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Substrate specificity and inhibition of human kallikrein-related peptidase 3 (KLK3 or PSA) activated with sodium citrate and glycosaminoglycans

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ABSTRACT

We report the enzymatic properties and substrate specificity of human recombinant KLK3 in the presence of glycosaminoglycans (GAGs) and sodium citrate. This salt is highly concentrated in prostate and in its presence KLK3 had a similar hydrolytic efficiency as chymotrypsin. In contrast to the latter peptidase, KLK3 activated by sodium citrate efficiently hydrolyzed substrates containing R, H and P at the P1 position. Activated KLK3 also cleaved peptides derived from the bradykinin domain of human kininogen at the same sites as human kallikrein KLK1, but presented low kininogenase activity. Angiotensin I has several sites for hydrolysis by KLK3; however, it was cleaved only at the Y–I bond (DRVY↓IHPFHL). Sodium citrate modulated KLK3 conformation as observed by alterations to the intrinsic fluorescence of phenylalanines and tryptophans. Activated KLK3 was reversibly inhibited by Z-Pro-Prolinal and competitively inhibited by *ortho*-phenantroline. Together, these are noteworthy observations for the future design of specific non-peptide inhibitors of KLK3 and to find natural substrates.

Introduction

Human kallikrein 1-related peptidase 3 (KLK3)[2] is a serine protease belonging to the clan PA(S) and family S1A, and is best known as "prostate-specific antigen" (PSA). This protein has received particular attention due to its clinical value for the early detection of prostate cancer (for review see [1]). Substrate specificity studies of KLK3 have emphasized its chymotrypsin-like activity due to cleavage after hydrophobic amino acids Y and F in proteins such as oxidized insulin, lysozyme, recombinant interleukin-2, semenogelins and in synthetic chromogenic or fluorogenic substrates [2–6]. Phage display analysis and positional scanning libraries show that substrates are cleaved by KLK3 mainly after hydrophobic amino acids [7] but also after K and R with low efficiency [8]. The hydrolyses after Q, as observed with semenogelin as substrate [5] and in synthetic peptides derived from it [9], are activities exclusive for KLK3 and not observed in chymotrypsin.

The elucidation of KLK3 enzymatic properties has proven difficult due to its relatively low activity compared to other serine proteases, however KLK3 hydrolytic activity is increased by NaCl [10], and in the presence of kosmotropic salts such as Na_2SO_4 it is activated more than three orders of magnitude [11]. Similarly, its inhibition by α_1-antichymotrypsin [12] and azapeptides [11] are also increased in the presence of salts. It was also reported that KLK3 is activated by peptides [13] and monoclonal antibodies [14].

Six insulin-like growth factors binding proteins (IGFBPs) control the insulin-like growth factors that are involved in the growth of both normal and malignant prostate cells. The hydrolysis of IGFBP-3 and IGFBP-4 by KLK3 was reported to be significantly enhanced by heparin, but not by other glycosaminoglycans [15]. Similarly, the complex formation between KLK3 and human protein C inhibitor (PCI) is significantly increased by heparin [16]. Dermatan sulfate was detected in human uterine cervix [17] and could be an activator of KLK3 from seminal fluid.

The present work provides biochemical data related to the hydrolytic activities of KLK3 on fluorescence resonance energy transfer (FRET) peptides in the presence of sodium citrate, a kosmotropic salt similar to Na_2SO_4 and glycosaminoglycans (GAGs). We chose sodium citrate as the activation salt because normal prostate tissue and seminal fluid contain a high concentration of

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[2] *Abbreviations used:* GAGs, glycosaminoglycans; FRET, fluorescence resonance energy transfer; LMWK, low molecular weight kininogen; Q-EDDnp, glutamine-[*N*-(2,4-dinitrophenyl)-ethylenediamine]; Abz, *ortho*-aminobenzoic acid; MCA, methyl coumarin amide; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; LCMS, liquid chromatography/mass spectrometry.

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this salt [18–20], and it can be a potential physiological modulator of KLK3 activity. We also assayed KLK3 in the presence of heparin, heparin sulfate, dermatan sulfate and chondroitin sulfate. The S_1 subsite specificity of KLK3 (Schechter and Berger nomenclature [21]) in the presence of sodium citrate was investigated using a series of FRET peptides Abz-KLXSSKQ-EDDnp that were previously used to investigate the specificity of the human kallikrein-related peptidase KLK6 and human tissue kallikrein (KLK1) [22]. In Abz-KLXSSKQ-EDDnp, X represents natural amino acids, Q-EDDnp is the fluorescence acceptor and Abz is the fluorescence donor (corresponding to glutamine-[*N*-(2,4-dinitrophenyl)-ethylenediamine] and *ortho*-aminobenzoic acid, respectively). The kininogenase activity of KLK3 on human low molecular weight kininogen (LMWK), and in peptides derived from the bradykinin-containing segment of LMWK, were also examined in the presence of sodium citrate. An unexpected and efficient hydrolysis of Abz-KLP↓SSKQ-EDDnp by KLK3 prompted us to assay angiotensin I (DRVYIHPFHL) and angiotensin II (DRVYIHPF) as substrates since these peptides contain potential cleavage sites for KLK3. We also describe the reversible slow-binding inhibition of KLK3 by Z-Pro-Prolinal and a surprising competitive inhibition by *ortho*-phenantroline.

Materials and methods

Enzymes

Recombinant KLK3 was expressed in an insect/baculovirus system and purified as previously described for KLK6 [23] and the protein concentration was determined photometrically at 280 nm. Chymotrypsin was obtained from Biobras Co. (Montes Claros, Minas Gerais, Brazil) batch 0598–TR 0027.

Glycosaminoglycans

Size-defined (10 kDa) bovine lung heparin (The Upjohn Co.) was used and prepared by size exclusion column chromatography [24]; heparan sulfate (16 kDa) from bovine lung was a generous gift from Dr. P. Bianchini (Opocrin Research Laboratories, Modena, Italy); dermatan sulfate (12 kDa) and chondroitin sulfate (25 kDa) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

Peptides

FRET peptides were synthesized by the solid-phase peptide synthesis method [25,26]. The molecular mass and purity of synthesized peptides were confirmed by analytical HPLC and by MALDI-TOF using a Microflex – LT mass spectrometer (Bruker – Daltonics, Billerica, MA, USA). Stock solutions of peptides were prepared in dimethyl formamide, and the concentrations were measured spectrophotometrically using a molar extinction coefficient of 17,300 $M^{-1}cm^{-1}$ at 365 nm. MCA peptides were synthesized and purified as earlier described [27].

Kinetic measurements

Hydrolyses of FRET peptides were assayed in a Shimadzu RF-1501 spectrofluorimeter, and the conditions for KLK3 assays are indicated in the text, in the legends of the figures and tables. Chymotrypsin was assayed in 100 mM Tris HCl, 10 mM Ca^{2+}, at pH 8.0. The fluorescence changes resultants of hydrolysis were monitored continuously at λ_{ex} = 320 nm and λ_{em} = 420 nm. The KLK3 concentration ranged over 10–15 nM and was chosen in order to hydrolyze less than 5% of the added substrate. Hydrolysis rates were converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The inner-filter effect was corrected using an empirical equation as previously described [28]. When fluorogenic MCA peptides were used, the excitation and emission wavelengths were changed to λ_{ex} = 380 and λ_{em} = 460 nm, respectively. The kinetic parameters K_m and k_{cat}, and respective standard errors, were obtained through fitting the Michaelis–Menten equation using Grafit® software-version 6.0.7 (Erithacus Software, U.K.). The errors were less than 5% for any of the obtained kinetic parameters in at least triplicate analyses.

