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Specificity of human tissue kallikrein towards substrates containing Phe–Phe pair of amino acids

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We have explored in detail the determinants of specificity for the hydrolysis by human tissue kallikrein (hK_1) of substrates containing the Phe–Phe amino acid pair, after which hK_1 cleaves kallistatin (human kallikrein-binding protein), a specific serpin for this protease, as well as somatostatin 1–14. Internally quenched fluorogenic peptides were synthesized with the general structure Abz-peptidyl-EDDnp [Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine], based on the natural reactive-centre loop sequence of kallistatin from P_9 to P'_{13}, and the kinetic parameters of their hydrolysis by hK_1 were determined. All these peptides were cleaved after the Phe–Phe pair. For comparison, we have also examined peptides containing the reactive-centre loop sequences of human protein-C inhibitor (PCI) and rat kallikrein-binding protein, which were hydrolysed after Phe–Arg and Leu–Lys bonds, respectively. Hybrid peptides containing kallistatin–PCI sequences showed that the efficiency of hK_1 activity on the peptides containing kallistatin and PCI sequences depended on both the nature of the P_1 amino acid as well as on residues at the P- and P′-sides. Moreover, we have made systematic modifications on the hydrophobic pair Phe–Phe, and on Lys and Ile at the P_3 and P_4 positions according to the peptide substrate, Abz-AIKFFSRQ-EDDnp. All together, we concluded that tissue kallikrein was very effective on short substrates that are cleaved after the Phe–Arg pair; however, hydrolysis after Phe–Phe or other hydrophobic pairs of amino acids was more restrictive, requiring additional enzyme–substrate interaction and/or particular substrate conformations.

Key words: fluorescent substrate, kallistatin, peptide, protease, serpin.

INTRODUCTION

Human tissue kallikrein (hK_1) is one member of a family of three closely related serine proteases [1,2], which also includes two enzymes expressed in prostate, namely, prostate-specific antigen (hK_3 or PSA) and a trypsin-like enzyme, hK_2. The genes that correspond to these proteins, *hKLK1*, *hKLK2* and *hKLK3* [3], have been mapped to chromosome 19 [4]. hK_1 is the only tissue kallikrein with already-established functions, which include release of Lys-bradykinin (kallidin) in several inflammatory processes, such as arthritis, asthma and rhinitis [5–8], and the processing of hormone and other peptide precursors [1,2]. Attempts to identify natural substrates for hK_2 and hK_3 have shown that coagulum-forming proteins from seminal plasma, semenogelins and fibronectin are cleaved at Arg sites by hK_2 and at Tyr, Phe or Leu sites by hK_3 [9,10].

hK_1 and the cognate enzymes in mammals release Lys-bradykinin from bovine and human kininogen by cleaving at Met–Lys and Arg–Ser bonds [11]. The only exception known so far is rat tissue kallikrein (rK_1), which cleaves a Lys–Arg bond and releases bradykinin from bovine and rat kininogens [12,13]. The efficiency of cleavage at the Met–Lys bond by hK_1 is dependent on the extension of the substrates, as demonstrated with internally quenched fluorescent peptides with the sequence of human kininogen [14,15]. hK_1 can also hydrolyse after a pair of phenylalanines, for example in the reactive-centre loop of hK_1-binding protein, in a specific serpin for tissue kallikrein called kallistatin [16,17], as well as in somatostatin [18]. Although hK_1 possesses significant similarity with trypsin, the crystal structures of human and porcine tissue kallikrein [19,20] show that their S_1 sites (see Schechter and Berger nomenclature, [21]) are enlarged, mainly owing to the insertion of an additional residue, Pro-219, which is in the *cis* configuration. This unique feature of tissue kallikrein allows its S_1 subsite to accept Arg as well as the larger and hydrophobic side chain of Phe.

hK_1 binds to α_1-antitripsin, the most abundant circulating proteinase inhibitor and an archetypal member of the serpin superfamily [22]; this interaction is slow when compared with interaction with human protein-C inhibitor (PCI) [23] and with kallistatin [24]. These two serpins contain Phe–Arg and Phe–Phe, respectively, at positions P_2 and P_1. Kallistatin is very specific for hK_1, in contrast with PCI, which has a broader inhibitory activity against other serine proteases [16,17]. It was demonstrated previously that the peptides derived from the reactive-centre loop of kallistatin and PCI were substrate for hK_1 [18,25].

The development of efficient and particularly specific inhibitors for hK_1 requires a detailed evaluation of hK_1's unique substrate specificity that accepts substrates with Phe at the P_1 position. In order to obtain this information, we synthesized internally quenched fluorogenic peptides with the general structure Abz-peptidyl-Gln-EDDnp [Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine] based on the reactive-centre loop sequence of kallistatin and PCI, and determined the kinetic parameters of their hydrolysis by hK_1. The effects of peptide chain length, of the amino acids Arg or Phe at the P_1 site and of hybrid kallistatin–PCI sequences on the susceptibility to hK_1 were examined as well.

Finally, it has been demonstrated that in the crystal structure of the complex hirustasin–porcine tissue kallikrein the inhibitor occupies the S_4–S'_2 sites of protease-extended-binding site [26].

Abbreviations used: PCI, human protein-C inhibitor; hK_1, human tissue kallikrein; Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)-ethylenediamine; pNA, *p*-nitroanilide; MCA, methylcoumarine amide; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight.

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In order to explore in detail the substrate specificity of hK_1 in these subsites, we used the peptide Abz-AIKFFSAQ-EDDnp as a lead substrate, which contains the reactive-centre loop sequence of kallistatin from P_4 to P'_3, and synthesized different combinations of the hydrophobic amino acid pair at P_2 and P_1. We also performed systematic modifications on Lys and Ile at the P_3 and P_4 positions.

