S(1)' and S(2)' Subsite Specificities of Human Plasma Kallikrein and Tissue Kallikrein 1 for the Hydrolysis of Peptides Derived from the Bradykinin Domain of Human Kininogen

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**S$_1'$** and **S$_2'$** subsite specificities of human plasma kallikrein and tissue kallikrein 1 for the hydrolysis of peptides derived from the bradykinin domain of human kinogen

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**Abstract**

The S$_1'$ and S$_2'$ subsite specificities of human tissue kallikrein 1 (KLK1) and human plasma kallikrein (HPK) were examined with the peptide series Abz-GFSPFRSRRK(PO$_4$)$_2$RI-NH$_2$ and Abz-GFSPFRSRRK(PO$_4$)$_2$RI-NH$_2$ [X=natural amino acids or S(PO$_4$)$_2$]: KLK1 efficiently hydrolyzed most of the peptides except those containing negatively charged amino acids at P$_1'$ and P$_2'$ positions. Abz-GFSPFRSRRK(PO$_4$)$_2$RI-NH$_2$, as in human kininogen, is the best substrate for KLK1 and exclusively cleaved the R-S bond. All other peptides were cleaved also at the R-F bond. The synthetic human kininogen segment Abz-MISLMKRPPGFSPFRS(PO$_4$)$_2$RI-NH$_2$ was hydrolyzed by KLK1 first at R-S and then at M-K bonds, releasing Lys-bradykinin. In the S$_{390}$ and S$_{391}$ phosphorylated analogs, this order of hydrolysis was inverted due to the higher resistance of the R-S bond. Abz-MISLMKRPPGFSPFRSRI-NH$_2$ was hydrolyzed by KLK1 at M-K and mainly at the F-R bond, releasing des-{Arg$_2$}-Lys-Bk which is a B1 receptor agonist. HPK cleaved all the peptides at R and showed restricted specificity for S in the S$_1'$ subsite, with lower specificity for the S$_2'$ subsite. Abz-MISLMKRPPGFSPFRSRI-NH$_2$ was efficiently hydrolyzed by HPK under bradykinin release, while the analogs containing S(PO$_4$)$_2$ were poorly hydrolyzed. In conclusion, S$_1'$ and S$_2'$ subsite specificities of KLK1 and HPK showed peculiarities that were observed with substrates containing the amino acid sequence of human kininogen.

**Keywords:** bradykinin; kinin; peptidase; peptides; phosphorylation; protease.

**Introduction**

Kinins are released by triggering two general proteolytic cascades that together are usually termed the kallikrein-kinin system. The simpler of the two cascades is constituted by tissue kallikrein 1 (KLK1) and by the protein substrates low-molecular-weight-kininogen (LK) and high-molecular-weight-kininogen (HK) (Mueller-Esterl et al., 1985). Human tissue KLK1 (Diamandis et al., 2000) is a serine protease from a family constituted of 15 members that are co-localized at human chromosome loci 19q13.3-q13.4 (Qin et al., 1991; Riegman et al., 1992; Yousef and Diamandis, 2001). The human kininogens, LK and HK, have identical amino acid sequences from the N-terminus to 12 amino acids beyond the bradykinin (Bk) sequence. After this point they differ in C-terminal domains due to alternative splicing (Kitamura et al., 1985; Takagaki et al., 1985). KLK1 release the decapeptide lysyl-bradykinin (Lys-Bk) by specific and limited hydrolysis on the peptide bonds M$_{397}$-K$_{380}$ (chymotrypsin-like activity) and R$_{390}^{-}$-S$_{390}$ (trypsin-like activity) from the sequence ...PLGMISLM$_{379}$K$_{380}$RPPGFSPFR$_{381}$S$_{390}$SRIG... in the Bk domain of human LK or HK. This pattern of kinogenase activity is observed in all studied mammals to date, except for mouse and rat tissue kallikrein that release Bk from mouse and rat kininogen, respectively (Bhoola et al., 1992; Olsson and Lundwall, 2002; Diamandis et al., 2004; Fogaça et al., 2004). The efficiency of the hydrolytic activities of tissue kallikreins 1 on the kininogens are species specific (Bhoola et al., 1992; Del Nery et al., 1995).

