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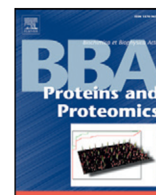

Activity of human kallikrein-related peptidase 6 (KLK6) on substrates containing sequences of basic amino acids. Is it a processing protease?

Roberta N. Silva [a], Lilian C.G. Oliveira [a], Carolina B. Parise [a], Juliana R. Oliveira [a], Beatrice Severino [b], Angela Corvino [b], Paola di Vaio [b], Piero A. Temussi [c,d], Giuseppe Caliendo [b], Vincenzo Santagada [b], Luiz Juliano [a], Maria A. Juliano [a,*]

[a] Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil
[b] Dipartimento di Farmacia, Università degli Studi di Napoli "Federico II", Via D. Montesano, 49, 80131 Napoli, Italy
[c] The Wohl Institute, King's College London, 5 Cutcombe Rd, London SE5 9RT, UK
[d] Dipartimento di Scienze Chimiche, Università di Napoli Federico II, Comp. Univ. Monte Sant'Angelo Via Cintia 21, 80126 Naples, Italy

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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.

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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 P_1 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],

* Corresponding author at: Departamento de Biofísica, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Três de Maio, 100, São Paulo 04044-020, Brazil.
E-mail addresses: map.juliano@gmail.com, ma.juliano@unifesp.br (M.A. Juliano).

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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^{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 fluorenyl-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 × 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, 10 μM 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

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

No	Peptides	K_M (μM)	k_{cat} $(s)^{-1}$	k_{cat}/K_M $(mM\ s)^{-1}$
	Peptidyl-ACC substrates			
1	**Bz-A-R↓R-ACC***	4.0 ± 0.4	8.0 ± 0.9	2000
2	**K-A-R↓R-ACC***	55 ± 5	9.7 ± 0.8	190
3	**Bz-A-K↓R-ACC***	23 ± 2	2.5 ± 0.1	107
4	**Z-R↓R-MCA***	26 ± 2	6.5 ± 0.6	250
5	**Bz-A-F-R↓ACC**	47 ± 4	10 ± 1	210
	FRET substrates			
6	**Abz-K-L-R↓S-S-K-Q-EDDnp**	1.2 ± 0.1	2.4 ± 0.2	1983
7	**Abz-K-L-R↓1R↓2S-K-Q-EDDnp**	17 ± 1	9.0 ± 0.3	529
8	**Abz-K-R-R↓S-S-K-Q-EDDnp**	27 ± 2	4.6 ± 0.4	170
9	**Abz-K-L-R↓K-S-K-Q-EDDnp**	2.6 ± 0.2	1.5 ± 0.1	570
10	**Abz-K-K-R↓S-S-K-Q-EDDnp**	25 ± 2	18 ± 1	720
11	**Abz-F-R↓R-Q-EDDnp**	43 ± 4	3.0 ± 0.3	70
12	**Abz-F-R↓K-Q-EDDnp (Ki = 31 ± 3 μM)**	23 ± 2	0.09 ± 0.01	3.8
13	**Abz-A-R↓R-Q-EDDnp**	73 ± 7	2.0 ± 0.1	27
14	**Abz-A-R↓K-Q-EDDnp**	53 ± 5	0.04 ± 0.01	0.72
15	**Abz-A-K-R↓Q-EDDnp**	76 ± 7	1.95 ± 0.09	26

All the reactions were done in 20 mM Tris-HCl, 1 mM EDTA, and 10 μM heparin, pH 7.5 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 nonlinear regression by the Grafit program.

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