OBzl)		-Gly-Arg-NH-Mec	160	62	27	1.9	50	20
21			Boc-Ser(Bzl)		-Gly-Arg-NH-Mec	103	122	28	2.0	75	57
22			Boc-	Lys(z)	-Gly-Arg-NH-Mec	42	54	15	1.2	45	121
23			Boc-Tyr(Bzl)		-Gly-Arg-NH-Mec	70	16	13	0.1	29	9
24					Gly-Arg-NH-Mec	47	11	44	0.6	248	25
25		Boc-Leu-		Gly	-Gly-Arg-NH-Mec	18	27	11	0.5	21	-
26		Boc-Ile-		Glu	-Glu-Arg-NH-Mec	-	45	16	1.0	35	-
27			Boc-	Gly	-Ala-Arg-NH-Mec	951	12	100	0.5	230	-
28				Boc	-Ala-Arg-NH-Mec	-	-	-	-	5	-
29			Boc-	Gly	-Val-Arg-NH-Mec	305	3	250	0.1	113	105
30			Boc-	Val	-Val-Arg-NH-Mec	146	6	100	0.3	224	116
31				Boc	-Val-Arg-NH-Mec	36	-	-	-	6	-
32			Boc-	Gly	-Pro-Arg-NH-Mec	>990	6	168	0.5	260	15
33			Boc-	Val	-Pro-Arg-NH-Mec	>990	4	48	0.5	419	28
34			Boc-	Asp	-Pro-Arg-NH-Mec	>990	7	57	0.6	ND	57
35				Boc	-Pro-Arg-NH-Mec	30	-	-	-	11	-
36				Z	-Pro-Arg-NH-Mec	647	3	15	0.1	57	21
37			Boc-Asp(OBzl)		-Pro-Arg-NH-Mec	>990	19	343	1.7	ND	68
38			Boc-	Ala	-Pro-Arg-NH-Mec	>990	3	47	0.5	254	39
39			Boc-	Gln	-Pro-Arg-NH-Mec	>990	5	111	2.0	808	152
40			Z -	<Glu	-Pro-Arg-NH-Mec	>990	ND[c)]	ND	ND	639	ND
41	Boc-Ala-Pro-			Arg	-Pro-Arg-NH-Mec	>990	ND	ND	ND	46	ND

a) The units of specific activites are nmol per min per nmol of enzyme.
b) -, not detectable.
c) ND, not determined.

Supplement Table 1. (continued)

