Human Tissue Kallikrein S_1 Subsite Recognition of Non-Natural Basic Amino Acids†

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ABSTRACT: We explored the unique substrate specificity of the primary S_1 subsite of human urinary kallikrein (hK1), which accepts both Phe and Arg, using internally quenched fluorescent peptides Abz-F-X-S-R-Q-EDDnp and Abz-G-F-S-P-F-X-S-S-R-P-Q-EDDnp [Abz is *o*-aminobenzoic acid; EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine], which were based on the human kininogen sequence at the C-terminal region of bradykinin. Position X, which in natural sequence stands for Arg, received the following synthetic basic non-natural amino acids: 4-(aminomethyl)phenylalanine (Amf), 4-guanidine phenylalanine (Gnf), 4-(aminomethyl)-*N*-isopropylphenylalanine (Iaf), N^{im}-(dimethyl)histidine [H(2Me)], 3-pyridylalanine (Pya), 4-piperidinylalanine (Ppa), 4-(aminomethyl)cyclohexylalanine (Ama), and 4-(aminocyclohexyl)alanine (Aca). Only Abz-F-Amf-S-R-Q-EDDnp and Abz-F-H(2Me)]-S-R-Q-EDDnp were efficiently hydrolyzed, and all others were resistant to hydrolysis. However, Abz-F-Ama-S-R-Q-EDDnp inhibited hK1 with a K_i of 50 nM with high specificity compared to human plasma kallikrein, thrombin, plasmin, and trypsin. The Abz-G-F-S-P-F-X-S-S-R-P-Q-EDDnp series were more susceptible to hK1, although the peptides with Gnf, Pya, and Ama were resistant to it. Unexpectedly, the peptides in which X is His, Lys, H(2Me), Amf, Iaf, Ppa, and Aca were cleaved at amino or at carboxyl sites of these amino acids, indicating that the S_1' subsite has significant preference for basic residues. Human plasma kallikrein did not hydrolyze any peptide of this series except the natural sequence where X is Arg. In conclusion, the S_1 subsite of hK1 accepts amino acids with combined basic and aromatic side chain, although for the S_1-P_1 interaction the preference is for aliphatic and basic side chains.

Human tissue kallikrein (hK1,[1] EC 3.4.21.35) 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 (hK3 or PSA) and a trypsin-like enzyme, hK2. This classical view has been recently changed by inclusion of 14 genes in the human kallikrein gene family, and some of them have been correlated to carcinogenesis (*3*−*5*). hK1 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 (*7*−*9*), and the processing of hormone and other peptide precursors (*1*, *2*). Tissue kallikreins comprise a subfamily of trypsin-like serine proteinases, widely distributed in the animal tissues with closely related structures, and share common biological features, including a highly specific kininogenase activity. In the limited proteolysis of high- and low-molecular weight kininogens by tissue kallikreins, the vasoactive decapeptide Lys-bradykinin (Lys-BK) is released by cleavage at the $Met^{379}-Lys^{380}$ and $Arg^{389}-Ser^{390}$ bonds. The human kininogen sequence from residue 370 to 399, using the human prekininogen numbering, is Cys^{370}-Gln-Pro-Leu-Gly-Met-Ile-Ser-Leu-Met^{379}-Lys^{380}-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg^{389}-Ser^{390}-Ser-Arg-Ile-Gly-Glu-Lys-Ile-Glu-Glu^{399}.

hK1 and the cognate enzymes in mammals release Lys-bradykinin from bovine and human kininogen by cleaving the Met−Lys and Arg−Ser bonds (*10*). The only exceptions known so far are rat tissue kallikrein (rK1), which cleaves a Lys−Arg bond and releases bradykinin from bovine and rat kininogens (*11*, *12*) and mouse submandibular tissue kallikrein (*13*). The efficiency of cleavage at the Met−Lys bond by hK1 is dependent on the extension of the substrates, as demonstrated with internally quenched fluorescent peptides with the sequence of human kininogen (*14*, *15*). hK1 can also hydrolyze after a pair of phenylalanines, for example,

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[1] Abbreviations: hK_1, human tissue kallikrein; Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; Amf, 4-(aminomethyl)phenylalanine; Gnf, 4-guanidine phenylalanine; H(2Me), N^{im}-(dimethyl)histidine; Iaf, 4-(aminomethyl)-*N*-isopropylphenylalanine; Pya, 3-pyridylalanine; Ppa, 4-piperidinylalanine; Ama, 4-(aminomethyl)cyclohexylalanine; Aca, 4-(aminocyclohexyl)alanine.

