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Human tissue kallikrein 9: production of recombinant proteins and specific antibodies

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Abstract

Human tissue kallikreins (genes, *KLK*s; proteins, hKs) are a subgroup of hormonally regulated serine proteases. Two tissue kallikreins, namely hK2 and hK3 (prostate-specific antigen, PSA), are currently used as serological biomarkers of prostate cancer. Human tissue kallikrein 9 (*KLK9*) is a newly identified member of the tissue kallikrein gene family. Recent reports have indicated that *KLK9* mRNA is differentially expressed in ovarian and breast cancer and has prognostic value. Here, we report the production of recombinant hK9 (classic form) using prokaryotic and mammalian cells and the generation of polyclonal antibodies. Total testis tissue mRNA was reverse-transcribed to cDNA, amplified, cloned into a pET/200 TOPO plasmid vector, and transformed into *E. coli* cells. hK9 was purified and used as an immunogen to generate polyclonal antibodies. Full-length *KLK9* cDNA was also cloned in the vector pcDNA3.1 and was expressed in CHO cells. The identity of hK9 was confirmed by mass spectrometry. hK9 rabbit antiserum displayed no cross-reactivity with other tissue kallikreins and could specifically recognize *E. coli*- and CHO-derived hK9 on Western blots. hK9 was mainly detected in testis and seminal vesicles by Western blotting. The reagents generated here will help to define the physiological role of this tissue kallikrein and its involvement in human disease.

Keywords: antibody generation; cancer biomarkers; human tissue kallikreins; protein expression; protein purification; serine proteases.

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.

Introduction

Human tissue kallikreins are a group of 15 closely related genes, which are tandemly located in a 300-kb region on chromosome 19q13.3-19q13.4 and encode for serine proteases with various physiological functions (Diamandis et al., 2000a; Yousef and Diamandis, 2001). In addition to the three well-known human tissue kallikreins, namely, pancreatic/renal tissue kallikrein (hK1), human glandular tissue kallikrein 2 (hK2) and prostate-specific antigen (hK3, PSA) which are now considered as the classical human tissue kallikreins, in recent years, 12 additional tissue kallikrein genes have been identified in the same locus (Borgono and Diamandis, 2004; Clements et al., 2004).

Numerous reports indicate that human tissue kallikreins are associated with hormone-dependent malignancies. *KLK2*, *KLK3* (PSA) and *KLK11* are established or candidate serologic biomarkers for prostate cancer (Diamandis, 1998; Rittenhouse et al., 1998; Stephan et al., 2002; Nakamura et al., 2003). A variety of the newly identified tissue kallikreins have been shown to be differentially expressed in ovarian (Tanimoto et al., 1999; Dong et al., 2001; Yousef et al., 2001a; Diamandis et al., 2003; Luo et al., 2003a), breast (Chang et al., 2002; Luo et al., 2002; Yousef et al., 2002a,b, 2003; Talieri et al., 2004), and testicular (Chang et al., 2001; Luo et al., 2001a, 2003b; Yousef et al., 2001b, 2002c) cancer.

Human tissue kallikrein 9 (*KLK9*) was originally identified by Yousef and Diamandis (2000) as *KLK-L3* using the positional-candidate cloning approach (Collins, 1995). With the adoption of new nomenclature for human tissue kallikreins (Diamandis et al., 2000b), *KLK-L3* is now known as *KLK9*. The full sequence of the gene (GenBank accession no. AF135026) and its precise chromosomal localization have been characterized (Yousef and Diamandis, 2000).

KLK9 has five coding exons, spans an area of 7.1 kb on the long arm of chromosome 19 and is flanked by *KLK8* and *KLK10* genes (centromere–…*KLK8-KLK9-KLK10*…–telomere).

Similar to other tissue kallikreins, the encoded protein, hK9, is predicted to be synthesized as a pre-proenzyme (251 amino acids) which is processed into a mature form (229 amino acids). hK9 harbors a signal peptide of 19 amino acids and a 3-aa pro-segment (Yousef and Diamandis, 2000, 2001). *KLK9* mRNA, by RT-PCR analysis, is found in a wide variety of tissues (Yousef and Diamandis, 2000).

