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Research paper

Substrate specificity of kallikrein-related peptidase 13 activated by salts or glycosaminoglycans and a search for natural substrate candidates

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ABSTRACT

KLK13 is a kallikrein-related peptidase preferentially expressed in tonsils, esophagus, testis, salivary glands and cervix. We report the activation of KLK13 by kosmotropic salts and glycosaminoglycans and its substrate specificity by employing a series of five substrates derived from the fluorescence resonance energy transfer (FRET) peptide Abz-KLRSSKQ-EDDnp. KLK13 hydrolyzed all these peptides only at basic residues with highest efficiency for R; furthermore, the S_3 to S_2' subsites accepted most of the natural amino acids with preference also for basic residues. Using a support-bound FRET peptide library eight peptide substrates were identified containing sequences of proteins found in testis and one with myelin basic protein sequence, each of which was well hydrolyzed by KLK13. Histatins are salivary peptides present in higher primates with broad antifungal and mucosal healing activities that are generated from the hydrolysis from large precursor peptides. KLK13 efficiently hydrolyzed synthetic histatin 3 exclusively at R^{25} (DSHAKRHHGYKRKFHEKHHSHRGYR^{25}↓SNYLYDN) that is the first cleavage observed inside the salivary gland.

In conclusion, the observed hydrolytic activities of KLK13 and its co-localization with its activators, glycosaminoglycans in the salivary gland and high concentration of sodium citrate in male reproductive tissues, indicates that KLK13 may play a role in the defense of the upper digestive apparatus and in male reproductive organs.

1. Introduction

The tissue kallikrein members represent a cluster of 15 serine peptidases of chymotrypsin-like S1A family clan PA(S) localized in the human chromosome locus 19q13.4, and present a high degree of identity [1–7]. Human kallikrein-related peptidase 13 (KLK13) is one of these peptidases that is expressed in many tissues with preferences in glandular epithelia, tonsils, esophagus, trachea, testis, salivary glands, vagina and cervix [8–10]. The physiological roles of KLK13 remain unknown, in contrast to KLK1 that is involved in kinin release [11]; KLK3 hydrolyzes seminogelin I and II in semen and contributes to its liquefaction after ejaculation [12,13]; KLK4 in prostate activates KLK3 [14,15], is modulated by a specific zinc binding site [16] and is involved in dental enamel formation [17]; KLK6 activates PAR2 and cleaves glutamatergic receptors and myelin basic protein [18–22]; KLK5 and KLK7 are involved in skin desquamation (for a recent review see [23]) and KLK8 participates in human epidermis and sweat in a proteolytic cascade contributing to the skin barrier [24]. The associations of KLK13 with some pathologies were earlier reported, and include psoriasis vulgaris and atopic dermatitis [25] (where the peptidase is expressed in significant amounts), it is observed to be a favorable prognosis marker in ovarian cancer [26], is expressed in high levels in some types of salivary gland tumors [27], is down regulated in testicular cancer [28,29] and is also reported to degrade the extracellular matrix [30].

Abbreviations: Q-EDDP, (glutamyl-N-[ethylenediamine] 2,4-dinitrophenyl); Abz, *ortho*-aminobenzoic acid; HOBt, N hydroxybenzotriazole; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluorborate; NMM, N-methyl-morpholine; (PMC), 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt, trityl; DIPEA, diisopropylethylamine; TFA, Trifluor acetic acid; EDT, ethanodithiol; DMF, dimethylformamide; NMM, 4-methylmorpholine; DCM, dichloromethane; GAGs, glycosaminoglycans; FRET, fluorescence resonance energy transfer; LMWK, low molecular weight kininogen; MCA, methyl coumarin amide; MALDI-TOF, Matrix-assisted laser desorption/ionization-Time of flight; LCMS, liquid chromatography/mass spectrometry.

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Detailed analyses of substrate specificity of peptidases are essential to identify their roles in mammalian organisms, to aid in the development of selective assays, and to guide the design of novel inhibitors. The substrate specificity of KLK13 was earlier investigated by screening of fluorogenic 7-amino-4-carbamoylcoumarin positional scanning-synthetic combinatorial libraries modified at the P_1 to P_4 positions [31] and KLK13 was observed to have selectivity of the S_1 subsite for R, preference for hydrophobic amino acids by the S_2 and S_4 subsites, and S_3 selectivity mainly for R (Schechter and Berger nomenclature [32]). Though these synthetic combinatorial libraries have provided valuable information about the specificity of numerous peptidases [33,34] they are restricted to the non-prime side of the peptidases, its application can be impaired for peptidases where the active site requires extended prime site substrates. In addition, cleavages of these substrates other than at the amino site of the 7-amino-4-methylcoumarin group (peptidyl-↓-AMC) are not detected with this library.

Glycosaminoglycans (GAGs) and kosmotropic salts such as sodium citrate and sodium sulfate were reported to strongly activate the hydrolytic activities of KLK3 and KLK6 [18,35,36] in contrast to KLK1 that is inhibited by salts [37]. In the present work we examined the modulation of salts and GAGs on hydrolytic activity of KLK13 and its S_3 to S_2' subsite substrate specificity taking as reference the fluorescence resonance energy transfer (FRET) peptide Abz-KLRSSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid and EDDnp = N-[2,4-dinitrophenyl]-ethylenediamine), which was designed based on previously reported subsite requirements of KLK1 and KLK6 [18]. Five series of analogs of this peptide were synthesized with substitution of each amino acid, except Gln, and assayed as substrates of KLK13.

Histatins are a class of salivary peptides present in higher primates, rich in basic amino acids [38] and have broad antifungal activity [39–41]. The systematic search by tandem mass spectroscopy of human saliva showed that the generation of histatin 3-related peptides resulted mainly from the action of trypsin-like proteases, and the first cleavage being at R^{25} (DSHAKRHHGYKRKF-HEKHHSHRGYR^{25}↓SNYLYDN) [42]. The selectivity of KLK13 for hydrolysis at R and K and its co-localization with histatins in salivary glands [10] prompted us to examined the hydrolytic activity of KLK13 on synthetic histatin 3 that is mainly processed in human saliva into histatin 6 (DSHAKRHHGYKRKFHEKHHSHRGYR^{25}) and then to histatin 5 by removing of C-terminal R^{25} [42]. Due to selectivity of KLK13 to cleave at basic amino acids, the kininogenase activity was also evaluated using low molecular weight (LMW) kininogen, and a synthetic human kininogen fragment, Abz-MIS-LMKRPPGFSPFRSSRI-NH_2 that contains a bradykinin sequence.

We further tried to find other possible candidates as natural substrates for KLK13 using a support-bound FRET peptide library prepared on PEGA resin (a beaded polyethylene glycol dimethylacrylamide copolymer) by the process of split-combine synthesis, which results in a single peptide sequence on each resin bead [43–46]. The FRET peptide bound to a resin bead when hydrolyzed by the peptidase turned the bead fluorescent; such beads were manually picked up and the remained peptide in the bead was sequenced by Edman degradation methodology. The identified peptides were synthesized and the cleavage sites by KLK13 were subsequently determined in solution and the kinetic parameters for their hydrolysis were evaluated.