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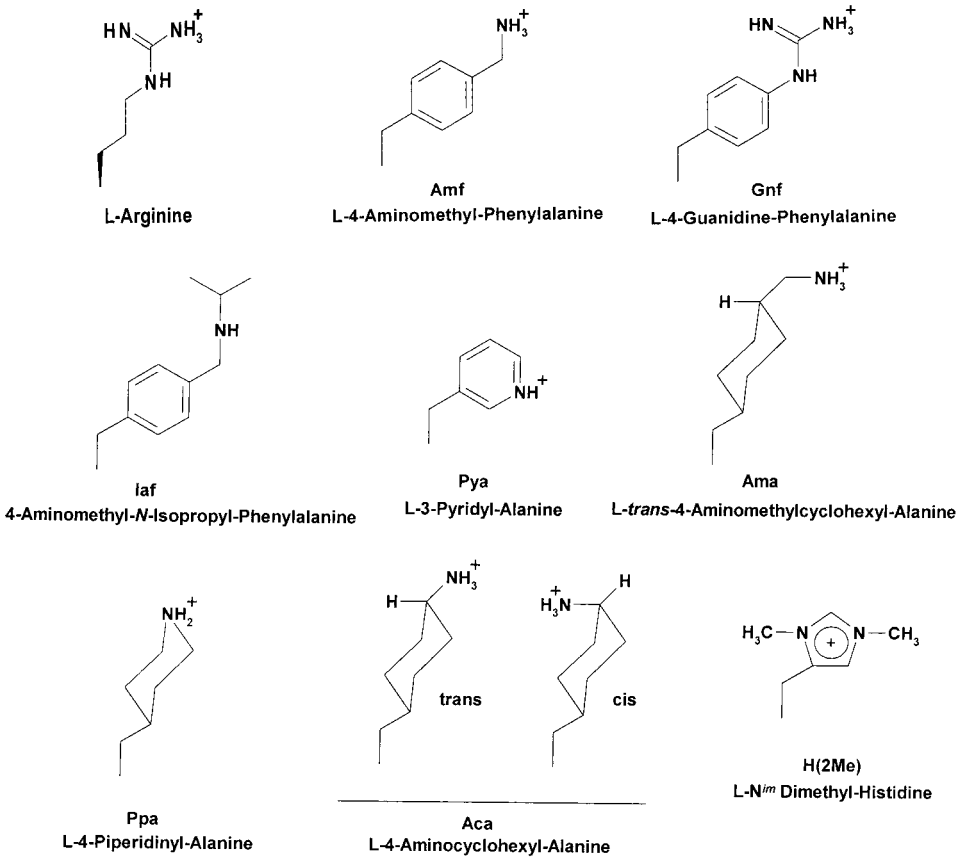

FIGURE 1: Side chain structure of the non-natural basic amino acids employed as substitutes for Arg in the peptides assayed in this paper.

in the reactive site loop of human tissue kallikrein-binding protein, which is a specific serpin for tissue kallikrein called kallistatin (*16*, *17*), and in somatostatin (*18*). Although hK1 is significantly homologous with trypsin, the crystal structures of human and porcine tissue kallikrein (*19*, *20*) show that their S_1 [see the nomenclature of Schechter and Berger (*21*)] sites are enlarged, mainly because of 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 a side chain larger than and as hydrophobic as that of Phe.

A quite specific inhibitor (phenylacetyl-Phe-Ser-Arg-EDDnp) for hK1 was demonstrated to present significant analgesic and anti-inflammatory activities (*22*); however, the K_i value in vitro for this peptide was 0.7 μM. In our program of developing efficient inhibitors for hK1, it seems relevant to explore in more detail the unique substrate specificity of the primary S_1 subsite that accepts amino acids Phe and Arg (*23*). We synthesized two sets of internally quenched fluorogenic peptides derived from the sequences Abz-F-R-S-R-Q-EDDnp and Abz-G-F-S-P-F-R-S-S-R-P-Q-EDDnp, which are based on the human kininogen sequence at the C-terminal region of bradykinin, and substituted Arg for non-natural basic amino acids which were designed to combine a large hydrophobic and/or aromatic group with a positively charged group at their side chains. The following amino acids were synthesized and introduced in these peptides: 4-(aminomethyl)phenylalanine (Amf), 4-guanidine phenylalanine (Gnf), 4-(aminomethyl)-*N*-isopropylphenylalanine (Iaf), N^{im}-(dimethyl)histidine [H(2Me)], 3-pyridylalanine (Pya), 4-piperidinylalanine (Ppa), 4-(aminomethyl)cyclohexylalanine (Ama), and 4-(aminocyclohexyl)alanine (Aca) (see Figure 1 for the structures of the side chains). The kinetic parameters for hK1 and the substrate cleavage sites were determined, and for comparison, the obtained peptides were also assayed with human plasma kallikrein and bovine trypsin.