Quantitative real-time RT-PCR in ovarian cancer tissues of different stages, grades and histological types indicated that higher *KLK9* expression has favorable prognostic value in ovarian cancer. *KLK9* expression in early-stage, low-grade, and optimally debulked ovarian cancer patients was significantly higher than in late-

stage, high-grade and sub-optimally debulked patients, and patients with *KLK9*-positive tumors had longer progression-free and overall survival compared to those who were *KLK9*-negative (Yousef et al., 2001a). In a similar study, higher *KLK9* expression was associated with longer disease-free and overall survival of breast cancer patients (Yousef et al., 2003). Owing to a lack of methods for detection of hK9 protein, the prognostic and diagnostic significance of this tissue kallikrein in cancer remains elusive.

Among the 15 members of the *KLK* gene family, *KLK9* is the only tissue kallikrein for which production of recombinant protein has not been reported to date. In this study we describe the production of recombinant hK9 and generation of antibodies against this tissue kallikrein. Initially, we produced the pro-form of hK9 containing an N-terminal His-tag using the PET-200 *E. coli* expression system. The hK9 was purified and used as an immunogen to generate polyclonal antibodies. We then cloned the full length mRNA (pre-pro form) in pcDNA3.1. The construct was used to transfect Chinese hamster ovary (CHO) cells and a stable cell line secreting pro-hK9 with no tag was generated. The identity of the purified mammalian hK9 was confirmed by mass spectroscopy and N-terminal sequencing. Using Western blotting, the hK9 rabbit antiserum specifically recognized the mammalian hK9, showing no cross-reactivity with other tissue kallikreins. Western blotting of cytosolic extracts of various human tissues detected hK9 mainly in testis and seminal vesicle.

Results

Production and purification of pro-hK9 in *E. coli* cells

RT-PCR with primers specific for *KLK9* cDNA from testis resulted in a single band corresponding to the expected length of 696 bp. DNA sequencing of the *KLK9*/pET200 construct confirmed the presence of a pro-*KLK9* insert with no mutation and its correct position, in-frame with the N-terminal $(His)_6$-tag. Protein expression could be detected as early as 1 h after IPTG stimulation, but was maximal at 4 h after addition of 0.5 mM IPTG. In Figure 1, results of SDS-PAGE for whole cell bacterial extracts from BL21 cells transformed with the *KLK9* construct or control cultures are presented. hK9 was not produced in BL21 cells without the insert (lane 2) or in BL21 cells transformed with the *KLK9* construct in the absence of IPTG (lane 3). However, hK9 was visible as the most prominent band in cell extracts from BL21 cells that were transformed with the pET200-*KLK9* construct at 4 h after IPTG stimulation (lane 4). hK9 fusion protein dissolved in 8 M urea remained bound to Ni-NTA beads at pH values 5.5, which allowed extensive washing and removal of unbound impurities. However, the hK9 fusion protein was readily released from Ni-NTA beads at pH 5.0 (lane 5). The eluted hK9 was further purified using reversed-phase HPLC (lane 6), which also facilitated urea removal. Using these procedures, a final yield of 2 mg of highly purified hK9 per liter of BL21 culture was obtained. Western blot using anti-$(His)_6$-tag antibody (data not shown) and tandem mass spectrometry analysis of the purified protein unequivocally confirmed the identity of the recombinant fusion protein as hK9.