MATERIALS AND METHODS

Enzymes

hK_1 was purified from pooled urine according to Shimamoto et al. [27]. The molar concentrations of enzyme solutions were determined by active-site titration with 4-nitrophenyl-4-guanidinobenzoate [28].

Synthetic substrates

All the intramolecularly quenched fluorogenic peptides contained EDDnp attached to glutamine, a necessary result of the solid-phase peptide-synthesis strategy employed, the details of which are provided elsewhere [29]. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the fluoren-9-ylmethoxycarbonyl (Fmoc) procedure. Chromogenic peptidyl-*p*-nitroanilide (peptidyl-pNA) and fluorescent peptidyl-methylcoumarine amide (peptidyl-MCA) substrates, used to titrate the enzymes and to calibrate the equipments, were synthesized as described previously [30]. Amino acid analyses were carried out in a Beckman 6300 amino acid analyser following hydrolysis in 6 M HCl with 5 % phenol at 110 °C for 48 h. The final deprotected peptides were purified by semi-preparative HPLC using an Econosil C_{18} column (10 μm mesh; 22.5 mm × 250 mm) and a two-solvent system: (A) trifluoroacetic acid/H_2O (1:1000); and (B) trifluoroacetic acid/acetonitrile/H_2O (1:900:100). The column was allowed to elute at a flow rate of 5 ml/min with either a 10–50 % gradient of solvent B over 30 min or a 30–60 % gradiant of solvent B over 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV/visible-light detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C_{18} column (5 μm mesh; 4.6 × 150 mm), which was allowed to elute with solvent systems A_1 (H_3PO_4/H_2O, 1:1000) and B_1 (acetonitrile/H_2O/H_3PO_4, 900:100:1) at a flow rate of 1.7 ml/min and a 10–80 % gradient of solvent B_1 over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm, following excitation at 320 nm. The molecular masses and purities of synthesized peptides were checked by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS (TofSpec-E, Micromass).

Fluorimetric enzyme assay

Hydrolysis of the fluorogenic peptidyl substrates at 37 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA, 10 % DMSO and 0.1 % Triton X-100, was followed by measuring the fluorescence at $\lambda_{emission}$ 420 nm and $\lambda_{excitation}$ 320 nm in a Hitachi F-2000 spectrofluorometer. The 1-cm path-length cuvette containing 2 ml of the substrate solution was placed in a thermostatically controlled cell compartment for 5 min before the enzyme solution was added and the increase in fluorescence with time was recorded continuously for 10 min. The slope was converted into mol of substrate hydrolysed/min based on the fluorescence curves of standard peptide solutions before and

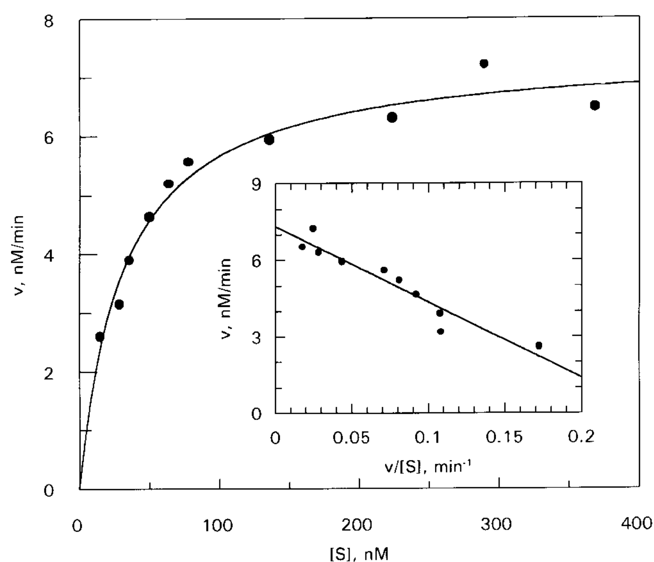

Figure 1 Hydrolysis of Abz-AIKFFSAQTNRHILRFNR-EDDnp (peptide IV) by hK_1

The kinetic parameters obtained for this assay were K_m 0.031 μM and V_{max} 7.4 nM/min (Table 1). Insert, the data were fitted into a Eadie–Hofstee plot, which yielded the values of K_m as 0.030 μM and of V_{max} as 7.3 nM/min. For this experiment, substrate concentration ranged as shown and enzyme concentration was 0.09 nM.

after total enzymic hydrolysis. Their concentration in solution could be determined from the fluorescence obtained following total tryptic hydrolysis for 2 h. The concentration of a standard solution of Abz-Phe-Arg-pNA (in 0.2 M NaOH) was calculated based on the amount of *p*-nitroaniline liberated by trypsin as measured by the absorbance at 405 nm. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyse less than 5 % of the substrate present [31]. The kinetic parameters were calculated by non-linear regression according to Wilkinson [32]. In order to check the reliability of K_m values lower than 0.05 μM, Eadie–Hofstee plots were used as shown in Figure 1.

The K_i values for competitive-inhibition assays of the peptides were determined according to Nicklin and Barrett [33].

Determination of bonds cleaved

The bonds cleaved were identified by isolation of the fragments by HPLC, and the retention times of the fluorescent Abz-containing fragments were compared with authentic synthetic sequences and/or by molecular mass, which was determined by MALDI-TOF MS.