2. Materials and methods

2.1. Recombinant KLK13

Mature KLK13 was expressed and purified from a baculovirus/insect cell line system as previously described [22]. Briefly, the KLK13 gene which encodes for the mature form of KLK13 was inserted in frame, C-terminal of an enterokinase propeptide. To facilitate the purification procedure, a 6× His-tag was also included on the N-terminal side of the enterokinase propeptide. The purification took advantage of the His-tag by Ni-NTA affinity chromatography. After purification, KLK13 was activated by extended (i.e. 24 h) incubation with enterokinase at 1:100 ratio of enterokinase:pro-KLK13 (w/w). Hydrolysis of the enterokinase propeptide proceeded to apparent completion and the released propeptide and enterokinase were removed from the mature KLK13 by size exclusion chromatography. The final yield of purified mature KLK13 was typically 20–25 mg per liter of culture. The final purity of mature KLK13 was assessed by Coomassie Brilliant Blue staining of a 15% SDS-PAGE gel, N-terminal sequencing, and mass spectrometry, and yielded a purity of at least 98% with no visible degradation (Fig. 1A) and the correct mature N-terminus. The purified, mature form of KLK13 remained a single chain and migrated as closely-spaced doublet bands under both reducing and non-reducing conditions. The results of N-terminal sequencing revealed that both bands had identical termini consistent with mature KLK13 (VLNTXGTSGFLP). The 5th cycle of the reaction did not give an amino acid assignment, and this is presumably due to glycosylation of Asn. The doublet bands were separated by Phenomenex C_4 reverse phase HPLC (Fig. 1-B) and analyzed by MALDI-TOF mass spectrometry (Fig. 1-C), and the peak of each fragment presented molecular masses 29,213 and 30,270 Da, respectively. These values are higher than the calculated mass of un-modified KLK13 presumably due to the result of heterogeneous glycosylation

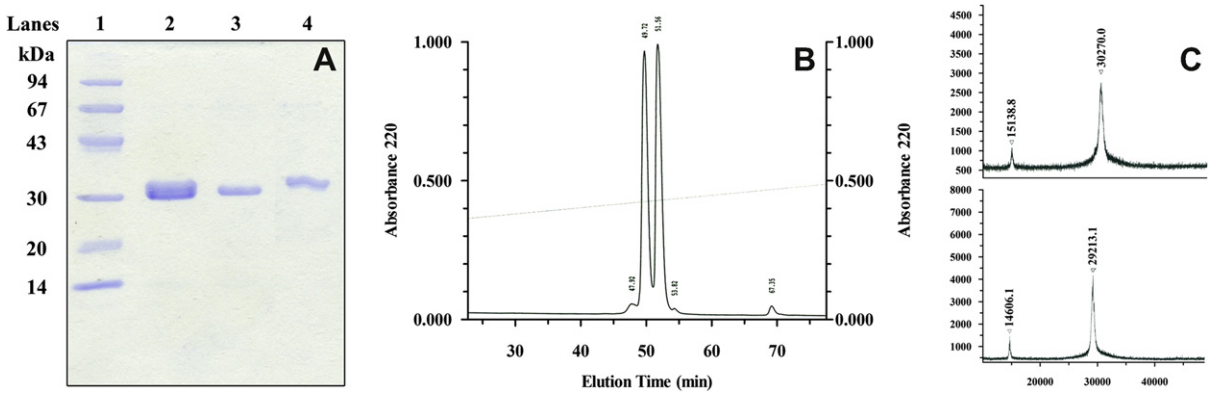

Fig. 1. (A) Purified recombinant KLK13 analysis by 15% SDS-PAGE in reducing conditions. Lanes: 1-Molecular weight marker; 2-Mature form of KLK13 with doublet fragments; 3- and 4-Mature forms of KLK13 isolated by C_4. (B) C_4 reverse phase HPLC separation of doublet fragment. (C) MALDI-TOF analysis of KLK13 fragments.

(with the higher-mass form containing approximately 6 additional hexose units). Both fragments exhibited similar enzymatic activity against Tos-GPR-AMC. Molar concentration of active KLK13 was determined by titration with MUGB (4-Methylumbelliferyl p-guanidinobenzoate hydrochloride) by spectrofluorimetric tritation as previously described [47]. Bovine myelin basic protein was purchased from (AbD Serotec, Raleigh, NC, USA).

2.2. Peptide synthesis

All FRET peptides and histatin-3 were obtained by solid-phase peptide synthesis as previously described [48,49] and using the Fmoc-procedure in an automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu,-Tokyo, Japan). The peptides were synthesized in TGR-resin (loading 0.2 mmol/g) using HBTU/HOBt as coupling reagent and the cleavage of peptide-resin was accomplished with TFA:anisol:EDT:water (85:5:3:7). All peptides obtained were purified by semi-preparative HPLC on an Econosil C-18 column. The molecular weight and purity (94% or higher) of synthesized peptides were checked by amino acid analysis and MALDI-TOF mass spectrometry, using a Microflex – LT mass spectrometer (Bruker – Daltonics, Billerica, MA, USA). Stock solutions of the peptides were prepared in DMF and the concentrations were measured spectrophotometrically using the molar extinction coefficient of 17,300 M^{-1} cm^{-1} at 365 nm.

2.3. Synthesis of support-bound FRET peptide library

The syntheses of libraries were carried out manually as previously described [46]. Briefly, the libraries were synthesized on 1 g of $PEGA_{1900}$ resin [50] in a 20 column Teflon synthesis block, using protected Fmoc amino acids. The resin was evenly distributed in the 20 wells of the Teflon synthesis block, and the Fmoc groups were removed. Prior to coupling the Fmoc amino acids (1 equiv.) were pre-activated with HOBt (1 equiv.), TBTU (1 equiv.) and NMM (2 equiv.) in DMF (1 ml) for 6 min and the activated amino acids were added, one to each of the 20 wells. After the completion of the coupling, the block was filled with DMF up to 1 cm above the top of the wells and inverted, and the resin was mixed vigorously by agitation for 30 min in the mixing chamber. The block was again inverted, evenly distributing the resins into the wells for washing and removal of the Fmoc group. This procedure was repeated for the incorporation of all the randomized positions. After the randomized positions, the Fmoc-K(Abz-Boc) and Fmoc-K(Dnp) were incorporated. The side chain protecting groups were removed by treatment with a mixture of TFA:thioanisole:ethanedithiol:water (87:5:5:3) for 8 h. The resin was washed with 95% acetic acid (4×), DMF (4×), 5% DIPEA in DMF (3×), DMF (3×), DCM (6×) and dried under vacuum.

2.4. Glycosaminoglycans

Size-defined (12,000 Da) bovine lung heparin (The Upjohn Co.) was prepared by using a size exclusion column approach [51]; heparan sulfate (16,000 Da) from bovine lung was a generous gift from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy); dermatan sulfate (12,000 Da) and chondroitin sulfate (25,000 Da) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

2.5. Kinetic measurements

The FRET peptides were assayed in a Shimadzu RF-1501 spectrofluorometer, at 35 °C. The assays were performed in 50 mM Tris, 1 mM EDTA, pH 7.5 and 35 °C (the temperature of highest activity was at 38 °C) with 0.5 μM heparin or 1.5 M sodium citrate. The enzymes were pre-incubated in the assay buffer for 3 min before the addition of substrate. Fluorescence changes were monitored continuously at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm. When fluorogenic MCA peptides were used, the excitation and emission wavelengths were changed to $\lambda_{ex} = 380$ and $\lambda_{em} = 460$ nm, respectively. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyze less than 5% of the 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.

2.6. Support-bound FRET peptide library screening

For all assays, the library beads were washed with water (3×) and the assay buffer (3×) before the addition of the enzyme. The reactions were stopped by dilution with 3 M HCl, and the mixtures were washed thoroughly until pH 5–6 was reached. The beads were transferred to a glass dish and inspected by fluorescence microscopy (Stereomicroscope Stemi-Zeiss), and the fluorescent beads were collected and transferred to a TFA-treated cartridge filter for on-resin sequence analysis. The amino acid sequence and cleavage point were determined by Edman degradation using a PPSQ/23 protein sequencer (Shimadzu, Japan). KLK13 was assayed as follows: 20 mg of resin in 50 mM Tris, 1 mM EDTA, pH 7.5, at 25 °C for 5 h with 75 nM of enzyme. The identified peptide sequences susceptible to hydrolysis by KLK13 were synthesized as FRET Abz-peptidyl-Q-EDDnp peptides and assayed in solution with the enzyme.

2.7. Digestion of myelin basic Protein by KLK13

Bovine myelin basic protein (MBP) was added to KLK13 at a 2000:1 mass ratio in 50 mM Tris and 1 mM EDTA, pH 7.5. This mixture was incubated at 35 °C, and time points were taken at 2, 4, 8, and 12 h. The MBP and degradative fragments were resolved using Tricine SDS-PAGE (16.5%) and developed by Comassie-Blue and two bands were isolated and the N-terminal sequenced by Edman degradation to identify the cleavage sites.

