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Human kallikrein 4: enzymatic activity, inhibition, and degradation of extracellular matrix proteins

Chistina V. Obiezu[1,2], Iacovos P. Michael[1,2], Michael A. Levesque[1] and Eleftherios P. Diamandis[1,2,*]

[1] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto M5G 1X5, ON, Canada

[2] Department of Laboratory Medicine and Pathobiology, University of Toronto, 100 College Street, Toronto M5G 1L5, ON, Canada

* Corresponding author
e-mail: ediamandis@mtsinai.on.ca

Abstract

Human kallikrein 4 (hK4) is a member of the expanded family of human kallikreins, a group of 15 secreted proteases. While this protein has been associated with ovarian and prostate cancer prognosis, only limited functional information exists. Therefore, we have undertaken an investigation of its enzymatic properties regarding substrate preference, degradation of extracellular matrix proteins, and its inhibition by various inhibitors. We successfully expressed and purified active recombinant hK4 from supernatants of the *Pichia pastoris* expression system. This enzyme seems to cleave more efficiently after Arg compared to Lys at the P1 position and exhibits modest specificity for amino acids at positions P2 and P3. hK4 forms complexes with α_1-antitrypsin, α_2-antiplasmin and α_2-macroglobulin. The protease mediates limited degradation of extracellular matrix proteins such as collagen I and IV, and more efficient degradation of the α-chain of fibrinogen. The cleavage of extracellular matrix proteins by hK4 suggests that this enzyme may play a role in tissue remodeling and cancer metastasis.

Keywords: cancer metastasis; extracellular matrix; human kallikrein 4; ovarian cancer; prognostic markers; prostate cancer; serine protease; serine protease inhibitors.

Introduction

Serine proteases play key roles in diverse physiological processes such as digestion, blood coagulation, cellular and humoral immunity, fibrinolysis, fertilization and embryonic development (Rawlings and Barrett, 1993). Some serine proteases, including members of the complement system, have highly specific, ordered roles in a single cascade of reactions (Sim and Laich, 2000); others, such as urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA), influence a wide variety of key physiological process, including extracellular matrix (ECM) remodeling, wound healing, angiogenesis, tumor invasion and metastasis (van Zonneveld et al., 1986; Sheng, 2001). Serine proteases vary widely with respect to substrate specificity. Some members, such as the digestive enzyme trypsin, cleave virtually any polypeptide chain, whereas others, such as the complement proteins, have acquired very specialized substrate recognition via protein domains or extra modules that confer specific localization and stringent specificity (Doolittle and Feng, 1987; Farries and Atkinson, 1991). Abnormal activity of serine proteases through deregulation leads to a myriad of pathological conditions such as asthma (Schwartz, 1992), clotting disorders (Ratnoff, 1972), chronic bronchitis and emphysema (Kanazawa et al., 1992).

The human tissue kallikreins comprise a multigene subfamily of the S1A serine proteases and are encoded by a tandemly arrayed set of genes co-localized to chromosome 19q13.4 (Yousef and Diamandis, 2000; Yousef et al., 2000a; Gan et al., 2000; Clements et al., 2001). Although hK1, hK2 and hK3 (prostate-specific antigen, PSA) were initially thought to be the only members of this gene family, the Human Genome Project has facilitated the mapping of previously characterized serine protease genes (Lin et al., 1993; Hansson et al., 1994; Anisowicz et al., 1996; Liu et al., 1996; Little et al., 1997; Yamashiro et al., 1997; Luo et al., 1998; Yoshida et al., 1998a,b; Brattsand and Egelrud, 1999; Ekholm and Egelrud, 1999; Yousef et al., 1999a, 2000b,c) as well as seven novel serine protease genes (Nelson et al., 1999; Stephenson et al., 1999; Yousef and Diamandis, 1999; Yousef et al., 1999b, 2000d,e, 2001a,b) to the same locus. These 15 human kallikreins make up a group of homologous (40–80%) serine proteases with either trypsin- or chymotrypsin-like specificity (Yousef and Diamandis, 2001; Borgono et al., 2004). Since PSA is the most useful biomarker for prostate cancer screening and monitoring for recurrence, several other human kallikreins have been evaluated for their utility as cancer diagnostic or prognostic markers (Borgono et al., 2004; Obiezu and Diamandis, 2005), and as markers of central nervous system (Yousef et al., 2003) and skin disorders (Komatsu et al., 2003; Clements et al., 2004). As yet, their direct role, if any, in the underlying pathological mechanisms of these conditions remains to be elucidated.

Human kallikrein gene 4 (*KLK4*), is a member of this expanded human kallikrein gene family. Initially identified as having prostate-specific expression (Nelson et al., 1999), it is now clear that this gene is expressed in a variety of other tissues, albeit at much lower levels (Stephenson et al., 1999; Yousef et al., 1999b). Its prostatic

Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

expression established a possible physiological connection to two other prostate-specific members of the same gene family, namely hK3 (PSA) and hK2, with the latter also an emerging marker in prostate cancer diagnostics (Rittenhouse et al., 1998). KLK4 is speculated to have mRNA forms coding for both extracellular (Nelson et al., 1999; Stephenson et al., 1999; Yousef et al., 1999b) and intracellular (Korkmaz et al., 2001) proteins; the corresponding proteins themselves have been immunohistochemically localized to the cytoplasm of normal human prostate tissue and to primary and metastatic prostate tumor tissues (Day et al., 2002; Obiezu et al., 2002), as well as to the nuclei of prostate cancer cells (Xi et al., 2004). The protein encoded by the classical form of *KLK4* mRNA was predicted to be a 254-aa zymogen with a conserved serine protease catalytic triad (Nelson et al., 1999). Sequence analysis predicted a 26-aa signal peptide with a cleavage site on the carboxyl side of Gly26 and a pro-protein cleavage site on the carboxyl side of Gln30, suggesting that classical hK4 is likely a secreted protein that is processed into a 224-aa active protein. As the human hK4 pro-peptide (SCSQ) differs markedly with respect to the terminal Lys or Arg present in other kallikreins and those established in its murine (VSSR) or porcine homologs (FINGGH), the physiological activator of human hK4 is still unknown. In addition, since there is one putative N-linked glycosylation site at Asn110-Val111-Ser112 (Nelson et al., 1999), the size of the mature native hK4 may differ slightly from the predicted 27 kDa. This is in contrast to porcine EMSP1 (enamel matrix serine proteinase 1), where *N*-glycosylation occurs at either two or three residues, conferring the mature protein apparent molecular weights of either 34 or 37 kDa, respectively (Ryu et al., 2002).

