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Activity of human kallikrein-related peptidase 6 (KLK6) on substrates containing sequences of basic amino acids. Is it a processing protease?

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Proteolytic enzymes, plasma kallikrein, tissue kallikrein, neurotrophins, matrix metalloproteases, enkephalins.

Abstract
Human kallikrein 6 (KLK6) is highly expressed in the central nervous system and with elevated level in demyelinating disease. KLK6 has a very restricted specificity for arginine (R) and hydrolyses myelin basic protein, protein activator receptors and human ionotropic glutamate receptor subunits. Here we report a previously unreported activity of KLK6 on peptides containing clusters of basic amino acids, as in synthetic fluorogenic peptidyl-Arg-7-amino-4-carbamoylmethylcoumarin (peptidyl-ACC) peptides and FRET peptides in the format of Abz-peptidyl-Q-EDDnp (where Abz= ortho-aminobenzoic acid and Q-EDDnp = glutaminyl-N-(2,4-dinitrophenyl) ethylenediamine), in which pairs or sequences of basic amino acids (R or K) were introduced. Surprisingly, KLK6 hydrolyzed the fluorogenic peptides Bz-A-R↓R-ACC and Z-R↓R-MCA between the two R groups, resulting in non-fluorescent products. FRET peptides containing furin processing sequences of human MMP-14, nerve growth factor (NGF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4) were cleaved by KLK6 at the same position expected by furin. Finally, KLK6 cleaved FRET peptides derived from human proenkephalin after the KR, the more frequent basic residues flanking enkephalins in human proenkephalin sequence. This result suggests the ability of KLK6 to release enkephalin from proenkephalin precursors and resembles furin a canonical processing proteolytic enzyme. Molecular models of peptides were built into the KLK6 structure and the marked preference of the cut between the two R of the examined peptides was related to the extended conformation of the substrates.
1. Introduction

Human kallikrein-related peptidase 6 (KLK6), the most abundant of all KLKs within the brain and spinal cord [1, 2] is present at demyelination processes of active multiple sclerosis (MS) lesions [3]. KLK6 is also present and is elevated in the serum [4] and in the cerebrospinal fluid [5] of progressive MS patients. Activated T cells and monocytes secrete KLK6, while its neutralizing antibodies block the migration of these cells across a matrigel barrier and reduce the development of CNS inflammatory infiltrates in animal models of MS [6, 7]. In human spinal cord injury, elevation of KLK6 is observed in oligodendroglia, astrocytes, and monocytes-microglia [2, 8]. In addition, KLK6 was cloned from other tissues and was identified by other names as protease M from breast tissue [9] and neurosin from a colon carcinoma cell line and oligodendrocytes [10, 11]. These data suggest KLK6 plays a role in inflammatory and degenerative diseases or even in traumatic lesions of CNS (review in [12]); therefore, details of KLK6 substrate specificity are relevant to establish its physiological roles and also to design inhibitors [13].

The enzymatic properties of KLK6 were described in detail by the screening of fluorescence resonance energy transfer (FRET) peptide families [14], by analysis of extended substrate specificity for the nonprime side using a positional scanning combinatorial library of tetrapeptide substrates [15] and by random library of peptides displayed on the surface of bacteriophage [16, 17]. KLK6 has restricted specificity for basic amino acids at the P₁ position of the substrates (Schechter and Berger nomenclature [18]) with large preference for Arg. All the reported peptidyl-para-nitroanilide and peptidyl-7-amino-4-methylcoumarin substrates were poorly hydrolyzed by KLK6 [19, 20] compared with other arginyl hydrolases such as KLK1 [21-23].
indicating that the prime sites of enzyme-substrate interactions are important for KLK6 efficient activity.

The identification of potentially physiological substrates and efficient inhibitors for KLK6 are relevant goals to elucidate the role of KLK6 in physiological and pathological processes. Besides the hydrolytic activity of KLK6 on myelin basic protein, protein activator receptors and human ionotropic glutamate receptor subunits [14], the peculiar activity of KLK6 on peptides containing clusters of basic amino acids raises the possibility of KLK6 furin-like functional activity, as already previously suggested [16]. This seems a reasonable hypothesis since serine proteases were reported to be involved in the activation of metalloproteases [24] and activation of pro-BDNF [25] that are classically considered to be activated by furin. A further possibility is the release by KLK6 of enkephalin from the pro-enkephalin precursor, where each enkephalin is flanked by basic amino acids. Additionally, hog pancreatic kallikrein [26] and human plasma kallikrein (HPK) [27] were reported to release enkephalin from pro-enkephalin precursor.