RESULTS

Hydrolysis by hK_1 of internally quenched fluorescent peptides with reactive-centre loop sequences of human serpins

The internally quenched fluorescent peptides containing the reactive-centre loop sequence of serpins were assayed as substrates of hK_1 (Table 1). The serpins used were human kallistatin (peptides I–IV), PCI (peptides V and VI) and rat kallikrein-binding protein (peptide VII). The substrate structures and the kinetic data for the hydrolysis of all susceptible peptides to hK_1 are also presented in Table 1. HPLC and mass spectrometric analyses showed that the substrates with the kallistatin sequence

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Table 1 Kinetic parameters for hydrolysis by hK_1 of internally quenched fluorescent peptides with reactive-centre loop sequences of some human serpins

Hydrolysis conditions: 20 mM Tris/HCl/1 mM EDTA/10% DMSO/0.1% Triton X-100 buffer, at pH 9.0 and 37 °C. Results are presented as means ± S.E. ↓ indicates the position of cleavage. Abbreviations: HKBP: human kallikrein-binding protein (kallistatin); RKBP: rat kallikrein-binding protein.

No.	Peptide	Sequence of Abz-peptidyl-Q-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
I	$HKBP^{350-361}$	A-T-T-F-A-I-K-F-F ↓ S-A	2.23 ± 0.09	0.50 ± 0.01	224
II	$HKBP^{354-361}$	A-I-K-F-F ↓ S-A	1.79 ± 0.11	0.54 ± 0.01	302
III	$HKBP^{354-364}$	A-I-K-F-F ↓ S-A-Q-T-N-R	0.72 ± 0.04	1.71 ± 0.01	2375
IV	$HKBP^{354-371}$	A-I-K-F-F ↓ S-A-Q-T-N-R-H-I-L-R-F-N-R	0.031 ± 0.004	1.21 ± 0.02	40333
V	$PCI^{354-363}$	I-F-T-F-R ↓ S-A-R-L-N	0.045 ± 0.004	0.91 ± 0.02	18200
VI	$PCI^{354-371}$	I-F-T-F-R ↓ S-A-R-L-N-S-Q-R-L-V-F-N-R	0.046 ± 0.003	2.29 ± 0.03	45800
VII	$RKBP^{354-371}$	T-A-A-L-K ↓ S-L-P-Q-T-I-P-L-L-N-F-N-R	1.99 ± 0.09	0.17 ± 0.0.1	85

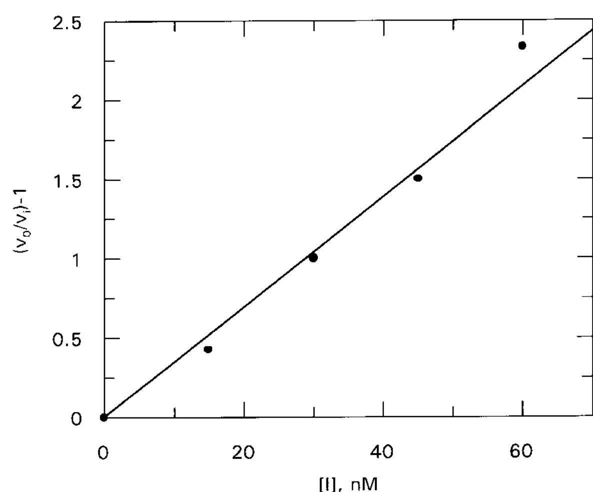

Figure 2 Competitive inhibitory assay of Abz-AIKFFSAQTNRHILRFNR-EDDnp (peptide IV)

Peptide IV was assayed as a competitive inhibitor over the hydrolytic activity of hK_1 on the substrate (D)Pro-Phe-Phe-MCA (K_m 70 μM, k_{cat} 12.5 s^{-1}, [18]) according to the method described by Nicklin and Barrett [33]. For this experiment, 30 μM (D)Pro-Phe-Phe-MCA and 5 pM hK_1 were used.

were cleaved at the Phe–Ser bond, and those with PCI and rat kallikrein-binding protein sequences were hydrolysed at Arg–Ser and Lys–Ser bonds, respectively.

The kinetic data for hydrolysis of peptides I–IV show that a C-terminal extension from the cleavage site of peptides with the kallistatin sequence significantly improved the hydrolytic efficiency by increasing the k_{cat} and decreasing the K_m values. However, peptide IV, with a larger C-terminal extension, was hydrolysed with similar k_{cat} value to peptide III, but with a low, sub-micromolar, K_m value. The reliability of this very low K_m value was then verified by transforming the kinetic data into an Eadie–Hofstee plot (Figure 1, insert), which confirmed the kinetic parameters obtained. Furthermore, the K_i value of 30 nM obtained for inhibition by peptide IV of (D)Pro-Phe-Phe-MCA hydrolysis (Figure 2) confirms the high affinity of hK_1 for this peptide.

The substrates with the PCI sequence (peptides V and VI) are hydrolysed by hK_1 with similar, and also low, K_m values. The extension on the C-terminal side (peptide VI) increased the k_{cat} value 2-fold in comparison with peptide V. The kinetic parameters for hydrolysis of peptide containing rat kallikrein-binding protein sequence from residues 354–371 (peptide VII) showed that the resulting substrate was poorly hydrolysed by hK_1.

The effect of the nature of the amino acid at the primary specificity site (P_1) in both kallistatin and PCI peptide sequences was examined by substituting Phe with Arg in the substrates containing kallistatin sequences (peptides VIII and IX); the opposite substitution was made in the substrates containing PCI sequences (peptides X and XI; Table 2). All of these four peptides were hydrolysed with k_{cat}/K_m values lower than the parent substrates (compare peptides III with VIII, IV with IX, V with X and VI with XI). Therefore, these results indicate that the efficiency of hydrolysis after Phe–Phe or Phe–Arg pairs by hK_1 is dependent on the nature of the other residues, probably on both sides of these dipeptides.