Functional studies involving hK4 have only recently begun to emerge. Strong evidence suggests that the murine and porcine homologs of *KLK4* function in the maturation of dental enamel in concert with enamelysin and amelogenin (Hu et al., 2000, 2002; Nagano et al., 2003). Recently, a point mutation in the human *KLK4* gene was proposed as a likely cause of a recessive form of a dental enamel defect known as hypomaturation amelogenesis imperfecta, but it was excluded as a candidate defect in the autosomal dominant form of this disease (Hart et al., 2003, 2004). Other studies point to a physiological function of hK4 in prostate tissue and seminal plasma. At least *in vitro*, hK4 was shown to be a better activator of pro-PSA than hK2 (Takayama et al., 2001), raising the possibility that hK4, in concert with other serine proteases, has a role in a cascade of proteolytic reactions that involve activation of hK3 (PSA) and dissolution of the seminal clot (Takayama et al., 1997). Another possible functional role of hK4 in seminal plasma includes regulation of the seminal clot via prostatic acid phosphatase (PAP), since hK4 was demonstrated to degrade PAP completely *in vitro* (Takayama et al., 2001). So far, hK4 has been shown to be inhibited by aprotinin, benzamidine, soybean trypsin inhibitor, and tosyl-lysyl chloromethyl ketone (TLCK; Takayama et al., 2001).

The differential expression pattern of hK4 in prostate and ovarian cancer (Dong et al., 2001; Obiezu et al., 2001; Xi et al., 2004) raises the possibility that this serine protease may be involved in promoting cancer invasion and metastasis. Therefore, we hypothesized that hK4, a secreted serine protease, may facilitate degradation of extracellular matrix proteins. To gain further insight into the physiological role of hK4, we characterized its enzymatic activity using chromogenic tri-peptides, full-length proteins and serpins.

Results

Production of enzymatically active hK4

The recombinant protein produced in *P. pastoris* had apparent molecular masses of 30 and 25 kDa on an SDS gel, before and after deglycosylation, respectively. N-Terminal sequencing (Glu-Ala-Glu-Phe-Ile-Ile-Asn-Gly) indicated that the protein had undergone cleavage within the signal peptide, resulting in two extra amino acids (Glu-Ala), in addition to the two amino acids generated by the EcoRI cloning site (Glu-Phe). The underlined amino acids show the mature enzyme sequence. Zymography indicated that the purified recombinant hK4 was enzymatically active (see below), despite the extra four amino acids at its N-terminus. In the subsequent sections, we present data that correspond to our modified hK4 protein (Glu-Ala-Glu-Phe-Ile-Ile-Asn-Gly…hK4). These data may not be identical to those for native hK4 (Ile-Ile-Asn-Gly…hK4).

Enzymatic activity of hK4

Buffer optimization During initial testing, we found that Tris was slightly better than phosphate, as determined by the rate of fluorescence increase, although the difference was not statistically significant. Since Tris-based buffers were also used during purification, we selected it for further enzymatic kinetic experiments. Addition of EDTA also improved results slightly. The optimal pH was 8.0 (Figure 1). Therefore, in subsequent experiments, 0.1 M Tris-HCl buffer at pH 8.0, supplemented with 0.1 mM EDTA and 100 mM NaCl, was used. Tween-20 (0.01%) was also added to the kinetics buffer to reduce protein binding to the reaction tubes.

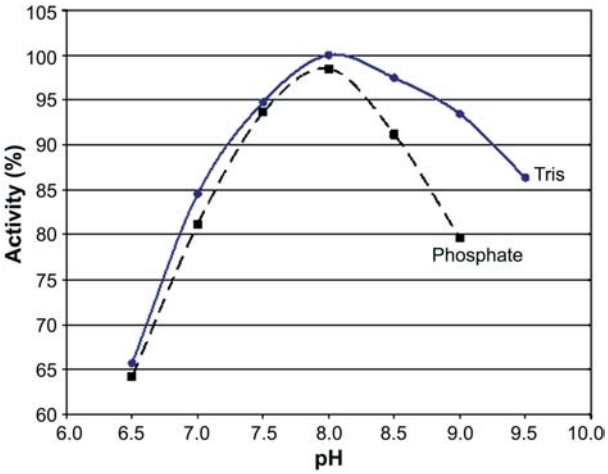

Figure 1 Percentage activity of hK4 with VPR-AMC substrate in Tris and phosphate buffers at various pH values.

Zymography Gelatin zymography (Figure 2) indicated production of active enzyme, but with much lower activity in comparison to trypsin.

Enzymatic specificity of hK4 using synthetic substrates

To assess preferences of hK4 at the P1, P2 and P3 substrate positions, we used 14 AMC-conjugated tri-peptides with arginine or lysine at the P1 position, as well as two tetrapeptides specific for chymotryptic activity. As shown in Table 1, the best substrates were VPR-AMC and VLK-AMC. The enzymatic activity of hK4 seems to be higher for substrates with Arg compared to Lys at P1 (e.g., compare GPR-AMC to GPK-AMC). hK4 did not demonstrate any enzymatic activity on either of the two chymotrypsin substrates (AAPF-AMC, LLVY-AMC). These results indicate that hK4 has a trypsin-like enzymatic activity with preference for arginine compared to lysine at the P1 site. Some general conclusions can also be drawn regarding preferences at P2 and P3 sites. Comparison of rates obtained for VPR-AMC and GPR-AMC indicates a marked preference for Val over Gly at P3. The least preferred substrates for hK4 are those with Leu (LRR, LKR, LGR) at P3, despite the presence of Arg at P1; indeed, the presence of Gln instead of Leu at P3 (QGR vs. LGR) favors the activity of hK4. The order of preference at P2 is Pro/Lys/Ala>Gly>Arg as deduced from the relative activity for the AMC conjugates QAR vs. QGR, LKR vs. LGR, GPR vs. GGR and QGR vs. QRR.