The other pathway of kinin release is more complex and involves initiating elements of intrinsic coagulation pathway as factor XII and plasma prekallikrein complexed with HK together with factor XI. The factor XII is activated to factor XIIa at a negatively charged surface and specifically hydrolyzes prekallikrein activating it to plasma kallikrein. This enzyme activates more factor XII and also hydrolyses the K$_{380}$, R$_{381}$ and R$_{390}$-S$_{390}$ bonds in human HK releasing Bk. Human plasma kallikrein (HPK) has only trypsin-like activity and has little structural homology with KLK1 because they come from separate genes (Kaplan et al., 2002), but they have the kinin releasing activity in common. More recently, it was reported that there is a proteolytic pathway on cells for plasma kallikrein activation independent of factor XII. This pathway for plasma kallikrein with subsequent factor XII activation indicates physiologic activities as blood pressure regulation and modulation of thrombosisis risk independently of hemostasis (for reviews, see Schmaier and McCrae, 2007; Schmaier, 2008).

Detailed analyses of KLK1 specificity using synthetic peptides demonstrated that it cleaves preferentially substrates containing R at P$_1$ position (Schechter and Berger nomenclature; Schechter and Berger, 1967) but also accepts F and M, and has restricted requirement for hydrophobic amino acids at P$_2$ (Chagas et al., 1995; Del
Nery et al., 1995; Bourgeois et al., 1997; Pimenta et al., 1999, 2003; Melo et al., 2001b). The S′, and S″′ subsites were reported to prefer S and R, respectively, in a series of peptides derived from kallistatin, in which the hydrolysis occurs after a pair of F (Pimenta et al., 1997). HPK substrate specificity was less studied, but it was reported to have preference for R or K at the P position of the substrates and also to have marked activity on peptides with basic amino acid at P′′, (Chagas et al., 1991; Almeida et al., 2000; Melo et al., 2001a; Gosalia et al., 2005). Phylogenetic analysis of vertebrate kininogen genes demonstrated that the Bk sequence (RPPGFSPFR) is highly conserved from amphibians to mammals (Zhou et al., 1999, 2003; Melo et al., 2001b). The S′′ and S″ subsites of KLK1 obtained so far. Similar para-
topic and S″′ in human kininogens and Abz at the N-terminal side of the substrate specificity analysis of KLK1 using the series Abz-KLRXSQ-EDDnp in which the peptide X=R was one of the best substrates (Angelo et al., 2006). In contrast, negatively charged amino acids at P′″ position results in the worst substrates.

KLK1 hydrolyzed most of the peptides of the series Abz-GFSPFRSXRQ-EDDnp with high efficiency and almost exclusively at R-S bond; however, the peptides Abz-GFSPFRSSRQ-EDDnp and Abz-GFSPFRSQRQ-EDDnp that have negatively charged residues at P′″ position are the noteworthy exceptions. They are poorly hydrolyzed and the chymotrypsin-like hydrolysis at F-R became expressive (Table 1). These results indicate that KLK1 does not tolerate negative charge at S′ and S″ subsites. The peptides containing P and Q in the series Abz-GFSPFRSXRQ-EDDnp were also good substrates for KLK1, but the F-R bond was also hydrolyzed significantly. This effect of P at P′″ position inducing chymo-
trypsin-like activity of KLK1 was also observed earlier with peptides derived from rat kininogen that contain P two amino acid after Bk sequence (Fogaça et al., 2004).

HPK efficiently hydrolyzed only the reference peptide Abz-GFSPFRRSSRQ-EDDnp in the series Abz-GFSPFR XSRQ-EDDnp, and the R-S bond was the exclusive cleavage site. It is noteworthy that the kcat/Km value for this hydrolysis is almost 500 times lower than the kcat/Km observed with KLK1 (Table 1). The peptides from the series Abz-GFSPFRSXSRQ-EDDnp were hydrolyzed with similar efficiency, except those containing E and A that were less susceptible to HPK and the peptide with P that was resistant to hydrolysis. Therefore, the presence of S at P′″ position for Bk release by HPK appears to be essential.