The scissile bonds of hydrolyzed FRET peptides were identified by isolation of the fragments using analytical HPLC and the molecular mass of each product was determined by MALDI-TOF as described above and also by LC/MS using LCMS-2010 equipped with the ESI-probe (Shimadzu, Japan). Analytical HPLC conditions were: Ultrasphere C18 column (5 μM, 4.6 × 250 mm) which was eluted with the solvent system A (water/TFA, 1:1000) and B (ACN/water/TFA 900:100:1) at a flow rate of 1 mL/min and a 0–80% gradient for 20 min monitored by absorbance at 220 nm.

Inhibition assays

The inhibition assays with *ortho*-phenantroline were carried out at 37 °C in 50 mM Tris/HCl, 1.5 M sodium citrate, pH 7, 8. KLK3 (10 nM) was incubate in the absence and presence of different concentrations of *ortho*-phenantroline (0.25–1.2 mM) using Abz-KLPSSKQ-EDDnp (2–35 μM) as substrate. Enzyme and inhibitor were pre-incubated by 5 min before the addition of the substrate. The kinetic analysis was performed based on Lineweaver–Burk plots for competitive inhibition.

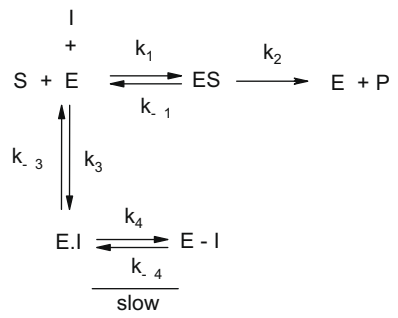
The inhibition by Z-Pro-Prolinal was done in the same conditions as described above for *ortho*-phenantroline, and Abz-KLY-SSKQ-EDDnp (9 μM) was used as substrate with 10 nM of KLK3. Substrate and inhibitor were added simultaneously. The progress curves for the inhibition of KLK3 were obtained during 40 min for each inhibitor concentration. The kinetic data were treated as reversible slow-binding inhibition as reported [29] and showed in Scheme 1.

The integrated rate Eq. (1), expressed as product concentration *vs.* time is as below

$$[P] = v_s t + \frac{(v_s - v_z)(1 - e^{-kt})}{k} + d \quad (1)$$

where, [*P*] is the product concentration formed by the hydrolysis of the FRET peptide, v_s and v_z are the velocities in steady state and at zero time, respectively, and *k* is the first-order rate constant. The values of K_i, k_4 and k_{-4} were derived from the relation of the first-order rate constant (*k*) with the inhibitor concentration [*I*] as in Eq. (2)

$$k = k_{-4} + \frac{k_4 \times [I]}{ki \times \left(1 + \frac{[S]}{K_m}\right) + [I]} \quad (2)$$



Scheme 1. Reversible slow-binding enzyme inhibition.

pH-profile of KLK3 activity

pH profiles were carried out in a constant ionic strength four component buffer comprised of 25 mM acetic acid, 25 mM Mes, 75 mM Tris and 25 mM glycine for the influence of heparin and in Tris 50 mM for sodium citrate, both adjusted with 2 M NaOH and HCl as required. The KLK3 (15 nM) activity was measured at 37 °C using the peptide Abz-KLYSSKQ-EDDnp. The experiments were performed in the peptide concentration 5 times higher than its km. The values of the velocities were fitted to theoretical curve for the bell-shaped pH-rate profiles using nonlinear regression as in Eq. (3) using the Grafit 6.0.7 software

$$V_{max} = V_{max}(limit)[1/(1 + 10^{pK_1 - pH} + 10^{pH - pK_2})] \quad (3)$$

where, V_{max} (limit) stands for the pH-independent maximum V_{max} constant and pK_1 and pK_2 are the dissociation constants of the catalytically components at acidic and basic limbs, respectively.

Heparin–Sepharose affinity chromatography and effects of sodium citrate upon KLK3 intrinsic fluorescence

KLK3 (1.9 μM) was applied on a heparin–Sepharose column (1 ml) previously equilibrated with 10 mM phosphate buffer (pH 7.3). After a washing step (10 volumes of column) with the same buffer, bound materials were eluted using a NaCl gradient (0–1 M) and monitored by measuring the fluorescence (λ_{ex} = 280 nm and λ_{em} = 330 nm).

The effects of sodium citrate upon KLK3 (2 μM) intrinsic fluorescence in 50 mM Tris, pH 7.8, was evaluated before and after additions different amounts of sodium citrate at fixed excitation wavelength (λ_{ex} = 270 nm) in Shimadzu RF-1501 spectrofluorimeter.

Radioimmunoassay of kinin released from kininogen

Stoichiometric amounts (1.6 pmol) of enzyme and low molecular weight kininogen (LMWK) were used to study the kininogenase activity in absence or presence of citrate ions (0.1–1.5 M). The buffer systems used were 50 mM Tris pH 7.5 containing 1 mM EDTA. The reaction mixtures, in a final volume of 40 μL, were incubated for 1 h at 37 °C and kinin was extracted in ethanol (four times the reaction's final volume) for 10 min at 70 °C. Solutions were freeze-dried and dissolved in 170 μL of egg albumin buffer (0.1% egg albumin in 10 mM phosphate buffer pH 7.0 containing 0.14 M NaCl, 0.1% NaN_3, 30 mM EDTA, 3 mM *ortho*-phenantroline). Triplicate aliquots (50 μL) were incubated with 100 μL of antibody anti-BK (1:80,000) and 100 μL of ^{125}I-labeled Tyr-bradykinin for 20 h at 4 °C. Four hundred microliters of 0.1% bovine γ-globulin in 10 mM phosphate buffer pH 7.0 containing 0.14 M NaCl, 0.1% NaN_3 and 800 μL of 25% PEG 6000 solution were added to the samples, which were incubated for 10 min at 4 °C. Finally, the samples were centrifuged at 3000 rpm for 20 min at 4 °C; the supernatants were removed and the pellets submitted to radiation counting [30,31].

Results

Effects of glycosaminoglycans and salts on KLK3 activity

Fig. 1 shows the effects of heparin, heparan sulfate and dermatan sulfate on the hydrolysis of the peptide Abz-KLY↓SSKQ-EDDnp by KLK3, which was exclusively cleaved at the Y–S bond. KLK3 activity was increased more than 100-fold with the addition of heparin up to 10 μM. At heparin concentrations higher than 10 μM the peptidase activity dropped and remained constant at concentrations higher than 40 μM. Heparan sulfate has a lower activation effect compared to heparin but with a similar profile. In contrast, dermatan sulfate activated KLK3 only at high concentrations and chondroitin sulfate had no effect.