Kinetic studies For the two best tri-peptide substrates, we determined enzyme kinetic parameters to allow quantitative comparisons under Michaelis-Menten conditions. As illustrated in Table 2, the K_m value was only slightly lower for VPR-AMC compared to VLK-AMC, but the k_{cat} value was 4.2-fold higher, resulting in a 4.4-fold higher k_{cat}/K_m ratio.

Cleavage of protein substrates

As hK4 is at least partially secreted, we assessed its enzymatic activity with extracellular matrix protein substrates. As shown in Figure 3, hK4 cleaves intact fibrinogen efficiently. hK4 had highest activity against the α-chain of fibrinogen, as a visible reduction in band intensity was observed after 2 h, with complete degradation after 24 h. hK4 activity with the β-chain of fibrinogen resulted in a visible reduction of band intensity by 6 h, while the γ-chain was not cleaved, even after 24 h of incubation. Cleavage of fibrinogen by hK4 was confirmed by the appearance of cleavage products and by cleavage of fluorescent fibrinogen (Figure 3); this protein was a better substrate for hK4 in comparison to fluorescent collagens I and IV (data not shown).

Figure 2 Zymography of 1, 2 and 4 µg of hK4 on a 10% gelatin gel stained with SimplyBlue™ SafeStain.
Enzyme activity is indicated by clear bands. Trypsin (0.1 µg) was included for comparison.

Table 1 Estimated activity of hK4 on AMC conjugates.

Substrate	Relative activity
VPR	1.00
VLK	0.60
QAR	0.38
GPR	0.20
EKK	0.09
PFR	0.07
QGR	0.07
FSR	0.03
LKR	0.005
QRR	0.004
GGR	0.003
GPK	0.0002
LRR	0.00
LGR	0.00
AAPF	0.00
LLVY	0.00

Relative activity was estimated based on comparison of the rate of fluorescence increase per minute using 0.5 µg of hK4 with 1.6 mM AMC conjugate, designating activity for VPR as 100%. All amino acids are shown in single-letter code.

Inhibition of hK4 by protease inhibitors

As shown in Table 3, the hK4-specific monoclonal antibody did not significantly affect the activity of hK4, suggesting that it does not bind at or in the vicinity of the catalytic site. Inhibition by ACT (8%), ATIII (47%) α_1-AT (100%) and α_2-AP (98%) was also observed. More efficient inhibition by α_1-AT and α_2-AP compared to ACT reflects the trypsin-like substrate preference of hK4.

Initially, ATIII, α_2-AP and α_1-AT were incubated with hK4 for 0, 0.5, 1, 2 and 4 h to investigate the rate of SDS PAGE-stable complex formation. A complex was detected by Coomassie blue staining after 4 h of incubation with α_1-AT and α_2-AP, even at molar ratios of 1:2 (Figure 4). ATIII was not able to form detectable complexes (data not shown).

Confirmation that the complexes observed in Figure 4 are due to hK4 addition and not to the presence of a contaminant in the inhibitor preparations comes from the observation that the complex band is not evident in the absence of hK4 at the same inhibitor loading (cf. lanes 3 and 4 in Figure 4B). The incomplete complexation of hK4 in Figure 4A,B and the complete inhibition of hK4 activity by these inhibitors (Table 3) suggest that a portion of the hK4-inhibitor complex disassociates under SDS-PAGE conditions. This phenomenon has previously been observed for hK4-ATIII complex formation.

Using kinetic analysis, we showed that α_2-AP inhibition of hK4 activity was much faster (within 1–2 min) than inhibition by α_1-AT (>10 min; data not shown). In the

Table 2 Enzyme kinetic parameters for hK4 with AMC conjugates of VPR and VLK.

Substrate	V_{max}[a] (nmol min^{-1} l^{-1})	K_m[b] (mM)	k_{cat}[c] (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	hK4 (nM)
VPR	109.20	0.049	1.99	39.8	55
VLK	26.04	0.052	0.47	9.00	55

[a]V_{max} was obtained from a plot of initial velocity versus substrate concentration at 0.05, 0.1, 0.25, 0.5, 1.0, 1.5 and 3 mM (Michaelis-Menten and Lineweaver-Burk plots).
[b]K_m was obtained from Michaelis-Menten and Lineweaver-Burk plots.
[c]$k_{cat}=V_{max}$/hK4 concentration.

same analysis, α_2-M did not appreciably inhibit hK4 activity (data not shown).

Binding of hK4 (without inhibition of its activity) to α_2-M was demonstrated by fractionating serum samples and determining the recovery of added hK4 in the fractions. After spiking serum fractions with a fixed amount of hK4, we observed a significant loss of hK4 immunoreactivity (59–68% recovery) in fractions 22–24, corresponding to a molecular mass of 600–700 kDa (Figure 5). When radiolabel led hK4 was incubated with serum fractions, a large shift was observed (Figure 5) under denaturing and reducing conditions, suggesting covalent interaction of hK4 with a large (ca. 700 kDa) protein. The complex was not detectable on Western blots.