No.	Substrates				Human α-thrombin	Bovine Xa	Bovine activated protein C	Bovine XIIa	Human XIa	Bovine plasma kallikrein
	P5	P4	P3	P2 P1						
42		Boc-	Leu	-Thr-Arg-NH-Mec	17	3	44	0.2	150	186
43		Boc-	Met	-Thr-Arg-NH-Mec	68	7	290	0.5	277	379
44		Boc-	Gly	-Asn-Arg-NH-Mec	81	5	101	0.3	177	69
45			Boc	-Asn-Arg-NH-Mec	13	4	13	0.1	42	87
46	Boc-Leu-		Ser	-Thr-Arg-NH-Mec	37	2	151	0.3	97	9
47	Boc-Val-		Ile	-His-Arg-NH-Mec	22	11	69	0.2	297	54
48			Z	-Arg-Arg-NH-Mec	-	-	-	-	13	-
49		Boc-	Gly	-Phe-Arg-NH-Mec	35	28	180	0.7	266	438
50		Boc-	Pro	-Phe-Arg-NH-Mec	-	3	7	0.1	23	441
51		Boc-	Asp	-Phe-Arg-NH-Mec	14	9	86	0.5	153	>505
52			Z	-Phe-Arg-NH-Mec	-	10	-	0.1	23	>505
53		Z -	Pro	-Phe-Arg-NH-Mec	-	4	11	0.1	26	>505
54		Boc-Asp(OBzl)		-Phe-Arg-NH-Mec	29	8	33	0.4	55	>505
55			Pro	-Phe-Arg-NH-Mec	-	13	34	0.8	11	>505
56			Boc	-Trp-Arg-NH-Mec	-	6	-	-	ND	60
57		Boc-	Tyr	-Phe-Arg-NH-Mec	32	29	22	0.6	68	460
58				Bz-Arg-NH-Mec	-	-	13	-	2	-
59		Boc-	Glu	-Lys-Lys-NH-Mec	-	-	-	-	-	14
60		Boc-	Val	-Leu-Lys-NH-Mec	-	-	-	-	1	8
61		Boc-	Gly	-Lys-Arg-NH-Mec	13	-	82	0.1	38	73
62		Boc-	Leu	-Lys-Arg-NH-Mec	-	-	55	0.1	47	127
63		Boc-	Gly	-Arg-Arg-NH-Mec	-	-	55	0.1	21	21
64		Boc-	Leu	-Arg-Arg-NH-Mec	-	-	23	0.1	19	25
65		Boc-	Gln	-Arg-Arg-NH-Mec	-	-	90	0.2	123	135
66		Boc-Asp(OBzl)		-Ala-Arg-NH-Mec	>990	7	99	0.5	142	ND
67		Boc-Glu(OBzl)		-Ala-Arg-NH-Mec	>990	20	128	1.4	755	106
68		Boc-	Glu	-Ala-Arg-NH-Mec	278	5	37	0.5	420	40
69		Boc-Ser(Bzl)		-Ala-Arg-NH-Mec	769	40	45	0.8	512	112
70		Boc-	D-Gln	-Ala-Arg-NH-Mec	432	24	100	0.6	193	28
71		Boc-	Gln	-Ala-Arg-NH-Mec	>990	9	97	1.7	209	106
72		Boc-	Met	-Ala-Arg-NH-Mec	671	12	54	0.7	404	45
73		Boc-	Gly	-Ser-Arg-NH-Mec	53	30	95	0.4	149	44
74		Boc-	Phe	-Ser-Arg-NH-Mec	82	23	22	0.3	156	18