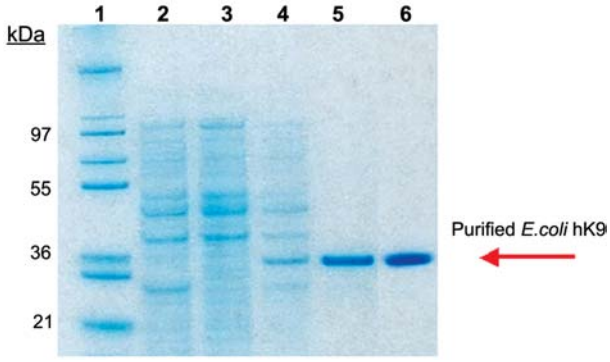

Figure 1 Detection of hK9 fusion protein in bacterial cell pellets and during purification steps by SDS-PAGE (Coomassie Blue stain).
Lane 1, Mark-12 molecular mass marker; lane 2, BL21 cell pellet (no insert); lane 3, BL21 cell pellet (containing hK9 insert) in the absence of IPTG, at 4 h; lane 4, BL21 cell pellet (containing hK9 insert) 4 h after IPTG stimulation; lane 5, purified hK9 after Ni-NTA chromatography; lane 6, purified hK9 after reversed-phase HPLC.

Antibody production

Using rabbit antiserum raised against the *E. coli* fusion protein, hK9 was readily detected by Western blotting, as shown in Figure 2. Whole cell extracts from BL21 *E. coli* cells without the insert (lane 2) and bovine serum albumin (BSA; lane 3) used as negative controls did not result in detection of any protein bands. hK9 rabbit antiserum could readily detect 20 ng of hK9 (lane 5), even when used at a 1:10 000-fold dilution. No protein was detected when hK9 rabbit antiserum was replaced with pre-immune rabbit antiserum. The specificity of hK9 rabbit antiserum was further tested by evaluating its possible cross-reactivity with 10 other recombinant tissue kallikrein proteins available in-house. Western blot analysis (Figure 3) indicated that hK9 (lane 8) was the only tissue kallikrein detected when probed with hK9 rabbit serum. Since these 10 control recombinant tissue kallikreins

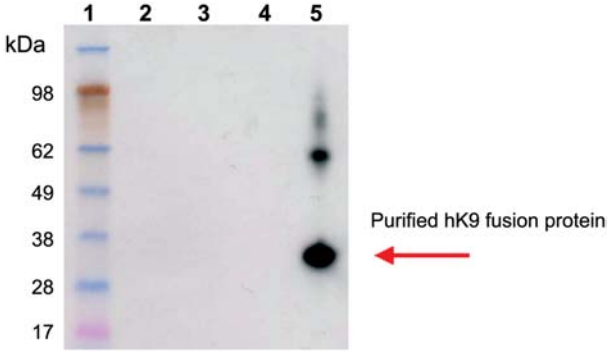

Figure 2 hK9 fusion protein as detected by Western blotting using 10 000-fold dilution of the hK9 rabbit serum.
Lane 1, SeeBlue plus2 molecular mass marker; lane 2, BL21 cell pellet (no insert); lane 3, BSA; lane 4, blank; lane 5, purified hK9 fusion protein.

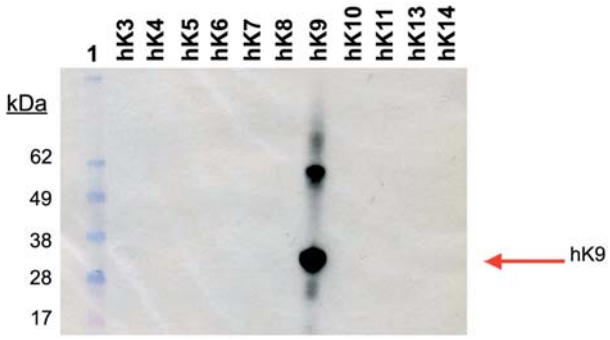

Figure 3 Western blot to test for cross-reactivity of hK9 rabbit antiserum with some other members of the tissue kallikrein family.
Samples of 100 ng of each protein were loaded into wells of a 12-well polyacrylamide gel. Note that no cross-reactivity with other tissue kallikreins is detectable.

were generated in various protein expression systems (*E. coli*, yeast, baculovirus, mammalian), the lack of any non-specific bands indicates that the hK9 rabbit antibody is highly specific. The antibody titer and specificity of the hK9 rabbit polyclonal were also tested using an enzyme-linked immunosorbent assay (ELISA). hK9 rabbit antibody, up to a dilution as high as 800 000-fold in dilution buffer (containing 6% BSA), produced a fluorescence signal that was significantly higher than non-immune rabbit control serum (data not shown).