These potentially relevant hydrolytic activities of KLK6 were explored in the present report by synthetic fluorogenic peptidyl-Arg-7-amino-4-carbamoylmethylcoumarin (peptidyl-ACC) peptides and by FRET peptides in the format of Abz-peptidyl-Q-EDDnp (where Abz= ortho-aminobenzoic acid and Q-EDDnp = glutaminyl- N-(2,4-dinitrophenyl) ethylenediamine), in which pairs or sequences of basic amino acids (Arg or Lys) were introduced. Other FRET peptides containing furin processing sequences of human MMP-14 (common name MT-MMP1) [28] and of the human neurotrophins NGF (Nerve Growth Factor), BDNF (Brain-Derived Neurotrophic Factor), NT-3 (Neurotrophin-3), and NT-4 (Neurotrophin-4) were synthesized and assayed as substrates for KLK6. For comparison, these FRET peptides
with furin processing sequences were also assayed as substrates for KLK1 and human plasma kallikrein (HPK). The potential KLK6 enkephalin releasing activity was also explored with FRET peptides containing human pro-enkephalin sequences with at least one complete enkephalin pentapeptide.

2. Material and methods

2.1 Enzymes.

Mature KLK6 was expressed and purified from a baculovirus/insect cell line system [29] that is glycosylated at Asn\textsuperscript{132} and the concentration of active enzyme was determined by spectrofluorimetric titration with 4-methylumbelliferyl p-guanidinobenzoate hydrochloride (MUGB) [30]. Recombinant KLK1 was obtained as previously described [31] and the molar concentration of active enzyme was determined with MUGB, as performed with KLK6. HPK was obtained from human plasma Cohn’s fraction IV [32] and the concentration of active HPK was determined as previously reported [33].

2.2 Peptides

Solid-phase peptide synthesis with the fluorenly-9-methyloxycarbonyl (Fmoc) methodology strategy was used to obtain both the FRET Abz-peptidyl-Q-EDDnp peptides [34, 35] and the fluorogenic peptidyl-7-Amino-4-carbamoylmethylcoumarin peptides (peptidyl-ACC) [36]. All the protected amino acids were purchased from Calbiochem-Novabiochem (San Diego, CA, USA) and the syntheses were performed in automated bench-top simultaneous multiple solid phase peptide synthesizers (PSSM 8
system; Shimadzu, Tokyo, Japan). All peptides were purified by semi-preparative HPLC using an Econosil C-18 column (10 μm particle size, 22.5 mm × 250 mm) and a two-solvent system: (A) trifluoroacetic acid/water (1:1000, v/v) and (B) trifluoroacetic acid/acetonitrile/water (1:900:100, by vol.). The molecular weight and purity of the obtained peptides were validated by MALDI-TOF mass spectrometry, using a LT mass spectrometer (Bruker Daltonics, Billerica MA). Stock solutions of peptides were prepared in dimethylformamide (DMF) and the concentrations of FRET peptides were measured spectrophotometrically using the molar extinction coefficient of EDDnp as 17,300 M⁻¹ cm⁻¹ at 365 nm. The concentrations of the peptidyl-ACC peptides were obtained by colorimetric quantitation of ACC concentration at 354 using 16,000 M⁻¹ as extinction coefficient.