Supplement Table 2. Kinetic parameters of human α-thrombin

Substrates				kcat	Km	kcat/Km
				s^{-1}	μM	$M^{-1}\ s^{-1}$
P6 P5	P4	P3	P2 P1			
	Boc-Asp(OBzl)	-Pro-Arg-NH-Mec		160	11	15,000,000
	Boc-	Ala	-Pro-Arg-NH-Mec	130	13	10,000,000
	Boc-	Gln	-Pro-Arg-NH-Mec	130	14	9,300,000
	Boc-	Val	-Pro-Arg-NH-Mec	120	22	5,500,000
	Boc-	Gly	-Pro-Arg-NH-Mec	39	11	3,500,000
	Boc-	Asp	-Pro-Arg-NH-Mec	110	41	2,700,000
	Z -	<Glu	-Pro-Arg-NH-Mec	37	15	2,500,000
Boc-Ala-Pro-		Arg	-Pro-Arg-NH-Mec	46	19	2,400,000
	Boc-Asp(OBzl)	-Ala-Arg-NH-Mec		52	44	1,200,000
	Boc-Glu(OBzl)	-Ala-Arg-NH-Mec		51	73	700,000
	Boc-	Gln	-Ala-Arg-NH-Mec	51	82	620,000
	Boc-	Gly	-Ala-Arg-NH-Mec	20	48	420,000
	Boc-Ser(Bzl)	-Ala-Arg-NH-Mec		16	40	400,000
	Z -	<Glu	-Gly-Arg-NH-Mec	11	89	120,000
	Boc-Glu(OBzl)	-Gly-Arg-NH-Mec		3.7	36	100,000
	Boc-	Phe	-Phe-Arg-NH-Mec	2.7	43	63,000
	Boc-	Tyr	-Gly-Arg-NH-Mec	2.1	35	60,000
	Boc-	Gly	-Val-Arg-NH-Mec	5.6	120	47,000
	Boc-	Gln	-Gly-Arg-NH-Mec	2.2	49	45,000
	Boc-	Gly	-Gly-Arg-NH-Mec	2.5	56	45,000
	Boc-	Pro	-Gly-Arg-NH-Mec	1.7	56	30,000
	Boc-	Lys	-Gly-Arg-NH-Mec	4.3	150	29,000
	Boc-	Met	-Gly-Arg-NH-Mec	2.3	79	29,000
	Z -	Leu	-Gly-Arg-NH-Mec	3.3	120	28,000
	Boc-Ser(Bzl)	-Gly-Arg-NH-Mec		1.9	68	28,000
	Boc-	Ala	-Gly-Arg-NH-Mec	2.2	83	27,000
	Boc-	Asn	-Gly-Arg-NH-Mec	2.6	110	23,000
	Boc-	Leu	-Gly-Arg-NH-Mec	1.2	66	18,000
	Boc-	Glu	-Gly-Arg-NH-Mec	1.2	68	17,000
	Boc-Leu-	Ser	-Thr-Arg-NH-Mec	0.76	47	16,000
	Boc-	Val	-Gly-Arg-NH-Mec	2.0	120	16,000
	Boc-	Ile	-Gly-Arg-NH-Mec	0.95	62	15,000
	Boc-	Gly	-Asn-Arg-NH-Mec	1.5	110	14,000
	Boc-	Met	-Thr-Arg-NH-Mec	1.9	180	10,000
	Boc-	Gly	-Phe-Arg-NH-Mec	0.23	39	5,900
		<Glu	-Gly-Arg-NH-Mec	0.49	180	2,800

Supplement Table 3. Kinetic parameters of bovine factor Xa

Substrates				kcat	Km	kcat/Km
				s^{-1}	μM	$M^{-1}\ s^{-1}$
P5 P4	P3	P2 P1				
	Z -	<Glu	-Gly-Arg-NH-Mec	19	59	320,000
	Boc-Ser(Bzl)	-Gly-Arg-NH-Mec		6.1	60	100,000
	Z -	Leu	-Gly-Arg-NH-Mec	3.5	43	81,000
	Boc-Glu(OBzl)	-Gly-Arg-NH-Mec		1.6	23	70,000
Boc-Ile-	Glu	-Gly-Arg-NH-Mec		4.4	77	57,000
	Boc-	Ile	-Gly-Arg-NH-Mec	3.7	69	54,000
	Boc-	Leu	-Gly-Arg-NH-Mec	2.1	66	32,000
	Boc-	Met	-Gly-Arg-NH-Mec	2.8	100	28,000
	Boc-	Val	-Gly-Arg-NH-Mec	2.4	91	26,000
	Boc-	Phe	-Gly-Arg-NH-Mec	3.1	120	26,000
	Boc-	Gly	-Gly-Arg-NH-Mec	1.7	69	25,000
	Boc-	Glu	-Gly-Arg-NH-Mec	0.78	40	19,000
	Boc-	Gln	-Gly-Arg-NH-Mec	3.3	170	19,000
	Boc-	Tyr	-Gly-Arg-NH-Mec	2.8	170	16,000
	Boc-	Asn	-Gly-Arg-NH-Mec	1.2	82	15,000
	Boc-	Lys	-Gly-Arg-NH-Mec	0.50	51	9,800
		<Glu	-Gly-Arg-NH-Mec	4.4	510	8,700
	Boc-	Gly	-Phe-Arg-NH-Mec	1.0	120	8,700
	Boc-Asp(OBzl)	-Pro-Arg-NH-Mec		0.79	120	6.500
	Boc-	Ala	-Gly-Arg-NH-Mec	0.51	80	6,400
	Boc-	Pro	-Gly-Arg-NH-Mec	0.27	85	3,000
	Boc-	Gly	-Ala-Arg-NH-Mec	0.20	77	2,600
	Boc-	Asp	-Pro-Arg-NH-Mec	0.13	60	2,200
	Boc-	Gly	-Pro-Arg-NH-Mec	0.21	220	970
	Boc-	Met	-Thr-Arg-NH-Mec	0.14	160	880
	Boc-	Gly	-Asn-Arg-NH-Mec	0.12	270	450
	Boc-	Gly	-Val-Arg-NH-Mec	0.076	200	390

