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Enzymatic properties of human kallikrein-related peptidase 12 (KLK12)

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Abstract

Human kallikrein-related peptidase 12 (KLK12) is a new member of the human tissue kallikrein family. Preliminary studies suggest that KLK12 is differentially expressed in breast cancer and may have potential use as a cancer biomarker. It has been predicted that KLK12 is a secreted serine protease. However, the enzymatic properties of this protein have not been reported so far. Here, we report the production of recombinant KLK12 and analyses of its enzymatic characteristics, including zymogen activation, substrate specificity, and regulation of its activity. KLK12 is secreted as an inactive pro-enzyme, which is able to autoactivate to gain enzymatic activity. Through screening of a panel of fluorogenic and chromogenic peptide substrates, we establish that active KLK12 possesses trypsin-like activity, cleaving peptide bonds after both arginine and lysine. Active KLK12 quickly loses its activity due to autodegradation, and its activity can also be rapidly inhibited by zinc ions and by α_2-antiplasmin through covalent complex formation. Furthermore, we demonstrate that KLK12 is able to activate KLK11 zymogen *in vitro*. Our results indicate that KLK12 may participate in enzymatic cascades involving other kallikreins.

Keywords: enzyme activity; enzyme cascades; serine proteases; tissue kallikreins; trypsin-like activity; tumor markers.

Introduction

Human tissue kallikreins are a group of 15 trypsin- or chymotrypsin-like serine peptidases that are encoded by homologous genes residing in a contiguous cluster on human chromosome 19q13.4 (Yousef and Diamandis, 2001; Borgono and Diamandis, 2004; Clements et al., 2004). The first member, human tissue kallikrein 1 (hK1, also known as tissue/pancreatic/renal kallikrein), was initially identified in the 1930s by Werle and colleagues (Kraut et al., 1930). Subsequently in the 1980s, two other members, human tissue kallikrein 2 (hK2, also named glandular kallikrein) and human tissue kallikrein 3 (hK3, also named prostate-specific antigen), were identified (Rittenhouse et al., 1998). These three proteins are now known as the 'classical kallikreins'. However, not until several years ago were another 12 members of this family discovered (Stephenson et al., 1999; Yousef et al., 2000a). The term 'kallikrein' was originally used to refer specifically to an enzyme that can release a vasoactive peptide, Lys-bradykinin, from a larger protein precursor (kininogen). Among the tissue kallikrein family, only kallikrein 1 has this activity, while the rest do not (Borgono and Diamandis, 2004). Therefore, a new nomenclature, in which kallikrein 1 retained its original name and the rest of the members were termed 'kallikrein-related peptidases', was recently proposed for these proteins (Lundwall et al., 2006).

Kallikrein-related peptidase 12 (KLK12) was originally cloned using the positional candidate gene approach (Yousef et al., 2000b) and is predicted to encode a putative secreted serine protease (Unigene cluster Hs.411572). This gene has five coding exons and one 5′ untranslated exon with at least three alternatively spliced forms known to date (Kurlender et al., 2005). RT-PCR analysis showed that this gene is highly expressed in a variety of human tissues, such as the salivary gland, stomach, prostate, colon, brain, breast, and trachea (Yousef et al., 2000b). Preliminary data indicate that the gene may be down-regulated in breast cancer tissues (Yousef et al., 2000b). Despite increased interest in using many other members of the kallikrein family as novel cancer biomarkers (Diamandis and Yousef, 2002), examination of KLK12 in this respect has been minimal to date. We therefore expressed this enzyme in a mammalian expression system to study its enzyme activity and compare it to the activities of other known kallikreins as a first step in the further characterization of this novel enzyme.

Results

Production and purification of recombinant KLK12

When purified KLK12 was analyzed on SDS-PAGE under reducing conditions, it migrated with a molecular mass of approximately 38 kDa. N-Terminal sequencing revealed that it had the following sequence: A_{18}TPKIFNGTECG. Previously, based on homology comparison between the KLK12 protein sequence and those of other members of the kallikrein family, cleavage of pro-KLK12 from its signal peptide was predicted to occur between A_{17} and A_{18} (Yousef et al., 2000b). We thus conclude that the majority of KLK12 purified from conditioned medium corresponds to the pro-form of the enzyme.

Autoactivation and autodegradation of KLK12

Purified pro-KLK12 displayed trivial amounts of enzyme activity (<200 pmol/min μg). However, when incubated at 37°C without adding any other additional proteases,

its activity gradually increased. At 5 h, it reached a maximum level of approximately 12 000 pmol/min μg (Figure 1A). N-Terminal sequencing of KLK12 with this level of activity revealed the sequence I_{22}FNGTECG. These results suggest that pro-KLK12 can likely undergo autoactivation and that active KLK12 starts from amino acid I_{22}. When KLK12 exhibited maximum activity, it was estimated that more than 90% of pro-KLK12 had been converted to the active form, as determined by active site titration with α_2-antiplasmin (data not shown). When active KLK12 was further incubated at 37°C, it progressively lost its activity. At 16 h, only 50% of the activity remained (Figure 1A). At 24, 48, and 72 h, only 35%, 20%, and 10% of the activity was detected (data not shown), indicating that active KLK12 is prone to autolysis.