2.3 Assays of enzymes.

The hydrolyses of the peptidyl-ACC and FRET (Abz-peptidyl-Q-EDDnp) peptides were monitored continuously in a Shimadzu RF 6000 spectrofluorometer with constant stirring at 37 °C. The fluorescence changes due to hydrolysis of FRET peptides were measured at λ_ex = 320 nm and λ_em = 420 nm, and the hydrolysis of peptidyl-ACC peptides at λ_ex = 380 nm and λ_em = 460 nm. The enzyme concentrations for determination of the kinetic parameters were chosen at a level intended to hydrolyze less than 5% of added substrate over the time course of data collection. The slope of the generated fluorescence signal over time was converted into micromoles of substrate
hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. Proteases were pre-incubated in the assay buffer for 3 min before the addition of substrate. The $K_M$ values were obtained using substrate concentrations spanning 0.5 – 2.0 x $K_M$ for each protease. The kinetic parameters $K_M$ and $k_{cat}$ were calculated by nonlinear regression using Grafit software (Erithacus Software, Horley, Surrey, UK). Errors were less than 5% for each of the obtained kinetic parameters. The buffer compositions for the hydrolyses of the substrates were 20 mM Tris-HCl, 1 mM EDTA, 10μM heparin at pH 7.5 for KLK6; 20 mM Tris-HCl, 1 mM EDTA, at pH 9.0 for KLK1 and at pH 7.5 for HPK. In used conditions the inner filter effect with the FRET peptides were lower than 5% of the total fluorescence and with practically negligible effects on the determination kinetic parameters of their hydrolysis.

The cleavage site of FRET or peptidyl-ACC peptide substrates by each of the three protease were identified by HPLC analysis of 100 μL aliquots taken from the reaction mixture at different times of the reaction using a Prominence liquid chromatography LC-20AD unit (Shimadzu, Japan). Each product was detected by its UV absorption at 220 nm and its molecular weight was determined by LC/MS using an LCMS-2010 equipped with an ESI-probe (Shimadzu, Japan) connected to the HPLC effluent after the UV-detector. An Ultrasphere C-18 column (5 μm, 4.6 250 mm) was used, and eluted with the solvent systems A (H$_3$PO$_4$/water, 1:1000) and B (ACN/water/H$_3$PO$_4$, 900:100:1) at a flow rate of 0.8 ml/min and a 0–80% gradient of solvent B for 60 min.

2.4 Molecular models.
Molecular models of peptides were built with the aid of Chem3D and fitted to the structure of KLK6 (RCSB accession 1L2E) using the program MOLMOL [37]. The fit aimed mainly at achieving best shape complementarity and optimizing electrostatic interactions. Polar or hydrophobic interactions were not optimized because the goal was only a proof of concept rather than finding the best possible complex. The energy of side chain conformations of the peptide was optimized by minimization by Chem3D (trial version 16). The Ramachandran plots corresponding to the two models of peptide V-A-R-R-A-A and the electrostatic surface representation of KLK6 were also generated with MOLMOL [37].
3. Results and Discussion

3.1 Hydrolysis of peptidyl-ACC and FRET substrates by KLK6.

Our initial purpose was the design of peptidyl-ACC peptides based on KLK6 substrate specificity reported with peptidyl-ACC substrate libraries [15] in order to find a convenient substrate to assay potential KLK6 inhibitors. Due to the reported preference of KLK6 for Arg at P$_1$ and P$_2$ positions, the peptide Bz-A-R-R-ACC (Pep-1) was prepared and it was efficiently cleaved (see parameters in Table 1), but unexpectedly, the cleavage occurred between the two arginines (Bz-A-R$^\dagger$R-ACC) as shown in Figure 1. This KLK6 pattern of cleavage between pairs of basic residues was also observed with the peptides K-A-R$^\dagger$R-ACC (Pep-2), Bz-A-K$^\dagger$R-ACC (Pep-3) and with the commercially available Z-R$^\dagger$R-MCA (Pep-4) peptide. As peptidyl-ACC substrates containing pairs of basic amino acids do not release the ACC fluorophore after cleavage by KLK6, Bz-A-F-R$^\dagger$ACC (Pep-5) was synthesized and found to efficiently release ACC by KLK6 cleavage (Table 1) and is therefore a convenient fluorogenic substrate for inhibitor assays.