MATERIALS AND METHODS

Synthesis of Protected Non-Natural Basic Amino Acids. All the amino acids were characterized by ^{1}H NMR (Bruker AMX-500) and mass spectroscopy (LCQ Thermoquest-Ion Trap), and the data were consistent with the considered structure (for details of the synthesis, see the Supporting Information).

L-4-(Aminomethyl)phenylalanine (Amf). Fmoc-L-Phe(4-CH_2NH-Boc) was prepared according to the procedure, opportunely modified (*24*). The key step of the AMF synthesis, introduction of the 4-[*N*-(trichloroacetyl)amino]-methyl group is accomplished readily via acid-catalyzed, nuclear amidoalkylation of L-Phe and is followed by acid hydrolysis to yield AMF dihydrochloride in modest yield (≈30%) but in a high state of chiral purity. Treatment with di-*tert*-butyldicarbamate in butyl alcohol afforded the 4-{[*N*-(benzyloxycarbonyl)amino]methyl}-L-phenylalanine. The Fmoc group was introduced via Fmoc-OSu.

L-4-(Aminomethyl)-N-isopropylphenylalanine (Iaf). Fmoc-L-Phe[4-CH_2NH-^{i}Pr-Boc] was obtained starting from Fmoc-L-Phe(4-CH_2NH_2) by reductive alkylation with acetone. Treatment with di-*tert*-butyldicarbonate in *tert*-butyl alcohol afforded the final product in good yield.

L-trans-4-(Aminomethyl)cyclohexylalanine (Ama). Fmoc-L-*trans*-Cha(4-CH_2NH-Boc) was prepared starting from Fmoc-L-Phe(4-CH_2NH-Boc) according to a previously de-

scribed procedure (*25*). Catalytic hydrogenation of the starting compound in the presence of platinum oxide gave a mixture of Fmoc-L-*cis*- and Fmoc-L-*trans*-Cha(4-CH_2NH-Boc). The trans isomers were separated by preparative liquid chromatography.

L-cis/trans-4-(Aminocyclohexyl)alanine (Aca). Fmoc-L-*cis*/*trans*-Cha-(4-NH-Boc) was prepared starting from Fmoc-4-nitro-L-phenylalanine as previously described (*25*). Catalytic hydrogenation of the nitro derivative in the presence of platinum oxide gave a mixture of Fmoc-*cis*- and Fmoc-*trans*-(aminocyclohexyl)-L-alanine. Treatment with di-*tert*-butyl dicarbonate in *tert*-butyl alcohol afforded the final product in good yield.

L-4-Guanidine Phenylalanine (Gnf). Fmoc-L-phenylalanine[4-guanidino($Boc)_2$] was prepared according to a previously reported procedure that had been opportunely modified (*26*), starting from *p*-nitrophenylalanine which, after its amine group has been protected by a Fmoc group, was reduced to convert the NO_2 group into an NH_2 group. The resulting derivative was reacted with a guanidination agent, such as 1-guanidino-3,5-dimethylpyrazole nitrate, to obtain Fmoc-GNF derivative. The guanidine moiety was successively protected as Boc by di-*tert*-butyl dicarbonate in *t*-BuOH (60% yield).

L-3-Pyridylalanine (Pya). Fmoc-L-(3-Pyr)-Ala was prepared as previously described (*27*). Treatment of 3-bromopyridine with methyl 2-acetamidoacrylate in the presence of $Pd_2(dba)_3$ under an inert atmosphere gives a good yield of prochiral enamide. The enamide is hydrogenated in the presence of a chiral Rh catalyst to give L-methyl 2-acetamido-3-(3-pyridyl)propanate. The free amino acid was released by hydrolysis, and treatment with Fmoc-Osu afforded the final compound in good yield.

L-4-Piperidinylalanine (Ppa). Fmoc-L-Ppa(Boc)-OH was prepared according to a previously reported procedure (*28*, *29*). The title compound was prepared in nine steps from 3-(4-pyridyl)acrylate acid using Evans chiral auxiliary.

L-N^{im}-(Dimethyl)histidine [H(2Me)]. Fmoc-L-N^{im}-(dimethyl)histidine was obtained from L-N^{im}-(dimethyl)histidine (*30*) which was prepared by treatment of N^{α}-benzoylhistidine methyl ester with a methyl sulfate/K_2CO_3 mixture under anhydrous conditions. The free amino acid was released by hydrolysis, and treatment with Fmoc-OSu afforded the final compound.