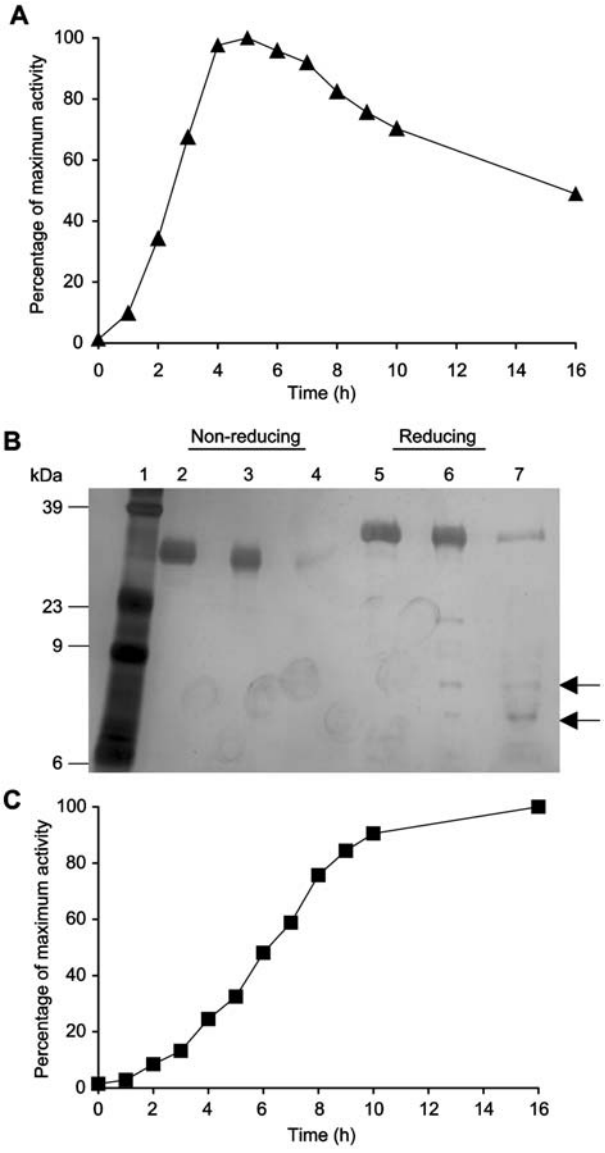

Figure 1 Autoactivation and autodegradation of KLK12. (A) Time course study demonstrating the change in KLK12 enzyme activity when incubated at 37°C. (B) Silver-stained SDS-PAGE showing unactivated (lanes 2 and 5), activated (lanes 3 and 6), and degraded KLK12 (lanes 4 and 7) under non-reducing and reducing conditions. Arrows indicate degraded KLK12 fragments. (C) Effect of Brij-35 and glycerol on the activation and degradation of KLK12. All values used to plot the graphs are mean values obtained from three independent experiments.

To further examine how KLK12 was modified during the autoactivation and autodegradation processes, pro-KLK12, active KLK12 (with optimal activity), and degraded KLK12 (with 10% maximum activity) were separated on 15% SDS-PAGE under reducing and non-reducing conditions, then stained with silver. As shown in Figure 1B, active KLK12 has a slightly smaller molecular mass than pro-KLK12. This is likely because active/mature KLK12 differs from inactive/pro-KLK12 by 4 aa. Under both reducing and non-reducing conditions, degraded KLK12 shows dramatically lower band intensity. No major band with smaller molecular masses are detected, except for two faint bands with molecular masses of ~10–15 kDa present only under reducing conditions. It appears that the majority of these self-digested fragments are relatively short and they are not retained on the gel.

To develop a formulation to preserve KLK12 enzyme activity, we tested glycerol and Brij-35. We found that adding 10–20% glycerol and 0.05% Brij-35 to the KLK12 autoactivation mixture could slow down activation, with KLK12 taking up to 16 h to reach its maximum activity (Figure 1C). However, under these conditions, at least 90% of the maximum activity could be preserved at 37°C for 72 h.

In our experiments, KLK12 could be activated without adding any additional proteases. This suggests that KLK12 is able to autoactivate. One method to prove this hypothesis is to use a KLK12-specific inhibitor to selectively inhibit its enzyme activity and examine whether KLK12 activation is affected. In the process of producing KLK12-specific antibodies, we identified a neutralizing monoclonal antibody that could specifically inhibit KLK12 enzyme activity. As shown in Figure 2A, when this antibody was incubated with optimally activated KLK12 at a molar ratio of 1:1, it was able to inhibit KLK12 enzyme activity by more than 95%. This monoclonal antibody likely inhibits KLK12 activity via steric hindrance of its active site. We therefore used it as a KLK12-specific inhibitor. As described earlier, purified pro-KLK12 has a small amount of activity, indicating the presence of low levels of active enzyme. By comparing the KLK12 activity before and after activation, the active KLK12 present in our pro-KLK12 preparation was estimated to be approximately 1% (data not shown). We hypothesized that if pro-KLK12 activation were primarily due to self-cleavage, adding just enough of this neutralizing antibody to block the active KLK12 pool present in the preparation would significantly slow down its activation; however, if KLK12 activation were a result of proteolytic cleavage by other enzymes, such as contaminating proteases present in the preparation, the presence of the neutralizing antibody would not affect KLK12 activation. In these experiments, we still used the enzyme assay to monitor KLK12 activation because the small amounts of antibody added were trivial compared to the gradually accumulating active KLK12 during activation. We found that when a small amount of monoclonal antibody was mixed with KLK12 to reach a molar ratio of antibody to active KLK12 of 0.2, resulting in a 50% reduction in the initial KLK12