2.8. Kininogenase activity of KLK13

Low molecular weight human kininogen (LK) was purchased from Calbiochem. The ability of KLK13 to cleave the human LK was evaluated incubating 1 ml of LK (100 mg/ml) and KLK13 (1.8 nM) in reaction mixtures containing 50 mM Tris, 0–1.5 M sodium citrate, pH 7.5, at 35 °C for 4 h. Ethanol (3:1, v/v) was added, and the mixture was centrifuged at 1000 × g for 15 min. The kinin content in the supernatant was measured by radioimmunoassay, as previously described [52] KLK1 was also assayed as comparative control.

3. Results

3.1. Conditions for high KLK13 activity

The FRET peptide Abz-KLRSSKQ-EDDnp was taken as the initial substrate to search for the best conditions required for efficient KLK13 hydrolytic activity. The peptide Abz-KLRSSKQ-EDDnp was hydrolyzed only at the R–S bond, and this activity of KLK13 at pH 7.5 was highly activated by sodium citrate or sodium sulfate, as shown in Fig. 2A. High activation was also observed with heparin up to 0.5 μM (Fig. 2-B) and chondroitin and dermatan sulfate also activated KLK13 but at concentration range 20–40 μM (data not showed). KLK13 bound to heparin–Sepharose resin and was eluted with 100 mM NaCl,

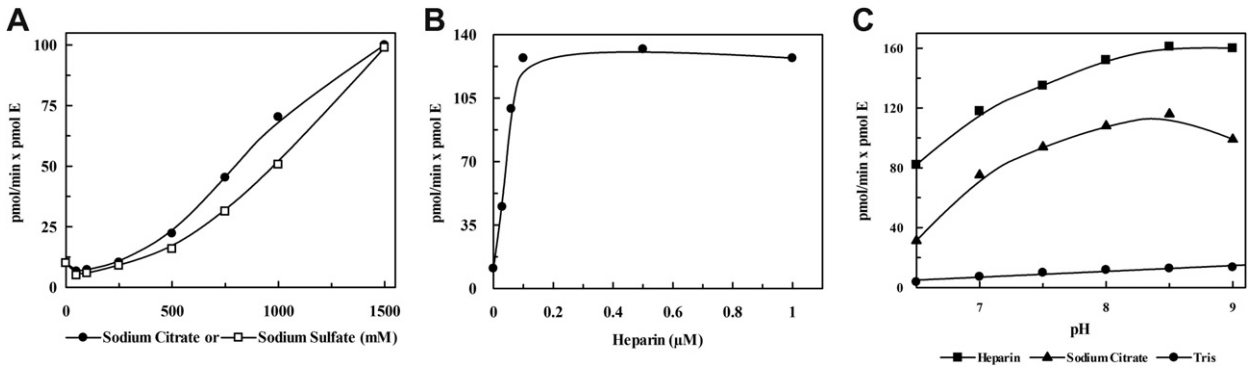

Fig. 2. (A) Activation of KLK13 by sodium citrate (●) and sodium sulfate (□). (B) Activation of KLK13 by heparin (C) pH profiles of KLK13 activity in the presence of sodium citrate (▲), heparin (■) and only with buffer (●). The reactions were performed in 50 mM Tris, 1 mM EDTA, pH 6.5–9.0, using as substrate 7.5 μM Abz-KLRSSKQ-EDDnp, and [E] = 3.8 nmol at 35 °C.

indicating a significant favorable electrostatic interaction between heparin and KLK13 that was not observed with KLK1 and KLK6. The pH profiles of KLK13 activities in the presence of 1 M sodium citrate and heparin are showed in Fig. 2-C. The KLK13 activity was reduced close to zero in the presence of 50 mM Tris HCl (with no sodium citrate or heparin). The presence of NaCl, KCl, $MgCl_2$ and $CaCl_2$ up to 1 M concentration did not activate the enzyme.

3.2. Substrate specificity of KLK13 using FRET peptides derived from Abz-KLRSSKQ-EDDnp

The peptide Abz-KLRSSKQ-EDDnp was taken as reference, and a series of five related FRET peptides were synthesized and assayed as substrates for KLK13 in 50 mM Tris at pH 7.5 and 35 °C in the presence of 0.5 μM heparin, and for some peptides the assays were done in the presence of 1.5 M sodium citrate. The specificity of the S_1 subsite was explored with the peptide series Abz-KLXSSKQ-EDDnp (where X = R, K, F, Y, M, L, V, W, D, E, G, H, I, N, P, Q, S, and T). Only the peptides Abz-KLR↓SSKQ-EDDnp and Abz-KLK↓SSKQ-EDDnp (i.e. X = R or X = K) were hydrolyzed and only at the peptide bonds R–S and K–S, respectively. The kinetic parameters of these hydrolyses in the presence of heparin and sodium citrate at pH 7.5 are presented in Table 1. All other peptides were resistant in both conditions up to 60 nM of KLK13. These results indicate that the S_1 subsite of KLK13 has a very restricted specificity for basic amino acids. The resistance to hydrolysis of peptide Abz-KLNSSKQ-EDDnp contrasts with the reported hydrolysis of peptides containing N at the P_1 position in the screening of fluorogenic 7-amino-4-carbamoylcoumarin positional scanning-synthetic combinatorial libraries [31].

The FRET peptides in the series Abz-XLRSSKQ-EDDnp, Abz-KXRSSKQ-EDDnp, Abz-KLRXSKQ-EDDnp and Abz-KLRSXKQ-EDDnp, were synthesized in order to explore the specificity of the subsites S_3, S_2, S_1' and S_2', respectively. The kinetic parameters for their hydrolysis by KLK13 are shown in Tables 2 and 3. All the peptides of the four series were hydrolyzed only at the R–S (or R–X) bond.

The parameters for the hydrolysis of the series Abz-KXRSSKQ-EDDnp (Table 2) shows that the S_2 subsite of KLK13 did not present restricted specificity; however, higher k_{cat}/K_m values were observed with substrates containing basic or hydrophobic amino acids at the P_2 position. Similarly, an S_3 subsite preference for basic and hydrophobic residues was observed as demonstrate from the parameters of hydrolysis of the series Abz-XLRSSKQ-EDDnp (Table 2). However, the peptide with the negatively charged residue E at the P_3 position was resistant to hydrolysis.

The kinetic parameters for the hydrolysis of the peptide series Abz-KLRXSKQ-EDDnp (Table 3) show that the S_1' subsite of KLK13 has a particular preference for K in contrast to R, as indicated by the k_{cat}/K_m value for the hydrolysis of the peptide Abz-KLR↓KSKQ-EDDnp that is 170-times higher than that of the peptide Abz-KLR↓RSKQ-EDDnp. The second best substrate of this series contains N and the peptides with F, S and H at the P_1' position were still efficiently hydrolyzed. Not unexpectedly, the peptide with X = P at the S_1' subsite was completely resistant to hydrolysis due to the imide bond of P. The kinetic parameters for hydrolysis of the series Abz-KLRSXKQ-EDDnp (Table 3) also show that the S_2' subsite of KLK13 does not have restricted specificity but does present preference for the basic amino acid R, and the resistance to hydrolysis of the peptide Abz-KLRSEKQ-EDDnp indicates that the S_2' of KLK13 does not accepted negative charge.

3.3. Screening of support-bound FRET peptide library

The peptides selected from the screening of this library were synthesized as FRET Abz-peptidyl-Q-EDDnp peptides and assayed with KLK13. Their sequences and velocities of hydrolysis in the presence of 1 M sodium citrate are shown in Table 4. The susceptibility to hydrolysis by KLK13 of the selected peptides was higher in the presence of sodium citrate than in the presence of 50 mM Tris–HCl, and most of these peptides were cleaved at the carboxyl side of R. Only two peptides were cleaved at K and each with lower velocities. It is noteworthy that the basic amino acids have significant frequency in all positions besides the P_1 position and eight out of twenty sequences were found in proteins from testis.