In order to explore these specificity requirements on the P′ side, we synthesized a hybrid peptide containing kallistatin sequence (Ala-Ile-Lys-Phe-Phe) followed by PCI sequence (Ser-Ala-Gln-Thr-Asn-Ser), shown in Table 2 as peptide XII. We also synthesized two other analogues of this peptide, one with a substitution of Arg by Gln (peptide XII) and the other with a substitution of Arg and Leu by Gln and Thr, respectively (peptide XIII). These substitutions gradually transformed the hybrid substrate (peptide XII) into one containing only kallistatin sequence (peptide III); the kinetic parameters for the hydrolysis of these peptides are shown in Table 2. The hybrid peptide was the poorest substrate for hK_1 in this series, being hydrolysed with the lowest k_{cat}/K_m value (due essentially to the high K_m value). The restoration of Gln and/or Thr in peptides XIII and XIV reduced the K_m value by 1 order of magnitude. However, the highest k_{cat}/K_m values were observed with peptide III, which contained amino acids only from kallistatin. These results suggest that Gln at P'_3 and Arg at P'_6 assume significant roles in determining the specificity of hK_1 for cleavage after the Phe–Phe pair in kallistatin.

Hydrolysis of peptides of the Abz-AIKX_2X_1SRQ-EDDnp series

The importance of the Phe–Phe pair at the P_1 and P_2 positions for efficient hydrolysis by hK_1 was evaluated by synthesizing a series of peptides containing one or both Phe residues substituted for other hydrophobic amino acids in Abz-AIKFFSRQ-EDDnp (peptide XV; Table 3). This peptide, with an Arg residue at the P'_2 position instead of Ala (peptide II), was chosen as the lead substrate because it is hydrolysed with a high k_{cat} and k_{cat}/K_m, and exhibits a low K_m value. We made this choice in order to increase the possibility of detection of minimal hydrolysis of the worst substrates of this series. In addition, the higher solubility

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Table 2 Kinetic parameters for hydrolysis by hK_1 of internally quenched fluorescent peptides with reactive-centre loop sequences of kallistatin and PCI, as well as hybrid peptides

Hydrolysis conditions were as for Table 1. Results are presented as means ± S.E. ↓ indicates the position of cleavage. Abbreviation: HKBP, hK_1-binding protein (kallistatin). Underlined amino acids in peptides XII–XIV indicate the segment that pertains to PCI sequence.

No.	Peptide (with mutation shown)	Sequence of Abz-peptidyl-Q-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
VIII	$HKBP^{354-364}$, $F \rightarrow R^{358}$	A-I-K-F-R ↓ S-A-Q-T-N-R	0.27 ± 0.02	0.46 ± 0.02	1704
IX	$HKBP^{354-371}$, $F \rightarrow R^{358}$	A-I-K-F-R ↓ S-A-Q-T-N-R-H-I-L-R-F-N-R	0.098 ± 0.006	0.37 ± 0.02	3700
X	$PCI^{354-363}$, $R \rightarrow F^{358}$	I-F-T-F-F ↓ S-A-R-L-N	–	–	130 ± 6*
XI	$PCI^{354-371}$, $R \rightarrow F^{358}$	I-F-T-F-F ↓ S-A-R-L-N-S-Q-R-L-V-F-N-R	–	–	1848 ± 55*
XII	$HKBP^{354-360}PCI^{361-364}$	A-I-K-F-F ↓ S-A-R-L-N-S	11.94 ± 0.24	0.32 ± 0.01	27
XIII	$HKBP^{354-361}PCI^{362-364}$	A-I-K-F-F ↓ S-A-Q-L-N-S	1.19 ± 0.03	0.48 ± 0.02	403
XIV	$HKBP^{354-364}$, $R \rightarrow S^{364}$	A-I-K-F-F ↓ S-A-Q-T-N-S	1.09 ± 0.05	0.16 ± 0.01	146
III	$HKBP^{354-364}$	A-I-K-F-F ↓ S-A-Q-T-N-R	0.72 ± 0.04	1.71 ± 0.01	2375

* Determined in pseudo first-order condition, due to low solubility of the peptides.

Table 3 Kinetic parameters for hydrolysis by hK1 of internally quenched fluorescent peptides with reactive-centre loop sequences of kallistatin containing some modifications at P_1 and P_2 positions with hydrophobic amino acids

Hydrolysis conditions were as in Table 1. Results are presented as means ± S.E. ↓ indicates the position of cleavage. Underlined residues represent those that have been modified in a given series.

No.	Sequence of Abz-peptidyl-Q-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
XV	A-I-K-F-F ↓ S-R	0.36 ± 0.01	3.33 ± 0.15	9250
XVI	A-I-K-M-M ↓ S-R	1.35 ± 0.09	0.28 ± 0.01	207
XVII	A-I-K-L-L ↓ S-R	1.63 ± 0.12	0.14 ± 0.01	86
XVIII	A-I-K-M-F ↓ S-R	0.80 ± 0.06	0.65 ± 0.01	813
XIX	A-I-K-L-F ↓ S-R	0.35 ± 0.01	0.74 ± 0.02	2114
XX	A-I-K-F-M ↓ S-R	0.68 ± 0.02	0.15 ± 0.01	221
XXI	A-I-K-L-M ↓ S-R	0.36 ± 0.01	0.18 ± 0.01	500
XXII	A-I-K-M-L ↓ S-R	5.37 ± 0.14	0.39 ± 0.01	73
XXIII*	A-I-K-I-I-S-R	–	–	–
XXIV*	A-I-K-V-V-S-R	–	–	–
XXV*	A-I-K-W-W-S-R	–	–	–
XXVI*	A-I-K-Y-Y-S-R	–	–	–

* Substrates not hydrolysed at enzyme concentration higher than 5 nM.

in water due to the presence of Arg was desirable for obtaining an appropriate concentration range in a systematic study.