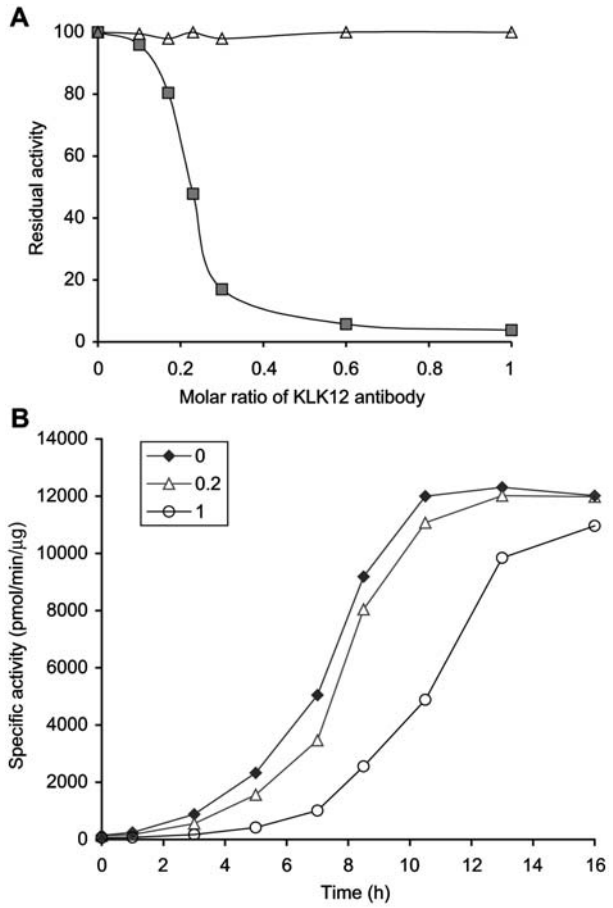

Figure 2 Inhibition of KLK12 enzymatic activity and autoactivation by its specific monoclonal antibody.
(A) Inhibition of enzyme activity. Optimally activated KLK12 was mixed with different molar ratios of its monoclonal antibody (squares) or an unrelated antibody (triangles) and the residual activity was measured; see the materials and methods section for details. (B) Inhibition of autoactivation. pro-KLK12 was incubated with small amounts of its monoclonal antibody and its activation time course was monitored. Molar ratios between the antibody and active KLK12 are indicated in the inset. See the text for a detailed discussion.

activity (Figure 2A), it took longer for KLK12 to reach optimal activity than in the absence of antibody (Figure 2B). When the amount of antibody increased further to optimally block the initial KLK12 activity, the lag phase of KLK12 activation increased even further. These results demonstrate that under our experimental conditions, modulation of KLK12 enzyme activity can affect its activation, suggesting that KLK12 is able to autoactivate.

Hydrolysis of peptide substrates by KLK12

The substrate specificity of KLK12 was examined using a panel of fluorogenic and chromogenic peptide substrates. KLK12 most efficiently cleaved the peptide substrates D-VLK-SBzl, Boc-QAR-AMC, and Boc-VPR-AMC, and to a lesser extent Boc-DPR-AMC and Boc-FSR-AMC. The kinetic constants are presented in Table 1. No cleavage was observed for Boc-QGR-AMC, Z-GGR-AMC, PFR-AMC, and Suc-AFK-AMC. These data demonstrate that KLK12 possesses trypsin-like enzyme activity and cleaves peptide bonds after both arginine and lysine. KLK12 did not show any activity against chymotrypsin-like peptide substrates, including Suc-AAPF-AMC, Suc-ALPF-AMC, Suc-LLVY-AMC, L-Y-AMC, and S2586.

Effect of pH and ionic strength on KLK12 enzyme activity

The effect of pH, salt, calcium, and zinc on KLK12 enzyme activity was examined. The optimal pH was between 7.5 and 8.0 (Figure 3). When the pH was decreased to <5.0, KLK12 enzyme activity became undetectable. It is noteworthy that KLK12 activity is very sensitive to pH changes in the range 6.5–7.0. Within this range, a decrease of 0.5 pH units resulted in a more than 60% reduction in activity. KLK12 activity was tolerant to NaCl concentration variations. It was higher when NaCl was absent and could be reduced by approximately 30% in the presence of 0.8 M NaCl. On the other hand, KLK12 activity was enhanced by calcium ions. Compared to the absence of calcium, a 2-, 2.5-, and 3-fold increase in activity could be obtained by adding $CaCl_2$ at concentration of 0.1, 1, and 10 mM, respectively. However, it was not increased further by higher concentrations of $CaCl_2$. KLK12 enzyme activity was strongly inhibited by zinc ions (Figure 4), being very sensitive to changes in zinc concentration in the range 0–25 μM. As little as 10 μM $ZnCl_2$ was sufficient to inhibit 50% of the KLK12 enzyme activity. More than 95% suppression could be achieved by 0.2 mM $ZnCl_2$.

Table 1 Kinetic parameters for the hydrolysis of some peptide substrates by KLK12.

Peptides	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	Activity[a] (%)
D-VLK-SBzl	29.4±0.5	744.9±5.4	25.3	100
Boc-QAR-AMC	68.6±3.6	1035.8±20.5	15.1	98
Boc-VPR-AMC	200±8.7	1548.4±17.8	7.7	85
Boc-DPR-AMC	350±12.3	1959.2±37.4	5.6	55
Boc-FSR-AMC	565±23.7	603.6±25.2	1.1	13

Mean and standard deviation for three experiments are presented for the K_m and k_{cat} values. The mean values were used to calculate k_{cat}/K_m ratios.
[a] Specific activities obtained with each substrate were used to calculate the relative activity.

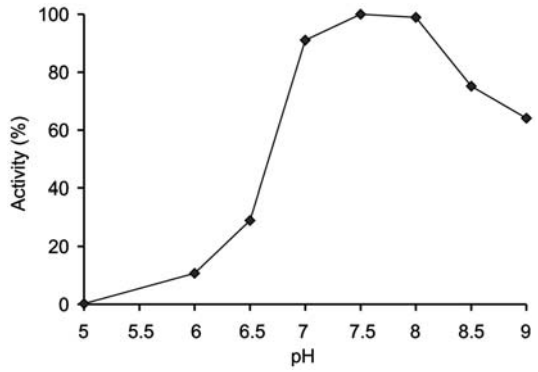

Figure 3 pH dependence of KLK12 enzymatic activity. Activity is presented as a percentage of the activity at pH 7.5.

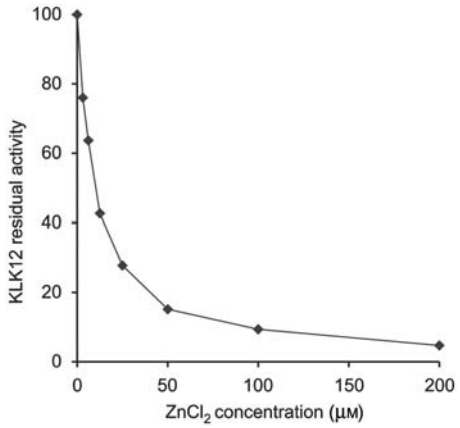

Figure 4 Inhibition of KLK12 enzymatic activity by zinc ions.