For details about the synthesis procedures, see the Supporting Information.

Enzymes. Homogeneous preparations of human tissue kallikrein obtained according to the method of Shimamoto et al. (*31*) were kindly provided by J. Chao of the Medical University of South Carolina (Charleston, SC). The molar concentrations of enzyme solutions were determined by active site titration with 4-nitrophenyl-4-guanidinobenzoate (*32*). Human plasma kallikrein was obtained and titrated as previously described (*32*−*34*). β-Trypsin was purified as described elsewhere (*35*) from a twice-crystallized bovine trypsin from Biobras Co. (Montes Claros, Minas Gerais, Brazil) previously treated with TPCK, and the operational molarities were determined by active site titration (*36*).

Synthetic Substrates. All the intramolecularly quenched fluorogenic peptides contain *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) attached to glutamine, a necessary result of the solid-phase peptide synthesis strategy that was employed, the details of which are provided elsewhere (*37*). An automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the Fmoc procedure. The final deprotected peptides were purified by semipreparative HPLC using an Econosil C-18 column (10 μm, 22.5 mm × 250 mm) and a two-solvent system: A, trifluoroacetic acid (TFA) and H_2O (1:1000); and B, TFA, acetonitrile (ACN), and H_2O (1:900:100). The column was eluted at a flow rate of 5 mL/min with a 10% (or 30%) to 50% (or 60%) gradient of solvent B over the course of 30 or 45 min. Analytical HPLC was performed using a binary HPLC system from Shimadzu with a SPD-10AV Shimadzu UV−vis detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μm, 4.6 mm × 150 mm) which was eluted with solvent systems A_1 (1:1000 H_3PO_4/H_2O mixture) and B_1 (900:100:1 ACN/H_2O/H_3PO_4 mixture) at a flow rate of 1.7 mL/min and a 10 to 80% gradient of B_1 over the course of 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 weight and purity of synthesized peptides were checked by MALDI-TOF mass spectrometry (TofSpec-E, Micromass) and/or peptide sequencing using a PPSQ-23 protein sequencer (Shimadzu, Tokyo, Japan)

Fluorometric Enzyme Assay. The hydrolyses of the fluorogenic peptide substrates were followed by measuring the fluorescence at $\lambda_{em} = 420$ nm and $\lambda_{ex} = 320$ nm in a Hitachi F-2000 spectrofluorometer, at 37 °C in 20 mM Tris-HCl (pH 9.0) containing 1 mM EDTA for hK1, 100 mM Tris-HCl buffer (pH 8.0) for plasma kallikrein, and 10 mM Ca^{2+} (pH 8.0) for trypsin. The inhibition of thrombin and plasmin was performed in 50 mM Tris buffer and 0.15 M NaCl (pH 8.0) using as a substrate D-Pro-Phe-Arg-MCA for the former and D-Val-Leu-Lys-pNa for the latter. A 1 cm path length cuvette containing 2 mL of the substrate solution was placed in the thermostated cell compartment for 5 min before the enzyme solution was added, and the increase in fluorescence with time was continuously recorded for 10 min. The slope was converted into moles of substrate hydrolyzed per minute based on the fluorescence curves for standard peptide solutions before and after total enzymatic hydrolysis. Provided HPLC analysis of the different fluorogenic substrates did not show any significant contamination; their concentration in solution could be determined from the fluorescence obtained following total tryptic hydrolysis and by colorimetric quantitation of the 2,4-dinitrophenyl group (extinction coefficient at 365 nm was 17 300 M^{-1} cm^{-1}). The enzyme concentrations for initial rate determinations were chosen so as to hydrolyze less than 5% of the substrate present. The kinetic parameters were calculated according Wilkinson (*38*) as well as by using Eadie−Hofstee plots. The K_i values for competitive inhibition assays of the peptides were determined according to the method of Nicklin and Barrett (*39*). The standard errors for K_i, K_m, and k_{cat} determinations were less than 5%. The points of cleavage of each substrate by each protease were determined by isolation of the substrates and their structures determined by MALDI-TOF mass spectrometry (TofSpec-E, Micromass) and/or peptide sequencing using a protein sequencer.