One human myelin basic protein sequence (GPVKKRNM) was found as a potential substrate of KLK13 in the support-bound peptide library that was confirmed by the hydrolysis of the FRET

Table 1
Kinetic parameters for the hydrolysis by KLK13 of the reference peptides Abz-KL**R**SSKQ-EDDnp and Abz-KL**K**SSKQ-EDDnp in the presence of heparin and sodium citrate.

Activator	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m $(mM\ s)^{-1}$
Abz-KL**R**↓SSKQ-EDDnp			
0.5 μM heparin	3.2 ± 0.1	2.2 ± 0.1	1455 ± 80
1.5 M sodium citrate	3.4 ± 0.2	1.2 ± 0.05	2834 ± 204
Abz-KL**K**↓SSKQ-EDDnp			
0.5 μM heparin	0.50 ± 0.03	31 ± 2	16 ± 3
1.5 M sodium citrate	0.10 ± 0.01	5.3 ± 0.3	19 ± 2

The reactions were performed in 50 mM Tris, 1 mM EDTA, pH 7.5, 0.5 μM heparin and [KLK13] = 3–10 nmol at 35 °C. The parameters and the standard errors were obtained from three determinations. The errors associated to k_{cat}/K_m were obtained by error propagation of the k_{cat} and K_m errors.

Table 2
Kinetic parameters for the hydrolysis by KLK13 of the series of peptides Abz-K**X**RSSKQ-EDDnp and Abz-**X**LRSSKQ-EDDnp in the presence of heparin (*i.e.* evaluating the specificity of S_2 and S_3 subsites, respectively).

X	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m $(Mm\ s)^{-1}$
Abz-K**X**RSSKQ-EDDnp (P_2)			
R[#]	5.60 ± 0.03	1.74 ± 0.03	3218 ± 58
	(0.50 ± 0.05)	(0.74 ± 0.02)	(676 ± 70)
I	8.84 ± 0.04	2.96 ± 0.05	3027 ± 53
K	8.96 ± 0.05	4.74 ± 0.03	1890 ± 16
L(ref)	3.22 ± 0.02	2.28 ± 0.03	1412 ± 21
P	7.56 ± 0.07	5.23 ± 0.01	1445 ± 14
V	6.05 ± 0.04	5.05 ± 0.05	1200 ± 12
F	7.04 ± 0.05	7.04 ± 0.06	1000 ± 11
E	–	–	860*±10
Y	6.9 ± 0.1	10.7 ± 0.1	635 ± 11
H	2.9 ± 0.1	5.7 ± 0.1	509 ± 20
M	–	–	531*±12
T	4.9 ± 0.08	8.9 ± 0.02	550 ± 9
S	5.7 ± 0.06	11.0 ± 0.07	518 ± 10
A	4.0 ± 0.03	8.5 ± 0.02	469 ± 4
Q	1.6 ± 0.07	6.0 ± 0.04	267 ± 12
G	2.0 ± 0.06	12.7 ± 0.02	161 ± 5
N	1.1 ± 0.1	9.9 ± 0.1	111 ± 10
Abz–**X**LRSSKQ-EDDnp (P_3)			
R	6.45 ± 0.05	3.92 ± 0.04	1135 ± 17
K(ref)	3.20 ± 0.02	2.25 ± 0.03	1422 ± 20
I	10.6 ± 0.1	7.80 ± 0.02	1359 ± 13
F	7.24 ± 0.05	6.9 ± 0.1	1049 ± 17
V	–	–	800*±25
H	6.32 ± 0.03	8.0 ± 0.1	790 ± 11
A	6.06 ± 0.04	10.3 ± 0.1	588 ± 7
Q	5.51 ± 0.01	10.40 ± 0.06	529 ± 3
N	5.03 ± 0.03	14.6 ± 0.1	344 ± 3
L	3.86 ± 0.03	12.6 ± 0.1	306 ± 3
E	–	NH	–

The reactions were performed in 50 mM Tris, 1 mM EDTA, pH 7.5, 0.5 μM heparin and [KLK13] = 3–10 nmol at 35 °C. The values in parenthesis were obtained in the presence of 1 M sodium citrate. Reference peptide Abz-KLRSSKQ-EDDnp (ref). *These k_{cat}/K_m values were obtained in pseudo-first order conditions because the K_m values were too high and impaired the kinetic parameter determination due to inner filter of fluorescence. [#]Two cleavages was observed with Abz-KR*R↓SSKQ-EDDnp, the cleavage indicated by ↓ was 80% and the cleavage indicated by * was 20%. NH = no hydrolysis. The parameters and the standard errors were obtained from three determinations. The errors associated to k_{cat}/K_m were obtained by error propagation of the k_{cat} and K_m errors.

peptide Abz-GPVKKRNMQ-EDDnp as indicated in Table 4. This finding and the detection of KLK13 in neurons and glial cells [8] prompted us to assay commercially available bovine myelin basic protein as a substrate for KLK13. Fig. 3 shows the SDS-PAGE of the highly digested myelin basic protein and at least two cleavage sites were indentified, namely …YGGR↓ASDYK… and …PRHR↓DTGI…

3.4. Hydrolysis of histatin 3 by KLK13

Histatin 3 was synthesized Abz at the N-terminal end in order to increase the detection of the peptide and its products of hydrolysis by HPLC analysis with fluorescence detector (Abz-DSHAKRHH-GYKRKFHEKHHSHRGYR^{25}SNYLYDN). The time course of its hydrolysis by KLK13 in the presence of 0.5 μM heparin is shown in Fig. 4 with the HPLC profiles of the reaction mixture sampled at different times. The hydrolysis of histatin 3 occurred only at the R^{25}–S peptide bond, with a velocity = 35 nmol/min/nmol of enzyme. It is noteworthy that even after 24 h of reaction only the R^{25}–S peptide bond was cleaved, which indicates a remarkable specificity of KLK13 for this segment of histatin 3. Similar results were obtained by the activation of KLK13 with 1 M sodium citrate (data not shown).

Table 3
Kinetic parameters for the hydrolysis by KLK13 of the series of peptides Abz-KLR**X**SKQ-EDDnp and Abz-KLRS**X**KQ-EDDnp in the presence of heparin (*i.e.* evaluating the specificity of S_1' and S_2' subsites, respectively).

X	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$
Abz-KLR**X**SKQ-EDDnp (P_1')			
K	26.6 ± 0.1	3.10 ± 0.02	8581 ± 64
	(0.55 ± 0.01)	(0.93 ± 0.01)	(591 ± 12)
N	11.8 ± 0.1	5.33 ± 0.03	2224 ± 23
F	4.1 ± 0.06	2.40 ± 0.01	1708 ± 26
S(ref)	3.21 ± 0.02	2.24 ± 0.03	1433 ± 21
H	4.24 ± 0.05	3.00 ± 0.06	14,013 ± 33
Q	4.8 ± 0.1	7.9 ± 0.1	607 ± 15
A	2.46 ± 0.01	4.06 ± 0.05	606 ± 8
I	0.60 ± 0.01	1.35 ± 0.02	444 ± 10
V	1.52 ± 0.03	3.60 ± 0.02	422 ± 9
L	1.20 ± 0.03	3.81 ± 0.01	314 ± 8
G	2.05 ± 0.06	10.20 ± 0.02	200 ± 6
R	0.34 ± 0.02	5.9 ± 0.1	58 ± 10
	(3.07 ± 0.05)	(1.54 ± 0.03)	(2000 ± 51)
E	–	NH	–
Abz-KLRS**X**KQ-EDDnp (P_2')			
R	8.9 ± 0.1	1.03 ± 0.01	8640 ± 128
L	29.5 ± 0.1	7.60 ± 0.03	3881 ± 20
F	14.8 ± 0.1	4.02 ± 0.03	3681 ± 37
Q	17.0 ± 0.1	8.30 ± 0.04	2048 ± 16
A	6.9 ± 0.05	3.83 ± 0.02	1816 ± 16
S(ref)	3.2 ± 0.02	2.20 ± 0.03	1454 ± 22
V	6.9 ± 0.03	7.02 ± 0.05	982 ± 8
H	2.9 ± 0.07	3.45 ± 0.03	840 ± 24
N	4.5 ± 0.07	6.00 ± 0.05	750 ± 13
G	3.3 ± 0.02	5.34 ± 0.07	618 ± 9
P	0.7 ± 0.01	6.42 ± 0.02	109 ± 9
I	0.5 ± 0.02	5.54 ± 0.02	91 ± 3
E	–	NH	–

The reaction conditions were as in Table 2 and NH = no hydrolysis. The values in parenthesis were obtained in the presence of 1 M sodium citrate.