Production and purification of hK9 in CHO cells

Unlike the pro-form of hK9 produced in *E. coli*, which contained an N-terminal His-tag, the mammalian form of full-length hK9 did not contain any additional amino acids. A concentration of 0.4 μg/ml geneticin was found to be optimal for selection of the pcDNA3.1/*KLK9* construct in CHO cells. A yield of 200 μg/l hK9 could be obtained from stably transformed CHO cells at the end of the culture period. hK9 was eluted from the FPLC column at NaCl concentration of 300 mM and from the C4 HPLC column at 40% acetonitrile. hK9 was predominantly detected at a molecular mass of 45 kDa. Tandem mass spectrometry unequivocally confirmed the identity of the 45-kDa band as hK9. N-terminal sequencing results (DTRAI) indicated that hK9 is secreted in its pro-form. In some purified culture supernatants, in addition to the 45-kDa band, two other bands (38 and 24 kDa) were also present. These bands (possibly degradation products of full-length hK9) were also identified as hK9 by mass spectrometry. hK9 rabbit antiserum generated against *E. coli* hK9 was capable of specifically detecting the mammalian from of hK9 (having no tag) obtained from transformed CHO cells (Figure 4, lane 1), whereas using non-immune rabbit serum resulted in detection of no bands.

Tissue Western blotting using hK9 rabbit serum

Results of the Western blot analysis for the detection of hK9 in cytosolic extracts of various normal human tissues are shown in Figure 4. The strong band in lane 1 of Figure 4 corresponds to CHO-derived mammalian hK9. This band is undetectable when hK9 rabbit serum is replaced with control non-immune rabbit serum (data not shown). hK9 could readily be detected in cytosolic cell extracts obtained from seminal vesicle and testis (Figure 4; lanes 7 and 8, respectively) as bands co-migrating with mammalian hK9. These bands were absent when hK9 rabbit antibody was replaced with non-immune rabbit antibody (data not shown). Upon prolonged exposure, a band of the same size, but with a much weaker signal, could be observed in breast, muscle, salivary gland, kidney and liver extracts. In addition, in pancreas a single band with a molecular mass of 17 kDa and in prostate, a very weak band with a molecular mass of 34 kDa could be observed. The presence of hK9 could not be detected in any of the other tissues tested.

Discussion

Two of the classical tissue kallikreins, hK2 and hK3, currently have utility in cancer diagnostics. With the identification of additional human tissue kallikrein genes, the potential value of this hormonally regulated family of serine proteases as biomarkers of cancer has been the focus of intense research. Numerous reports indicate that the mRNA of many of the newly identified tissue kallikreins is differentially expressed in hormone-dependent malignancies. To study the expression of tissue kallikreins at the protein level, the production of recombinant

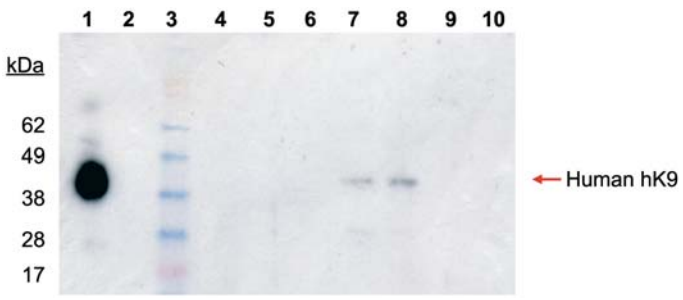

Figure 4 Presence of hK9 in various cytosolic extracts of human tissue as detected by Western blotting using hK9 rabbit serum as the primary antibody.
Lane 1, purified CHO-derived hK9; lane 2, blank; lane 3, SeeBlue plus2 molecular mass marker; lane 4, colon; lane 5, pancreas; lane 6, prostate; lane 7, seminal vesicle; lane 8, testis; lane 9, trachea; lane 10, uterus. Note the hK9 detection in seminal vesicle (lane 7) and testis (lane 8).