The kinetic parameters for the hydrolysis of FRET peptides presented in Table 1 allow a further evaluation of the susceptibility of basic amino acid sequences to KLK6. Abz-K-L-R-S-S-K-Q-EDDnp (Pep-6) that was previously assayed with KLK6 [14], but in different conditions, was introduced as a hydrolysis reference. Abz-K-L-R-S-K-Q-EDDnp (Pep-7) (the modifications in relation to Pep-6 is double underlined) was efficiently hydrolyzed but in two sites with higher preference between the two Arg. In contrast, Abz-K$^\underline{R}$-R-S-S-K-Q-EDDnp (Pep-8) is cleaved only at R-S bond but with
$k_{cat}/K_M$ 10 times lower than Pep-6 due to higher $K_M$ value. The substitutions in the same positions of Pep-6 but with Lys giving Abz-K-L-R-Ke-S-K-Q-EDDnp (Pep-9) and Abz-K-K-R-S-S-K-Q-EDDnp (Pep-10) resulted in only one cleavage in the carboxyl side of Arg with $k_{cat}/K_M$ lower than that for Pep-6, however it is noteworthy for its high $k_{cat}$ value. The shorter FRET sequences in Abz-F-R-R-Q-EDDnp (Pep-11) and Abz-F-R-K-Q-EDDnp (Pep-12) were specifically designed to compare the susceptibility to KLK6 of R-R and R-K bonds, and there is a clear preference for R-R hydrolysis due high $k_{cat}$ value; although R-K in Pep-12 exhibits a very low $k_{cat}$ value, it maintains $K_M$ value as observed for R-R hydrolysis. Similar results were observed with Pep-13 and Pep-14, which reinforces the view that KLK6 definitively prefers R-R cleavage, independently of the amino acid at P$_2$ position in the substrate. Abz-A-K-R-Y-Q-EDDnp (Pep-15) was only cleaved at R-Q bond, contrasting with the cleavage of Bz-A-K-R-Y-R-ACC (Pep-3) that was hydrolyzed only at K-R bond. These results suggest that KLK6 does not accommodate adequately the fluorophore ACC at its S$_{1}´$ subsite as also observed with the hydrolysis of Z-R-Y-R-MCA (Pep-4). In addition, KLK6 accommodates quite well R at S$_{1}´$ subsite as particularly observed with the phage display analysis of KLK6 subsite specificity [16].

### 3.2 Hydrolysis of FRET peptides containing furin processing sequences.

The peptide Abz-N-V-R-Y-R-K-R-Y-A-I-Q-EDDnp contains the furin processing sequence derived from MMP-14 and it is cleaved by KLK6 first at R-R peptide bond and at R-Y after a longer time of incubation as showed in Figure 2, and the kinetic constants are presented in Table 2. These results are in accord with the KLK6 hydrolytic
properties discussed above, since peptides containing the pair of basic amino acids R-R are preferentially cleaved by KLK6 between the two Arg and the substrates with K-R sequence are cleaved by KLK6 only at Arg carboxyl site of substrates containing this pair of basic residues. These observations qualify KLK6 as a potential activating serine protease of MMP-14, which is one of four type I transmembrane proteins (also known as MT1-MMP), well known by its collagen I, II, and III degrading activities [38] and by its ability to activate MMP-2 [39] (a key element in tumor invasion and metastasis [40, 41]). In addition, MMP-14 plays physiological roles in extracellular matrix remodeling, and its deficiency in mice results in connective tissue disorders [42]. For comparison, we assayed Abz-N-V-R-R-K-R-Y-A-I-Q-EDDnp with KLK1, which was not able to cleave it but was inhibited with a $K_i$ value in the nM range. In contrast, HPK hydrolyzed Abz-N-V-R-R-K-Y-A-I-Q-EDDnp with high efficiency and at the R-Y bond that is the cleavage site of furin.

The cleavage sites and the kinetic parameters of hydrolysis by KLK6 of the peptides derived from furin processing sequence of human NGF, BDNF, NT-3 and NT-4 are shown in Table 3. The peptides derived from NGF and NT-3 are hydrolyzed by KLK6 in the same place as expected for furin and these hydrolyses are in accordance with the KLK6 hydrolytic properties presented above. The peptides with BDNF and NT-4 sequences are cleaved between the two R-R as also expected from the results presented in Table 1. All these peptides were also substrates for KLK1 and HPK, except the peptide Abz-R-T-S-R-R-K-R-Y-A-E-H-K-Q-EDDnp that was resistant to KLK1 and inhibited the enzyme in the nM range. HPK hydrolyzed efficiently all the peptides in the same place as expected for furin, except the peptide derived from BDNF.