The highest specificity constant (k_{cat}/K_m) among the hydrophobic amino acid pairs was observed for the hydrolysis of the lead substrate (peptide XV), which contained the Phe–Phe pair, followed by peptides XIX and XVIII, which also contained Phe at position P_1. The peptide with the Met–Met pair was a better substrate than the one containing Leu–Leu (compare peptides XVI and XVII). To explore the susceptibility of peptides containing a Leu–Leu pair we synthesized two peptides containing the α_1-antichymotrypsin reactive-centre loop: Abz-ATAVKITLLSA-EDDnp and Abz-KITLLSALVETRTIVRFNR-EDDnp. These peptides were completely resistant to hK_1. All other hydrophobic pairs, Ile–Ile, Val–Val, Trp–Trp and Tyr–Tyr (peptides XXIII–XXVI), were not hydrolysed by hK_1. The combination of Phe, Met and Leu was also examined. The substrate with Leu–Met (peptide XXI) was more susceptible to hK_1 than that with Met–Leu (peptide XXII), which was hydrolysed with the highest K_m value of this series. In contrast, the lowest values for K_m were obtained with the Phe–Phe-, Leu–Met- and Leu–Phe-containing peptides (peptides XV, XXI and XIX). It is noteworthy that somatostatin and human kininogen were also hydrolysed by hK_1 after the dipeptides Phe–Phe and Leu–Met, respectively [15,18].

Hydrolysis of peptides of the series Abz-AIX_3FFSRQ-EDDnp and Abz-AX_4KFFSRQ-EDDnp

The modification at the P_3 position, which corresponds to the series Abz-AIX_3FFSRQ-EDDnp, resulted in substrates that were all susceptible to hK_1; however, the lead peptide of this series (X = Lys, peptide XV) was the best-hydrolysed substrate (Table 4). Higher K_m values were observed with Ser, Thr, Asn and Gln (peptides XXXI–XXXIV), indicating an unfavourable effect on the substrate binding of hydroxyl and carboxamide functions. Ala-, Leu-, Glu- and Pro-containing substrates were hydrolysed with lower K_m values. However, despite this increase in the substrate affinity, a negative effect was observed for their hydrolysis, due to lower k_{cat} values (peptides XXVII–XXX). It is worthwhile to note that the substrate with Thr at P_3 (peptide XXXII), which is also present in PCI at the same position, was hydrolysed with the second-best k_{cat} value, followed by Ser (peptide XXXI).

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Table 4 Kinetic parameters for hydrolysis by hK_1 of internally quenched fluorescent peptides with reactive-centre loop sequences of kallistatin and some modifications at the P_3 position

Hydrolysis conditions were as for Table 1. Results are presented as means ± S.E. ↓ indicates the position of cleavage. Underlined residues represent those that have been modified in a given series.

No.	Sequence of Abz-peptidyl-Q-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
XV	A-I-K-F-F↓S-R	0.36 ± 0.01	3.33 ± 0.15	9250
XXVII	A-I-A-F-F↓S-R	0.29 ± 0.01	0.60 ± 0.02	2069
XXVIII	A-I-L-F-F↓S-R	0.14 ± 0.01	0.18 ± 0.01	1289
XXIX	A-I-E-F-F↓S-R	0.60 ± 0.02	0.58 ± 0.01	967
XXX	A-I-P-F-F↓S-R	0.40 ± 0.02	0.06 ± 0.01	150
XXXI	A-I-S-F-F↓S-R	3.52 ± 0.09	1.49 ± 0.06	423
XXXII	A-I-T-F-F↓S-R	2.36 ± 0.11	2.06 ± 0.09	873
XXXIII	A-I-Q-F-F↓S-R	1.21 ± 0.08	1.19 ± 0.05	983
XXXIV	A-I-N-F-F↓S-R	1.40 ± 0.09	0.38 ± 0.02	271

Table 5 Kinetic parameters for hydrolysis by hK_1 of internally quenched fluorescent peptides with reactive-centre loop sequences of kallistatin and some modifications at the P_4 position

Hydrolysis conditions as described in Table 1. Results are presented as means ± S.E. ↓ indicates the position of cleavage. Underlined residues represent those that have been modified in a given series.

No.	Sequence of Abz-peptidyl-Q-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
XV	A-I-K-F-F↓S-R	0.36 ± 0.01	3.33 ± 0.15	9250
XXXV	A-V-K-F-F↓S-R	1.10 ± 0.05	1.52 ± 0.08	1381
XXXVI	A-L-K-F-F↓S-R	0.12 ± 0.01	0.07 ± 0.01	583
XXXVII	A-A-K-F-F↓S-R	1.40 ± 0.02	0.59 ± 0.02	421
XXXVIII	A-P-K-F-F↓S-R	0.31 ± 0.01	0.43 ± 0.01	1387
XXXIX	A-E-K-F-F↓S-R	20.0 ± 1.22	0.96 ± 0.03	48
XL	A-Q-K-F-F↓S-R	2.00 ± 0.05	1.33 ± 0.05	665
XLI	A-N-K-F-F↓S-R	1.50 ± 0.06	0.37 ± 0.01	247
XLII	A-T-K-F-F↓S-R	3.80 ± 0.09	2.46 ± 0.02	647
XLIII	A-S-K-F-F↓S-R	1.10 ± 0.05	0.85 ± 0.02	772
XLIV	A-K-K-F-F↓S-R	0.36 ± 0.02	0.14 ± 0.01	388