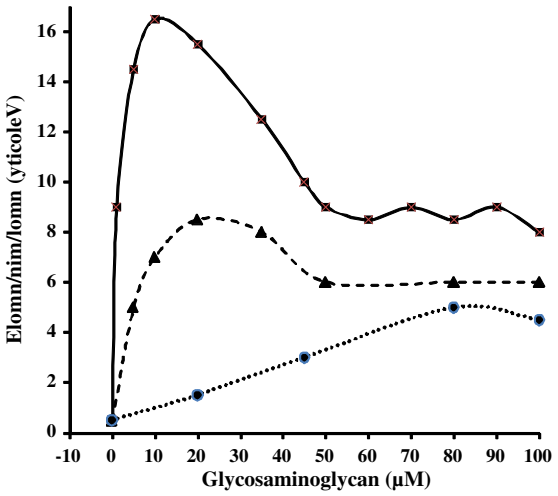


Fig. 1. Effects of the glycosaminoglycans heparin (–■–), heparan sulfate (–●–) and dermatan sulfate (–▲–) on the hydrolysis of Abz-KLYSSKQ-EDDnp by KLK3. Assays were carried out in 50 mM Tris HCl, pH 7.8, 7.5 μM substrate and [*E*] = 15 nM at 37 °C.

NaCl drastically reduced the hydrolysis of Abz-KLY↓SSKQ-EDDnp by heparin-activated KLK3 and the activity returned only at high NaCl concentrations (Fig. 2). The interaction of KLK3 with heparin was evaluated by heparin–Sepharose affinity chromatography; in which KLK3 was eluted at 0.3 M NaCl (Fig. 3). The peptides Abz-GLYSSKQ-EDDnp and Abz-GLYSSPQ-EDDnp did not bind to heparin–Sepharose and were not hydrolyzed by heparin-activated KLK3. These results indicate that the observed hydrolysis of Abz-KLY↓SSKQ-EDDnp in the presence of heparin depends on the simultaneous binding of KLK3 and the peptide to the glycosaminoglycan.

Table 1 shows the kinetic parameters for hydrolyses of Abz-KLY↓SSKQ-EDDnp, and its analogue peptides with Y substituted by other amino acids, in the presence of high molecular weight heparin. In this condition the S_1 subsite of KLK3 has preference for P_1 residues with aromatic side chains, although slow hydrolyses were observed for the peptides with P, Q, R, H and S.

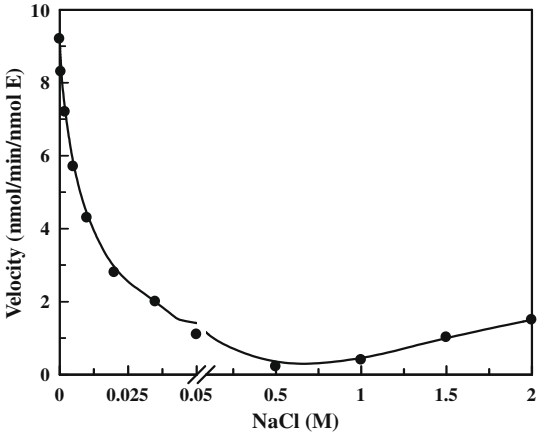


Fig. 2. Effect of NaCl on the hydrolysis of Abz-KLYSSKQ-EDDnp by KLK3 pre-activated with heparin (16 μM). Assays were carried out in 50 mM Tris HCl, 1 mM EDTA, pH 7.8, 7.5 μM substrate and [*E*] = 15 nM at 37 °C.

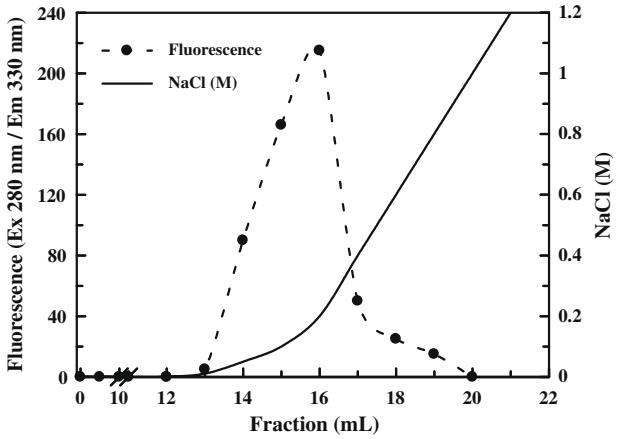


Fig. 3. Heparin–Sepharose chromatography analysis of KLK3. Elution by NaCl gradient 0–1.2 M and the fractions were monitored by tryptophan fluorescence (λ_{ex} = 280 nm/λ_{em} = 330 nm). Experimental details are described in Material and methods.

Table 1
Kinetic parameters for the hydrolysis by KLK3 of FRET peptide Abz-KLXSSKQ-EDDnp and some analogues in the presence of heparin.

	k_{cat} (min)$^{-1}$	K_m (μM)	k_{cat}/K_m (mM.s)$^{-1}$
Abz-KLY↓SSKQ-EDDnp	72 ± 6	14 ± 1	84
Abz-KLF↓SSKQ-EDDnp	7 ± 0.8	15 ± 2	8
Abz-KLL↓SSKQ-EDDnp	8 ± 0.7	30 ± 4	4
Abz-KLP↓SSKQ-EDDnp	Slow hydrolysis, less than 15% in 24 h of reaction		
Abz-KLQ↓SSKQ-EDDnp			
Abz-KLR↓SSKQ-EDDnp			
Abz-KLH↓SSKQ-EDDnp			
Abz-KLS↓SSKQ-EDDnp			

Conditions of reactions: 15 nM KLK3, 50 mM Tris HCl, 16 μM high molecular weight heparin, pH 7.8, at 37 °C.

Effects of citrate and chloride salts on KLK3 activity

The hydrolysis of Abz-KLY↓SSKQ-EDDnp by KLK3 was substantially increased by sodium citrate compared to NaCl (Fig. 4). This stimulatory effect of sodium citrate on the hydrolysis of Abz-KLY-SSKQ-EDDnp, which contains both prime and non-prime sites, contrasts with a lower difference for activation of KLK3 by these two salts for hydrolysis of the fluorogenic peptide Suc-AAPF-MCA [11], which contains only non-prime residues. The activation of KLK3 by citrate does not depend on the substrate charge (as described above for heparin) since the activation profile of KLK3 for the hydrolysis of Abz-GLY↓SSPQ-EDDnp was similar to that presented in Fig. 4 for the hydrolysis of Abz-KLY↓SSKQ-EDDnp.

pH-profile of KLK3 activity in the presence of citrate and heparin

Fig. 5 displays the pH-profile for the hydrolysis of Abz-KLY↓SSKQ-EDDnp by KLK3 activated by heparin and by sodium citrate. In both conditions the optimum pH was between 7.5 and 8.0. The acid limb of the pH-profile of KLK3 activated by sodium citrate was displaced towards lower pH values (pK_1 = 6.7) compared with the pH-profile curve in the presence of heparin (pK_1 = 7.3), while the basic limbs of the two curves were superimposed (pK_2 = 8.5). The pH-profile of the FRET peptide Abz-GLY↓SSPQ-EDDnp hydrolysis was very similar to that observed with the substrate Abz-KLY↓SSKQ-EDDnp. The positive charges of this peptide did not interfere in the pH-profile of its hydrolysis by KLK3.