The neurotrophins are essential proteins for the development of the vertebrate nervous system, and they are initially synthesized as pro-neurotrophins or pre-pro-
neurotrophins, which are cleaved by furin to produce the mature neurotrophins [43]. However, pro-neurotrophins are also secreted and act as distinct ligands that involve these pro-proteins in relevant cell processes in central nervous system, as for instance, to initiate cell death (for review see [44]). Recombinant proNGF and proBDNF were reported to be susceptible to cleavage by plasmin, MMPs, [45, 46] and tryptase [47]. These results put KLK6 as a potential pro-neurotrophin processing protease due to its substrate specificities, and because KLK6 is the higher expressed kallikrein-related peptidase in the central nervous system [1, 2]. All together, these observations support the hypothesis of a furin-like activity for KLK6 as previously suggested by the qualitative hydrolysis of the peptide GGR-R^2-R^2-KVGG [16]. Special attention should be invested on the processing of pro-neurotrophins also by KLK1 and HPK not only due to their efficient hydrolytic activities on the FRET peptides containing furin recognized sequences in human proneurotrophin factors but also because these kallikreins are also detected in central nervous system [48-51].

3.3 Hydrolysis of FRET peptides containing human pro-enkephalin sequences.

The cleavage sites and the kinetic parameters of four FRET peptides containing human proenkephalin sequences are provided in Table 4. The peptide with two enkephalin sequences (double underlined), Abz-LLAKRYGGFMKRYGGFMKKMDE-Q-EDDnp was cleaved in three sites releasing the enkephalin peptides YGGFMKR and YGGFMR. The other three FRET peptides Abz-DYQKRYYGFLKRFAEAL-Q-EDDnp, Abz-EEVKRYGGFMRLKRS-Q-EDDnp and Abz-DMSKRYYGGFMRSLKRS-Q-EDDnp contain only one enkephalin sequence, and the peptides YGGFLKR and YGGFMR were released. The higher $k_{cat}/K_M$ value for the peptide with two enkephalin sequences is due to its low $K_M$ value compared with the
other sequences (possibly related to its larger size). The cleavage after the KR pair of basic amino acids were the preferential sites of cleavages in the four FRET peptides as observed following the products of hydrolysis by HPLC – mass spectrometry, and KR is the more frequent dibasic motif flanking the enkephalins in the human proenkephalin sequence.

The complete release of enkephalin requires the activity of a carboxypeptidase to remove the C-terminal basic amino acid from each peptide released by KLK6 and a possible candidate is carboxypeptidase M due its preference for basic residues and also its presence in CNS [52] as reviewed in [53,54]. Therefore, similar to hog pancreatic kallikrein [26] and HPK [27], enkephalin can be released from human proenkephalin by KLK6, particularly in the CNS. The furin-like activity by KLK6, KLK1 and HPK, evaluated with peptides derived from MMP-14 (Table 2) and from NGF, BDNF NT-3 and NT-4 (Table 3) indicate the higher efficiency of HPK followed by KLK1, however these kallikreins are present in the CNS in lower concentrations.

3.4 Modelling of KLK6 interactions with substrates containing clusters of basic amino acids.

As mentioned above, the peptide Bz-A-R-R-ACC was designed to obtain an efficient and convenient substrate for KLK6, on the assumption that the $S_2$ and $S_1$ subsites have both a high specificity for R residues, as suggested by the data reported by Debela et al. [15]. Based on this assumption, the R-R pair should occupy $S_2$ and $S_1$ catalytic subsites of KLK6 and the R-ACC bond should be cleaved with release of the ACC fluorophore. On the contrary, we observed in Bz-A-R-R-ACC and in several other peptides that hydrolysis generally occurs at the bond between the two basic residues.
Summarizing all results reported above, some traits emerge clearly: contrary to the simplified picture that was at the basis of the design, the $S_1$ site has an affinity for R stronger than previously suggested [15]. The affinity of subsite $S_2$ for K and R is probably lower than initially hypothesized, particularly for R as previously reported [14]. On the other hand, our results presented in this paper, also supported by phage display analysis of KLK6 substrate specificity [16], indicate that $S_1$ subsite can easily host R. These observations prompted us to re-evaluate the model of the complex between peptide V-A-R-R-A-A and the active site of KLK6 as previously proposed [15]. The conformation of this peptide bound to the catalytic site, even at first glance, appears to be far from an extended conformation. The internal rotation angles of the model of V-A-R-R-A-A fitted in the active site of KLK6 are modeled by imposing the same contacts with KLK6 residues reported in the original paper [15]. When we examined the phi/psi values of the four central residues (A2, R3, R4, A5) of this model, which define the conformation of the peptide, we found that not only the angles are far from those typical of an extended conformation, but apparently fall in forbidden zones of the Ramachandran plot (Figure 2a). However, the energy deficit originating from the unfavorable internal angles of the peptide can be easily compensated by favorable enthalpic interactions of the peptide with the enzyme. When judging the interaction of a flexible peptide with an enzyme, it is imperative to take into account all possible conformations existing in solution. In order to assess all possible interactions of the ensemble of peptide conformers with the active site of KLK6 it is useful to check whether low energy conformers of V-A-R-R-A-A in solution can possibly fit the KLK6 active site. As can be seen in Figure 2b, it is a simple matter to find an extended conformer of the peptide by starting from a fully extended conformation and running a few cycles of minimization. The four pairs of peptide bonds in the extended model fall
in the center of a conformational favored zone (Figure 2 b). Can such a conformer still fit the KLK6 site? The answer is yes, provided the first R residue (of the pair of basics) goes into S$_1$ and the second into S$_1'$ as shown in Figure 2 d, justifying the marked preference of the hydrolysis between the two R of the examined peptides as substrates for KLK6. This extended conformation of the substrate is in accordance to the previous evidences obtained from the conformational selection of inhibitors and substrates by proteases [55] and also extensively reviewed [56]. The hydrolysis of the peptide Acetyl-V-A-R-R-ACC only at R-R peptide bound, as showed in Figure 4, supports this interpretation.