Discussion

The lack of studies on hK4 protein was initially due to the unavailability of recombinant protein and hK4-specific antibodies; functional studies were further hampered by the difficulty of activation of hK4 zymogen because of to the presence of an unusual amino acid, glutamine, at the pro-peptide cleavage site. The physiological activator of hK4 has been elusive, although the possible involvement of metalloproteases has been suggested (Nelson et al., 1999). To circumvent the difficulty of activating the hK4 zymogen, we expressed active recombinant hK4 without any sequence tags in the *P. pastoris* yeast expression system. However, our purified protein possesses four additional amino acids at its amino-terminus, which may affect its catalytic activity in comparison to native hK4. Thus, the data presented in this paper correspond to the modified form of hK4 and not necessarily to the native enzyme. Based on our fluorigenic tri-peptide data, active hK4 has a trypsin-like specificity; hK4 was not able to cleave any of the chymotrypsin-like substrates. This is in accordance with previous data (Takayama et al., 2001; Matsumura et al., 2005) and was not unexpected, given that trypsin-like specificity could be predicted by the presence of Asp 201 in the binding pocket of hK4 (Nelson et al., 1999). Evidence for Arg preference at P1 was also provided using substrates that share the same P2 and P3 (e.g., GPR-AMC vs. GPK-AMC; Table 1). These results are in general agreement with other studies; the specific activity of hK4 for VLR-pNA was shown to be approximately 4.5-fold higher than for VLK-pNA (Takayama et al., 2001; Matsumura et al., 2005). In comparison to the activity of trypsin, which has little preference for Arg over Lys, and has a 20-fold higher

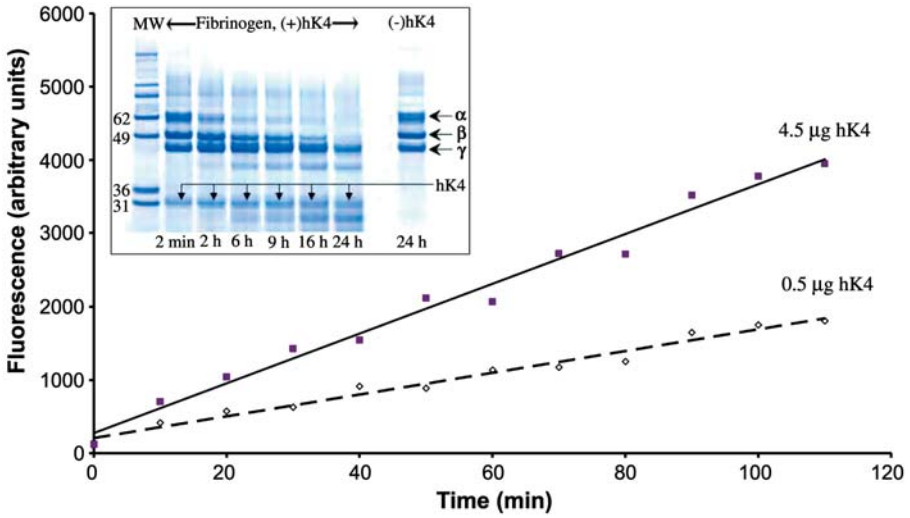

Figure 3 Cleavage of fluorescent fibrinogen (4 µg) by 0.5 and 4.5 µg of hK4.
Fluorescence induced by cleavage was monitored for 2 h. Insert: SDS-PAGE analysis of degradation of fibrinogen chains (10 µg) by 0.5 µg of hK4. Lane 1, molecular mass marker (MW) with corresponding masses in kDa; lanes 2–7, hK4 plus fibrinogen with varying incubations (shown) with vertical arrows marking hK4; last lane, fibrinogen-only reaction incubated for 24 h. The symbols α, β, γ indicate the fibrinogen chains. The gel was stained with SimplyBlue™ SafeStain to visualize bands. For more comments, see the results section.

Table 3 Percentage residual activity of hK4 with various inhibitors.

Inhibitor	Residual hK4 activity (%)
Monoclonal anti-hK4 antibody	77
ACT	92
ATIII	53
α_1-AT	0
α_2-AP	1.9

Percentage activity was tested after 1-h incubation at a 1:10 molar ratio of hK4/inhibitor using VPR as the substrate. For full inhibitor names, see the materials and methods section.

published k_{cat}/K_m value for VPR-AMC than we measured for hK4, hK4 appears to be a much weaker enzyme. For other kallikreins, such as hK6 and hK13, which have a preference for Arg at P1, the k_{cat}/K_m ratios of 84 and 353 mM^{-1} min^{-1}, respectively (Magklara et al., 2003; Kapadia et al., 2004), are approximately similar to hK4 with VPR-AMC as a substrate. As illustrated in Figure 1, hK4 activity was optimal at pH 8.0. This finding supports a possible function in seminal plasma, which has an average pH of 8.0. hK4 retains 40% of its activity at pH 10.3 (Takayama et al., 2001). Results with the fluorigenic tripeptides revealed that hK4 has some preferences among

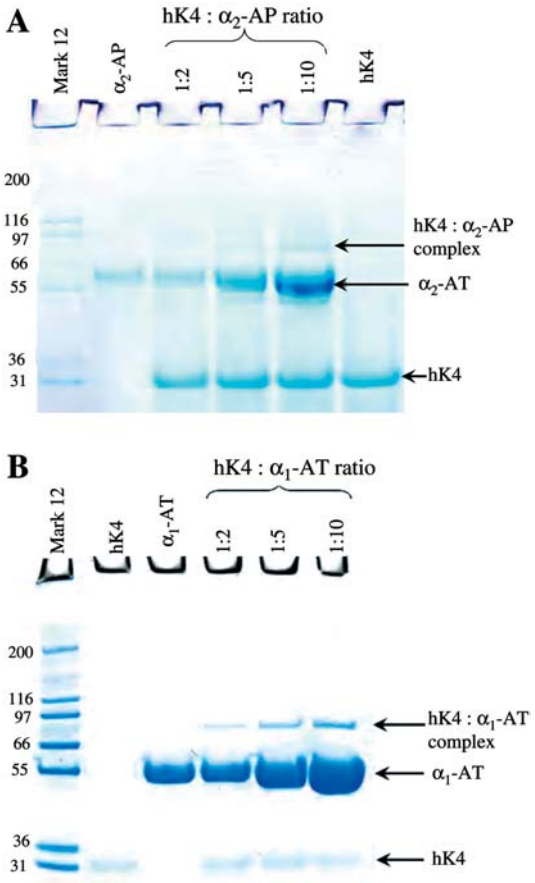

Figure 4 Visualization of SDS-PAGE stable complexes of hK4:α_2-AP (A) and hK4:α_1-AT (B) at molar ratios of 1:2, 1:5 and 1:10.
Reactions were incubated for 5 h prior to gel electrophoresis and gels were stained with SimplyBlue™ SafeStain. For more details, see the results section.