forms of these proteases (for functional studies as well as for antibody generation) has been very actively pursued. Generation of recombinant tissue kallikreins such as hK6, hK10, and hK11 in recent years and the consequent generation of antibodies against these tissue kallikreins has provided new avenues for characterizing their structure and function (Bernett et al., 2002; Gomis-Ruth et al., 2002), and has also resulted in the generation of highly sensitive serological assays. These assays confirm the potential utility of these tissue kallikreins as cancer biomarkers at the protein level (Borgono et al., 2004).

Among the 15 members of the *KLK* gene family, *KLK9* is the only tissue kallikrein gene for which production of recombinant protein has not been reported. We initially produced the pro-form of hK9 in *E. coli* as a fusion protein containing an N-terminal polyhistidine tag to facilitate protein detection and purification. Additional purification steps using reversed-phase HPLC provided some degree of added purity, but were mainly useful in the gradual removal of urea without precipitation of the recombinant protein.

The apparent molecular mass of hK9 fusion protein as determined by SDS-PAGE was slightly higher than its predicted molecular mass owing to the presence of the 3-kDa N-terminal fusion tag. Even though purified inclusion bodies can be used directly as an immunogen (Harlow and Lane, 1988), the additional purification steps used here resulted in production of hK9 with higher purity. hK9 rabbit antibody did not react with any of the proteins in whole-cell lysates of control BL21 cells. Among the tissue kallikreins tested, hK9 has the highest protein homology with hK11 and hK5 (40% and 38%, respectively). Western blot analysis (Figure 3) indicates that the hK9 rabbit antiserum has no cross-reactivity with either of these or any other tissue kallikreins tested. Since the recombinant tissue kallikreins used for Western blot were generated in various expression systems (*E. coli*, yeast, baculovirus, mammalian cells), the lack of any non-specific interaction indicates that the hK9 rabbit antibody is highly specific.

The mammalian form of hK9 was generated by transfection of CHO cells with the pre-pro-form of the *KLK9* construct. Since the hK9 rabbit antiserum generated earlier was suitable for the detection of CHO-derived hK9, we produced the mammalian hK9 without any additional fusion tag. Tissue kallikreins are secreted proteins and, as predicted, CHO-derived hK9 was detected in the culture supernatant in pro-form, and could be purified using FPLC and reversed-phase HPLC. The mammalian form of hK9 (as detected by Western blotting and confirmed by mass spectrometry) had a molecular weight of 45 kDa. However, in some purified culture supernatants, in addition to the 45-kDa band, two smaller bands (38 and 24 kDa) were present. Since these bands were also identified as hK9 fragments by mass spectrometry, they likely represent degradation products of the full-length hK9. The molecular mass of CHO-derived hK9 is significantly higher than the hK9 produced in *E. coli.* Similar discrepancies in the molecular mass of many other tissue kallikreins have been reported and can be explained by the fact that tissue kallikreins are glycosylated proteins (Yousef and Diamandis, 2001). In contrast to CHO cells, *E. coli* cells are not capable of glycosylation of recombinant hK9.