### Results

**Hydrolysis by KLK1 and HPK of the Abz-GFSPFRXSRQ-EDDnp and Abz-GFSPFRXSRQ-EDDnp series of peptides**

The specificities of S′, and S″ subsites of KLK1 and HPK were examined with the series of FRET peptides Abz-GFSPFRXSRQ-EDDnp and Abz-GFSPFRXSRQ-EDDnp. These peptides derived from GFSPFRSSRI correspond to the six C-terminal residues of Bk (GFSPFR) followed by the next four amino acids (SSRI) as in human kininogen. The kinetic parameters of their hydrolysis and the cleaved peptide bonds are shown in Table 1. The peptides in the series Abz-GFSPFRXSRQ-EDDnp were hydrolyzed by KLK1 at R-S or F-R bonds, with preference for the former bond (Table 1). Abz-GFSPFRXSRQ-EDDnp that has the natural human kininogen sequence was the only peptide in this series exclusively hydrolyzed at R-S bond. The high kcat/Km value observed for its hydrolysis by KLK1 puts this peptide as the best synthetic substrate for KLK1 obtained so far. Similar parameters of hydrolysis of this peptide were previously reported with the KLK1 isolated from human urine (Del Nery et al., 1995). The peptide Abz-GFSPFRXSRQ-EDDnp was poorly hydrolyzed but cleaved exclusively at F-R bond. This occurs because the imide bond of P impairs the hydrolysis at R-P bond and only the chymotrypsin-like activity in F-R bond appears. The high kcat/Km value for the hydrolysis of Abz-GFSPFRXSRQ-EDDnp indicates the well acceptance of basic residues by the S′, subsite of KLK1. We observed similar preference in the substrate specificity analysis of KLK1 using the series Abz-KLRXSQ-EDDnp in which the peptide X=R was one of the best substrates (Angelo et al., 2006). In contrast, negatively charged amino acids at P′″ position results in the worst substrates.

### Effect of negatively charged phosphorylated S(Po4H2) at positions P′, and P″ subsites

The deleterious effects for hydrolysis of peptides containing negatively charged residues as D and E at P′″ and...
P₁₋ for both KLK1 and HPK induced us to inquire the effect of phosphorylated serine S(PO₃H₂) at these positions. Using the world-wide-web-accessible servers for phosphorylation site predictions (reviewed in Kobe et al., 2005; Hjerrild and Gammeltoft, 2006; Ubersax and Ferrel, 2007), the scores founded for S₃⁹₀ and S₃⁹₁ are shown in Table 2. Although kininogens were not described so far to be phosphorylated, S₃⁹₁ has the highest score and frequencies for that. To verify the effect of S(PO₃H₂) on the hydrolysis of Abz-GFSPFRSRIQ-EDDnp and Abz-GFSPFRSRIQ-EDDnp by KLK1 and HPK, the serines, S₃⁰₉ and S₃⁹₁, after Bk sequence were substituted by S(PO₃H₂) in both peptides. Abz-GFSPFRS(PO₃H₂)SRIQ-EDDnp and Abz-GFSPFRS(PO₃H₂)RQ-EDDnp were poorly hydrolyzed by KLK1, a simple velocity (V) of product formation was determined in the first hour of reaction when it was linear with the time, and it is expressed in μmol of product/min×nmol of enzyme. Very low hydrolysis means that the peptide was hydrolyzed less than 10% of its initial amount by HPK after 24 h of incubation. HPK hydrolyzed all the peptides at the R-X or R-S bond. For peptides with two cleavages, in which one of them is higher than 10%, only k_cat/K_m was determined in pseudo-first order condition. Errors were less than 5% for each of the obtained kinetic parameters.

Table 1 Kinetic parameters for the hydrolysis by human tissue kallikrein (KLK1) and human plasma kallikrein (HPK) of a series of peptides derived from the peptides Abz-GFSPFRSSRIQ-EDDnp, with variations at the P₁₋ and P₂₋ positions.

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<tr>
<td></td>
<td>k_cat (s⁻¹)</td>
<td>K_m (μM)</td>
<td>k_cat/K_m (s⁻¹×μM⁻¹)</td>
<td>Cleavage site (%)</td>
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<table>
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<tr>
<td></td>
<td>k_cat (s⁻¹)</td>
<td>K_m (μM)</td>
<td>k_cat/K_m (s⁻¹×μM⁻¹)</td>
<td>Cleavage site (%)</td>
</tr>
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Conditions of hydrolysis: for the peptides poorly hydrolyzed, a simple velocity (V) of product formation was determined in the first hour of reaction when it was linear with the time, and it is expressed in μmol of product/min×nmol of enzyme. Very low hydrolysis means that the peptide was hydrolyzed less than 10% of its initial amount by HPK after 24 h of incubation. HPK hydrolyzed all the peptides at the R-X or R-S bond. For peptides with two cleavages, in which one of them is higher than 10%, only k_cat/K_m was determined in pseudo-first order condition. Errors were less than 5% for each of the obtained kinetic parameters.