substrates that shared Arg at P1. As such, hK4 is more likely to have a specific regulatory role than a general degradative role within the human degradome.

hK4 was able to cleave components of the extracellular matrix such as fibrinogen and collagens I and IV. Fibrinogen degradation products have been associated with breast, bladder and ovarian cancer (Lipinska et al., 1976; Paternoster et al., 1981) and significant fibrinogen elevation in plasma was reported in prostate cancer (Caine et al., 2004). At the tissue level, the involvement of fibrinogen in prostate cancer is less clear (Wojtukiewicz et al., 1991; Pereira et al., 2002). As hK4 expression seems to be upregulated in prostate cancer (Xi et al., 2004), its possible involvement in prostate cancer pathobiology may be via the degradation of collagen and fibrinogen fibrils in the extracellular matrix, facilitating local spread of the tumor, possibly alongside other matrix-degrading proteases.

The synthesis of serine proteases in pro-enzyme form is a well-established regulatory mechanism, ensuring that enzymatic activity only occurs at the desired location, where the pro-peptide is removed by an activating protease. Crystallographic studies indicated that the pro-peptide partially covers the active site of the enzyme, which becomes accessible to substrates only upon its removal (Neurath, 1989; Khan and James, 1998). Comparison of the structure of chymotrypsin and its zymogen chymotrypsinogen suggests that there is no difference in the orientation of the His, Asp and Ser of the catalytic triad; however, Ile16 of chymotrypsin, exposed as the new N-terminus, is free to electrostatically interact with Gly193, a critical residue responsible for correctly orienting the substrate (Kraut et al., 1967; Wright, 1973; Wang et al., 1985). While all this implies that conformational change is limited by the presence of the pro-peptide, it is possible that pro-peptides may take up a conformation that allows some zymogens to possess limited proteolytic activity (Wang et al., 1985). The presence of Gln preceding the first Ile of the active form means that possible autolytic activation or activation by another serine protease is unlikely. While such unusual amino acid endings of pro-peptides such as Glu or Gly are known to exist for mast-cell serine proteases (Caughey, 2002), a similar mechanism for the activation of hK4 is not likely given that secreted hK4 is thought to be activated in the extracellular milieu.

Recent recognition of both inhibitory and promoting roles of proteases in cancer progression prompted a focus on the human degradome (Puente et al., 2003), with an emphasis on inhibitory therapeutic strategies (Abbott, 2004). While previous studies established inhibition of hK4 enzymatic activity by aprotinin and soybean trypsin inhibitor (Takayama et al., 2001), we evaluated inhibition by serpins. Serpins act by presenting their flexible reactive-center loop as a bait substrate, where cleavage of the P1-P1′ bond of the reactive-center loop allows insertion of the loop in the P1 side (Ye and Goldsmith, 2001). Among the inhibitors tested, α_2-antiplasmin (α-2-plasmin inhibitor, serpin F2) demonstrated the most potent inhibition. α_2-Antiplasmin, even at a 1:1.5 molar ratio, was able to suppress enzymatic activity within

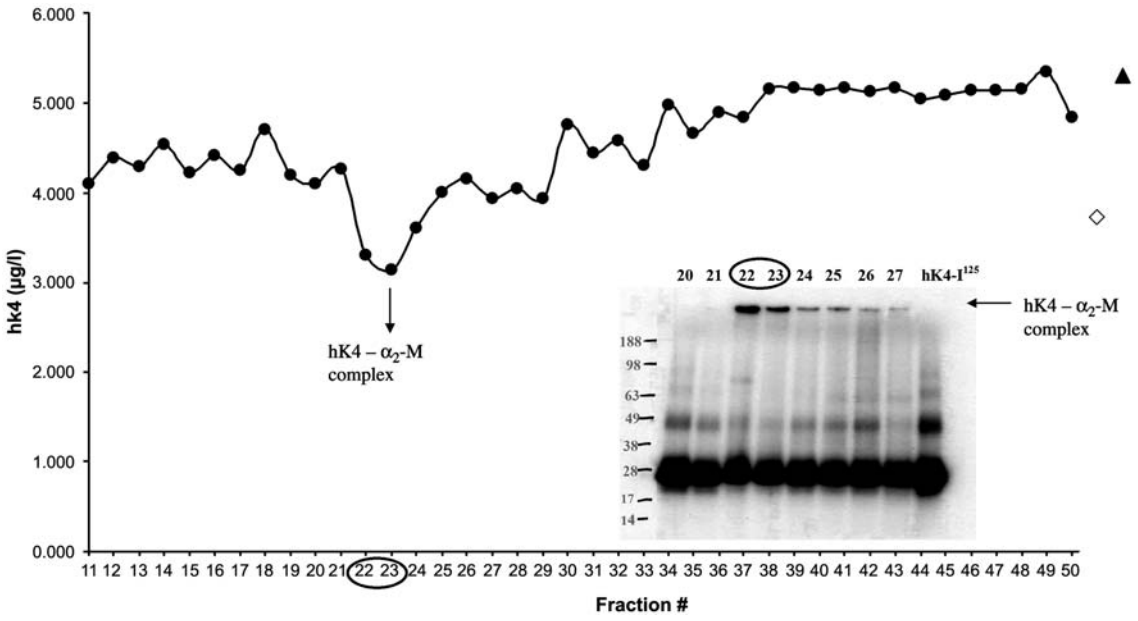

Figure 5 Plot of hK4 concentration versus fraction number for serum fractions spiked with a fixed amount of hK4, after gel filtration fractionation.
The plot reveals a significant loss of hK4 immunoreactivity (30–40%) in fractions 22–23 (circled). Insert: autoradiography of serum fractionated by gel filtration and spiked with [I^{125}]-labeled hK4. Last lane, labeled hK4 alone. Numbers on top correspond to serum fractions of the main figure. Fractions 22–23 correspond to a molecular mass of 600–700 kDa and likely represent α_2-macroglobulin (α_2-M) complexed with hK4. (▲) Recovery of added hK4 in a 6% BSA solution and (◇) recovery of hK4 added in unfractionated serum.

minutes. This inhibitor is unusual in its structure, as it has two reactive sites in its center loop (Arg 403-Met 404 and Met 404-S 405) for both trypsin- and chymotrypsin-like proteases (Potempa et al., 1988), as well as several stabilizing disulfide bonds not usually present in serpins (Christensen et al., 1997). Preference of hK4 for cleavage after arginine suggests that it likely interacts with α_2-antiplasmin via its Arg 403-Met 404 reactive site, known to be preferred by plasmin and trypsin as well.