S_1 subsite specificity of KLK3 in the presence of 1.5 M sodium citrate

The series of FRET peptides Abz-KLXSSKQ-EDDnp were assayed as substrates for KLK3 under conditions of maximum activity (namely, 50 mM Tris HCl, 1.5 M sodium citrate, pH 7.8, and 15 nM KLK3 and 6.5 μM FRET peptides). The products of hydrolysis of these peptides were separated by HPLC and characterized by

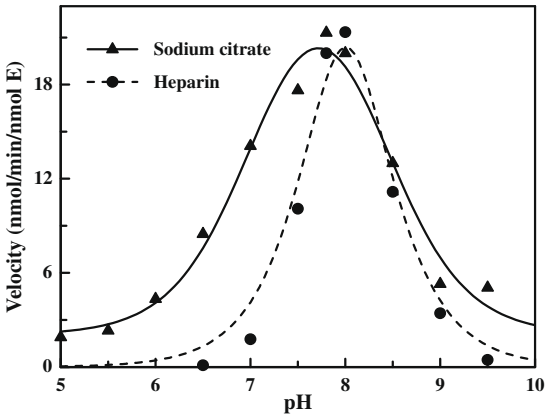


Fig. 5. pH-profile of KLK3 hydrolytic activity on Abz-KLYSSKQ-EDDnp in the presence of 16 μM heparin (–●–) and 1.5 M sodium citrate (–▲–). Experimental details are described in Material and methods.

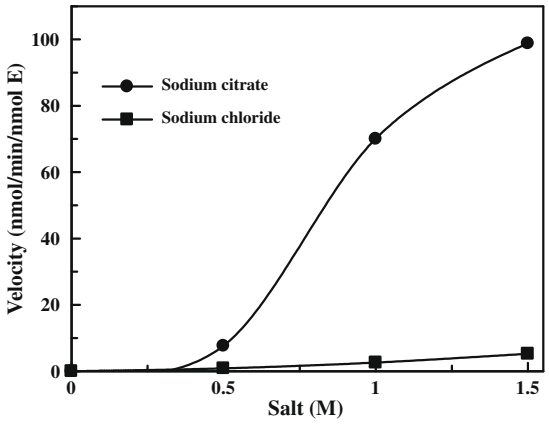


Fig. 4. Effect of NaCl and sodium citrate on the hydrolysis of Abz-KLYSSKQ-EDDnp by KLK3. Assays were carried out in 50 mM Tris HCl, pH 7.8, 7.5 μM substrate and [E] = 15 nM at 37 °C.

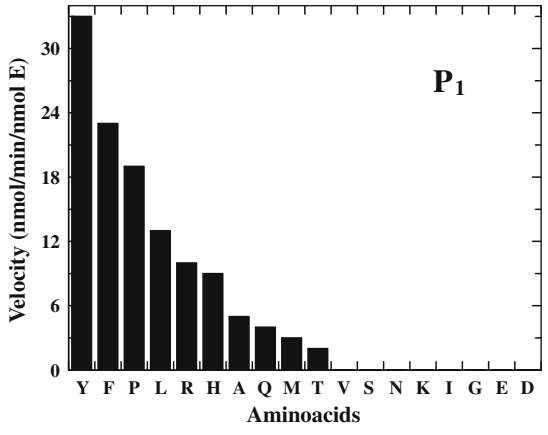


Fig. 6. S1 subsite specificity of KLK3 for the hydrolysis of the FRET peptide series Abz-KLXSSKQ-EDDnp. Assays were carried out in 50 mM Tris HCl, 1.5 M sodium citrate, pH 7.8, 7.5 μM substrate and [E] = 15 nM at 37 °C.

Table 2
Kinetic parameters for the hydrolysis by KLK3 of FRET peptides derived from Abz-KLX↓SSKQ-EDDnp in the presence of sodium citrate.

	k_{cat} $(min)^{-1}$	K_m (μM)	k_{cat}/K_m $(mM\ s)^{-1}$
Abz-KLF↓SSKQ-EDDnp	2.9 ± 0.01	0.04 ± 0.001	1250
Abz-KLY↓SSKQ-EDDnp	32 ± 2	2.7 ± 0.4	196
Abz-KLP↓SSKQ-EDDnp	22 ± 1.5	1.9 ± 0.3	195
Abz-KLR↓SSKQ-EDDnp	5.3 ± 0.1	0.97 ± 0.06	93
Abz-KLL↓SSKQ-EDDnp	2.4 ± 0.1	0.95 ± 0.01	42
Abz-KLA↓SSKQ-EDDnp	4.8 ± 0.3	2.5 ± 0.3	32
Abz-KLH↓SSKQ-EDDnp	0.56 ± 0.08	0.30 ± 0.09	30
Abz-KLQ↓SSKQ-EDDnp	0.69 ± 0.03	0.38 ± 0.06	30

Conditions of reactions: 15 nM KLK3, 50 mM Tris HCl, 1.5 M sodium citrate, pH 7.8, at 37 °C.

MALDI-TOF and electron-spray mass spectrometry. Only one cleavage at the X–S bond was observed in all assayed peptides. Fig. 6 shows the initial velocities for the hydrolysis of each peptide, whereby the most susceptible peptides were identified as X = Y, F, P, L, A, H, M, Q, T and R. The peptides containing Y and F were hydrolyzed with the highest initial velocities; however significant hydrolysis was observed in the peptides containing P and R. Table 2 shows the kinetic parameters for the hydrolysis of these peptides with variations at the P_1 position. The peptide Abz-KLF↓SSKQ-EDDnp was hydrolyzed with the highest k_{cat}/K_m value due to its very low K_m value. In contrast, the peptides Abz-KLY↓SSKQ-EDDnp and Abz-KLP↓SSKQ-EDDnp were hydrolyzed with the highest k_{cat} values. This surprising efficient hydrolysis of Abz-KLP↓SSK-Q-EDDnp at the P–S bond indicates that the S_1 subsite of KLK3 can accommodate P. This observation was confirmed by the reversible slow-binding inhibition of KLK3 by the commercially available prolyl-oligopeptidase inhibitor Z-Pro-Prolinal [32] (Fig. 7), with K_i = 40 μM and k_4 = 0.05 min^{-1} and 0.03 min^{-1}. The analysis of the kinetics of this inhibition is in accordance to two step mechanism (see Scheme 1) similar to those described for the inhibition of trypsin by leupeptin [33,34] and chymotrypsin by *N*-acyl-phenylalaninals [35]. First, it is established a weak bound complex of Z-Pro-Prolinal and KLK3 followed by slower formation of acetal bound to make the hemiacetal.

It is noteworthy that KLK3 hydrolyzed the peptides containing Q and H with very similar kinetic parameters and did not hydrolyze the peptide containing N. These results suggest that the S_1 subsite of KLK3 interacts with the N^{τ} of the imidazole group of H because the nitrogen of γ-carboxamide of glutamine (Q) is isosterically equivalent to N^{τ}-imidazole of H, while N^{π}-imidazole of H is equivalent to the nitrogen of the β-carboxamide group of asparagine (N) (nomenclature of histidine nitrogens as IUPAC-IUB recommendation [36]). Fig. 8 illustrates this concept of isosteric equivalence between the side-chains of the amino acids H, Q and N. In order to verify this concept we synthesized the peptides Abz-KL(π-methyl)HSSK-Q-EDDnp and Abz-KL(τ-methyl)HSSK-Q-EDDnp and assayed them with KLK3 under the same conditions used for the substrates described above. The peptide with (π-methyl)-H (see the structure in Fig. 8) was hydrolyzed by KLK3 with k_{cat} = 0.1 min^{-1}, whereas the peptide with (τ-methyl)-H was resistant to hydrolysis. These results confirm that N^{τ} of the imidazole ring of H interacts in the S_1 subsite of KLK3 as does the nitrogen of the γ-carboxamide group of Q.