3.5. Kininogenase activity of KLK13

Low molecular weight human kininogen (LK) incubated with KLK13 released a low amount of kinin compared with the activity of KLK1. This low kininogenase activity of KLK13 was dependent on the presence of sodium citrate as shown in Fig. 5. We also examined the KLK13 hydrolysis time course of the human kininogen fragment Abz-MISLMKRPPGFSPFRSSRI-NH_2 which was rapidly cleaved at the R–S bond with release of Abz-MISLMKRPPGFSPFR-OH, and this fragment was subsequently slowly hydrolyzed at the K-R bond with low yield of bradykinin (Fig. 6). These results were confirmed by KLK13 hydrolysis in the presence of 1 M sodium citrate of the peptide Abz-GFSPFR↓SSRIQ-EDDnp ($k_{cat} = 0.3\ s^{-1}$, $K_m = 0.6$ μM and $k_{cat}/K_m = 500\ mM^{-1}.s^{-1}$) and by the very slow hydrolysis of the peptide Abz-MISLMK↓RPQ-EDDnp.

4. Discussion

The hydrolytic activity of KLK13 is substantially activated by the kosmotropic salts sodium citrate and sodium sulfate, and in their absence the KLK13 activity was very low with most of the assayed peptide substrates. Although this observation may be puzzling, the also high activities of KLKs 3 and 6 in the presence of sodium citrate suggest that this KLK may be effectively modulated by *in vivo* microenvironment concentrations of ions. The macromolecular crowding concept seems to be adequate to interpret the activation of the KLKs 3, 6 and 13 by high sodium citrate concentrations. The macromolecular crowding obtained by high salt concentration was reported to compact KLK3 [35] with reduction of surface-to-volume ratio,

Table 4
Sequences and hydrolysis of FRET peptides obtained from support-bound peptide library.

Sequences	Velocity of Hydrolysis	Human protein containing the peptide sequence (with database access)
Abz-KLR↓SSKQ-EDDnp	130	Reference peptide
Abz-HK*TR↓SEAQ-EDDnp	120	c114 SLIT-like testicular protein isoform (XP_002344102.1)
Abz-GPKQR↓SR*RQ-EDDnp	50	Bromodomain adjacent to zinc finger domain protein 1A (Q9NRL2 (BAZ1A_human)
Abz-EVK*R↓KTYQ-EDDnp	50	Histone-lysine N-methyltransferase (Q8IZD2 MLL5_human)
Abz-RQIR↓KNEQ-EDDnp	40	Ankyrin repeat domain-containing protein (Q8N7Z5 ANR31_human)
Abz-GPNLR↓ARQQ-EDDnp	30	1) Sperm-associated antigen 17 (Q6Q759.1|SPG17_human) 2) Cancer/testis antigen 75 (Q6PK30.2|CT75_human)
Abz-GPKLYRR↓IQ-EDDnp	20	Not found
Abz-TMVK*QAR↓KQ-EDDnp	20	Neutrophil cytosolic factor 4 (Q15080 NCF4_human)
Abz-GPVK*KR↓NMQ-EDDnp	20	1) Spermatogenesis-associated protein (Q8NHS9|SPT22_human) 2) Myelin basic protein (MBP) (sp|P02686|MBP_human)
Abz-AMER*AR↓MQ-EDDnp	20	Sarcoma antigen NY-SAR-22 (Q86WF6_ human)
Abz-GPRMYR↓KLQ-EDDnp	15	IQ motif containing GTPase activating protein (Q59HA3_ human)
Abz-GPR↓VGGRVQ-EDDnp	15	Testis-specific Y-encoded-like protein (Q86VY4.2|TSYL5_human)
Abz-NKR↓HPNAQ-EDDnp	15	Dual specificity testis-specific protein kinase 1 (Q15569 TESK1_human)
Abz-GPVAK*NR↓SQ-EDDnp	15	1) Testis-expressed sequence 2 protein (Q8IWB9|TEX2_human) 2) Spermatogenic leucine zipper protein 1 (Q9BXG8|SPZ1_human)
Abz-HPR↓AMR*RQ-EDDnp	10	1) T-complex-associated testis-expressed protein 1 (Q5JU00.1_human) 2) Cancer/testis antigen 42 (Q9BXT5.1|TEX15_human)
Abz-GPFAYQR↓HQ-EDDnp	10	Not found
Abz-GPR↓QVK*KFQ-EDDnp	10	Glutamate receptor 4 precursor (P48058|GRIA4_human)
Abz-GPR↓SK*EIQ-EDDnp	5	Not found
Abz-NWR*ER↓SLQ-EDDnp	5	Not found
Abz-GPK↓K↓YSIAQ-EDDnp	5	Cancer/testis antigen 42 (Q9BXT5.1|TEX15_human)
Abz-EPHMK↓HQ-EDDnp	5	Neuropeptide Y receptor type (Q15761.1|NPY5R_human)

Conditions of hydrolysis: 50 mM Tris, pH 7.5, 35 °C, [KLK13] = 8.7 nM and a fixed concentration of 10 μM of each peptide. ↓ indicates the preferred cleavage site (at least 90%) and * indicates the a secondary cleavage site. The only exception was the peptide Abz-GPK↓K↓YSIAQ-EDDnp where both cleavage sites were equally hydrolyzed. Velocity unit = pmol/min × nmol E.

which results in an active conformation of the enzyme; for review and concept explanation of macromolecular crowding see [53]. Sodium citrate is present in normal prostate tissue and seminal fluid in high concentration [54,55,56], and it can be a potential physiological modulator of KLK13 activity since it is also present in prostate, testis and in female reproductive organs [10]. In addition, KLK13 was reported to activate KLK2 and KLK3 by cleaving the R–I bond in the common sequence …IQSR↓IVGG… [57], that is in accordance to our observed KLK13 specificity, and then sodium citrate can also modulate the activation cascade these kallikreins in prostate. The hydrolysis of histatin 3 exclusively at R^{25} (Fig. 4) by KLK13 activated by heparin seems to be a relevant observation due to the presence of high concentration chondroitin sulfate in tonsils [58] and in saliva [59] that also activates KLK13 (data not showed).

The S_1 subsite of KLK13 presented strict specificity for the basic amino acids R and K, with large preference for R compared to K as shown by the k_{cat}/K_m value for the cleavage of Abz-KLR↓SSKQ-EDDnp that was two orders of magnitude higher than that of Abz-KLK↓SSKQ-EDDnp. These observations are in agreement with the screening of the support-bound peptide library that selected only peptides hydrolyzed at R or K residues (Table 4). The subsites S_2, S_3, S_1' and S_2' of KLK13 also accepted basic amino acids as indicated from kinetic parameters of hydrolysis of the FRET peptides series derived from Abz-KLRSSKQ-EDDnp. The efficient hydrolysis of the

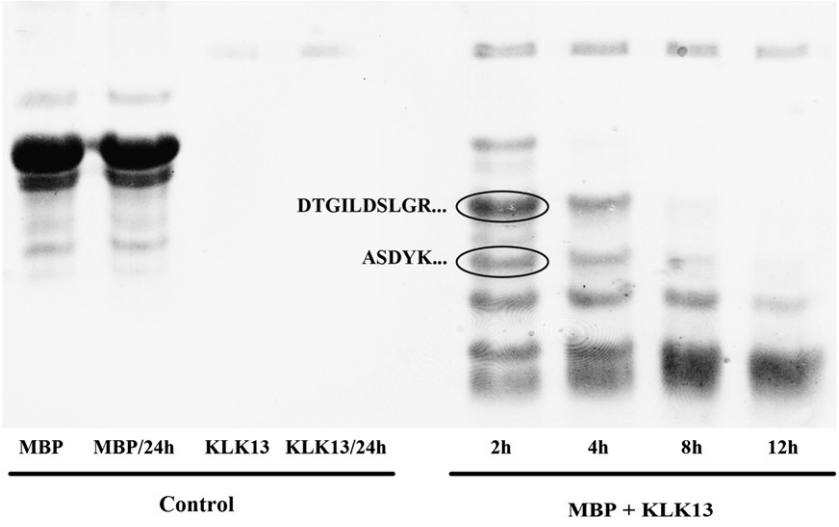

Fig. 3. SDS-PAGE of myelin basic protein digested by KLK13 in 18% polyacrylamide gel, developed by Coomassie Blue. The hydrolysis was performed in 50 mM Tris, 1 mM EDTA, 0.5 μM heparin, pH 7.5 at 35 °C. At least two cleavage sites were indentified: …YGGR↓ASDYK… and …PRHR↓DTGI… by N-terminal sequencing using Edman degradation.