Although kinetic experiments showed good inhibition of hK4 by ATIII, the formation of an SDS-PAGE-stable complex, a hallmark of the covalent nature of serpin-serine protease interaction (Longas and Finlay, 1980), could not be observed. This may have been due to formation of an SDS-PAGE unstable intermediate, similar to the complex between tPA and plasminogen activator inhibitor-1 (Declerck et al., 1992).

Among the inhibitors tested, one of the most potent was α_1-antitrypsin, forming SDS-PAGE-stable complexes with hK4. For α_1-antitrypsin, the major physiological inhibitor of elastase (Kalsheker, 1989), Met 358-Ser 359, acts as the bait peptide bond for proteases. However, inhibition of trypsin-like serine proteases by α_1-antitrypsin is not unexpected, as it can effectively inhibit other kallikreins such as hK3, hK6 and hK13 (Rittenhouse et al., 1998; Magklara et al., 2003; Kapadia et al., 2004). Moreover, α_1-antitrypsin is a potent inhibitor of trypsin, as shown by crystallographic studies, whereby the cleaved loop was observed to insert into trypsin (Huntington et al., 2000). In the case of hK4, less effective inhibition by α_1-antitrypsin compared to α_2-antiplasmin may be explained by a P1 Met in the former versus the preferred P1 Arg in the latter.

In this study, α_1-antichymotrypsin was not an effective inhibitor of hK4, although it can successfully inhibit other kallikreins (hK13, hK6, PSA and hK2) with trypsin- or chymotrypsin-like substrate specificities (Lilja et al., 1991; Lovgren et al., 1999; Magklara et al., 2003; Kapadia et al., 2004). Although the primary reactive bond of the α_1-antichymotrypsin reactive-center loop is Lys-Ser, serine proteases such as elastase actually cleave this inhibitor at the P5-P4 (Lys 378-Ile 379) position (Morii and Travis, 1983), suggesting the possibility of interaction of trypsin-like serine proteases at this site. Covalent complexing of serum α_2-macroglobulin and hK4 was demonstrated in this work by SDS-PAGE. However, using fluorigenic tripeptides, it was not possible to demonstrate inhibition to a great extent. This is likely due to the fact that small molecules still have access to the catalytic site of the protease engulfed by the α_2-macroglobulin homotetramer (Sottrup-Jensen, 1989). This unique mechanism of protease entrapment, together with the presence of three stretches of binding loops (Sottrup-Jensen et al., 1984) makes α_2-macroglobulin a highly effective inhibitor of proteases of a wide variety of specificity.

In conclusion, we demonstrate here for the first time the enzymatic activity of recombinant hK4 produced in a *P. pastoris* system. This kallikrein prefers Arg over Lys at the P1 position and displays some specificity for P2 and P3 amino acids. Its ability to form stable complexes with

several serpins further demonstrates its catalytic activity *in vivo*. The cleavage of extracellular matrix proteins by hK4 may pinpoint a link with prostate cancer progression.

Materials and methods

Production of enzymatically active hK4

Expression construct KLK4 cDNA, coding for the mature enzyme (amino acids 31–254; numbering based on GenBank accession no. NP_004908), was prepared in a 25-μl reaction volume using 1 μl of human prostate cDNA as template, 200 μM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 2.5 U of Pfu DNA polymerase (Stratagene, La Jolla, USA), 100 ng of the PCR forward primer 5′-AGC TGC GAA TTC ATC ATA AAC GGC-3′, and 100 ng of the PCR reverse primer 5′-TCC GCG GCC GCA GAG TTA ACT GGC CTG-3′. The PCR reaction was carried out in an Eppendorf thermocycler (initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 30 s; and final extension at 72°C for 10 min). Using the EcoRI and NotI restriction sites of the pPICZαA expression vector from the Easyselect™ *Pichia pastoris* yeast expression system (Invitrogen, Burlington, Canada), the PCR product was cloned using standard techniques (Sambrook et al., 1989) so that the expression construct contained the *KLK4* cDNA sequence cloned in-frame with the *P. pastoris* α factor secretion signal and its own TAA stop codon, but did not have the 3′ end myc epitope or the polyhistidine $(His)_6$-tag sequence.

Protein production The expression construct was transformed into the KM71H yeast strain and a stable transformant was selected as suggested by the manufacturer. Selected yeast clones were then grown in a 30°C shaking incubator to an OD_{600} of between 2 and 6 (approx. 24 h) in BMGH medium (100 mM potassium phosphate, pH 6.0, 13.4 g/l yeast nitrogen base, 0.4 mg/l biotin, 0.004% histidine and 1% glycerol). Cells were recovered by centrifugation at 3000 *g* for 5 min, and re-suspended in one-fifth of the original volume in BMMH medium (100 mM potassium phosphate pH 6.0, 13.4 g/l yeast nitrogen base, 0.4 mg/l biotin, 0.004% histidine, with methanol added daily to the medium to a final concentration of 0.5%). After 4 days, the supernatant was recovered from the culture by centrifugation. To test for the presence of recombinant hK4, the supernatant was run on an SDS-PAGE gel, followed by transfer of the protein samples onto a Hybond-C membrane (GE Healthcare, Piscataway, USA). Subsequently, the membrane was probed with hK4-specific rabbit antiserum [diluted 1:500 in Tris-buffered saline with Tween 20 (TBS-T) buffer] previously raised in-house (Obiezu et al., 2002). After extensive washing with TBS-T and application of a horseradish peroxidase-labeled goat anti-rabbit IgG (GE Healthcare), the signal was detected using the SuperSignal® West Pico chemiluminescent substrate (Pierce, Rockford, USA).