The peptides presented in Table 2 containing F, R, H, P and Q were also assayed with chymotrypsin, which only hydrolyzed the peptide Abz-KLF↓SSLQ-EDDnp as indicated by the arrow, and the

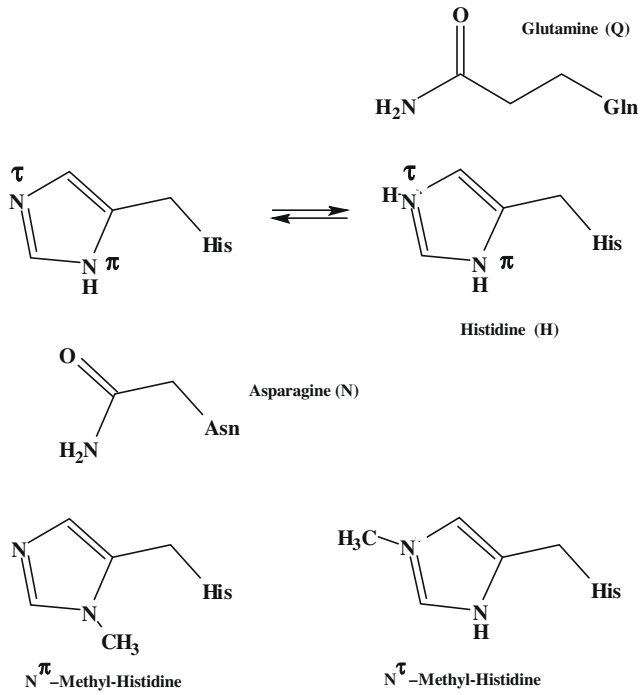


Fig. 8. Structural relationships among the side-chains of the amino acids histidine, asparagine and glutamine. The nitrogen of γ-carboxamide of glutamine (Q) is isosterically equivalent to N^{τ}-imidazole of histidine (H), while N^{π}-imidazole of histidine is equivalent to the nitrogen of the β-carboxamide group of asparagine (N). The nomenclature of histidina is as recommended in [36].

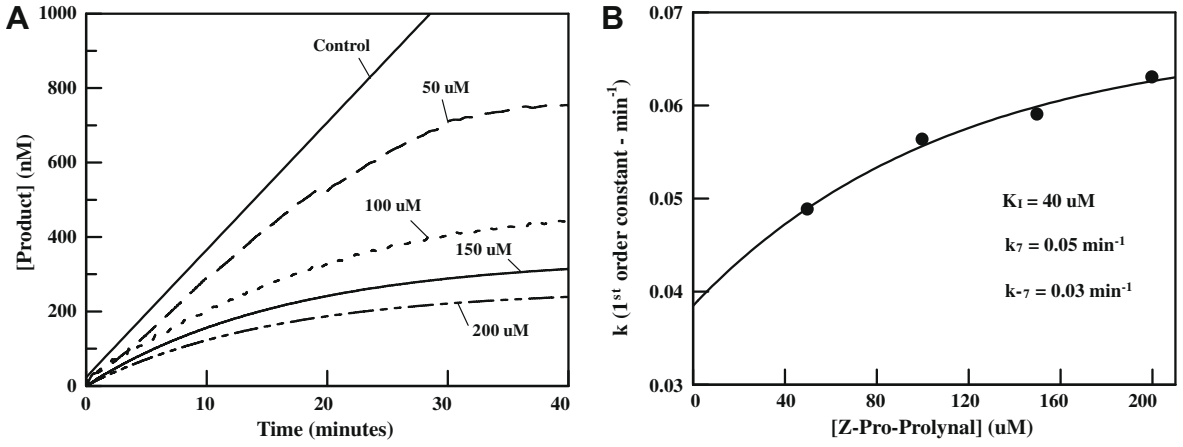


Fig. 7. Inhibition of KLK3 by Z-Pro-Prolinal. (A) Progress curve of product formation in different concentrations of inhibitor. (B) Plot of *k* (first-order rate constant) *vs.* inhibitor concentration. Experimental details are described in Material and methods.

kinetic constants were k_{cat} = 160 ± 12 min^{-1}, K_m = 2.0 ± 0.4 μM and k_{cat}/K_m = 1313 mM^{-1} s^{-1}.

We further explored the arginyl-hydrolase activity of KLK3 by assaying four series of FRET peptides derived from Abz-KLR↓SSKQ-EDDnp, where R at the P_1 position was fixed and K(P_3), L(P_2), S(P_1') and S(P_2') were substituted by natural amino acids. Fig. 9 presents the initial velocities for hydrolysis of all the series under the same conditions as those used for the analysis of S_1 subsite specificity (Fig. 6) in the presence of 1.5 M sodium citrate. All the FRET peptides were hydrolyzed only at R–S or R–X peptide bonds; therefore, all these results are related to specificity of subsites S_3, S_2, S_1' and P_2' with R at the P_1 position of the substrates. Under these conditions, the S_2 subsite prefers hydrophobic amino acids although it also accepts basic hydrophilic amino acids; while the S_3 subsite presented lesser selectivity. The S_1' presented a preference for hydrophilic amino acids, particularly for R; and the S_2' subsite prefers hydrophobic residues. Noteworthy, these results indicate that it is possible to optimize the argynyl-hydrolase activity of KLK3 by selecting different amino acids at P_3, P_2, P_1' and P_2' positions of the substrate.

We further examined the activity of KLK3 in 1.5 M sodium citrate towards commercial fluorogenic substrates Z-FR-MCA and Z-RR-MCA and observed that KLK3 was not able to release AMC with either of them; however, the HPLC analysis of the reaction mixture of Z-RR-MCA with KLK3 confirmed the hydrolysis at the R–R bond (Fig. 10) while no hydrolysis was detected with Z-FR-MCA.

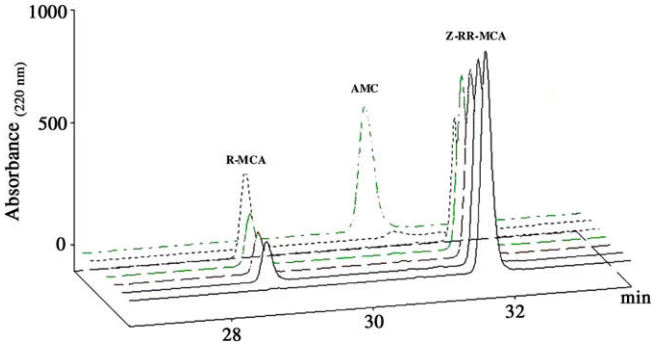


Fig. 10. HPLC chromatogram of the reaction mixture of Z-RR-MCA hydrolysis by KLK3. Assays were carried out in 50 mM Tris HCl, pH 7.8, 1.5 M sodium citrate, and 7.5 μM substrate and [*E*] = 15 nM at 37 °C. HPLC conditions are as described in Material and methods.