Western blot analysis for the detection of hK9 in cytosolic extracts of various normal human tissues indicated the presence of hK9 at highest levels in testis and seminal vesicle. Testis is among the tissues in which *KLK9* mRNA is maximally expressed; however, *KLK9* mRNA expression is not tissue-specific, since it has been detected in a wide array of tissues by RT-PCR (Yousef and Diamandis, 2000). It is likely that hK9 will be detected in additional tissues when more sensitive assays (sandwich ELISAs) for the detection of this tissue kallikrein become available.

mRNAs of many tissue kallikreins, including *KLK5, KLK9, KLK10, KLK12, KLK13* and *KLK14*, are highly expressed in normal testicular tissue (Luo et al., 2003b). Even though the physiological functions of these tissue kallikreins in testis are not clear, the expression of multiple tissue kallikreins in this tissue may be an indication that they participate in cascade enzymatic reactions to mediate processes such as spermiogenesis that require the function of proteases for extensive morphological and functional differentiation of newly formed spermatids. Many *in vitro* studies indicate that tissue kallikreins are able to activate other tissue kallikrein members (Kumar et al., 1997; Takayama et al., 2001a; Brattsand et al., 2005). Some tissue kallikreins are capable of degrading seminal fluid proteins such as seminogelins and prostatic acid phosphatase or activating seminal-fluid tissue kallikreins (such as PSA) *in vitro* (Takayama et al., 2001b). A possible role of hK9 in the activation of seminal-fluid tissue kallikreins, or its potential involvement in degrading seminal vesicle proteins, needs to be determined. Recent reports indicate that mRNA expression of *KLK5, KLK10, KLK13* and *KLK14* is universally down-regulated in testicular cancer (Chang et al., 2001; Luo et al., 2001a, 2003b; Yousef et al., 2001b, 2002c). The association, if any, of *KLK9* with testicular cancer merits further investigation.

Production of the different forms of hK9 reported in this study provides the research tools required to characterize the structure and function of this tissue kallikrein. Utilization of hK9-specific antibodies may be valuable in cancer diagnosis.

Materials and methods

Production and purification of hK9 in *E. coli*

PCR amplification of proKLK9 Commercially available total human testis-tissue RNA (Clontech, Palo Alto, CA, USA) was reverse-transcribed to cDNA using the Superscript™ pre-amplification system (Gibco-BRL, Rockville, MD, USA) according to the manufacturer's recommendations. Polymerase chain reaction was conducted using the proof reading enzyme Pfu (Stratagene Cloning Systems, La Jolla, CA, USA) and oligonucleotide primers (forward primer: 5′-CAC CGA CAC CCG TGC CAT CGG G-3′; reverse primer: 5′-TCA GTT CTC CAT GAT TTC TTG GAT-3′) specific to the pro-form of human *KLK9* (GenBank accession no. AF135026). PCR was performed using a thermal cycler (Eppendorf gradient Mastercycler). The PCR conditions were 95°C for 1.5 min, 39 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 50 s,

followed by a final extension at 72°C for 10 min. The PCR product was separated on a 1% agarose gel and purified. The amount of DNA generated by PCR was assessed by measuring the absorbance at 260 nm.

pET200-TOPO cloning of *KLK9* The blunt-ended PCR product was ligated into pET-200/D TOPO plasmid vector containing an N-terminal $(His)_6$-tag and was used to transform TOP10 chemically competent *E. coli* cells according to the vendor's instructions (Invitrogen, Carlsbad, CA, USA). Positive colonies were isolated on kanamycin agar plates and grown for plasmid isolation. Plasmid DNA containing the pET200-*KLK9* construct was isolated using a Qiagen (Valencia, CA, USA) plasmid purification system according to the vendor's instructions. The construct was sequenced in both directions using T7 forward and reverse primers with an automated DNA sequencer. The sequences were subjected to a homology search using the BLASTN algorithm.

Protein production using BL21 *E. coli* cells The pET200-*KLK9* construct was used to transform *E. coli* strain BL21(DE3) for protein production. Protein production was induced using 0.5 mM isopropyl β-D-thiogalactoside (IPTG). Samples were collected prior to the addition of IPTG and every hour thereafter. *E. coli* cultures were harvested 4 h after IPTG stimulation and the cell pellet was kept frozen at -20°C until use. Whole-cell extracts of cultured BL21 cells were subjected to SDS-PAGE and Western blotting. Western blots using anti-histidine (Anti-His G/AP; Invitrogen) antibody followed by tandem mass spectrometry were used to confirm the identity of hK9.