Table 1: Kinetic Parameters of Hydrolysis by Human Tissue Kallikrein of Substrates Derived from the Sequence F-**X**-S-R-Q[a]

no.	sequence Abz-peptidyl-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
I	F-**R**↓S-R-Q	1.4 ± 0.1	1.6 ± 0.1	1143
II	F-**F**↓S-R-Q	1.2 ± 0.2	3.6 ± 0.3	3000
III	F-**K**↓S-R-Q	1.3 ± 0.1	3.7 ± 0.3	2846
IV	F-**H**↓S-R-Q	0.70 ± 0.06	0.040 ± 0.003	57
V	F-**Amf**↓S-R-Q	3.6 ± 0.3	1.0 ± 0.1	278
VI	F-**Gnf**-S-R-Q	resistant, K_i = 1.4 μM		
VII	F-H**(2Me)**↓S-R-Q	0.70 ± 0.06	1.2 ± 0.1	1714
VIII	F-**Pya**-S-R-Q	resistant, K_i = 11.6 μM		
IX	F-**Iaf**-S-R-Q	resistant, K_i = 3.5 μM		
X	F-**Ppa**-S-R-Q	resistant, K_i = 0.3 μM		
XI	F-**Ama**-S-R-Q	resistant, K_i = 0.05 μM		
XII	F-**Aca**-S-R-Q	resistant, K_i = 0.7 μM		

[a] Conditions: 50 mM Tris-HCl buffer and 1 mM EDTA at pH 9.0 and 37 °C.

RESULTS

Hydrolysis by hK1 of Peptides Derived from Abz-F-R-S-R-Q-EDDnp. Human tissue kallikrein (hK1) hydrolyzed efficiently Abz-F-R-S-R-Q-EDDnp at the R−S bond. The Arg residue at position P_1 of this substrate was substituted with Phe, Lys, His, Amf, Gnf, Iaf, Pya, Ama, Ppa, Aca, and H(2Me), resulting in peptides **II**−**XII**. The susceptible peptides were cleaved only at the X−S bond, where X is any of the amino acids used instead of Arg. The kinetic parameters for hydrolysis by hK1 of these peptides are shown in Table 1.

The substrates with Phe and Lys (peptides **II** and **III**, respectively) were hydrolyzed with the higher k_{cat}/K_m in the series that is a consequence of high k_{cat} values. The peptides containing His and His(2Me) (peptides **IV** and **VII**, respectively) were hydrolyzed and presented the lowest K_m values in this series. The k_{cat} value for the latter peptide with a fixed positive charge at the imidazole ring was almost 2 orders of magnitude higher than that for the former. This observation indicates that the permanent positive charge in the imidazole ring is highly favorable for hydrolysis, resulting in a k_{cat}/K_m value that is higher than that for peptide **I** with Arg at P_1. Peptide **V** with Amf that combines the aromaticity of the benzene ring with the positive charge of methylamino group is hydrolyzed with a lower k_{cat} and a higher K_m in comparison to those of peptide **I**. All other substitutions of Arg (peptides **VI** and **VIII**−**XII**) resulted in peptides resistant to hydrolysis until the hK1 concentration reached 15 nM. However, all of then presented a significant competitive inhibitory activity on the enzyme. Particularly relevant was the inhibitory effect of peptide **XI** with the amino acid Ama, which presented a K_i of 50 nM.

Hydrolysis by hK1 of Peptides Derived from Abz-G-F-S-P-F-R-S-S-R-P-Q-EDDnp. The substrate Abz-G-F-S-P-F-R-S-S-R-P-Q-EDDnp (peptide **XIII**) is hydrolyzed at the R−S bond (Table 2) more efficiently than its short homologue Abz-F-R-S-R-Q-EDDnp (peptide **I**, Table 1), and their k_{cat}/K_m values differ by more than 1 order of magnitude. The substitution of Arg at P_1 with Phe, Lys, or His (peptides **XIV**−**XVI**, respectively) drastically reduced the k_{cat}/K_m values. The presence of the positively charged aminomethyl group in the para position of the Phe side chain, as in Amf (peptide **XVIII**), recovered part of the substrate susceptibility to hK1. However, the kinetic parameters for this substrate are apparent because it was hydrolyzed at F−Amf or Amf−S bonds with an indistinguishable preference (Figure 2). However, as the kinetic curve in the Eadie−Hofstee plot presented one hyperbolic curve, which adjusts only one K_m, indicates that this parameter is quite similar for both cleavages, the k_{cat} for each cleavage could be obtained by multiplying the cleavage percentage. Similar double cleavage was also observed with the substrates containing H and H(2Me) (peptides **XVI** and **XIX**, respectively), whereas the substrates with amino acids Iaf, Ppa, and Aca (peptides **XXI**, **XXII**, and **XXIV**, respectively) are cleaved in two sites, the preference being for the X−S bond. In contrast to the effect of the aminomethyl group (peptide **XVIII**), the guanidine function in the para position of the Phe side (Gnf) made peptide **XVII** resistant to hK1. Similarly, the peptides with Pya and Ama (**XX** and **XXIII**, respectively) were also resistant, but all these nonsubstrate peptides competitively inhibited hK1. As in shorter peptide series (Table 1), peptide **XXIII** containing the amino acid Ama was the more efficient inhibitor of the series.