Inhibition of KLK12 activity by protease inhibitors

The inhibition of KLK12 by a panel of protease inhibitors is summarized in Table 2. When serpins at a five-fold molar excess were incubated with autoactivated KLK12 at 37°C for 2 h, complete suppression of KLK12 activity was observed for protein C inhibitor, α_2-antiplasmin, and C1 inhibitor. Antithrombin III and α_1-antitrypsin could inhibit KLK12 activity by approximately 80% and 50%, respectively. No inhibition was observed for α_1-antichymotrypsin, kallistatin, and plasminogen activator inhibitor-1 (PAI-1).

To further characterize the interaction between KLK12 and protein C inhibitor, α_2-antiplasmin, and C1 inhibitor, their association rate constants (k_{ass}) were determined. Since our preliminary experiments showed that the interaction between KLK12 and α_2-antiplasmin was a fast reaction (k_{ass} estimated to be $>10^5$ M^{-1} s^{-1}), we followed second-order rate conditions to determine k_{ass}. On the other hand, to obtain k_{ass} values between KLK12 and protein C inhibitor/C1 inhibitor, we adopted pseudo-first-order rate conditions (Salvesen and Nagase, 2001). Examples of the graphs used to generate the k_{ass} values are shown in Figure 5. Among these serpins, α_2-antiplasmin had the fastest binding rate with KLK12. When they were incubated at an equal concentration, more than 95% of KLK12 activity was inhibited within 2 min. The k_{ass} value was determined to be 2.1×10^5 M^{-1} s^{-1}. C1 inhibitor and protein C inhibitor exhibited much slower binding rates with KLK12, with k_{ass} values of 2.2×10^3 M^{-1} s^{-1} and 1.0×10^3 M^{-1} s^{-1}, respectively.

In addition, complex formation between KLK12 and α_2-antiplasmin, C1 inhibitor, protein C inhibitor, and antithrombin III was investigated using SDS-PAGE. As Figure 6 shows, for mixtures of KLK12 and α_2-antiplasmin/C1 inhibitor/protein C inhibitor, new bands with molecular masses of approximately 80–110 and 20 kDa are evident. In the mixture of KLK12 and α_2-antiplasmin, additional cleaved fragments of 45–60 kDa are also detected. Surprisingly, in the mixture of KLK12 and antithrombin III, no obvious high-molecular-mass complex was present. However, many fragments with molecular masses between 10 and 30 kDa are evident. These data suggest that KLK12 not only forms covalent complexes with these serpins, but also cleaves the serpins or the newly formed complexes.

For the other protease inhibitors examined, 3,4-dichloroisocoumarin and benzamidine were both strong inhibitors for KLK12. At concentrations of 11 and 280 μM, respectively, they were able to inhibit 50% of the activity. The inhibitory effect of aprotinin was relatively weak. No inhibition was observed for soybean trypsin inhibitor at concentrations up to 500 nM. Although α_2-macroglobulin was able to form a complex with PSA in parallel experiments, it failed to do so with KLK12 (data not shown).

Activation of recombinant pro-KLK11 by KLK12

In the literature, it has been reported that some members of the kallikrein family are able to activate some other members (Borgono and Diamandis, 2004). Since both recombinant KLK12 and KLK11 are available, we also examined the activation of KLK11 by KLK12. In these *in vitro* experiments, KLK11 enzyme activity was monitored using a peptide substrate after incubation with KLK12. It is noteworthy that since both of these enzymes possess trypsin-like enzyme activity and share similar peptide substrate profiles, before measuring KLK11 activity after KLK12 processing, it is critical to use a KLK12-specific

Table 2 Inhibition of KLK12 enzymatic activity by some common protease inhibitors.

	Inhibitory conc.	k_{ass} (M^{-1} s^{-1})
α_2-Antiplasmin		2.1×10^5
C1 inhibitor		2.2×10^3
Protein C inhibitor		1×10^3
Antithrombin III		$<10^{3a}$
α_1-Antitrypsin		$<10^{3a}$
α_1-Antichymotrypsin	ND	
Kallistatin	ND	
PAI-1	ND	
α_2-Macroglobulin	ND	
Soybean trypsin inhibitor	ND	
Aprotinin	2 μM	
3,4-Dichloroisocoumarin	0.25 mM	
Benzamidine	5 mM	

For the inhibitors, either the concentration required to inhibit more than 95% of KLK12 activity or the association rate constant (k_{ass}) with KLK12 are presented. ND, not detected. For experimental conditions, see the materials and methods section.
[a] Not experimentally determined, based on our observation that KLK12 interacted with antithrombin III and α_1-antitrypsin at slower rates than it did with protein C inhibitor.