KLK3 activity on LMWK and angiotensins

KLK3 activated by 1.5 M sodium citrate released kinin from human LMWK as shown in Fig. 11, but this kininogenase activity is 10^3-fold lower than that of human kallkrein 1 (KLK1). The kinin released from human LMWK was detected by radioimmunoassay and probably the kinin is Lys-bradykinin (Lys-Bk) because KLK3 releases this kinin from the human LMWK bradykinin-containing segment Abz-MISLMKRPPGFSPFRSSRI-NH_2, as shown in Fig. 12.

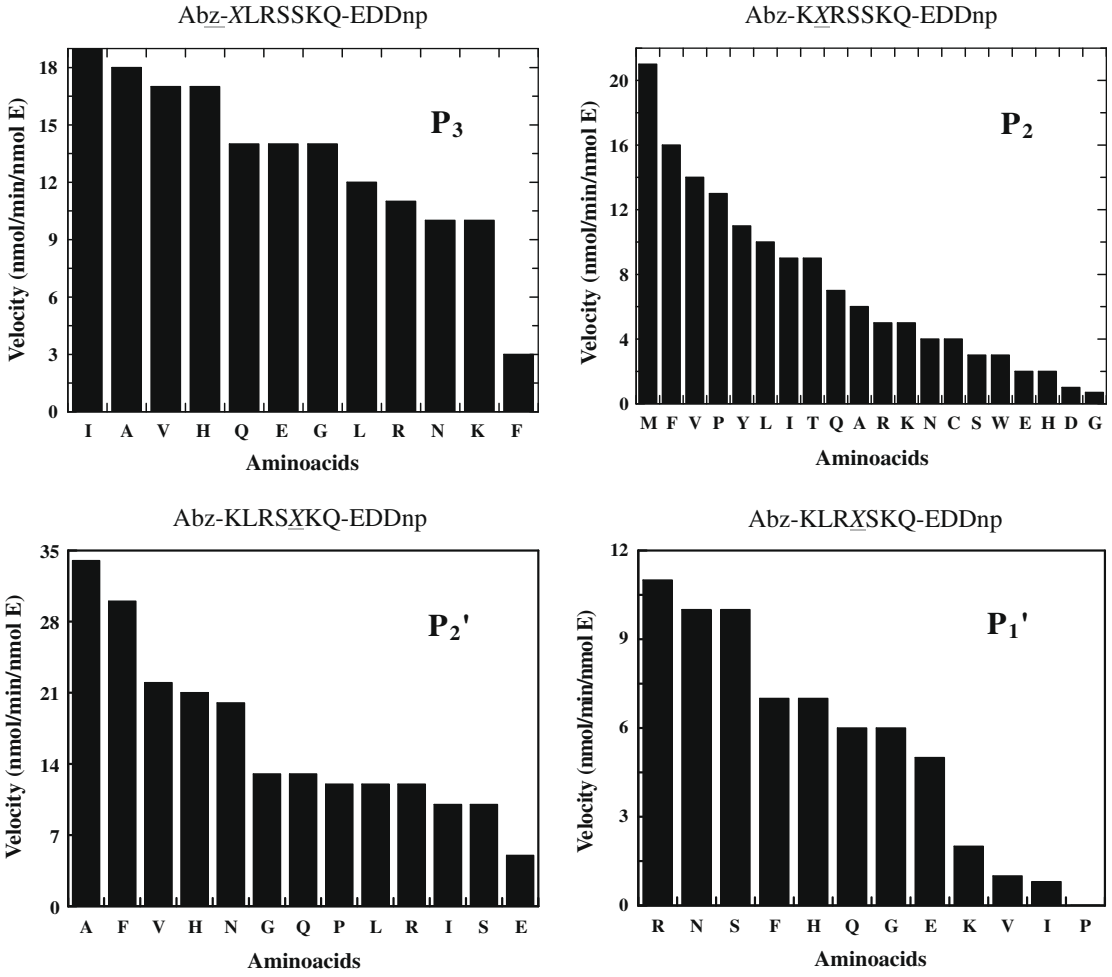


Fig. 9. Specificity of KLK3 subsites S_3, S_2, S_1', and S_2' on the hydrolysis of FRET peptide series Abz-KXRSSKQ-EDDnp (P_2), Abz-KLRSXKQ-EDDnp (P_2') and Abz-KLRXSKQ-EDDnp (P_1'). Assays were carried out in 50 mM Tris HCl, 1.5 M sodium citrate, pH 7.8, 7.5 μM substrate and [*E*] = 15 nM at 37 °C.

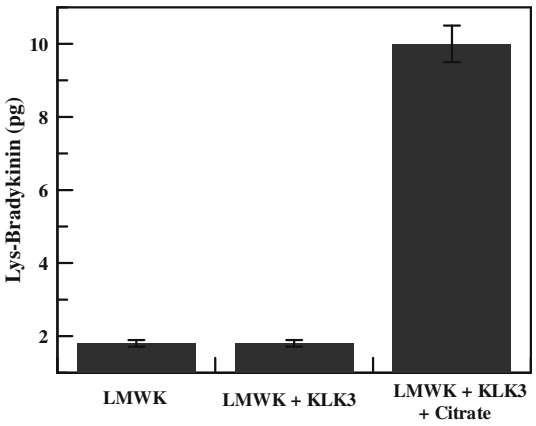


Fig. 11. Kinin release from LMWK by stoichiometric amounts of KLK3 (16 pmol). Assays were carried out in 50 mM Tris HCl, pH 7.8, 1.5 M sodium citrate, 7.5 μM substrate and [*E*] = 15 nM after 1 h of reaction at 37 °C.

We further examined the activity of KLK3 on the FRET peptides Abz-GFSPFRSSRIQ-EDDnp and Abz-MISLMKRPQ-EDDnp that are also derived from the human LMWK bradykinin-containing segment. Table 3 shows the kinetic parameters for their hydrolysis at the R–S and M–K bonds. The activities of KLK3 on the peptides Abz-GFSPFRSSRIQ-EDDnp and Abz-MISLMKRPQ-EDDnp were significantly lower compared to earlier reported parameters with KLK1 [37] as shown in Table 3.

Angiotensin I (DRVY↓IHPFHL) has several potential cleavage sites for KLK3 based on the data we presented above. The peptide bond Y–I was the only observed cleavage site even after 60 min of incubation (Fig. 13). Also, angiotensin II was assayed as substrate for KLK3, which was cleaved only at the Y–I bond (data not shown).

Competitive inhibition of KLK3 by ortho-phenantroline

Unexpectedly, we observed KLK3 inhibition by *ortho*-phenantroline of the preliminary studies of the best conditions to work with

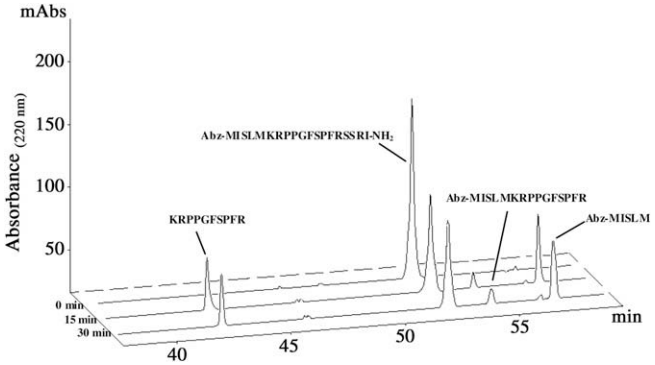


Fig. 12. HPLC chromatogram of the reaction mixture hydrolysis of Abz-MISLMKRPPGFSPFRSSRI-NH_2 by KLK3. Assays were carried out in 50 mM Tris HCl, pH 7.8, 1.5 M sodium citrate, 7.5 μM substrate and [*E*] = 15 nM at 37 °C.