Hydrolysis by Trypsin and Human Plasma Kallikrein of Peptides Derived from Abz-F-R-S-R-Q-EDDnp and Abz-G-F-S-P-F-R-S-S-R-Q-EDDnp. Trypsin hydrolyzed very efficiently the substrate Abz-F-Amf-S-R-Q-EDDnp (peptide **V**, Table 3) but at the R−Q bond, precluding the analysis of the effect of the non-natural basic amino acids at position P_1 in the series of short peptides. This analysis was performed on the series of larger peptides because Pro after the Arg prevented hydrolysis at this site. Trypsin was able to hydrolyze only the peptides containing Arg, Gnf, and Ppa (**XIII**, **XVII**, and **XXII**, respectively) at the X−S bond

Table 2: Kinetic Parameters of Hydrolysis by Human Tissue Kallikrein of Substrates Derived from the Sequence G-F-S-P-F-**X**-S-R-R-P-Q[a]

no.	sequence Abz-peptidyl-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	% double hydrolysis
XIII	G-F-S-P-F-R↓S-S-R-P-Q	0.13 ± 0.01	6.7 ± 0.5	51538	
XIV	G-F-S-P-F-F↓S-S-R-P-Q	2.6 ± 0.2	3.7 ± 0.1	1423	
XV	G-F-S-P-F↓K↓S-S-R-P-Q	0.67 ± 0.06	3.4 ± 0.3	5074	20 (F↓K), 80 (K↓S)
XVI	G-F-S-P-F↓H↓S-S-R-P-Q	0.80 ± 0.07	0.50 ± 0.4	625	46 (F↓H), 54 (H↓S)
XVII	G-F-S-P-F **Gnf** S-S-R-P-Q		resistant, K_i = 1.5 μM		
XVIII	G-F-S-P-F↓**Amf**↓S-S-R-P-Q	0.30 ± 0.2	1.8 ± 0.1	6000	44 (F↓Amf), 56 (Amf↓S)
XIX	G-F-S-P-F↓**H(2Me)**↓S-S-R-P-Q	1.0 ± 0.1	2.1 ± 0.2	2100	58 [F↓H(2Me)], 42 [H(2Me)↓S]
XX	G-F-S-P-F-**Pya**-S-S-R-P-Q		resistant, K_i = 9.6 μM		
XXI	G-F-S-P-F↓**Iaf**↓S-S-R-P-Q	3.8 ± 0.3	0.81 ± 0.2	1279	30 (F↓Iaf), 70 (Iaf↓S)
XXII	G-F-S-P-F↓**Ppa**↓S-S-R-P-Q	0.64 ± 0.04	0.61 ± 0.2	2542	25 (F↓Ppa), 75 (Ppa↓S)
XXIII	G-F-S-P-F-**Ama**-S-S-R-P-Q		resistant, K_i = 0.13 μM		
XXIV	G-F-S-P-F↓**Aca**↓S-S-R-P-Q	0.70 ± 0.6	0.51 ± 0.2	4800	18 (F↓Aca), 82 (Aca↓S)

[a] See Table 1 for conditions.