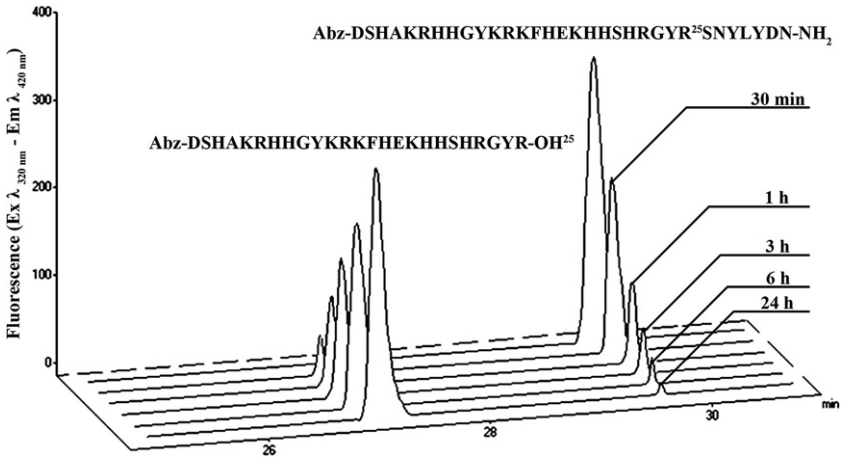

Fig. 4. Hydrolysis of histatin 3 by KLK13 in the presence of heparin. Reaction conditions 50 mM Tris, 1 mM EDTA pH 7.5 [E] = 13 nmol with 0.5 μM heparin. The substrate is 38 μM Abz labeled histatin 3 (Abz-DSHAKRHHGYKRKFHEKHHSHRGYR^{25}↓SNYLYDN-NH_2). The products were identified by mass spectrometry.

peptides with basic amino acids at P_2 position suggested that the commercial peptide Z-RR-MCA would be a convenient substrate for KLK13, however this peptide was cleaved at the R—R bond instead of at the R-MCA bond as detected by HPLC analysis of the hydrolysis products. This result suggests that for a short peptide constituted of a pair of R flanked by hydrophobic groups put the R residues at S_1 and S_1' subsites of KLK13.

The identification of eight substrates of KLK13 in the screening of the support-bound peptide library with sequences that are present in proteins from testis appears to be a significant observation because only in testis were found five splice variants in addition to the normal KLK13. In testicular cancer only normal KLK13 is expressed, while KLK13 variants are expressed only in normal testis [28,29]. At the moment it would be very speculative to correlate possible physiological or pathological roles of the hydrolysis by KLK13 of the testis proteins indicated in Table 4, however this finding merits further investigation. The identification in the support-bound peptide library of a peptide with sequence of myelin basic protein as a potential substrate of KLK13 was confirmed by the efficient hydrolysis of bovine equivalent protein (Fig. 3). It is possible that KLK13 plays a role in the cleavage of myelin basic protein because this kallikrein was found in neurons and glial cells by immunohistochemical technique [8]. In addition, glycosaminoglycans that activate KLK13 are associated with central nervous system remodeling, development, and disease (for reviews see [60,61]).

The co-localization of KLK13 and its activators in different tissues reported above incite questions about how the *in vitro* conditions used in the present work would mimic physiological or pathological conditions *in vivo*. Our data spanned a large range of concentration of the activators that possibly included those found in the normal or pathological tissues.

The kininogenase activity of KLK13 on human LMW kininogen is very poor compared with that of KLK1, although KLK13 was able to digest it. As shown in the hydrolysis of the human kinogen fragment peptide Abz-MISLMKRPPGFSPFRSSRI-NH_2 (Fig. 6) the low kininogenase activity is due to the resistance of hydrolysis of the K—R bond in the N-terminal side of the bradykinin sequence in the kininogen.

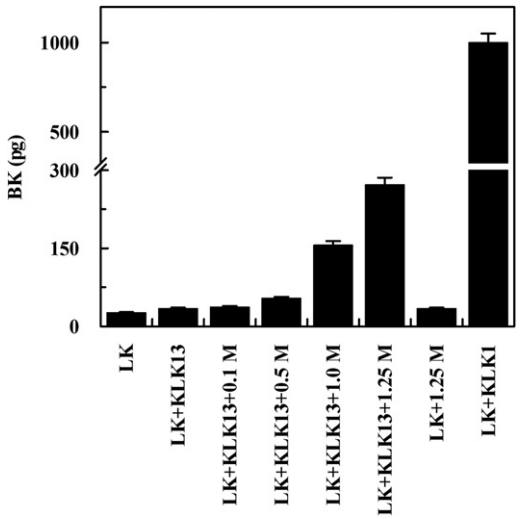

Fig. 5. Kininogenase activity of KLK13 towards human LMW kininogen in the presence of different concentrations of sodium citrate. Stoichiometric amounts (16 pmol) of KLK13 and kininogen were used in the absence or presence of citrate ions (0.1—1.25 M). The buffer system used was 50 mM Tris, pH 7.5 containing 1 mM EDTA. The reaction mixtures, in a final volume of 80 μL, were incubated for 4 h at 35 °C.

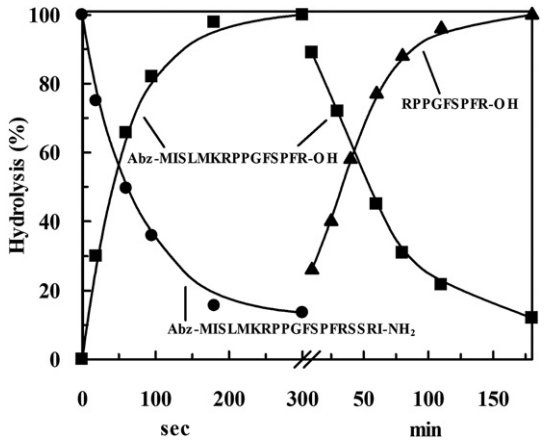

Fig. 6. Time course of hydrolysis by KLK13 of the human kininogen fragment Abz-MISLMKRPPGFSPFRSSRI-NH_2. The substrate and their products of hydrolysis were quantified by HPLC and are shown as percentage of product formation or substrate degradation. The products were identified by mass spectrometry.

5. Conclusions

KLK13 cleaved peptide substrates only at the carboxyl side of basic residues and with a large preference for R compared to K; in contrast the S_3, S_2, $S_1'$and S_2' subsites do not present strict specificity but accept basic amino acids. The high activation of KLK13 by sodium citrate that is present in normal prostate tissue and seminal fluid in high concentration, the presence of KLK13 in the male and female reproductive organs and the presence of proteins that are potential substrates of KLK13 substrates in testis suggest that KLK13 plays roles in the physiology of these organs that could be modulated by sodium citrate. Finally, besides the hydrolysis of myelin basic protein our data strongly suggest that the KLK13 expressed in salivary glands and/or tonsils is involved in the initial cascade of histatin 3 processing and can be involved in the defense of the upper digestive apparatus.