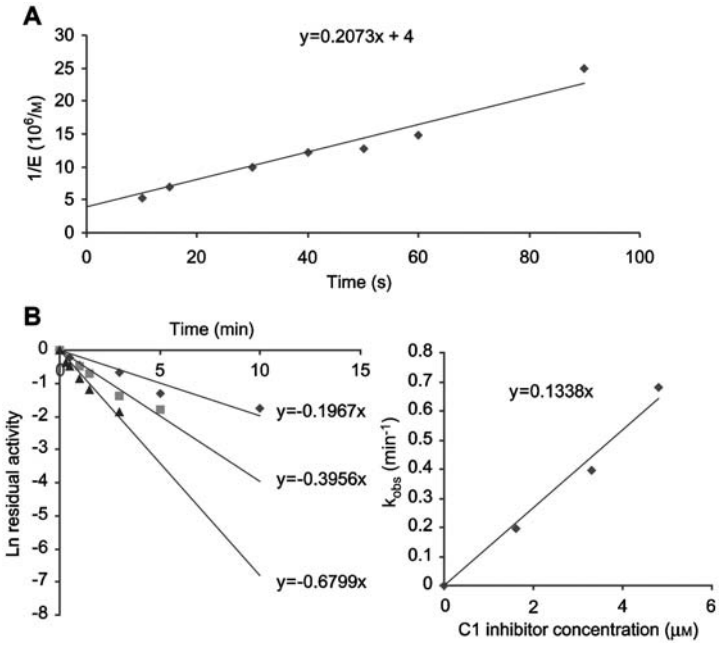

Figure 5 Typical graphs showing the determination of association rate constants (k_{ass}) between KLK12 and its inhibitors.
(A) Determination of the k_{ass} value between KLK12 and α_2-antiplasmin using second-order rate conditions. Under these conditions, the kinetics can be described by the following equation: $1/[E]=1/[E]_0+k_{ass}\times t$ (E, residual KLK12 concentration; E_0, initial KLK12 concentration; *t*, time). When 1/[E] is plotted against time, the slope (2.1×10^5 M^{-1} s^{-1}) represents the k_{ass} value between KLK12 and α_2-antiplasmin. (B) Determination of the k_{ass} value between KLK12 and C1 inhibitor using pseudo-first-order rate conditions. Autoactivated KLK12 (0.25 μM) was incubated with C1 inhibitor at concentrations of 1.5 (diamonds), 3.75 (squares), and 5 μM (triangles). The pseudo-first-order rate constants, k_{obs}, were obtained according to the following equation: Ln (residual activity)$=-k_{obs}\times t$. For a plot of k_{obs} against the C1 inhibitor concentration, the slope represents the k_{ass} value between KLK12 and C1 inhibitor (2.2×10^3 M^{-1} s^{-1}).

inhibitor to completely abolish KLK12 activity without affecting KLK11 activity. As described above, α_2-antiplasmin is a fast-acting inhibitor for KLK12. However, it does not inhibit KLK11 (Luo et al., 2006). α_2-Antiplasmin was therefore used in these experiments to inhibit KLK12 activity. The activation time course of pro-KLK11 by autoactivated KLK12 is shown in Figure 7A. Pro-KLK11 was activated by KLK12 in a dose-dependent manner. When KLK11 and KLK12 were incubated at an equal molar ratio, KLK11 activity rapidly increased. By 30 min, it reached maximum activity of approximately 2000 pmol/min μg. It was estimated that more than 50% of pro-KLK11 had been converted to the active form by comparison of its activity with that of natural KLK11 purified from seminal plasma (Luo et al., 2006). To determine whether KLK12 specifically cleaves KLK11 at the pro-enzyme activation site, optimally activated KLK11 was separated on SDS-PAGE under reducing and non-reducing conditions. Our results showed that the majority of KLK12-activated KLK11 appeared similar to the unactivated form. However, a faint band of approximately 20 kDa was also observed under reducing conditions (Figure 7B). A recent study has shown that the active form of KLK11 isolated from seminal plasma migrates similarly on SDS-PAGE to the pro-form owing to its small pro-peptide. In addition, an internally cleaved form of KLK11 has also been identified that appears as a 20-kDa band under reducing conditions (Luo et al., 2006). It seems that on SDS-PAGE, KLK12-activated KLK11 exhibits the same characteristics as the native active KLK11. These results suggest that KLK12 can specifically cleave KLK11 at the pro-enzyme activation site, leading to KLK11 activation. However, whether the degraded KLK11 fragment is a result of KLK12 cleavage or autolysis is not clear.

Discussion

In this investigation, we produced recombinant KLK12 and analyzed its enzymatic characteristics. Using homology comparison between the KLK12 protein sequence and those of other members of the kallikrein family, the

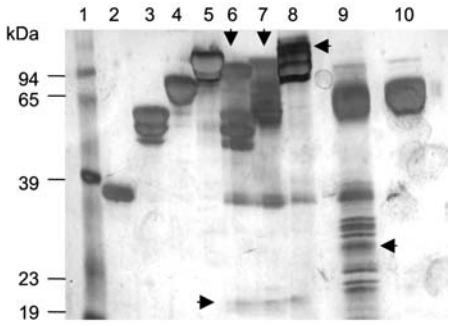

Figure 6 Silver-stained SDS-PAGE showing complex formation between KLK12 and serpins.
Lane 1, molecular mass marker; lane 2, KLK12 alone; lane 3, protein C inhibitor alone; lane 4, α_2-antiplasmin alone; lane 5, C1 inhibitor alone; lane 6, KLK12 with protein C inhibitor; lane 7, KLK12 with α_2-antiplasmin; lane 8, KLK12 with C1 inhibitor; lane 9, KLK12 with antithrombin III; lane 10, antithrombin III alone. The complexes formed and fragments cleaved are indicated by arrows. For discussion, see the text.