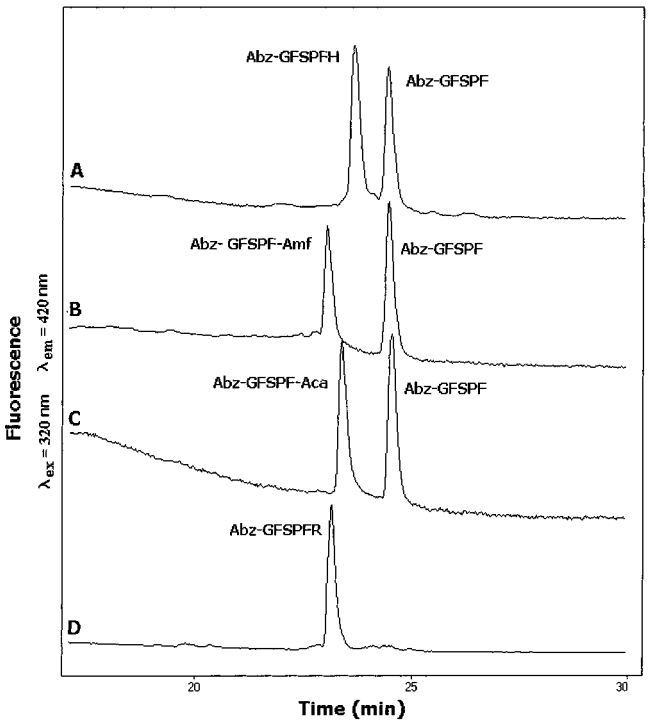

FIGURE 2: HPLC profiles of the fluorescent fragments containing Abz (λ_{ex} = 320 nm, λ_{em} = 420 nm) after complete hydrolysis by hK1 of Abz-GFSPFHSSRPQ-EDDnp (A), Abz-G-F-S-P-F-Amf-S-S-R-P-Q-EDDnp (B), Abz-G-F-S-P-F-Aca-S-S-R-P-Q-EDDnp (C), and Abz-G-F-S-P-F-R-S-S-R-P-Q-EDDnp (D). All these peacks were isolated, and the structures of the peptides were confirmed by mass spectrometry.

Table 3: Kinetic Parameters of Hydrolysis by Trypsin of Substrates from Abz-F-X-S-R-Q-EDDnp and Abz-G-F-S-P-F-X-S-S-R-P-Q-EDDnp Series[a]

no.	sequence Abz-peptidyl-EDDnp	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
V	F-**Amf**-S-R↓Q	0.9 ± 0.04	12.0 ± 0.5	13483
XIII	G-F-S-P-F-**R**↓S-S-R-P-Q	9.1 ± 0.9	33 ± 1	3626
XVII	G-F-S-P-F-**Gnf**↓S-S-R-P-Q	3.5 ± 0.3	3.0 ± 0.1	857
XXII	G-F-S-P-F-**Ppa**↓S-S-R-P-Q	3.0 ± 0.3	0.82 ± 0.6	273

[a] Conditions: 0.1 M Tris·HCl buffer and 10 mM Ca^{2+} at pH 8.0 and 37 °C.

(Table 3). While the peptide with Arg was hydrolyzed with a high k_{cat} value, the peptides with Gnf and Ppa presented K_m values that were 3 times lower. All the other peptides listed in Table 2 were also assayed but were completely resistant until the trypsin concentration reached 15 nM.

Human plasma kallikrein hydrolyzed only the peptides Abz-F-R-S-R-Q-EDDnp (k_{cat} = 0.045 s^{-1}, K_m = 1.5 μM, and k_{cat}/K_m = 30 mM^{-1} s^{-1}) and Abz-G-F-S-P-F-R-S-S-R-Q-EDDnp (k_{cat} = 1.3 s^{-1}, K_m = 9.8 μM, and k_{cat}/K_m = 133 mM^{-1} s^{-1}) at the R−S bond. All other peptides listed in Tables 1 and 2 were resistant until the enzyme concentration reached 15 nM. The resistant peptides were assayed as inhibitors for this enzyme, and the K_i values for competitive inhibition are presented in Table 4. They inhibited human plasma kallikrein with a K_i > 1 μM. In general, all the shorter peptides presented higher affinities than the larger ones.

As Abz-F-Ama-S-R-Q-EDDnp inhibited hK1 with a K_i value in the nanomolar range, we assayed this peptide as a substrate as well as an inhibitor with human plasma kallikrein, trypsin, thrombin, and plasmin. None of these proteases hydrolyzed the peptide and were inhibited with K_i values significantly higher than that observed for hK1 (Table 5).

Table 4: Inhibition Constants (K_i) of Peptidase Activity of Plasma Kallikrein with Peptides Derived from Abz-F-X-S-R-Q-EDDnp and Abz-G-F-S-P-F-X-S-S-R-P-Q-EDDnp Series

X	Abz-F-X-S-R-Q-EDDnp K_i (μM)[a]	Abz-G-F-S-P-F-X-S-S-R-P-Q-EDDnp K_i (μM)[b]
F	2.0	6.0
H	4.0	26
Amf	1.3	8.0
Gnf	5.6	9.0
H(2Me)	ND	6.0
Pya	6.1	6.6
Iaf	2.9	10.0
Ppa	4.9	10.0
Ama	3.0	5.5
Aca	5.0	7.0

[a] Abz-F-R-S-R-Q-EDDnp (k_{cat} = 0.045 s^{-1}, K_m = 1.5 μM, k_{cat}/K_m = 30 mM^{-1} s^{-1}). [b] Abz-G-F-S-P-F-R-S-S-R-P-Q-EDDnp (k_{cat} = 1.3 s^{-1}, K_m = 9.8 μM, k_{cat}/K_m = 133 mM^{-1} s^{-1}) were hydrolyzed at the R−S bond with.