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References

[1] G.M. Yousef, L.Y. Luo, S.W. Scherer, G. Sotiropoulou, E.P. Diamandis, Molecular characterization of Zyme/Protease M/Neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease, Genomics 62 (1999) 251–259.
[2] C.A. Borgoño, E.P. Diamandis, The emerging roles of human tissue kallikreins in cancer, Nat. Rev. . Cancer 4 (2004) 876–890.
[3] T.J. Harvey, J.D. Hooper, S.A. Myers, S.A. Stephenson, L.K. Ashworth, J.A. Clements, Tissue-specific expression patterns and fine mapping of the human kallikrein (KLK) locus on proximal 19q13.4, J. Biol. Chem. 275 (2000) 35397–35406.
[4] G.M. Yousef, E.P. Diamandis, The new human tissue kallikrein gene family: structure, function and association to disease, Endocr. Rev. 22 (2001) 184–204.
[5] C.A. Borgoño, I.P. Michael, E.P. Diamandis, Human tissue kallikreins: physiologic roles and applications in cancer, Mol. Cancer Res. 2 (2004) 257–280.
[6] K. Oikonomopoulou, E.P. Diamandis, M.D. Hollenberg, Kallikrein-related peptidases: proteolysis and signaling in cancer, the new frontier, Biol. Chem. 391 (2010) 299–310.
[7] A. Lundwall, M. Brattsand, Kallikrein-related peptidases, Cell Mol. Life Sci. 65 (2008) 2019–2038.
[8] C.D. Petraki, V.N. Karavana, E.P. Diamandis, Human kallikrein 13 expression in normal tissues: an immunohistochemical study, J. Histochem. Cytochem. 51 (2003) 493–501.
[9] C.D. Petraki, P.A. Papanastasiou, V.N. Karavana, E.P. Diamandis, Cellular distribution of human tissue kallikreins: immunohistochemical localization, Biol. Chem. 387 (2006) 653–663.
[10] J.L.V. Shaw, E.P. Diamandis, Distribution of 15 human kallikreins in tissues and biological fluids, Clin. Chem. 53 (2007) 1423–1432.
[11] K.D. Bhoola, C.D. Figueroa, K. Worthy, Bioregulation of kinins–kallikreins, kininogens, and kininases, Pharmcol. Rev. 44 (1922) 1–80.
[12] H. Lilja, A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein, J. Clin. Invest. 76 (1985) 1899–1903.
[13] J. Malm, J. Hellman, P. Hogg, H. Lilja, Enzymatic action of prostate-specific antigen (PSA or hK3): substrate specificity and regulation by $Zn^{(2+)}$, a tight-binding inhibitor, Prostate 45 (2000) 132–139.
[14] T.K. Takayama, B.A. McMullen, P.S. Nelson, M. Matsumura, K. Fujikawa, Characterization of hK4 (Prostase), a prostate-specific serine protease: activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase, Biochemistry 40 (2001) 15341–15348.
[15] M. Matsumura, A.S. Bhatt, D. Andress, N. Clegg, T.K. Takayama, C.S. Craik, P.S. Nelson, Substrates of the prostate-specific serine protease Prostase/KLK4 defined by positional-scanning peptide libraries, Prostate 62 (2005) 1–13.
[16] M. Debela, V. Magdolen, V. Grimminger, C. Sommerhoff, A. Messerschimidt, R. Huber, R. Friedrich, W. Bode, P. Goettig, Crystal structures of human tissue kallikrein 4: activity modulation by a specific zinc binding site, J. Mol. Biol. 362 (2006) 1094–1107.
[17] Y. Lu, P. Papagerakis, Y. Yamakoshi, J.C.-C. Hu, J.D. Bartlett, J.P. Simmer, Functions of KLK4 and MMP-20 in dental enamel formation, Biol. Chem. 389 (2008) 695–700.
[18] P.F. Ângelo, A.R. Lima, F.M. Alves, S.I. Blaber, I.A. Scarisbrick, M. Blaber, L. Juliano, M.A. Juliano, Substrate specificity of human kallikrein 6: salt and glycosaminoglycan activation effects, J. Biol. Chem. 281 (2006) 3116–3126.
[19] I.A. Scarisbrick, S.I. Blaber, J.T. Tingling, M. Rodriguez, M. Blaber, G.P. Christophi, Potential scope of action of tissue kallikreins in CNS immune-mediated disease, J. Neuroimmunol. 178 (2006) 67–176.
[20] A.G. Vandell, N. Larson, G. Laxmikanthan, M. Panos, S.I. Blaber, M. Blaber, I.A. Scarisbrick, Protease-activated receptor dependent and independent signaling by kallikreins 1 and 6 in CNS neuron and astroglial cell lines, J. Neurochem. 107 (2008) 855–870.
[21] K. Oikonomopoulou, K.K. Hansen, M. Saifeddine, I. Tea, M. Blaber, S.I. Blaber, I. Scarisbrick, P. Andrade-Gordon, G.S. Cottrell, N.W. Bunnett, E.P. Diamandis, M.D. Hollenberg, Proteinase-activated receptors, targets for kallikrein signaling, J. Biol. Chem. 281 (2006) 32095–32112.
[22] M.J. Bernett, S.I. Blaber, I.A. Scarisbrick, P. Dhanarajan, S.M. Thompson, M. Blaber, Crystal structure and biochemical characterization of human kallirein 6 reveals that a trypsin-like kallikrein is expressed in the central nervous system, J. Biol. Chem. 277 (2002) 24562–24570.
[23] G. Sotiropoulou, G. Pampalakis, E.P. Diamandis, Functional roles of human kallikrein-related peptidases, J. Biol. Chem. 284 (2009) 32989–32994.
[24] A. Eissa, V. Amodeo, C.R. Smith, E.P. Diamandis, Kallikrein-related peptidase-8 (KLK8) is an active serine protease in human epidermis and sweat and is involved in a skin barrier proteolytic cascade, J. Biol. Chem. 286 (2011) 687–706.
[25] N. Komatsu, K. Saijoh, T. Toyama, R. Ohka, N. Otsuki, G. Hussack, K. Takehara, E.P. Diamandis, Multiple tissue kallikrein mRNA and protein expression in normal skin and skin diseases, Br. J. Dermatol. 153 (2005) 274–281.
[26] A. Scolaris, C.A. Borgoño, N. Harbeck, J. Dorn, B. Schmalfelt, M. Schimitt, E.P. Diamandis, Human kallikrein 13 protein in ovarian cancer cytosols: a new favorable prognostic marker, J. Clin. Oncol. 22 (2004) 678–685.
[27] M.R. Darling, L. Jakson-Boeters, T.D. Daley, E.P. Diamandis, Human kallikrein 13 expresssion in salivary gland tumors, Int. J. Biol. Markers 21 (2006) 106–110.
[28] A. Chang, G.M. Yousef, K. Jung, E. Rajpert-De Meyts, E.P. Diamandis, Identification and molecular characterization of five novel kallikrein gene 13 (KLK13; KLK-L4) splice variants: differential expression in testicular cancer, Anticancer Res. 21 (2001) 3147–3152.
[29] L.-Y. Luo, G. Yousef, E.P. Diamandis, Human tissue kallikreins and testicular cancer, Acta Pathol. Microbiol. Immunol. Scand. 111 (2003) 225–233.
[30] C. Kapadia, M.C. Ghosh, L. Grass, E.P. Diamandis, Human kallikrein 13 involvement in extracellular matrix degradation, Biochem. Biophys. Res. Comm. 323 (2004) 1084–1090.
[31] C.A. Borgoño, J.A. Gavigan, J. Alves, B. Bowles, J.L. Harris, G. Sotiropoulou, E.P. Diamandis, Defining the extended substrate specificity of kallikrein 1-related peptidases, Biol. Chem. 388 (2007) 1215–1225.
[32] I. Schecheter, A. Berger, On the size of the active site in proteases. I. Papain, Biochem. Biophys. Res. Comm. 27 (1967) 157–162.
[33] J.L. Harris, B.J. Backes, F. Leonetti, S. Mahrus, J.A. Ellman, C.S. Craik, Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries, Proc. Natl. Acad. Sci. U S A 97 (2000) 7754–7759.
[34] B.J. Backes, J.L. Harris, F. Leonetti, C.S. Craik, J.A. Ellman, Synthesis of positional-scanning libraries of fluorogenic peptide substrates to define the extended substrate specificity of plasmin and thrombin, Nat. Biotechnol. 18 (2000) 187–193.
[35] X. Huang, C.T. Knoell, G. Frey, M. Hazegh-Azam, A.H. Tashjian, L. Hedstrom, R.H. Abeles, S.N. Timasheff, Modulation of recombinant human prostate-specific antigen: activation by Hofmeister salts and inhibition by azapeptides. Appendix: thermodynamic interpretation of the activation by concentrated salts, Biochemistry 40 (2001) 11734–11741.
[36] D. Andrade, D.M. Assis, A.R. Lima, J.R. Oliveira, M.S. Araujo, S.I. Blaber, M. Blaber, M.,A. Juliano, L. Juliano, Substrate specificity and inhibition of human kallikrein-related peptidase 3 (KLK3 or PSA) activated with sodium citrate and glycosaminoglycans, Arch. Biochem. Biophys. 498 (2010) 74–82.
[37] E. Del Nery, J.R. Chagas, M.A. Juliano, E.S. Prado, L. Juliano, Evaluation of the extent of the binding site in human tissue kallikrein by synthetic substrates with sequences of human kininogen fragments, Biochem. J. 312 (1995) 233–238.
[38] L.M. Sabatini, T. Ota, E.A. Azen, Nucleotide sequence analysis of the human salivary protein genes HIS1 and HIS2, and evolution of the STATH/HIS gene family, Mol. Biol. Evol. 10 (1993) 497–511.
[39] H. Tsai, L.A. Bobek, Human salivary histatins: promising anti-fungal therapeutic agents, Crit. Rev. Oral Biol. Med. 9 (1998) 480–497.
[40] E.J. Helmerhorst, R.F. Troxler, F.G. Oppenheim, The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species, Proc. Natl. Acad. Sci. U S A 98 (2001) 14637–14642.
[41] A.L. Ruissen, J. Groenink, E.J. Helmerhorst, E. Walgreen-Weterings, W. Van't Hof, E.C. Veerman, A.V. Nieuw Amerongen, Effects of histatin 5 and derived peptides on Candida albicans, Biochem. J. 356 (2001) 361–368.
[42] M. Castagnola, R. Inzitari, D.V. Rossetti, C. Olmi, T. Cabras, V. Piras, P. Nicolussi, M.T. Sanna, M. Pellegrini, B. Giardina, I. Messana, A cascade of 24 histatins (histatin 3 fragments) in human saliva, J. Biol. Chem. 279 (2004) 41436–41443.
[43] E. Del Nery, M.A. Juliano, M. Meldal, I. Svendsen, J. Sharfstein, A.R. Walmsley, Characterization of the substrate specificity of the major cysteine protease (cruzipain) from *Trypanosoma cruzi* using a portion-mixing combinatorial library and fluorogenic peptide, L. Juliano, Biochem. J. 323 (1997) 427–433.