The time courses of the hydrolysis by KLK1 and HPK of the peptide Abz-MISLMKRPPGFSPFRSRIQ-EDDnp were determined in the first hour of reaction when it was linear with the time, and it is expressed in μmol of product/min×nmol of enzyme. Very low hydrolysis means that the peptide was hydrolyzed less than 10% of its initial amount by HPK after 24 h of incubation. HPK hydrolyzed all the peptides at the R-X or R-S bond. For peptides with two cleavages, in which one of them is higher than 10%, only k_cat/K_m was determined in pseudo-first order condition. Errors were less than 5% for each of the obtained kinetic parameters.

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Figure 1  Time course of hydrolysis by KLK1 and HPK of the human kininogen fragment, Abz-MISLMKRPPGFSPFRSSRI-NH$_2$.

Each product was isolated and characterized by MALDI-TOF-MS. The substrate and their products of hydrolysis were quantified by HPLC and are shown as percentage of product formation or substrate degradation. Analytical HPLC conditions were: Ultrasphere C18 column (5 µm, 4.6×250 mm) which was eluted with the solvent systems A (H$_3$PO$_4$/water, 1:1000) and B1 (ACN/water/H$_3$PO$_4$, 900:100:1) at a flow rate of 0.8 ml/min and a 0–80% gradient of B for 60 min monitored by fluorescence emission at 420 nm following excitation at 320 nm. Hydrolysis conditions were: for KLK1 50 mM Tris/HCl (pH 9.0), 1 mM EDTA, 37°C, and the same conditions for HPK except that the buffer had a pH of 7.5.

R-S bond at a ratio of 9:1. This result shows that KLK1 can release des-(Arg$^9$)-Lys-Bk (KRPPGFSPF) directly from human kininogen, if S$^{391}$ was phosphorylated. HPK also hydrolyzed the K-R bond first and the second cleavage occurs exclusively at R-S bond that takes several hours to complete the release of Bk (Figure 2D).

Discussion

KLK1 hydrolyzed quite well almost all the series of peptides derived from Abz-GFSPFRSXRIQ-EDDnp and Abz-GFSPFRSXRIQ-EDDnp, except those with negative-charged residues. KLK1 avoids negative charges in S$_2$ subsite as E, D and even S(PO$_3$H$_2$) in the hydrolysis of Abz-GFSPFRSXRIQ-EDDnp using its ability to work as a chymotrypsin-like peptidase. The synthetic human kininogen fragment Abz-MISLMKRPPGFSPFRSSRI-NH$_2$ was hydrolyzed by KLK1 cleaving first the R-S bond, with intermediate accumulation of Abz-MISLMKRPPGFSPFR-OH that is then hydrolyzed at the M-K bond and Lys-Bk is released. This synthetic human kininogen fragment has mimicked the kininogen, in which the cleavages of the same two peptide bonds are required for kinin release. Very little is known about the mechanisms of these hydrolyses and so far nothing has been reported about chemical or biochemical controls of the hydrolytic activities of the kallikreins. The three-dimensional (3D) structures of porcine and human tissue kallikrein (Chen and Bode, 1983; Katz et al., 1998; Laxmikanthan et al., 2005) show that they have just one active site, although their kininogenase functions require activities, such as trypsin (R$\_x$S cleavage) and chymotrypsin (M$\_x$K cleavage), for the release of Lys-Bk. In contrast, we have observed, with the hydrolysis of Abz-MISLMKRPPGFSPFRSSRI-NH$_2$, evidence from the reaction time course and competition experiments using the integer natural kininogen that indicate both hydrolyses occur in the same enzyme-substrate complex because no intermediates were detected in the reaction of the integer kininogen and tissue kallikreins 1 (Fiedler et al., 1986; Fiedler and Hinz, 1992). Therefore, there are kininogen-kallikrein interactions that are missed in the hydrolysis of the synthetic peptide Abz-MISLMKRPPGFSPFRSSRI-NH$_2$. Information on this reaction is of interest because tissue KLK1 is usually considered as a prototype for other kallikrein gene family members. The phosphorylation of S$^{320}$ and S$^{391}$ in Abz-MISLMKRPPGFSPFRSSRI-NH$_2$ resulted in peptides with significant impairment on the hydrolysis at R-S bond by KLK1. As shown in Figure 2A and B, the first cleavage in the phosphorylated peptides was at the M-K bond with accumulation of the C-terminal fragment. Des-(Arg$^9$)-Lys-Bk is the main kinin after the complete hydrolysis of the peptide Abz-MISLMKRPPGFSPFRSSRI-NH$_2$. As observed with Abz-GFSPFRSXRIQ-EDDnp when X=D, E or S(PO$_3$H$_2$), KLK1 puts F in S$_1$, S$_2$ and S$_3$ subsites, respectively. This
displacement of the cleavage site from R-S to F-R bond by KLK1 in the human kininogen-derived peptides, particularly by the phosphorylation of S\(^{390}\), suggests a direct release of des-(Arg\(^9\))-Lys-Bk. A direct release route of this B1 receptor of Bk would be more efficient because it avoids the carboxypeptidase B activity on Lys-Bk that is also quickly digested by angiotensin-converting enzyme, nepriyisin, dipeptidyl peptidase IV and aminopeptidase P (Oliveira et al., 2007). To date, there is no report on phosphorylation of LK or HK \(\text{in vivo}\) however it is reasonable to speculate some alternatives as the capture of kininogens by endothelial cells, phosphorylation and then secretion, or phosphorylation outside the cells by ecto-protein kinase (Redegeld et al., 1999) as in prostasomes, the prostate secreted granules (Babiker et al., 2006), or even in the plasma by ATP and kinases from platelets (Ekdahl and Nilsson, 1999).