Purification of hK9 from BL21 *E. coli* cells BL21 *E. coli* cultures expressing hK9 were centrifuged (3000 *g*, 15 min, 4°C) to pellet the bacterial cells. The pellet was suspended in lysis buffer [50 mM K_2HPO_4, 0.4 M NaCl, 0.1 M KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole (Bioshop, Burlington, ON, Canada), pH 7.8]. The suspension was vortexed and centrifuged as above. The bacterial pellet was then re-suspended in 20 ml of the same lysis buffer, vortexed and sonicated on ice. Lysozyme was added to a final concentration of 1 mg/ml and the mixture was allowed to incubate for 30 min. The lysate was then freeze-thawed in liquid nitrogen three times. RNase A was added to the lysate to a final concentration of 5 μg/ml. After incubation for 30 min at room temperature DNase I (5 μg/ml) was added and the lysate was allowed to incubate for an additional 15 min at room temperature. The lysate was then centrifuged at 2000 *g* in a Sorvall centrifuge for 15 min at room temperature. The pellet (inclusion bodies) was re-suspended in 20 ml of 50 mM Tris, 0.1 M NaCl, pH 8.0, containing 2% Triton X-100, vortexed, allowed to incubate for 10 min and centrifuged. The pellet was further washed in the same buffer containing 2 M urea, followed by three washes with sterile water to remove Triton X-100. The final pellet (purified inclusion bodies) was either stored at -80°C or directly used for additional purification using nickel metal affinity chromatography as follows. hK9 was batch-purified from inclusion bodies under denaturing conditions by dissolving the inclusion bodies in pre-warmed (37°C) buffer A (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.8) for 1 h at room temperature. The solution was then centrifuged at 22 000 *g* for 15 min and the supernatant was collected and filtered using a 0.2-μm syringe filter. The filtrate was then added to 2.0 ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) resin previously equilibrated with buffer A. The filtrate/resin mixture was incubated at room temperature for 2 h while shaking at 200 rpm. Ni-NTA resin bound to His-tagged hK9 was then separated from the solution by low-speed centrifugation (800 *g*, 2 min, room temperature). Solution B (8 M urea, 20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.8) was then added to the Ni-NTA resin and allowed to mix for 5 min on a rotary shaker at 200 rpm. The Ni-NTA resin was then separated from the solution by low-speed centrifugation. In a similar manner, the resin was further washed using solution B adjusted to pH 6.0 and then pH 5.5 to remove impurities and unbound proteins. hK9 bound to Ni-NTA resin was then eluted by lowering the pH to 5.0. Following centrifugation, the supernatant containing pure hK9 was collected. To remove urea, hK9 was further purified by reversed-phase high-performance liquid chromatography (HPLC) on a C4 Vydac column (see below).

Production of polyclonal antibodies against hK9

Purified hK9 was used as an immunogen for production of polyclonal antibodies in female New Zealand white rabbits and female BALB/c mice. A sample of 100 μg of purified hK9 was diluted in complete Freund's adjuvant for the first subcutaneous (s.c.) injection and in incomplete Freund's adjuvant for subsequent s.c. injections. Test bleeds from immunized animals were drawn and the serum was tested for antibody generation. The ability of hK9 rabbit serum to detect hK9 was initially tested using an antibody capture immunoassay as follows: 50 ng of purified hK9 diluted in 100 μl of coating buffer (50 mM Tris-HCl, pH 7.8) was dispensed (in duplicate) into wells of a 96-well white polystyrene microtiter plate and incubated overnight without shaking at room temperature. The following day the plates were washed three times with washing buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4). hK9 rabbit serum (100 μl) used as the primary antibody in dilutions ranging from 1:1000 to 1:800 000-fold in dilution buffer (50 mM Tris-HCl, 6% BSA, 0.05% sodium azide, pH 7.8) was then dispensed into each well. After incubation for 2 h and washing, 100 μl of alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG diluted 3000-fold in dilution buffer was added to each well and allowed to incubate for 30 min. At the end of the incubation, the plates were washed six times. For detection of AP enzyme activity, a time-resolved immunofluorometric assay using an established method (Christopoulos and Diamandis, 1992; Luo et al., 2001b) was employed. In brief, 100 μl of 1 mM diflunisal phosphate (DFP) diluted in substrate buffer (0.1 mM Tris-HCl, 0.1 M NaCl, 1 mM $MgCl_2$, pH 9.1) was added to each well and allowed to incubate for 10 min. Then 100 μl of developing solution (1 M Tris-HCl, 0.4 M NaOH, 2 mM $TbCl_3$, 3 mM EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorimeter (Cyberfluor 615 Immunoanalyzer; MDS Nordion, Kanata, Canada). Serum obtained from non-immune rabbits was used in parallel as a negative control. Sera from animals with high antibody titers were further tested for specificity using Western blotting.