Protein purification Recombinant hK4 was purified from the medium using a combination of anion exchange and benzamidine column affinity chromatography. The 250-ml supernatant was diluted 1:4 in 10 mM Tris-HCl buffer, pH 8.0 and the pH was maintained at 8.0 with 3 M KOH. DEAE Sepharose™ Fast Flow beads (GE Healthcare) were washed according to the manufacturer's instructions. Beads were packed into an Econo-Pac (Bio-Rad, Hercules, USA) open column filled with buffer to give a 5-ml bed volume. The pH-adjusted supernatant was loaded onto the DEAE column, washed extensively with wash buffer (100 mM KCl, 10 mM Tris-HCl, pH 8.0), and eluted with 10 ml of each of 200, 300 and 400 mM KCl in 10 mM Tris-HCl, pH 8.0. These ion-exchange elution fractions were then loaded separately onto a Hi-Trap™ benzamidine FF column (GE Healthcare) previously washed and equilibrated with 500 mM KCl in 10 mM Tris-HCl, pH 8.2. The column was eluted 10 times with 1 ml of 1 M KCl in 10 mM Tris-HCl, pH 8.2. The purity and molecular weight of hK4 were assessed using SDS-PAGE, and gels were stained with Simply Blue™ SafeStain (Invitrogen) or the SilverXpress Silver staining kit (Invitrogen). The purest fractions were collected and concentrated using a Centricon™ centrifugal filter device (Millipore, Billerica, USA). Total protein content was determined using the BCA protein assay kit (Pierce) with BSA protein standards. The identity of the hK4 protein was confirmed by tandem mass spectrometry, as well as by N-terminal sequencing using protocols developed by Edman (1970). To characterize glycosylation of hK4, 5 U of peptide-N-Glycosidase F (PNGaseF) was incubated with purified hK4 for 1 h at 37°C according to manufacturer's instructions (Sigma-Aldrich, St. Louis, USA). Untreated and treated hK4 were then visualized by SDS-PAGE and SimplyBlue™ SafeStain.

Assessment of hK4 enzymatic activity

Buffer optimization The optimum buffer for analysis of hK4 enzymatic activity was determined using VPR-7-amino-4-methylcoumarin (AMC) as substrate in Tris (0.1 M NaCl, 0.1 M Tris-HCl) and phosphate buffers (0.1 M NaCl, 0.1 M Na_2HPO_4) at pH 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 in the presence of 0.01% Tween 20 and 0.2% BSA with or without 0.1 mM EDTA. Since hK4 demonstrated highest enzymatic activity in buffer containing 0.1 M Tris-HCl, 0.1 M NaCl and 0.1 mM EDTA at pH 8.0, this buffer was used in the kinetic studies.

Zymography Novex™ non-prestained Zymogram 10% gels (gelatin and casein) were used in conjunction with Tris-Glycine SDS Sample Buffer, Tris-Glycine SDS Running Buffer, Zymogram Renaturing Buffer and Zymogram Developing Buffer, as instructed by the manufacturer (Invitrogen). Briefly, samples of 1–4 μg of purified hK4 protein were mixed with 5 μl of Novex™ Tris-Glycine SDS (2×) sample buffer and the total volume was adjusted to 10 μl. For the positive control, 0.1 μg of trypsin was prepared in a similar manner. Protein samples were separated under non-reducing conditions for 90 min (125 V) on a 10% gelatin or casein gel. Protein samples in the gel were renatured with the buffer provided and then the gel was incubated for 4 h at 37°C in developing buffer. To visualize enzymatic activity, the gel was stained with SimplyBlue™ SafeStain (Invitrogen).

Determination of hK4 enzymatic specificity using fluorigenic peptides

Kinetic studies All amino acids are presented with single letter codes. AMC-conjugated synthetic peptides (PFR-AMC, QAR-AMC, QRR-AMC, VPR-AMC, FSR-AMC, QGR-AMC, GPR-AMC, GGR-AMC, VLK-AMC, EKK-AMC, GPK-AMC, LGR-AMC, LRR-AMC, LKR-AMC, LLVY-AMC, AAPF-AMC) were purchased from Bachem Bioscience (King of Prussia, USA). All substrates were diluted in DMSO to a final concentration of 80 mM and stored at -20°C. AMC was obtained from Sigma. AMC-conjugated peptide substrates (1.6 mM) were incubated either with or without 300 nM hK4 in kinetics buffer (100 mM Tris-HCl, 100 mM NaCl, 0.01 mM EDTA, pH 8.0). The final reaction volume was 100 μl, with each reaction set up in duplicate and incubated in 96-well microtiter plates at 37°C. The fluorescence generated by cleavage of the AMC-conjugated substrates was monitored for 20 min; measurements were taken every 1 min with a Wallac

Victor fluorimeter set at 355 nm for excitation and 460 nm for emission. Stock solutions (80 mM) of the two best substrates, VPR-AMC and VLK-AMC, were diluted in DMSO to obtain substrate concentrations ranging from 0.1 to 3 mM. Reaction volumes of 100 µl were set up as above using 50 nM hK4. In negative control reactions, buffer was substituted for hK4. Reactions were set up in triplicate and fluorescence was measured as above. Free AMC was incubated on its own at concentrations ranging from 100 to 5000 nM and a standard curve was constructed to convert the rate of fluorescence increase (fluorescence/min) to the rate of product formation (mM AMC conjugate cleaved/min). The Michaelis constants were calculated by nonlinear regression analysis using Enzyme Kinetics Module1.1 of Sigma Plot 8.0 (Sigma Plot, SPSS, St. Louis, USA).