The modifications at the P_4 position demonstrated that the hydrolytic activity of hK_1 on the series Abz-AX_4KFFSRQ-EDDnp was quite sensitive to the S_4–P_4 interaction. Again, the lead substrate (peptide XV; Table 5), with Ile at the P_4 position, was hydrolysed with the highest k_{cat}/K_m value in the series. However, it is particularly noteworthy that the k_{cat}/K_m value for this substrate was 7 and 17 times higher than for the substrates with Val (peptide XXXV) or Leu (peptide XXXVI) respectively, due to the sensitivity to the structure of aliphatic side chain of the amino acids. In addition, the latter substrates were hydrolysed with the lowest k_{cat} and K_m values in the series. The substrates with Pro and Lys were hydrolysed with K_m values similar to that of the lead compound, but with significantly lower k_{cat} values (compare peptide XV with peptides XXXVIII and XLIV). All other substitutions, Ala, Glu, Gln, Asn, Thr and Ser (peptides XXXVII and XXXIX–XLIII) were hydrolysed with higher K_m and lower k_{cat} values than the lead substrate (peptide XV). The worst substrate was obtained with Glu (peptide XXXIX), indicating the very unfavourable effect of a carboxyl negative charge at the P_4 position.

DISCUSSION

The development of specific inhibitors for tissue kallikreins and for other kallikrein-like enzymes depends on detailed information about their substrate–subsite interactions. Some of these determinants were identified in previous studies on the hydrolysis by human, pig, horse and rat tissue kallikreins of the arginyl and lysyl bonds in synthetic substrates. The following observations have been reported: (i) Arg in position P_1 is preferred over Lys [34]; (ii) the Phe side chain binds best to the enzyme's S_2 subsite [21,34,35]; (iii) D-amino acid residues at P_3 interact favourably with human and horse kallikreins [35,36]; and (iv) Pro in P'_2 impairs the cleavage by horse and pig, but not by rat, tissue kallikrein, whereas Arg in P'_1 or P'_2 increases the efficiency of cleavage by all three kallikreins [31,37–39].

Met and Phe were the only hydrophobic amino acids so far described at which hK_1 was able to cleave [14,15,18]. The specificity to Phe is higher than to Met because hK_1 was able to hydrolyse small chromogenic or fluorogenic peptides, such as (D)Pro-Phe-Phe-pNa or (D)Pro-Phe-Phe-MCA [15,18], in contrast with the resistance of the Met–X linkage in short synthetic peptides [40]. The results shown in this paper demonstrate that the efficiency of hK_1 hydrolytic activity is higher after Phe–Phe in comparison with other hydrophobic pairs assayed and is significantly influenced by the substrate size as well as by the nature of amino acids at secondary subsites.

All the synthetic peptides based on the sequence of the reactive-centre loop of human kallistatin were recognized as substrates by hK_1. The values of the kinetic parameters obtained were dependent on the size of the C-terminal extensions. The significant decrease in K_m values for hydrolysis of peptides I–IV, to 30 nM for peptide IV, indicates that the C-terminal amino acids of the extended substrate (residues P'_1–P'_{13}) profoundly influenced

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binding to hK_1. A similar behaviour, but with a less dramatic reduction in K_m values, was also observed for the cleavage of the Met–Lys bond by hK_1 in substrates with the human kininogen sequence [14], which also required larger sequences for a more efficient hydrolysis. On the other hand, the extension of substrate on the N-terminal side of the scissile bond beyond P_5 (peptide I; Table 1) seems to have no effect on its hydrolysis by hK_1. This contrasts with the hydrolysis of the Met–Lys bond in substrates with human kininogen sequence that is apparently sensitive to substrate extension up to P_7 [14]. Therefore, it seems that the requirements of secondary specificity for cleavage of substrates after hydrophobic amino acid pairs by hK_1 depend on the nature of the amino acid pairs.

The K_m values for the hydrolysis of peptides V and VI were alike and as low as the value for peptide IV. On the other hand, the k_{cat} value for the longer PCI-loop peptide (IX) was twice as high. Therefore, extension of the C-terminus in peptides V and VI did not have the same effect on K_m as observed with the hydrolysis of peptides III and IV. In general, short peptides were efficiently hydrolysed by hK_1 after a Phe–Arg pair, and the obtained kinetic parameters indicate that fewer amino acids were required to interact at S′ subsites of the enzyme than those with Phe–Phe at the same positions. A similar, but not-so-pronounced, behaviour was observed for the hydrolysis of Arg–Ser and Met–Lys bonds in substrates containing human kininogen sequence [14]. These results suggested that the electrostatic guanidinium–carboxylate interaction of Arg from substrate and Asp-189 at the S_1 protease pocket provides most of the required energy for enzyme–substrate interaction. On the other hand, in substrates containing Phe at P_1, the energy for binding is supplemented by the secondary subsite interactions. This view is in accordance with the kinetic parameters for the hydrolysis of Abz-AIKFRSR-EDDnp (K_m 0.05 μM, k_{cat} 2.07 s^{-1}) compared with those obtained with Abz-AIKFFSR-EDDnp (peptide XV; Table 3).