HPK presented restricted specificity for serine at P\(^1\), as observed with the assay of Abz-GFSFRX-EDDnp series, which contrasts with the broad specificity of S\(^9\) (Table 1). HPK is well studied from the physiological, pathological and pharmacological point of view (Marcondes and Antunes, 2005; Zhang et al., 2006; Sainz et al., 2007), but little information is found in the literature about the secondary substrate specificity of HPK (Chagas et al., 1991; Almeida et al., 2000; Gosalia et al., 2005). In fact, the restricted specificity of S\(^9\) of HPK and its complete resistance to hydrolyze the peptides containing S(PO\(_4\))\(_2\) at P\(^1\) position can be anticipated in the analysis of the particular features of S\(^9\) subsite, as observed in the 3D structure of the catalytic domain of this enzyme (Tang et al., 2005). In contrast to trypsin and hK6, additional loop residues inserted after residue 59 forms the ‘60s loop’ that provides another wall for S\(^9\) subsite for HPK. HPK presented similar behavior to KLK1 for the hydrolysis of all assayed peptides containing S(PO\(_4\))\(_2\), except that HPK presented restricted trypsin-like activity, cleaving all the substrates only at R or K in P\(^1\) position. It is noteworthy that the peptides from the two series Abz-peptidyl-Q-EDDnp (Table 1) were in general hydrolyzed with \(k_{\text{cat}}/K_m\) almost 500 times lower than those obtained with KLK1; however, the hydrolysis of Abz-MISLMKRPPGFSPFRSSRI-NH\(_2\) by HPK seems to be as efficient as or even better than the hydrolysis by KLK1, as can be seen when comparing Figure 1A and B. This result suggests that HPK could require further interactions, e.g., away from the catalytic center for its full kininogenase activity. Further substrate specificity analysis with larger synthetic peptides is required for a more detail analysis of substrate specificity of HPK and for the investigation of the existence of activation site out of the catalytic domain. This point is relevant since HPK is a large and complex molecule and their activities can be modulated by other molecules or by the substrate itself. Other noteworthy features of HPK are its optimum pH at 7.5–8.0 and insensitivity to ionic strength (data not shown) for the hydrolysis of small synthetic peptides. This behavior of HPK is in contrast with that of KLK1 that hydrolyzed synthetic peptides at optimum pH 9.0 and is very sensitive to ionic strength (Del Nery et al., 1995). This suggests that the interaction of KLK1 with its protein substrates could optimize its proteolytic activity to physiological conditions.

In conclusion, the S\(^9\) and S\(^9\) specificities of human KLK1 and HPK are well adapted to release of kinins from human kininogens and modifications at S\(^{390}\) and/or S\(^{391}\) residues can control kinin release, e.g., phosphorylation in physiological or pathological conditions.