Determination of enzymatic cleavage of protein substrates

Fluorigenic conjugates of collagen type I (from bovine skin), collagen type IV (from human placenta), and fibrinogen (from human plasma) were obtained from Molecular Probes (Burlington, Canada). To test the activity of hK4 on extracellular matrix proteins, mature hK4 was incubated with fluorigenic fibrinogen, collagen I and collagen IV in a 200-µl reaction volume in kinetics buffer. Negative control samples without hK4 were also set up, and the fluorescence (fluorescein) was measured every 10 min for 2 h in a Wallac Victor fluorimeter set at 492 nm for excitation and 535 nm for emission. The activity of hK4 on fibrinogen was also monitored using SDS-PAGE analysis followed by staining with SimplyBlue™ SafeStain, for which 10 µg of fibrinogen was incubated with 1 µg of hK4 in kinetics buffer supplemented with 0.01% Tween-20 for 2 min to 24 h. Controls of fibrinogen-only and hK4-only samples were also included.

Inhibition of hK4 by serine protease inhibitors

Initial assessment Serine protease inhibitors [α_2-antiplasmin (α_2-AP), α-1-antichymotrypsin (ACT), antithrombin (ATIII), α-1-antitrypsin (α_1-AT), α-2-macroglobulin (α_2-M)] were purchased from Calbiochem (Mississauga, Canada), diluted to 1 mg/ml and stored at -20°C until use. HK4 at a final concentration of 48 nM in the reaction mixture was used to assess the extent of inhibition by 480 nM (final concentration) of the above inhibitors. Our in-house anti-hK4 monoclonal antibody clone 10F4.5G6 was also tested at 240 nM. Reactions of 98 µl were set up in kinetics buffer (supplemented with 0.01% Tween 20) in duplicate, and reactions without hK4 were used for background readings. Reactions were incubated for 1 h at 37°C, followed by addition of 2 µl of 10 mM VPR-AMC. Fluorescence was measured in a Wallac Victor fluorimeter every min for 30 min.

Extent of inhibition After 5 h of incubation at 37°C, duplicate sets of reactions using a 1:5 enzyme/inhibitor molar ratio and corresponding negative control reactions (see above) were diluted into 270 µl of kinetics buffer, and split into three 95-µl reactions in microtiter plates. To each reaction, 5 µl of 80 mM VPR-AMC was added and the fluorescence was monitored for 40 min as above. In this set of experiments, the inhibitor-only reactions served as the background reading for the enzyme/inhibitor reactions, and the buffer-only reaction served as the background reading for the hK4-only reactions.

Speed of inhibition To monitor how quickly hK4 is inhibited, enzyme/inhibitor ratios of 1:1.5, 1:5 and 1:10 were prepared for α_2-AP and α_1-AT with hK4. Quadruplicate reactions of 98 µl were prepared by adding inhibitors, kinetics buffer and 0.2 mM VPR-AMC (final concentration) to microtiter plates and then preincubating the reactions at 37°C for 2 min. To two reactions of each inhibitor was added 2 µl of hK4 (48 nM final concentration) and the fluorescence was monitored for 45 min.

SDS-PAGE analysis of complex formation

Time course study Inhibition of hK4 by ATIII, α_2-AP, and α_1-AT was examined by incubating 30-µl reactions consisting of 4 µM inhibitor, 2 µM hK4 and kinetics buffer supplemented with 0.01% Tween 20 at various time points (0, 30 min, 1 h, 2 h and 4 h). Reactions were stopped by adding 10 µl of 4× SDS-PAGE sample buffer, followed by reduction with dithiothreitol (DTT) at 70°C for 10 min, and then separation by SDS-PAGE and staining with SimplyBlue™ SafeStain.

Extent of inhibition ATIII, α_2-AP, and α_1-AT were incubated with 42 µM hK4 at enzyme/inhibitor molar ratios of 1:2, 1:5 and 1:10 for 5 h at 37°C in 30-µl reaction volumes in kinetics buffer containing 0.01% Tween 20, except for the 1:5 ratio reactions and hK4-only reactions; these were initially set up in a volume of 60 µl and then split into halves before incubation. Two inhibitor-only (83.2 and 208 µM) reactions were also prepared to serve as the negative controls for SDS-PAGE and fluorimetric analysis, respectively. Following incubation, 10 µl of 4× SDS-PAGE sample buffer was added and the sample was reduced with DTT at 70°C for 10 min and separated by SDS-PAGE, followed by staining with SimplyBlue™ SafeStain. Duplicate sets of the 1:5 ratio, inhibitor-only (208 µM) and hK4-only reactions were diluted with 270 µl of kinetics buffer and 98 µl of each (in duplicate) were subjected to fluorimetric analysis with 0.2 mM VPR-AMC as substrate (final concentration).

Inhibition by serum components

Serum samples were leftovers from routine biochemical testing, stored at 4°C. For serum fractionation experiments, 250 µl of 10 male and 10 female serum samples were pooled and concentrated 10-fold in an Amicon Ultra-4 PLGC centrifugal concentrator (Millipore) at 4°C and 2000 *g*. Then 200 µl of the serum concentrate was separated on a silica-based TSK-GEL gel filtration column at a flow rate of 0.5 ml/min as described elsewhere (Yu and Diamandis, 1993), and 0.5-ml fractions were collected. Fractions were then diluted with 500 µl of 12% BSA solution and hK4 levels were measured using an in-house immunoassay described previously (Obiezu et al., 2002). In a parallel experiment, 2 µl of [I^{125}]-labeled hK4 (250 ng) and 12 µl of kinetics buffer were added to 10 µl of the serum fractions. The reactions were incubated for 2 h, followed by addition of 8 µl of SDS-PAGE 4× sample buffer (Invitrogen). Samples were heated for 10 min at 70°C, and 10 µl of the reactions were run on 4–12% Bis-Tris SDS-PAGE gels. Gels were then dried overnight after 5-min incubation with a 5% glycerol/30% ethanol solution. Autoradiograms were prepared by exposing Hyperfilm® (Invitrogen) to the gels for 24 h.

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