The efficiency of the substrate hydrolysis by hK_1 after Phe–Phe or Phe–Arg amino acid pairs is highly dependent on the nature of the residues on both sides of these pairs. The substrates with the kallistatin sequence, but with Phe-358 substituted by Arg (peptides VIII and IX), and peptides X and XI, derived from PCI sequence, in which Arg-358 was replaced by Phe, were hydrolysed with k_{cat}/K_m values (Table 2) significantly lower than those of parent peptides (III, IV, V and VI; Table 1). Therefore, the limited proteolytic activity of hK_1 is also extended to its arginyl-hydrolase function. Accordingly, the Arg–Phe bonds in peptide IV as well as in Abz-HILRFNRQ-EDDnp were resistant to hydrolysis to hK_1, although the sequences of these peptides have all the known requirements to be substrates of this enzyme. On the other hand, we observed that peptide Abz-VIAGRSLNP-NRVTFK↓ANR-EDDnp, which contains the reactive-centre loop of antithrombin III, is hydrolysed at the Lys–Ala bond (indicated by the arrow) with a K_m of 0.06 μM and a k_{cat} of 1.26 s^{-1}.

We further explored the specificity requirements for hydrolysis after the Phe–Phe pair using three approaches. The first was the synthesis of a hybrid peptide, containing kallistatin and PCI sequences, and its analogues, gradually turning the PCI segment into the kallistatin sequence. It was clear from the analysis of the kinetic parameters for hydrolysis of these substrates (peptides XII–XIV) that Gln at P'_3 and Arg at P'_6 play significant roles in the interaction and catalytic processes of hK_1 on the kallistatin reactive-centre loop. The second approach was to verify if other hydrophobic pairs of amino acids in the sequence of kallistatin could be hydrolysed by hK_1, and the third approach was a systematic variation at the P_3 and P_4 sites of the kallistatin reactive loop. The parent substrates were most susceptible to hK_1, as determined from the lowest K_m and the highest k_{cat} values of the series with substitution on the Phe–Phe pair (Tables 1, 2 and 3). In addition, P_3 and P_4 substrate sites also take part in the determination of specificity (Tables 4 and 5) for the hydrolysis after the Phe–Phe pair and, again, as in the lead substrate, Lys and Ile were the best residues at the P_3 and P_4 positions. These results, together with the previously described hydrolysis by hK_1 of somatostatin at the Phe–Trp bond (shown by an arrow; AGCKNFF↓WKTFTSC), and the resistance of substance P (RPKPQQFFGLM-NH_2) and of the human insulin β-chain (… G^{20}ERGFFYTPKA^{30}-COOH) [18], lead us to the hypothesis that the efficiency of hydrolysis after Phe–Phe by hK_1 does not depend on the presence of specific amino acids at both sides of this amino acid pair. Indeed, the small chromogenic or fluorogenic peptides (D)Pro-Phe-Phe-pNa or (D)Pro-Phe-Phe-MCA are hydrolysed by hK_1 with k_{cat}/K_m values of 462 and 179 $mM^{-1} \cdot s^{-1}$ respectively [15,18]. In addition, the presence of such diverse amino acids as Lys, Leu and Pro at the P_3 and P_4 positions resulted in substrates cleaved with the lowest K_m values. On the other hand, since the cleavage after Phe–Phe was more restricted in larger peptides, it is reasonable to speculate that this particular hydrolytic activity of hK_1 depends on secondary structures that the substrate could adopt. This situation would be similar to the previously described dependency of hydrolytic activity of THIMET, a thiol-metallo-protease that processes biologically active peptides [41,42], on general peptide structure rather than absolute sequence. Moreover, limited proteolysis seems to be a process in which dynamics, and not only the enzyme/substrate ratio, play a role in determining whether a particular cleavage will be observed. Nevertheless, the actual proteolytic cleavage of a peptide bond still depends on the prime determinants of specificity of the enzyme and on the flexibility and exposure of the putative scissile bond [43].

In conclusion, it is noteworthy that tissue kallikrein is very effective on substrates that are cleaved after the Phe–Arg pair; however, the hydrolysis after Phe–Phe, or other pairs of hydrophobic amino acids, is much more restricted, requiring secondary enzyme–substrate bindings and/or preferred sets of conformations of the substrates. Accordingly, hK_1 is more specific for hydrolysis after the Phe–Phe pair, and kallistatin, which contains Phe–Phe, is a more specific serpin for tissue kallikrein than PCI, which contains Phe–Arg [16,17,20].

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REFERENCES

1 Bhoola, K. D., Figueroa, C. D. and Worthy, K. (1992) Pharmacol. Rev. **44**, 1–80
2 MacDonald, R. J., Margolius, H. S. and Erdös, E. G. (1988) Biochem. J. **253**, 313–321
3 Berg, T., Bradshaw, R. A., Carretero, O. A., Chao, L., Clements, J. A., Fahnestock, M., Fritz, H., Gauthier, F., MacDonald, R. J., Margolius, H. S., Morris, B. J., Richards, R. I. and Scili, A. G. (1992) Agents Actions **38**, 19–25
4 Riegman, P. H. J., Vlietstra, R. J., Suurmeijer, L., Cleutjens, C. B. J. M. and Trapman, J. (1992) Genomics **14**, 6–11
5 Worthy, K., Figueroa, C. D., Dieppe, P. A. and Bhoola, K. D.(1990) Int. J. Exp. Pathol. **71**, 587–601
6 Christiansen, S. C., Proud, D. and Cochrane, C. G. (1987) J. Clin. Invest. **79**, 188–197
7 Proud, D., Togias, A., Naclerio, R. M., Crush, S. A., Norman, P. S. and Linchtenstein, L. M. (1983) J. Clin. Invest. **72**, 1678–1685
8 Braumgarten, C. R., Nichols, R. C., Naclerio, R. M. and Proud, D. (1986) J. Immunol. **137**, 1323–1328