### Materials and methods

#### Enzymes

Recombinant human tissue KLK1 was expressed and purified as previously described (Laxmikantham et al., 2005) and the molar concentration of active enzyme was determined by titration with MUGB (4-methylumbelliferyl p-guanidinobenzoate hydrochloride) by spectrophotometric titration as previously described (Jameson et al., 1973). Homogeneous HPK was purified from Cohn’s fraction IV (Oliva et al., 1982) and the enzyme concentration was determined as described earlier (Oliva et al., 1987).

#### Peptides

All the peptides were obtained by the solid-phase peptide synthesis strategy as previously described (Hirata et al., 1994) using the fluoren-9-yl-methoxy carbonyl (Fmoc) procedure in an automated bench-top simultaneous multiple solid-phase peptide
Figure 2  Time course of hydrolysis by HPK of the human kininogen fragment containing phosphorylated modified serines: Abz-MISLMKRPPGFSPFRS(PO$_3$H$_2$)SRI-NH$_2$ and Abz-MISLMKRPPGFSPFRSS(PO$_3$H$_2$)RI-NH$_2$.

All conditions are the same as those described in the legend of Figure 1.

The peptides were synthesized on dimethoxybenzhydrolamine-PEG (TGR)-resin (loading 0.2 mmol/g) using O-benzotriazole-N,N,N$_9$N$_9$-tetramethyl-uronium-hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBt) as coupling reagent, and the cleavage of peptide resin was carried out with trifluoroacetic acid (TFA):anisol:ethanedithiol:water (85:5:3:7). The building block N-$\alpha$-Fmoc-O-benzyl-L-phosphoserine [Fmoc-Ser(PO(OBzl)OH)-OH] was used to introduce S(PO$_3$H$_2$) in the peptide synthesis. The final deprotected peptides were purified by semi-preparative HPLC on an Econosil C-18 column (10 µm; 22.5 mm×250 mm) using a two-solvent system: (A) TFA/water (1:1000, v/v) and (B) TFA/acetonitrile/water (900:100:1) at a flow rate of 0.8 ml/min and a 10–80% gradient of B over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm. The FRET peptides were further monitored by fluorescence emission at 420 nm following excitation at 320 nm. The molecular mass and purity (94% or higher) was further confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using ToFSpec-E (Micromass, Manchester, UK). Stock solutions of the peptides

synthesizer (PSSM 8 system from Shimadzu, Tokyo, Japan).
were prepared in DMF and the concentrations were measured spectrophotometrically using the molar extinction coefficient of 17 300 m²·cm⁻¹ at 365 nm.

**Kinetic measurements**

The hydrolyses of Abz-MISLMKRPPGFSPFRSSRI-NH₂ were monitored and quantified by HPLC for each peptide. The substrate concentration for hydrolysis of each peptide was chosen at a level intended to hydrolyze less than 5% of the amount of added substrate over the time course of the reaction using a Prominance liquid chromatography LC-20AD (Shimadzu). The amount of each product was determined by its UV absorption at 220 nm using authentic samples of each product as reference. The molecular weight of each product was also determined by LC/MS using LCMS-2010 equipped with the ESI-probe (Shimadzu) that was connected to the HPLC circuit after the UV detector. The HPLC conditions were: Ultrashphere C18 column (5 μm, 4.6×250 mm) which was eluted with the solvent systems A (H₂O/water, 1:1000) and B (ACN/water/H₃PO₄, 900:100:1) at a flow rate of 0.8 ml/min and a 0–80% gradient of B for 60 min.

The FRET peptides were assayed in the same buffer conditions described above, using a Shimadzu RF-1501 spectrofluorometer, at 37°C. The enzymes were pre-incubated in the assay buffer for 3 min before the addition of substrate. Fluorescence changes were monitored continuously at λex=320 nm and λem=420 nm. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyze less than 5% of the amount of added substrate over the time course of data collection. The slope of the generated fluorescence signal was converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The substrate concentration for determination of the kinetic parameters was between two Kᵣ values higher and lower of the obtained value. The kinetic parameters Kᵣ and kₐ were calculated by non-linear regression using the Grafit program (Leatherbarrow, 1992). For peptides hydrolyzed at more than one site, the apparent kᵣ/Kᵣ values were determined under pseudo first-order conditions (where [S]<<Kᵣ) and performed under three different substrate concentrations. Errors were less than 5% for each of the obtained kinetic parameters.

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**References**


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