Table 5: Inhibition Constants (K_i) of Peptidase Activity of Tissue and Plasma Kallikrein, Trypsin, Thrombin, and Plasmin by Abz-F-Ama-S-R-Q-EDDnp

enzyme	K_i (μM)
human tissue kallikrein	0.05
human plasma kallikrein	3.0
trypsin	2.5
thrombin	0.6
plasmin	1.0

DISCUSSION

The work presented here is the first description of substrates or inhibitors for hK1 containing non-natural basic amino acids aimed at mapping the specificity of the S_1 subsite of this enzyme. The unique feature of the S_1 subsite of hK1, accepting Arg as well as Phe, remained when the basicity and the aromaticity of these amino acids were combined as in Amf. In contrast, the phenyl group with guanidine in the para position, as in Gnf, resulted in peptides resistant to hK1, and their K_i values were higher than 1 μM. Amf has been so far reported to substitute for Arg in a small number of compounds aimed to inhibit thrombin (*40*) or in a fibrinogen receptor antagonist as in MK-852 (*41*). On the other hand, Gnf derivatives and Gnf-containing peptides have been reported as substrates of serine proteases, particularly trypsin (*42*, *43*). In contrast, Gnf derivatives are poorly hydrolyzed by thrombin (*43*, *44*). Our results with trypsin are in accordance to these observations. The permanent charge at the imidazole ring in H(2Me) seems to generate an amino acid, the side chain of which fitted at the S_1 subsite of hK1, resulting in a short substrate that was well hydrolyzed, whose kinetic parameters were compared to those of substrate with Arg at position P_1 (Table 1, peptides **I** and **VII**). The higher affinities, measured by the K_i values, were observed in the series of shorter substrates (Table 1) with the peptides containing basic groups attached to aliphatic chains, as in amino acids Ppa, Ama, and Aca. All of them have cyclic aliphatic side chains, which fitted better than Lys at the S_1 subsite of hK1. Particularly relevant are the peptides with

the amino acid Ama that present the lowest K_i values and are resistant to hK1. This high affinity could be related to the particularities of the Ama side chain that is the longest of all non-natural basic amino acids examined in this paper. The enlargement of the S_1 pocket in human and porcine kallikrein was described to prevent the ammonium group of Lys at this location from being fully hydrogen bonded (*19*, *20*). Therefore, the larger volume and length of the Ama side chain compared to those of Arg or Lys could result in a better fitting of Ama at the hK1 S_1 subsite. Abz-F-Ama-S-R-Q-EDDnp seems to be promising, since besides its resistance to human plasma kallikrein, thrombin, and plasmin, it was also resistant to hydrolysis in preliminary assays with fresh human serum. In addition, few synthetic inhibitors have been previously described, and the K_i values were in micromolar range of higher (*45*−*48*).

The double hydrolyses by hK1 of the peptides of the series Abz-G-F-S-P-F-**X**-S-S-R-P-Q-EDDnp where X is Amf, His, H(2Me), Lys, Iaf, Ppa, and Aca, described in Table 2 and Figure 2, were unpredictable and seem to depend on the size of the substrates and, therefore, of extra interactions in the hK1 extended binding site. It is noteworthy that hydrolysis of the peptides at the F−X bond puts Pro at position P_2, which is very unfavorable (*49*, *50*) but also puts a basic residue at position P_1'. Indeed, Lys is located at the P_1' site of kininogen for its hydrolysis when Lys-bradykinin is released; therefore, it is reasonable to propose that a basic group could be quite well accepted at the S_1' subsite of hK1 compensating for the presence of Pro at the position P_2 of the substrates presented in Table 2.

All the assayed peptides with non-natural basic amino acids were resistant to plasma kallikrein and were poor inhibitors for it, indicating that its S_1 subsite is more restrictive than those of hK1 or trypsin.

In conclusion, the S_1 subsite of hK1 accepts amino acids with combined basic and aromatic side chains, although for S_1-P_1 interaction the preference is for aliphatic and basic side chains. We found a potent competitive inhibitor for hK1, Abz-F-Ama-S-R-EDDnp, that was specific for this enzyme if compared to other serine proteases. This molecule is a good candidate to be taken as a lead compound for modeling and design of more potent and specific inhibitors for hK1, aimed at developing compounds with analgesic and anti-inflammatory properties.

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SUPPORTING INFORMATION AVAILABLE

Details of syntheses. This material is available free of charge via the Internet at http://pubs.acs.org.

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