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9 Deperthes, D., Frenette, G., Brillard-Bourdet, M., Bourgeois, L., Gauthier, F., Tremblay, R. R. and Dubé, J. Y. (1996) J. Androl. **17**, 659–665
10 Mikolajczyk, S.D., Millar, L. S., Kumar, A. and Saedi, M. S. (1998) Prostate **34**, 44–50
11 Pierce, J. W. and Webster, M. E. (1961) Biochem. Biophys. Res. Commun. **5**, 353–357
12 Alhenc-Gelas, F., Marchetti, J., Alegrini, J., Carod, P. and Menard, J. (1981) Biochim. Biophys. Acta **677**, 477–488
13 Kato, H., Enjyoji, K., Miyata, T., Hayashi, I., Oh-ishi, S. and Iwagaga, S. (1985) Biochem. Biophys. Res. Commun. **127**, 289–295
14 Del Nery, E., Chagas, J. R., Juliano, M. A., Prado, E. S. and Juliano, L. (1995) Biochem. J. **312**, 233–238
15 Chagas, J. R., Portaro, F. C. V., Hirata, I. Y., Almeida, P. C., Juliano, M. A., Juliano, L. and Prado, E. S. (1995) Biochem. J. **306**, 63–69
16 Zhou, G. X., Chao, L. and Chao, J. (1992) J. Biol. Chem. **267**, 25873–25880
17 Chai, K. X., Chen, L.-M., Chao, J. and Chao, L. (1993) J. Biol. Chem. **268**, 24498–24505
18 Pimenta, D. C., Juliano, M. A. and Juliano, L. (1997) Biochem. J. **327**, 27–30
19 Katz, B. A., Liu, B. S., Bames, M. and Springman, E. B. (1998) Protein Sci. **7**, 875–885
20 Chen, Z. and Bode, W. (1983) J. Mol. Biol. **164**, 283–311
21 Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. **27**, 157–162
22 Loebermann, H., Tokuoka, R., Deisenhofer, J. and Huber, R. (1994) J. Mol. Biol. **177**, 531–556
23 Ecke, S., Geiger, M., Resch, I., Jerabek, I., Sting, L., Maier, M. and Binder, B. R. (1992) J. Biol. Chem. **267**, 7048–7052
24 Chen, L.-M., Chao, L., Mayfield, R. K. and Chao, J. (1990) Biochem. J. **267**, 79–84
25 Bourgeois, L., Brillard-Bourdet, M., Deperthes, D., Juliano, M. A., Juliano, L., Tremblay, R. R., Dubé, J. Y. and Gauthier, F. (1997) J. Biol. Chem. **272**, 29590–29595

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26 Mittl, P. R. E., Di Marco, S., Fendrich, G., Pohlig, G., Heim, J., Sommerhoff, C., Fritz, H., Priestle, J. P. and Grütter, M. G. (1997) Structure **5**, 253–264
27 Shimamoto, K., Chao, J. and Margolius, H. S. (1980) J. Endocrinol. Metab. **51**, 840–859
28 Sampaio, C. A. M., Sampaio, M. U. and Prado, E. S. (1984) Hoppe-Seyler's Z. Physiol. Chem. **365**, 297–302
29 Hirata, I. Y., Cezari, M. H. S., Nakaie, C., Boschcov, P., Ito, A. S., Juliano, M. and Juliano, L. (1994) Lett. Peptide Sci. **1**, 299–308
30 Alves, L. C., Almeida, P. C., Franzoni, L., Juliano, L. and Juliano, M. A. (1996) Peptide Res. **9**, 92–95
31 Chagas, J. R., Juliano, L. and Prado, E. S. (1991) Anal. Biochem. **192**, 419–425
32 Wilkinson, G. N. (1961) Biochem. J. **80**, 324–332
33 Nicklin, M. J. and Barrett, A. J. (1984) Biochem. J. **223**, 245–253
34 Fiedler, F. (1987) Eur. J. Biochem. **163**, 303–312
35 Araujo-Viel, M. S., Juliano, M. A., Oliveira, L. and Prado, E. S. (1988) Biol. Chem. Hoppe-Seyler **369**, 397–401
36 Oliveira, L., Araujo-Viel, M. S., Juliano, L. and Prado, E. S. (1987) Biochemistry **26**, 5032–5035
37 Prado, E. S., Prado de Carvalho, L., Araujo-Viel, M., Ling, N. and Rossier, J. (1983) Biochem. Biophys. Res. Commun. **122**, 366–371
38 Prado, E. S., Araujo-Viel, M. S., Juliano, M. A., Juliano, L., Stella, R. C. R. and Sampaio, C. A. M. (1986) Biol. Chem. Hoppe-Seyler **367**, 199–205
39 Chagas, J. R., Hirata, I. Y., Juliano, M. A., Xiong, W., Wang, C., Chao, J., Juliano, L. and Prado, E. S. (1992) Biochemistry **31**, 4969–4974
40 Fiedler, F. (1986) Adv. Exp. Med. Biol. **198A**, 283–289
41 Camargo, A. C. M., Gomes, M. D., Reichl, A. P., Ferro, E. S., Jacchieri, S., Hirata, I. Y. and Juliano, L. (1997) Biochem. J. **324**, 517–522
42 Jacchieri, S. C., Gomes, M. D., Juliano, L. and Camargo, A. C. M. (1998) J. Peptide Res. **51**, 452–459
43 Hubbard, S. J., Beynon, R. J. and Thornton, J. M. (1998) Protein Eng. **11**, 349–359

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