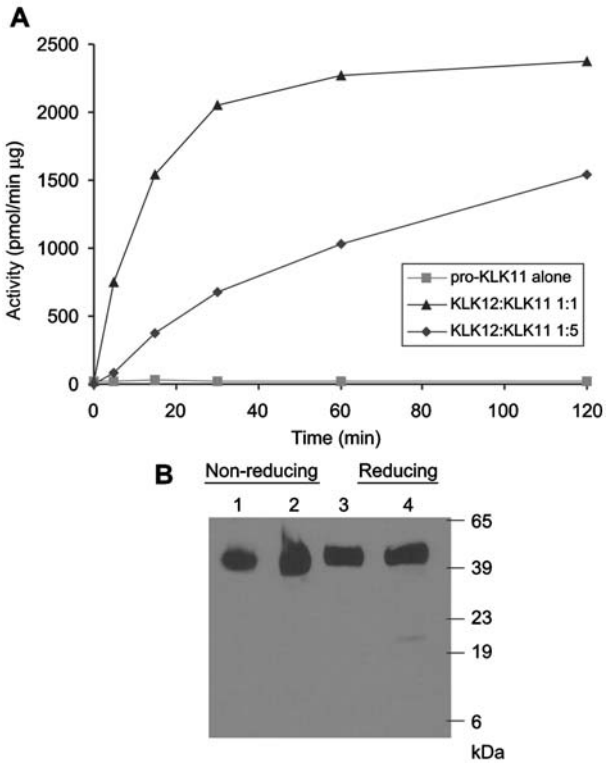

Figure 7 Activation of pro-KLK11 by autoactivated KLK12. (A) Time course studies. (B) Western blot analysis. Aliquots were taken from incubation mixtures containing equal molar concentrations of KLK11 and KLK12 at 0 and 60 min, separated on SDS-PAGE under non-reducing and reducing conditions, and detected with an anti-KLK11 polyclonal antibody. Lanes 1 and 3, 0 min; lanes 2 and 4, 60 min. All values used to plot the graphs are mean values obtained from three independent experiments.

pro-/inactive and mature/active forms of KLK12 have been predicted to start from A_{18} and I_{22}, respectively (Yousef et al., 2000b). Our experimental data confirm these predictions. We further demonstrated that KLK12 could be autoactivated *in vitro*. However, whether this process also occurs *in vivo* remains to be further determined.

KLK12 enzyme activity is most notably affected by pH and zinc. It displays the highest activity at physiological pH (~7.5). Its activity is very sensitive to pH changes in more acidic solutions. When the pH decreases below 7.0, the enzyme rapidly loses its activity. We also observed that when the pH in the activation buffer was lower than 7.0, pro-KLK12 did not autoactivate. It seems that pH is crucial in regulating KLK12 autoactivation. KLK12 enzyme activity is also modulated by zinc. KLK12 is expressed in epithelial cells lining glandular tissues, such as the salivary gland and prostate, and the central nervous system (Yousef et al., 2000b). In these tissues, zinc concentration can be as high as 9 mM in secretory granules (Kavanagh, 1985). Most of this zinc is complexed to other proteins or solvents, with a small percentage being in the free form. As our results indicated, 10 µM zinc was sufficient to inhibit 50% of KLK12 enzyme activity and more than 95% inhibition could be achieved with 0.2 mM zinc. Therefore, it is conceivable that zinc also plays an important role in regulating KLK12 activity. It appears that zinc ion is a common regulator for the kallikreins, since similar observations have been reported for KLK2, KLK3, and KLK5 (Lovgren et al., 1999a; Malm et al., 2000; Michael et al., 2006).

The major mechanisms underlying the inactivation of proteases include proteolytic cleavage and the formation of covalent complexes with protease inhibitors. Cleavage of a protease can be the result of self-digestion or proteolysis by other proteases. Our *in vitro* data clearly demonstrate that KLK12 can undergo rapid inactivation by self-digestion. This may represent one of the mechanisms that control KLK12 activity if this is confirmed with *in vivo* studies. Examples of other kallikreins inactivated by self-digestion or proteolysis have also been reported (Bayes et al., 2004; Luo et al., 2006). Serpins are the major protease inhibitors distributed among human tissues and biological fluids. Therefore, we investigated the regulation of KLK12 enzyme activity by the most abundant serpins found in the circulation. Although KLK12 could form covalent complexes with α_2-antiplasmin, protein C inhibitor, and C1 inhibitor *in vitro*, α_2-antiplasmin appeared to be the fastest-binding serpin for KLK12, based on association rate constants, while α_2-macroglobulin, another abundant protease inhibitor in serum, did not show any inhibition (Table 2). Consequently, if active KLK12 is released into the circulation, the protease inhibitor that would most likely complex to it is α_2-antiplasmin. Similarly, it has been shown that many kallikreins form complexes with various proteinase inhibitors in serum (Christensson et al., 1990; Stenman et al., 1991; Christensson and Lilja, 1994). Collectively, under physiological conditions, self-cleavage and complex formation with α_2-antiplasmin may contribute to reduce the proteolytic activity of KLK12.

It was predicted that KLK12 has trypsin-like activity owing to the presence of an aspartic acid residue in its substrate-binding pocket, which was confirmed by a subsequent study (Yousef and Diamandis, 2001; Shinmura et al., 2004). By screening a panel of peptide substrates, we have shown that KLK12 prefers arginine or lysine in the P1 position of its cleavage site. This substrate specificity of KLK12 could be further demonstrated by its autoactivation and the activation of pro-KLK11. The activation sites for pro-KLK12 and pro-KLK11 are ATP**K**↓IFNG and ET**R**↓IIKG, respectively (Yoshida et al., 1998). These activation sites, cleaved by KLK12, are in agreement with the substrate specificity of KLK12. However, with these peptide substrates, it is difficult to determine the influence of residues in other positions, such as P1′, P2, P3, and P4. To more precisely define the substrate specificity of KLK12, other technologies, such as positional scanning combinatorial libraries and substrate phage display, need to be used (Matthews and Wells, 1993; Harris et al., 2000). Such methods have already been used to investigate substrate specificity for some kallikreins and useful information was obtained (Cloutier et al., 2004; Felber et al., 2005; Matsumura et al., 2005).