3.5 Conclusion

All observed hydrolytic activities reported here with of KLK6 support the view that it can be considered a processing protease with very restricted specificity for R, that is potentiated if this amino acid residue is flanked by another basic residue. This conclusion is proposed because the cleavages in most of the cases were between R-R and very rare at K-R peptide bond. This behavior gives to KLK6 the potentiality to release enkephalin of the pro-enkephalin precursors and resembles furin that is a canonical processing proteolytic enzyme. Finally, it is noteworthy that KLK6 and plasma kallikrein presented very close similarity cleaving all the assayed peptides as substrates. Altogether, the reported data indicate that KLK6 is a multifunctional protease in CNS.

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References


Table 1.
Hydrolysis by KLK6 of peptidyl-ACC and FRET (Abz-peptidyl-Q-EDDnp) substrates *

<table>
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<th>Nº</th>
<th>Peptides</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
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<td><strong>Peptidyl-ACC Substrates</strong></td>
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</tr>
<tr>
<td>1</td>
<td>Bz-A-R↓R-ACC*</td>
<td>4.0 ± 0.4</td>
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<td>K-A-R↓R-ACC*</td>
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<tr>
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<td>2.5 ± 0.1</td>
<td>107</td>
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<tr>
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<tr>
<td>6</td>
<td>Abz-K-L-R↓S-S-K-Q-EDDnp</td>
<td>1.2 ± 0.1</td>
<td>2.4 ± 0.2</td>
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<td>7</td>
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<td>9.0 ± 0.3</td>
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<tr>
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<td>27 ± 2</td>
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<td>Abz-K-L-R↓K-S-K-Q-EDDnp</td>
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<td>Abz-A-R↓K-Q-EDDnp</td>
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<td>Abz-A-K-R↓Q-EDDnp</td>
<td>76 ± 7</td>
<td>1.95 ± 0.09</td>
<td>26</td>
</tr>
</tbody>
</table>

All the reactions were done in 20mM Tris-HCl, 1 mM EDTA, and 10µM heparin, pH7.5 at 37° C, with substrate concentrations in the range 0.1–10 µM and [KLK6] = 7.5–15.0 nM. ↓ indicates the cleaved peptide bond. For hydrolysis of Pep-7 cleavage ↓↓1 was preferred (80%) compared with ↓↓2 (20%) and the kinetic parameters do not distinguish the cleavages. *The kinetics of hydrolysis was followed by HPLC procedures. The showed standard errors were obtained from non linear regression by the Grafit program.
Table 2
Hydrolysis by KLK6, KLK1 and Human Plasma Kallikrein of MMP-14 furin processing sequence.