Table 3
Kinetic parameters for the hydrolysis by KLK3 of FRET peptides derived from the human LMWK bradykinin-containing segment.

	k_{cat} $(min)^{-1}$	K_m (μM)	k_{cat}/K_m $(mM\ s)^{-1}$
Abz-GFSPFR↓SSRIQ-EDDnp	1.2 ± 0.03	0.4 ± 0.02	56 (*18750*)
Abz-MISLM↓KRPQ-EDDnp	0.6 ± 0.05	1.4 ± 0.04	11 (*136*)

Conditions of reactions: 15 nM KLK3, 50 mM Tris HCl, 1.5 M sodium citrate, pH 7.8, at 37 °C. Values in parenthesis are from the KLK1, without salt [37].

the enzyme. This inhibitory activity of *ortho*-phenantroline was reverted by gel filtration chromatography using Sephadex G-25 in pre-packed PD-10 column eluted with the same buffer used in the assays of hydrolytic activity. Fig. 14 ilustrates the competitive nature of *ortho*-phenantroline inhibition of KLK3, with K_i = 130 μM. Although this K_i value is high, it is noteworthy that a simple and small molecule as *ortho*-phenantroline with three condensed aromatic rings presented this inhibitory activity on KLK3 and that was not observed with chymotrypsin.

Effects of sodium citrate upon KLK3 intrinsic fluorescence

Significant variations in the intrinsic fluorescence of phenylalanine (emission λ_{max} = 280 nm) and tryptophan (emission λ_{max} = 340 nm) were caused by increasing the sodium citrate concentration on a constant amount of KLK3 (Fig. 15). The rising of phenylalanine fluorescence with the increasing concentration of sodium citrate indicates that some phenylalanines to be buried deeper in the protein structure.

Discussion

KLK3 was activated by heparin, heparan sulfate and dermatan sulfate (in order of decreasing efficiency), and this activation was shown to be dependent on the presence of at least two positively charged amino acids in the assayed FRET substrates. In accordance with this observation, the hydrolysis of insulin-like growth factor binding protein-3 (IGFBP-3) by KLK3 is also highly enhanced by heparin. Mutants of IGFBP-3 modified at the heparin binding site are less prone to hydrolysis by KLK3 activated by heparin [15]. The binding of KLK3 on heparin–Sepharose (Fig. 3) confirms the existence of heparin binding sites that were identified on the KLK3 surface in modeling of α_1-antichymotrypsin-KLK3 complex [38]. Therefore, it is reasonable to accept that glycosaminoglycans stabilize the complex of KLK3 with positively charged substrates.

KLK3 is considered a poor proteolytic enzyme when compared with chymotrypsin; however, in the presence of 1.5 M sodium citrate the peptide Abz-KLF↓SSK-Q-EDDnp was hydrolyzed with k_{cat}/K_m = 1250 $mM^{-1}s^{-1}$ while chymotrypsin hydrolyzed the same peptide with k_{cat}/K_m = 1313 $mM^{-1}s^{-1}$. These results indicate that the hydrolytic activity of KLK3 is highly dependent on the environment composition, where salts, glycosaminoglycans and proteins can play an important role in KLK3 activation. It is worth mentioning that the original crystal structure of horse KLK3 [39] showed substantial conformational changes in the active site kallikrein loop region compared to other serine proteases. Also KLK3 can be activated by binding of an antibody that interacts with the kallikrein loop and can stabilize KLK3 in an open and active conformation [40]. Thus, KLK3 may be uniquely susceptible to conformational changes influencing its active site and consequently its activity. The macromolecular crowding concept seems to be very adequate to interpret the activation of KLK3 by high sodium citrate concentrations. The macromolecular crowding obtained by high salt concentration compacts KLK3 [11] with reduction of surface-to-volume ratio, which results in active conformation of the enzyme; for review and concept explanation of macromolecular crowding see [41]. The activation seems to include the stabilization of the enzyme-substrate complex since the kosmotropic salt sodium citrate is more effective than NaCl on the hydrolysis of substrate that contains both prime and non-prime sites when compared with the hydrolysis of substrates containing only non-prime amino acids.

KLK3 is categorized as a "chymotrypsin-like" protease but it was able to cleave substrates containing R at the P_1 position in the Abz-KLXSSK-Q-EDDnp peptide series. Arginyl-hydrolase

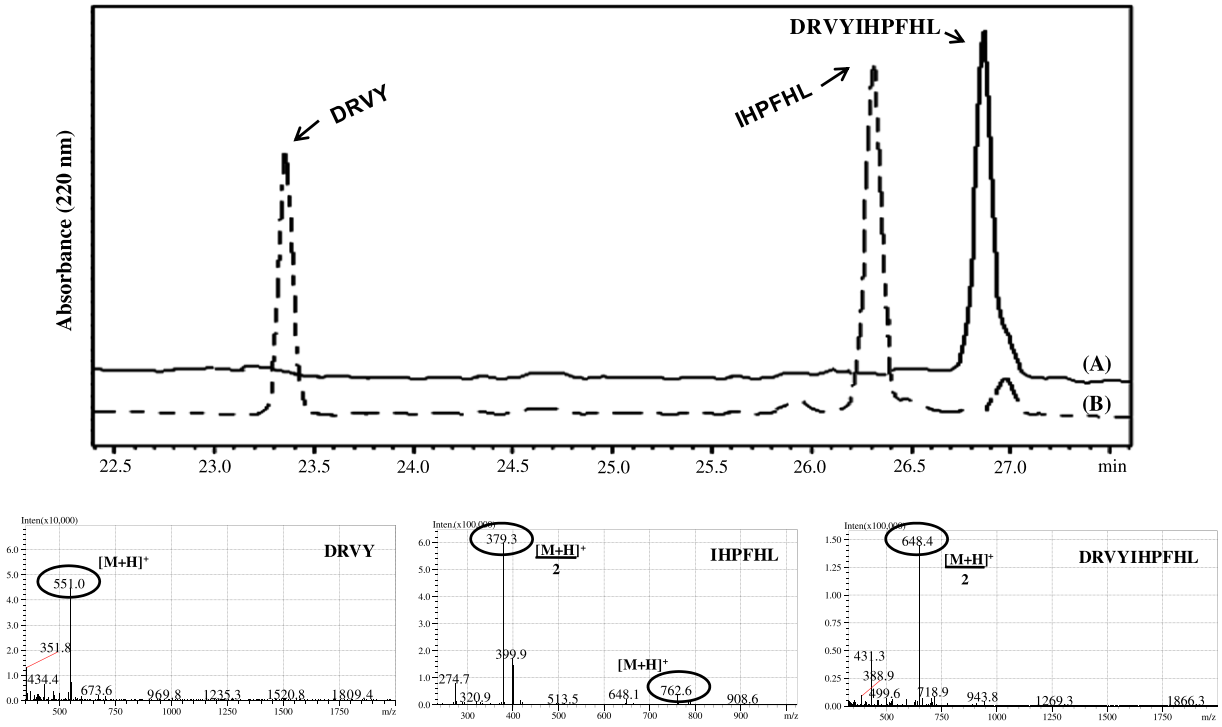


Fig. 13. HPLC chromatogram with LCMS profile of angiotensin I before (A) and after incubation by 1 h (B) with KLK3. The molecular weight of hydrolysis product was determined by LCMS-2010 equipped with the ESI-probe that was connected to HPLC circuit with UV detector. The assay was carried out 50 mM Tris–HCl buffer, pH 7.8, containing 1.5 M sodium citrate, [*E*] 50 nM and 50 μM angiotensin I.