Supplement Table 4. Kinetic parameters of bovine activated protein C

Substrates				kcat	Km	kcat/Km
				s^{-1}	μM	$M^{-1}\ s^{-1}$
P5 P4	P3	P2 P1				
	Boc-Asp(OBzl)	-Ala-Arg-NH-Mec		9.2	120	77,000
	Boc-Asp(OBzl)	-Pro-Arg-NH-Mec		22	760	29,000
	Boc-	Gly	-Val-Arg-NH-Mec	16	570	28,000
	Boc-	Met	-Thr-Arg-NH-Mec	26	1,100	24,000
Boc-Leu-		Ser	-Thr-Arg-NH-Mec	9.8	430	23,000
	Boc-	Gly	-Asn-Arg-NH-Mec	8.3	470	18,000
	Boc-Glu(OBzl)	-Ala-Arg-NH-Mec		6.8	380	18,000
	Boc-	Gln	-Pro-Arg-NH-Mec	10	640	16,000
	Boc-	Gly	-Pro-Arg-NH-Mec	14	1,200	12,000
	Boc-	Gly	-Ala-Arg-NH-Mec	12	1,100	11,000
	Boc-	Gly	-Phe-Arg-NH-Mec	13	1,200	11,000
	Boc-	Asp	-Phe-Arg-NH-Mec	4.5	790	5,800

Supplement Table 5. Kinetic parameters of bovine factor XIIa

Substrates				kcat	Km	kcat/Km
				s^{-1}	μM	$M^{-1}\ s^{-1}$
P6 P5	P4	P3	P2 P1			
	Boc-	Gln	-Gly-Arg-NH-Mec	5.8	140	42,000
	Boc-	Gln	-Pro-Arg-NH-Mec	2.4	110	22,000
	Boc-	Lys	-Gly-Arg-NH-Mec	3.4	180	19,000
	Boc-	Gln	-Ala-Arg-NH-Mec	1.7	100	17,000
	Boc-Asp(OBzl)	-Pro-Arg-NH-Mec		2.8	170	16,000
Boc-Ala-Pro-		Arg	-Pro-Arg-NH-Mec	2.3	150	15,000
	Boc-	Glu	-Gly-Arg-NH-Mec	2.0	160	13,000
	Boc-	Asn	-Gly-Arg-NH-Mec	1.7	190	8,900

Supplement Table 6. Kinetic parameters of human factor XIa

Substrates				kcat	Km	kcat/Km
				s^{-1}	μM	$M^{-1}\ s^{-1}$
P4	P3	P2 P1				
Boc-Glu(OBzl)	-Ala-Arg-NH-Mec			46	370	120,000
Boc-	Gln	-Pro-Arg-NH-Mec		54	640	84,000
Boc-Ser(Bzl)	-Ala-Arg-NH-Mec			29	350	83,000
Boc-	Met	-Ala-Arg-NH-Mec		26	320	81,000
Boc-	Glu	-Ala-Arg-NH-Mec		24	340	71,000
Boc-	Gln	-Ala-Arg-NH-Mec		76	1100	69,000
Boc-	Val	-Pro-Arg-NH-Mec		15	290	52,000
Z -	<Glu	-Pro-Arg-NH-Mec		33	760	43,000
Boc-	D-Gln	-Ala-Arg-NH-Mec		10	380	38,000
Boc-	Met	-Thr-Arg-NH-Mec		36	1500	24,000
Boc-	Gly	-Pro-Arg-NH-Mec		33	1400	24,000
Boc-	Gly	-Ala-Arg-NH-Mec		15	660	23,000
Boc-	Gly	-Asn-Arg-NH-Mec		17	830	20,000
Boc-	Leu	-Thr-Arg-NH-Mec		31	2500	12,000
Boc-	Gly	-Val-Arg-NH-Mec		21	1700	12,000