[44] P.M. St Hilaire, M. Willert, M.A. Juliano, L. Juliano, M. Meldal, Fluorescence-quenched solid phase combinatorial libraries in the characterization of cysteine protease substrate specificity, J. Comb. Chem. 1 (1999) 509–523.
[45] P.M. St Hilaire, L.C. Alves, S.J. Sanderson, J.C. Mottram, M.A. Juliano, L. Juliano, G.H. Coombs, M. Meldal, The substrate specificity of a recombinant cysteine protease from Leishmania mexicana: application of a combinatorial peptide library approach, Chembiochem 1 (2000) 115–122.
[46] F.M. Alves, I.Y. Hirata, I.E. Gouvea, M.F. Alves, M. Meldal, D. Brömme, L. Juliano, M.A. Juliano, Controlled peptide salvation in portion-mixing libraries of FRET peptides: inproved specificity determination for dengue 2 virus NS2B-NS3 protease and human cathepsin S, J. Comb. Chem. 9 (2007) 627–634.
[47] G.W. Jameson, D.V. Roberts, R.W. Adams, W.S.A. Kyle, D.T. Elmore, Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorimetric titration, Biochem. J. 131 (1973) 107–117.
[48] I. Hirata, M.H.C. Cezari, C.R. Nakaie, P. Boshcov, A.S. Ito, M.A. Juliano, L. Juliano, Internally quenched fluorogenic protease substrates: solid-phase synthesis and fluorescence spectroscopy of peptides containing ortho-aminobenzoyl/dinitrophenyl groups as donor-acceptor pairs, Lett. Pept. Sci. 1 (1994) 299–308.
[49] B. Korkmaz, S. Attucci, M.A. Juliano, T. Kalupov, M.L. Jourdan, L. Juliano, F. Gauthier, Measuring elastase, proteinase 3, and cathepsin G activities at the surface of human neutrophils with fluorescence energy transfersubstrates, Nat. Protoc. 3 (2008) 991–1000.
[50] M. Roice, I. Johannsen, M. Meldal, High capacity poly(ethylene glycol) based amino polymers for peptide and organic synthesis, QSAR Comb. Chem. 23 (2004) 662–673.
[51] C.P. Dietrich, I.L.S. Tersariol, L. Toma, C.T. Moraes, M.A. Porcionatto, F.W. Oliveira, H.B. Nader, Structure of heparin sulfate: identification of variable and Constant oligosaccharide domains in eight heparan sulfates of different origins, Cell Mol. Biol. 44 (1998) 417–429.
[52] K. Shimamoto, T. Ando, S. Tanaka, Y. Nakahashi, T. Nishitani, S. Hosoda, H. Ishida, O. Iimura, Na improved method for the determination of human blood kinin levels by sensitive kinin radioimmunoassay, Endocrinol. Jpn. 29 (1982) 487–494.
[53] R.J. Ellis, Macromolecular crowding: obvious but underappreciated, Trends Biochem. Sci. 26 (2001) 597–604.
[54] K.K. Singh, M.M. Desouki, R.B. Franklin, L.C. Costello, Mitochondrial aconitase and citrte metabolism in malignant and nonmalignant human prostate tissues, Mol. Cancer 5 (2006) 14–21.
[55] L.C. Costello, R.B. Franklin, P. Narayan, Citrate in the diagnosis of prostate cancer, The Prostate 38 (1999) 237–245.
[56] M.E. Mycielska, A. Patel, N. Rizaner, M.P. Mazurek, H. .Keun, A. Patel, V. Ganapathy, M.B.A. Djamgoz, Citrate transport and metabolism in mammalian cells, BioEssays 31 (2009) 10–20.
[57] H. Yoon, G. Laxmikanthan, J. Lee, S.I. Blaber, A. Rodrigues, J.M. Kogot, I.A. Scarisbrick, M. Blaber, Activation profiles and regulatory cascades of the human kallikrein-related peptidases, J. Biol. Chem. 282 (2007) 31852–31864.
[58] I.E. Triantaphyllidou, N.S. Mastronikolis, Th.A. Papadas, D.H. Vynios, HPLC identification of oversulphated chondroitin as the major glycosaminoglycan of human tonsils, Biomed. Chromatogr. (2004) 694–699.
[59] L.M. Loeb, M.G. Naffah-Mazzacoratti, M.A. Porcionatto, J.M.R. Martins, M. Kouyoumdjian, L.M. Weckx, H.B. Nader, Chondroitin sulfate and kallikrein in saliva: markers for glossodynia, Int. Immunopharmacol. 8 (2008) 1056–1058.
[60] C.E. Bandtlow, D.R. Zimmermann, Proteoglycans in the developing brain: new conceptual insights for old proteins, Physiol. Rev. 80 (2000) 1267–1290.
[61] U. Rauch, Extracellular matrix components associated with remodeling processes in brain, Cell Mol. Life Sci. 61 (2004) 2031–2045.