It has been postulated that some members of the human tissue kallikrein family may participate in proteolytic cascades in certain tissues to facilitate specific physiological processes (Yousef and Diamandis, 2002; Borgono and Diamandis, 2004). So far, the kallikreins for

which autoactivation has been reported include KLK2 and KLK5 (Mikolajczyk et al., 1997; Lovgren et al., 1999b; Brattsand et al., 2005; Michael et al., 2005). I*n vitro* experiments showed that KLK2 can activate pro-KLK3 (Lovgren et al., 1997; Vaisanen et al., 1999). Similarly, it has been reported that KLK5 can activate pro-KLK7 and pro-KLK14 (Brattsand et al., 2005). In this context, it has been hypothesized that KLK2 may be the physiological activator of KLK3 in seminal plasma and KLK5 may be the initiator of an enzyme cascade in the stratum corneum that involves KLK5, KLK7, and KLK14. In addition, both KLK4 and KLK15 have been shown to activate pro-KLK3 *in vitro* (Takayama et al., 2001a,b). As shown in our investigation, pro-KLK12 can also undergo autoactivation. We thus speculate that it may also be able to activate some other kallikreins. Our preliminary results clearly demonstrate that KLK12 can indeed rapidly activate pro-KLK11 *in vitro*. In addition, we also tested the activation of pro-KLK7 and pro-KLK14 with KLK12 using similar strategies. No activation was observed for KLK14 (data not shown). However, a slow activation was observed for pro-KLK7. When pro-KLK7 was incubated with active KLK12 at equal concentrations, approximately 30% activation was observed within 24 h (data not shown). Taken together, our results indicate that KLK12 can selectively activate only some of the kallikreins. KLK12 and KLK11 are expressed concomitantly in many tissues, such as the salivary gland, breast, gastrointestinal tract, and uterus (Yousef and Diamandis, 2001). Therefore, it is possible that KLK12 is the physiological activator of KLK11 in some tissues under certain physiological conditions. Our preliminary findings warrant further investigations.

Materials and methods

Production and purification of recombinant KLK12

The cDNA sequence encoding full-length pre-pro-KLK12 (aa 1–248, GenBank accession number NP_665901) was amplified from breast-derived cDNA by PCR and cloned into a eukaryotic expression vector. To facilitate detection and purification, a 10-His tag was added to the C-terminus. This construct was then sequenced to ensure that no variation was introduced during PCR. To generate stable cell lines for KLK12, the expression construct was transfected into the murine NS0 myeloma cell line according to standard techniques. Stable clones expressing KLK12 were selected by Western blot analysis. These cells were then expanded in H-SFM medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 2% fetal bovine serum for 10–14 days and the conditioned medium was harvested. KLK12 was purified by affinity chromatography and gel filtration using previously described procedures (Johnson and Jiang, 2005). The purified protein was then analyzed on SDS-PAGE and stained with silver to assess its purity. It was further subjected to N-terminal sequencing using the Edman degradation method.

Autoactivation and autodegradation of KLK12

To investigate the autoactivation of KLK12, purified KLK12 (stored frozen at -20°C in 25 mM MES, 0.15 M NaCl, pH 5.0) was diluted to 0.1 mg/ml in TCN buffer containing 0.1 M Tris, 10 mM $CaCl_2$, 0.15 M NaCl, pH 8.0 and incubated at 37°C. Aliquots were removed and KLK12 enzyme activity was measured using Boc-VPR-**7**-amino-4-methyl coumarin (Boc-VPR-**7**-AMC) as the substrate every hour for the first 10 h, then at 16, 24, 48, and 72 h. For detailed procedures, see related sections below. In addition, aliquots taken at 0, 5, and 72 h were further separated on 15% Tris-glycine SDS-PAGE under reducing (addition of fresh β-mercaptoethanol) and non-reducing conditions and stained with silver to further assess KLK12 modifications.

The effect of glycerol and Brij-35 on KLK12 enzyme activity was examined. Glycerol was added to purified KLK12 at a final concentration of 50% for storage (KLK12 storage buffer is now 12.5 mM MES, 75 mM NaCl, 50% glycerol, pH 5.0). For autoactivation, this KLK12 glycerol stock was diluted to 0.1 mg/ml (typical final glycerol concentration in the autoactivation mixture of 10–20%) in TCNB buffer (TCN buffer supplemented with 0.05% Brij-35). The KLK12 autoactivation time course was monitored as described above.

To determine whether the activation of KLK12 is due primarily to self-activation, we examined the effect of a KLK12 neutralizing monoclonal antibody (clone 364932, R&D Systems Inc., Minneapolis, USA) on KLK12 activation. To perform experiments, we first determined the minimal amount of this monoclonal antibody required to block KLK12 enzyme activity. KLK12 stored in glycerol was activated overnight as described above. It was then diluted to 10 μg/ml and mixed with 0–50 μg/ml of the antibody (antibody/KLK12 molar ratio of 0–1) in TCNB buffer. These mixtures were incubated at 37°C for 1 h and residual KLK12 activity was measured. An unrelated monoclonal antibody [anti-human glucosamine (*N*-acetyl)-6-sulfatase] at the same concentrations was used as a negative control. Next, we examined whether small amounts of this neutralizing antibody could block the activation of KLK12. Non-activated KLK12 at a concentration of 0.1 mg/ml was incubated with the antibody at 1 and 5 μg/ml in TCNB buffer at 37°C. KLK12 enzyme activity was monitored at different time intervals as described above.

Hydrolysis of peptide substrates by KLK12

The substrate specificity of KLK12 was determined using a panel of fluorogenic and chromogenic peptide substrates (amino acids are represented as single letter abbreviations), including Boc-VPR-AMC, Boc-QAR-AMC, Boc-DPR-AMC, Boc-FSR-AMC, Boc-QGR-AMC, Z-GGR-AMC, H-PFR-AMC, Suc-AFK-AMC, Suc-AAPF-AMC, Suc-ALPF-AMC, Suc-LLVY-AMC, LY-AMC, S2586 (MeoSuc-RPY-*p*-nitroanilide, MeoSuc-RPY-pNA), and D-VLK-SBzl. Boc-DPR-AMC was obtained from Sigma (St. Louis, MO, USA). Boc-VPR-AMC was from R&D Systems. All other AMC substrates were purchased from Bachem Bioscience (King of Prussia, PA, USA). S2586 and D-VLK-SBzl were from DiaPharma (West Chester, OH, USA) and MP Biochemicals (Aurora, OH, USA), respectively. Purified KLK12 (stored in 50% glycerol) was first activated in TCNB buffer at 37°C for 16 h as described above. Hydrolysis of peptide substrates by KLK12 was carried out in 96-well microtiter plates containing 10 ng of autoactivated KLK12 (final concentration 2.5 nM) and 100 μM peptide substrate in 100 μl of TCNB buffer. For D-VLK-SBzl, 0.1 mM DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)] was also added. Fluorescence (for AMC substrates) was then measured on a SpectraMax GeminiXS Microplate Spectrofluorimeter (Molecular Devices Corp., Sunnyvale, CA, USA) using excitation and emission wavelengths of 380 and 460 nm, respectively. Hydrolysis of the chromogenic substrates S2586 and D-VLK-SBzl was monitored at a wavelength of 405 nm on a SpectraMax Plus^{384} Microplate spectrophotometer (Molecular Devices Corp.). For all experiments, the initial rate of hydrolysis was monitored for 5 min at room temperature. The specific activity of KLK12, representing the amount of peptide substrate cleaved per μg of KLK12 per minute, was then calculated by comparison to standard curves. To determine the K_m and k_{cat} values, substrate concentrations ranging from 25 to 400 μM were used. The kinetic