Supplement Table 7. Kinetic parameters of bovine plasma kallikrein

Substrates				kcat	Km	kcat/Km
				s^{-1}	μM	$M^{-1}\ s^{-1}$
P4	P3	P2 P1				
Boc-	Gly	-Phe-Arg-NH-Mec		29	230	130,000
	Z	-Phe-Arg-NH-Mec		37	370	100,000
	Pro	-Phe-Arg-NH-Mec		24	270	89,000
Boc-	Asp	-Phe-Arg-NH-Mec		14	170	83,000
Boc-	Pro	-Phe-Arg-NH-Mec		12	230	53,000
Boc-	Met	-Thr-Arg-NH-Mec		11	260	42,000
Boc-	Leu	-Thr-Arg-NH-Mec		7.9	330	24,000
Boc-	Gln	-Pro-Arg-NH-Mec		13	790	16,000
Boc-Ser(Bzl)	-Ala-Arg-NH-Mec			6.0	470	13,000
Boc-	Gly	-Val-Arg-NH-Mec		4.2	490	8,600
Boc-	Gln	-Ala-Arg-NH-Mec		4.7	580	8,100
	Boc	-Trp-Arg-NH-Mec		4.8	1,500	3,200
Boc-	Gln	-Gly-Arg-NH-Mec		2.9	1,200	2,400
Z -	<Glu	-Gly-Arg-NH-Mec		0.87	360	2,400