<table>
<thead>
<tr>
<th>MMP-14 Furin Processing Sequence</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_M$ (mM. s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLK6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abz-N-V-R↑¹R-K-R↑²Y-A-I-Q-EDDnp</td>
<td>3.3 ± 0.2</td>
<td>0.26 ± 0.02</td>
<td>78</td>
</tr>
<tr>
<td>KLK1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abz-N-V-R-R-K-R-Y-A-I-Q-EDDnp</td>
<td>Not Hydrolyzed (Ki = 33 nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Plasma Kallikrein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abz-N-V-R-R-K-R↑²Y-A-I-Q-EDDnp</td>
<td>2.0 ± 0.2</td>
<td>0.87 ± 0.03</td>
<td>438</td>
</tr>
</tbody>
</table>

For the hydrolysis of Abz-NVRRKRYAI-Q-EDDnp by KLK6 both cleavages (1 and 2) were equivalent in terms of preference, approximately 50% each, and the kinetic parameters do not distinguish the cleavages. The showed standard errors were obtained from non linear regression by the Grafit program.
Table 3
Kinetic parameters of hydrolysis by KLK6, KLK1 and Human Plasma Kallikrein (HPK) of FRET peptides containing furin recognized sequences in human proneurotrophin factors.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Neutrophin Derived Substrates</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM.s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NGF - (Nerve Growth Factor)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLK6</td>
<td>Abz-T-H-R-S-K-R$\downarrow$S-S-H-P-I-Q-EDDnp</td>
<td>7.4 ± 0.7</td>
<td>0.20 ± 0.02</td>
<td>27</td>
</tr>
<tr>
<td>KLK1</td>
<td>Abz-T-H$\downarrow$S-K-R-S-S-H-P-I-Q-EDDnp</td>
<td>1.02 ± 0.02</td>
<td>0.37 ± 0.01</td>
<td>362</td>
</tr>
<tr>
<td>HPK</td>
<td>Abz-T-H-R-S-K-R$\downarrow$S-S-H-P-I-Q-EDDnp</td>
<td>0.80 ± 0.07</td>
<td>0.19 ± 0.01</td>
<td>238</td>
</tr>
<tr>
<td><strong>BDNF (Brain-Derived Neurotrophic Factor)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLK6</td>
<td>Abz-S-M-R-V-R$\downarrow$R-H-S-D-P-A-Q-EDDnp</td>
<td>14 ± 1</td>
<td>0.10 ± 0.01</td>
<td>7</td>
</tr>
<tr>
<td>KLK1</td>
<td>Abz-S-M-R$\downarrow$V-R-R-H-S-D-P-A-Q-EDDnp*</td>
<td>0.22 ± 0.02</td>
<td>0.057 ± 0.002</td>
<td>258</td>
</tr>
<tr>
<td>HPK</td>
<td>Abz-S-M-R-V-R$\downarrow$R-H-S-D-P-A-Q-EDDnp</td>
<td>1.67 ± 0.07</td>
<td>0.459 ± 0.009</td>
<td>275</td>
</tr>
<tr>
<td><strong>NT-3 (Neurotrophin-3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLK6</td>
<td>Abz-R-T-S-R-R-K-R$\downarrow$Y-A-E-H-K-Q-EDDnp</td>
<td>1.7 ± 0.1</td>
<td>0.070 ± 0.001</td>
<td>41</td>
</tr>
<tr>
<td>HPK</td>
<td>Abz-R-T-S-R-R-K-R$\downarrow$Y-A-E-H-K-Q-EDDnp</td>
<td>1.10 ± 0.21</td>
<td>0.33 ± 0.03</td>
<td>302</td>
</tr>
<tr>
<td><strong>NT-4 (Neurotrophin-4)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLK6</td>
<td>Abz-A-P-A-N-R-R-S-R-G-V-S-E-T-Q-EDDnp*</td>
<td>$k_{cat}/K_M = 3$ (mM. s$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLK1</td>
<td>Abz-A-P-A-N$\downarrow$S-R-R-G-V-S-E-T-Q-EDDnp</td>
<td>1.8 ± 0.2</td>
<td>0.141 ± 0.008</td>
<td>78</td>
</tr>
<tr>
<td>HPK</td>
<td>Abz-R-T-S-R-R-K-R$\downarrow$Y-A-E-H-K-Q-EDDnp</td>
<td>2.0 ± 0.1</td>
<td>0.110 ± 0.002</td>
<td>55</td>
</tr>
</tbody>
</table>

The following conditions for substrate hydrolysis were used:
pH 7.5, 20 mM Tris-HCl, 1 mM EDTA, and 10 µM heparin at 37 °C, with [substrate] - 0.1–10 µM and [KLK6] - 2.0 -7.5nM;
pH 7.5. Tris 20 mM, EDTA 1 mM at 37 °C with [substrate] - 0.1–10 µM and [HPK] - 4.0 -6.4 nM;
pH 9.0. Tris 20 mM, EDTA 1 mM at 37 °C with [substrate] - 0.1–10 µM and [KLK1] - 4.0 -6.0 nM;
↓ indicates the cleaved peptide bond.