parameters were then determined from Lineweaver-Burk double-reciprocal plots.

Effect of pH and ionic strength on KLK12 enzyme activity

The effect of pH, salt, calcium, and zinc was examined. To determine the optimal pH, buffers of pH 5.0 (0.1 M MES) and pH 6.0–9.0 (0.1 M Tris) were tested. The concentrations of NaCl, $CaCl_2$, and $ZnCl_2$ solutions, prepared in 0.1 M Tris, pH 7.5, ranged from 0 to 0.8 M, 0 to 100 mM and 0 to 0.5 mM, respectively. The enzyme activity of 10 ng of autoactivated KLK12 in these buffers was measured for Boc-VPR-AMC as described above.

Inhibition of KLK12 by serpins

The serpins investigated were α_1-antitrypsin, α_1-antichymotrypsin, antithrombin III, kallistatin, protein C inhibitor, α_2-antiplasmin, plasminogen activator inhibitor-1 (PAI-1), and C1 inhibitor (all from R&D Systems, Inc.) To perform the experiments, 0.25 μM autoactivated KLK12 was incubated with 1.25 μM serpin in TCNB buffer at 37°C for 2 h. For antithrombin III and protein C inhibitor, the buffer was supplemented with 0.5 μM heparin. KLK12 residual activity was measured for Boc-VPR-AMC as described above.

Association rate constants (k_{ass}) between KLK12 and α_2-antiplasmin, C1 inhibitor, and protein C inhibitor were further determined. To determine the k_{ass} value between KLK12 and α_2-antiplasmin, second-order rate conditions were used. Briefly, 0.25 μM autoactivated KLK12 was incubated with 0.25 μM α_2-antiplasmin at room temperature and KLK12 residual activity was measured at different time intervals. k_{ass} was derived from the following equation: $1/[E]=1/[E]_0+k_{ass}\times t$ (E, residual enzyme concentration; E_0, initial enzyme concentration; t, time) (Salvesen and Nagase, 2001). To determine the k_{ass} values between KLK12 and C1 inhibitor or protein C inhibitor, pseudo-first-order rate conditions were adopted. Under these conditions, 0.25 μM autoactivated KLK12 was mixed with a 5–20 molar excess of C1 inhibitor or protein C inhibitor and incubated at room temperature. KLK12 residual activity was measured at different time intervals. The kinetics can be described by the following equations: Ln (residual activity)$=-k_{obs}\times t$ (k_{obs}, pseudo-first-order rate constant) and $k_{obs}=k_{ass}\times[I]$ (I, serpin concentration) (Salvesen and Nagase, 2001). The k_{ass} values were obtained by plotting graphs according to these formulas.

Complex formation between KLK12 and antithrombin III, protein C inhibitor, C1 inhibitor, and α_2-antiplasmin was investigated by incubating autoactivated KLK12 with the inhibitors at an equal concentration of 0.25 μM at 37°C for 1 h. The reaction mixtures were then separated on SDS-PAGE under reducing conditions and stained with silver.

Inhibition of KLK12 by other protease inhibitors

The effect of some other common protease inhibitors on KLK12 enzyme activity was also examined, including α_2-macroglobulin, aprotinin, benzamidine, 3,4-dichloroisocoumarin, and soybean trypsin inhibitor. All of these reagents were purchased from Sigma, except for α_2-macroglobulin (R&D Systems, Inc.). The interaction between KLK12 and α_2-macroglobulin was evaluated by mixing at equal concentrations of 0.25 μM in TCNB buffer and incubation at 37°C for 16 h. The mixtures were then separated on SDS-PAGE under reducing and non-reducing conditions and stained with silver. To determine whether KLK12 was also inhibited by the other inhibitors, 10 ng of autoactivated KLK12 was mixed with different concentrations of inhibitors in 100 μl of TCNB buffer. KLK12 residual activity was measured as described above. The minimum concentrations required to inhibit more than 95% of the KLK12 activity were determined.

Activation of recombinant pro-KLK11 by KLK12

Recombinant pro-KLK11 produced in NS0 cells was obtained from R&D Systems Inc. Pro-KLK11 (50 μg/ml) was incubated with autoactivated KLK12 at concentrations of 50 and 10 μg/ml in TCNB buffer at 37°C. Aliquots were taken at time intervals of 0, 5, 10, 30, 60, and 120 min. To terminate KLK12 activity, the mixture was diluted five-fold in TCNB buffer and incubated with α_2-antiplasmin at a final concentration of 40 μg/ml at 37°C for 30min. To measure KLK11 enzyme activity, 20 ng of KLK11 was mixed with 100 μM D-VLK-SBzl and DTNB in 100 μl of 50 mM Tris, 0.25 M NaCl, pH 8.5. Hydrolysis of D-VLK-SBzl was measured as described above. The aliquots taken at 0 and 60 min were further separated on SDS-PAGE under reducing and non-reducing conditions and Western blot analysis was performed using a goat anti-KLK11 polyclonal antibody (R&D Systems, Inc.).

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