Supplement Table 8. Kinetic parameters of trypsin

Substrates					kcat	Km	kcat/Km	kcat/Km (calculated)[a]
					s^{-1}	µM	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$
P5	P4	P3	P2	P1				
	Boc-	Gln	-Ala-	Arg-NH-Mec	120	6.0	20,000,000	29,000,000
	Boc-Glu(OBzl)-		Ala-	Arg-NH-Mec	86	5.0	17,000,000	18,000,000
	Boc-	Gln	-Pro-	Arg-NH-Mec	90	6.3	14,000,000	17,000,000
	Boc-	Glu	-Ala-	Arg-NH-Mec	84	6.0	14,000,000	26,000,000
	Boc-	Met	-Ala-	Arg-NH-Mec	110	8.4	13,000,000	17,000,000
	Boc-Ser(Bzl)		-Ala-	Arg-NH-Mec	150	12	13,000,000	19,000,000
	Boc-Asp(OBzl)-		Pro-	Arg-NH-Mec	120	9.4	13,000,000	
	Boc-	D-Gln	-Ala-	Arg-NH-Mec	200	23	8,700,000	
	Boc-	Val	-Pro-	Arg-NH-Mec	31	3.8	8,200,000	6,200,000
	Boc-	Gln	-Arg-	Arg-NH-Mec	90	14	6,400,000	11,000,000
	Boc-	Ala	-Pro-	Arg-NH-Mec	56	9.4	6,000,000	4,800,000
	Boc-	Gly	-Ala-	Arg-NH-Mec	230	42	5,500,000	
	Boc-	Asp	-Pro-	Arg-NH-Mec	76	15	5,100,000	
	Boc-	Gln	-Gly-	Arg-NH-Mec	260	53	4,900,000	
	Boc-Asp(OBzl)-		Ala-	Arg-NH-Mec	125	26	4,800,000	
	Boc-	Glu	-Gly-	Arg-NH-Mec	230	52	4,400,000	
	Boc-	Gly	-Pro-	Arg-NH-Mec	240	74	3,200,000	
	Boc-Ser(Bzl)		-Gly-	Arg-NH-Mec	200	63	3,200,000	
	Boc-Glu(OBzl)-		Gly-	Arg-NH-Mec	120	40	3,000,000	
	Boc-	Met	-Gly-	Arg-NH-Mec	84	29	2,900,000	
	Boc-	Met	-Thr-	Arg-NH-Mec	45	16	2,800,000	
	Boc-	Gly	-Asn-	Arg-NH-Mec	130	50	2,600,000	
	Boc-	Phe	-Ser-	Arg-NH-Mec	120	47	2,600,000	2,500,000
	Boc-	Lys	-Gly-	Arg-NH-Mec	130	51	2,500,000	
	Boc-	Lys(Z)	-Gly-	Arg-NH-Mec	100	44	2,300,000	
	Boc-	Leu	-Arg-	Arg-NH-Mec	36	17	2,100,000	4,100,000
	Boc-	Gly	-Arg-	Arg-NH-Mec	47	24	2,000,000	
	Boc-	Gly	-Ser-	Arg-NH-Mec	250	130	1,900,000	
	Boc-	Ser	-Gly-	Arg-NH-Mec	210	110	1,900,000	
	Boc-	Leu	-Gly-	Arg-NH-Mec	70	37	1,900,000	
	Boc-	Val	-Gly-	Arg-NH-Mec	33	18	1,800,000	
Boc-Ile-		Glu	-Gly-	Arg-NH-Mec	174	98	1,800,000	
	Boc-	Leu	-Lys-	Arg-NH-Mec	33	19	1,700,000	3,300,000
	Boc-	Ile	-Gly-	Arg-NH-Mec	48	30	1,600,000	
	Boc-	Gly	-Lys-	Arg-NH-Mec	47	29	1,600,000	
	Boc-	Asn	-Gly-	Arg-NH-Mec	200	130	1,500,000	
	Z -	<Glu	-Gly-	Arg-NH-Mec	140	100	1,400,000	
	Boc-	Ala	-Gly-	Arg-NH-Mec	61	44	1,400,000	
	Boc-	Leu	-Thr-	Arg-NH-Mec	41	30	1,400,000	
	Boc-	Val	-Val-	Arg-NH-Mec	11	8.9	1,200,000	1,800,000
	Boc-	Phe	-Gly-	Arg-NH-Mec	11	90	1,200,000	
	Z -	Leu	-Gly-	Arg-NH-Mec	67	54	1,200,000	
Boc-Leu-		Ser	-Thr-	Arg-NH-Mec	57	49	1,200,000	
		<Glu	-Gly-	Arg-NH-Mec	330	300	1,100,000	
	Boc-	Asp	-Phe-	Arg-NH-Mec	42	40	1,100,000	
	Boc-	Tyr	-Gly-	Arg-NH-Mec	140	140	1,000,000	
	Boc-	Gly	-Val-	Arg-NH-Mec	61	64	950,000	
	Boc-	Gly	-Gly-	Arg-NH-Mec	130	140	930,000	
	Boc-	Gly	-Phe-	Arg-NH-Mec	63	100	630,000	
	Z -	Gly	-Gly-	Arg-NH-Mec	160	260	620,000	
Boc-Leu-		Gly	-Gly-	Arg-NH-Mec	68	140	490,000	
	Boc-Asp(OBzl)-		Phe-	Arg-NH-Mec	87	220	400,000	
	Boc-	Pro	-Phe-	Arg-NH-Mec	5	29	170,000	110,000
	Boc-	Pro	-Gly-	Arg-NH-Mec	16	99	160,000	
				Bz-Arg-NH-Mec	2.1	25	84,000	

a) Calculated as described under "DISCUSSION".