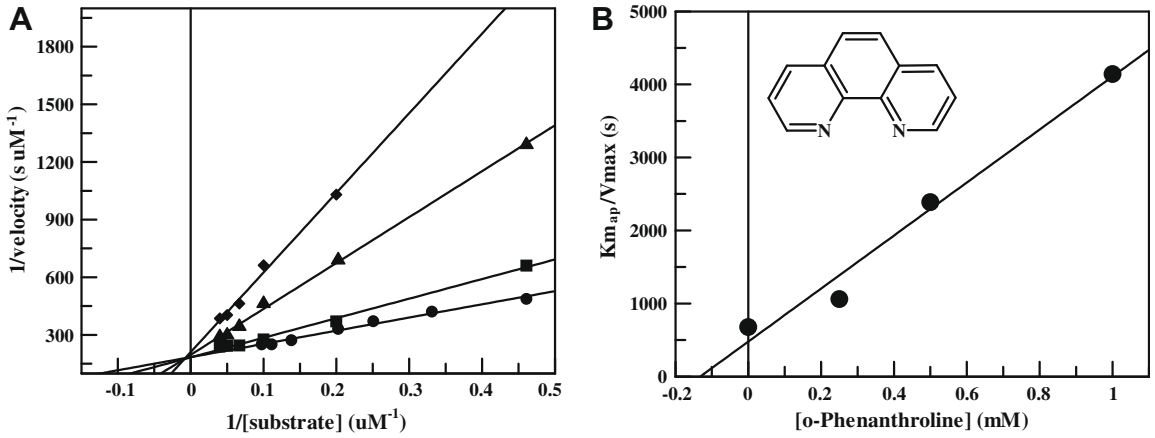


Fig. 14. The double-reciprocal plots of KLK3 inhibition by *ortho*-phenantroline. (A) Lineweaver–Burk plots for competitive inhibition in the absence (●) and presence of different concentrations of *ortho*-phenantroline: 0.25 mM (■), 0.5 mM (▲) and 1.2 mM (♦) using Abz-KLPSSKQ-EDDnp as substrate and KLK3 (10 nM). (B) Replot of apparent Km_{ap}/V_{max} *vs. ortho*-phenantroline concentration. K_i = 130 μM.

activity of KLK3 was also earlier observed with positional scanning libraries [8], additionally, KLK3 was shown to substantially cleave after R in its pro-sequence peptide after extensive incubation [42]. A noteworthy observation in the present work is the hydrolysis of peptide Abz-KLH↓SSK-Q-EDDnp with H at P_1 position that depends on the interaction of the N^{τ}-imidazole ring in the S_1 subsite of KLK3. Another surprising observation is the efficient hydrolysis of the peptide Abz-KLP↓SSK-Q-EDDnp by KLK3 and its reversible slow-binding inhibition by Z-Pro-Prolinal. The structural features of the S_1 pocket of KLK3 [43] based on its crystal structure [40] indicate a hydrophobic entrance that promotes the interaction of aromatic side-chains of Y or F and also of P. The bottom of the S_1 subsite is more hydrophilic due the presence of Ser^{189}, Ser^{226} and Thr^{190} and can provide the environment to fit hydrophilic groups such as the carboxamide of Q, the imidazole ring of H or even the guanidine group of R. The arginyl-hydrolase activity of KLK3 on the four series of FRET peptides derived from Abz-KLR↓SSKQ-EDDnp with R fixed at P_1 position demonstrated that it is possible to find substrates being efficiently hydrolyzed at R. The acceptance of R by the S'_1 subsite was confirmed by the hydrolysis of the peptide Z-R↓R-MCA at the R–R bond.

The kininogenase activity of KLK3 compared with KLK1 was very poor even in the presence of 1.5 M sodium citrate, and this is a result of the low hydrolysis of the peptide bond M–K and R–S as observed in the peptides derived from human LMWK bradykinin-containing segment (Table 3). Although KLK1 and salt activated KLK3 have trypsin- and chymotrypsin-like activities the former peptidase seems to be designed to release kinin with high efficiency.

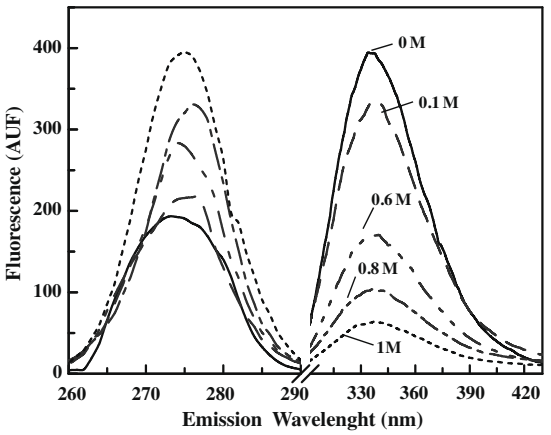


Fig. 15. Effects of sodium citrate upon KLK3 intrinsic fluorescence. The emission spectra of KLK3 (2 μM) in 50 mM Tris, pH 7.8 was evaluated before and with increasing of sodium citrate concentration. The fluorescence measurements were done at λ_{ex} = 270 nm.

The hydrolysis of angiotensin I or II only at the Y–I bond is in agreement with the preferences of KLK3 reported in the present work and in earlier studies and the resulting peptides DRVY and IHPFHL or IHPF that are so far considered inactive peptides [44].

Sodium citrate induced significant changes in the intrinsic fluorescence intensities of phenylalanines and tryptophanes of KLK3, which could indicates conformational changes earlier reported [11] that bury the benzyl groups of phenylalanines and expose the indole group of tryptophans. The competitive inhibition of KLK3 by *ortho*-phenantroline is particularly noteworthy for future design of specific non-peptide inhibitors for KLK3, particularly in terms of specificity due to the absence of inhibition of chymotrypsin by *ortho*-phenantroline. KLK3 seems to be a unique serine protease, particularly with regard to the dependence of its activity on the environment composition, and we present relevant observations about substrate specificity and inhibition of KLK3.

Conclusions

The large activation of KLK3 by sodium citrate, which is concentrated in prostate and seminal fluid in high amount, indicates that this salt can modulate the KLK3 activity in this organ. Although KLK3 hydrolyses with high efficiency substrates at aromatic amino acids, in the presence of sodium citrate the hydrolysis at R, Q and particularly P the hydrolytic activities of KLK3 are also efficient. Finally, this work suggests that promising inhibitors can be designed based on the structure of *ortho*-phenantroline and from compounds derived of proline.

Acknowledgments

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