*The $k_{cat}/K_M$ value for this peptide was determined in pseudo-first order condition

The showed standard errors were obtained from non linear regression by the Grafit program.
Table 4
Human proenkephalin derived peptides hydrolysis by KLK6*

<table>
<thead>
<tr>
<th>Peptides</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM.s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abz-L-L-A-K-R$^\perp$Y-G-G-F-M-K-R$^\perp$Y-G-G-F-M-K$^\perp$K-M-D-E-Q-EDDnp</td>
<td>0.36 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>228</td>
</tr>
<tr>
<td>Abz-D-Y-Q-K-R$^\perp$Y-G-G-F-L-K-R$^\perp$F-A-E-A-L-Q-EDDnp</td>
<td>21 ± 2</td>
<td>0.18 ± 0.01</td>
<td>8.6</td>
</tr>
<tr>
<td>Abz-E-E-V-K-R$^\perp$Y-G-G-F-M-R$^\perp$G-L-K-R-S-Q-EDDnp</td>
<td>4.6 ± 0.4</td>
<td>0.08 ± 0.01</td>
<td>16</td>
</tr>
<tr>
<td>Abz-D-M-S-K-R$^\perp$Y-G-G-F-M-R$^\perp$S-L-K-R$^\perp$S-Q-EDDnp</td>
<td>1.8 ± 0.1</td>
<td>0.06 ± 0.01</td>
<td>33</td>
</tr>
</tbody>
</table>

*The following conditions for substrate hydrolysis were used: for pH 7.5, 20 mM Tris-HCl, 1 mM EDTA, and 10 µM heparin. All the reactions were done at 37 °C, with [substrate] = 0.1–10 µM and [KLK6] = 6.4–8.0 nM. $^\perp$ indicates the cleaved peptide bond.
Figure 1

Figure 1 HPLC profiles of hydrolysis by KLK6 of Bz-ARR-ACC. The absorbance of the elutes were recorded at $\lambda = 214$ nm. The identification of the indicated fragment peaks was done by electron spray mass spectrometer LCMS-2010EV equipped with the ESI-probe on-line with the HPLC system. Conditions of hydrolysis as described in Experimental section.
Figure 2

Figure 2 HPLC profiles of hydrolysis by KLK6 of Abz-N-V-R²R-K-R²Y-A-I-Q-EDDnp that contains the furin processing sequence derived of MMP-14. The identification of the indicated fragment peaks was done by electron spray mass spectrometer LCMS-2010EV equipped with the ESI-probe on-line with the HPLC system. Conditions of hydrolysis as described in Experimental section.
Figure 3. Comparison of the interaction of two conformers of VARAA with KLK6. a) Ramachandran plot of the central residues of VARAA corresponding to Figure 4 of Debela et al. (2006) [15]; c) fit of VARAA with the active site of hk6, adapted from [15]; b) Ramachandran plot of the central residues of VARAA corresponding to a completely extended conformation; d) fit of conformer b of VARAA with the active site of hk6. The position on the plot of each pair of angles is indicated by a + sign flanked by the symbol of the corresponding residue.
Figure 4 HPLC profiles of hydrolysis by KLK6 of Ac-VARR-ACC. Panel A shows the elute fluorescence obtained in $\lambda_{ex}=326\text{nm}/\lambda_{em}=402\text{ nm}$, and panel B shows the elute absorbance at $\lambda=214\text{ nm}$. Ac-VAR and R-ACC fragments are overlapped in used conditions. The identification of the indicated fragment peaks was done by electron spray mass spectrometer LCMS-2010EV equipped with the ESI-probe on-line with the HPLC system. Conditions of hydrolysis as described in Experimental section.