Electrophoresis and Western blot analysis

Protein samples were mixed with loading buffer (NuPage LDS, Invitrogen). Reducing agent (dithiothreitol, DTT) was added to a final concentration of 0.1 M. Samples were boiled for 10 min, cooled to room temperature and subjected to SDS-PAGE using a Novex Mini-Cell gel apparatus. A Mark 12 molecular weight standard and the SeeBlue Plus2 pre-stained protein marker (Invitrogen) were used as protein standards for molecular mass determination. For Western blots only pre-stained protein markers were used. Electrophoresis was conducted using a gradient 4–12% Bis-Tris NuPAGE pre-cast polyacrylamide mini-gel system with MES-SDS running buffer (Invitrogen). Gels were stained using Coomassie Brilliant Blue G-250 solution or processed further for Western blotting. Proteins were transferred to nitrocellulose membranes electrophoretically. Electroblotting was conducted using NuPAGE transfer buffer (Invitrogen) containing 10% methanol at a constant voltage of 30 V for 1 h. Membranes

were then removed and incubated in 20 ml of blocking solution (PBS+5% non-fat dry milk, w/v), covered with plastic wrap and stored at 4°C overnight with gentle agitation to block non-specific binding. Nitrocellulose membranes were then washed three times in Tris-buffered saline containing Tween-20 (TBST) (20 mM Tris-HCl, 140 mM NaCl, pH 7.5 plus 0.1% Tween-20 v/v) for 10 min. For the initial detection of hK9 (tagged with polyhistidine tag), membranes were probed with anti-histidine (Anti-His G/AP; Invitrogen) antibody conjugated to AP. The antibody was diluted 1:3000 in diluting buffer (TBST+5% non-fat dry milk, w/v). The nitrocellulose membranes were transferred to a tray containing the diluted antibody and incubated with gentle agitation for 2 h at room temperature. The blots were then washed six times with TBST. AP activity was then detected on an X-ray film using a chemiluminescent substrate (Pierce, Rockford, IL, USA).

For molecular mass determination the X-ray film was superimposed on a nitrocellulose membrane containing the SeeBlue plus2 marker and then photographed. Western blotting using hK9 rabbit serum as the primary antibody was performed in a similar manner, except that nitrocellulose membranes were probed with hK9 rabbit serum and then detected using AP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:15 000 as the secondary antibody. Serum obtained from pre-immune rabbit was used in parallel as a negative control.

Production and purification of hK9 using a stable mammalian cell line

Cloning of full-length *KLK9* cDNA in mammalian cells The full-length *KLK9* cDNA was cloned without any additional bases. The pre-pro-form of *KLK9* cDNA was generated by PCR using similar conditions as for generation of the pro-form of hK9, with the exception that the forward primer: 5′-CAC CAT GAA GCT GGG ACT CCT CTG T-3′ was used. The PCR product was ligated into the pcDNA3.1 TOPO expression system according to the vendor's instructions (Invitrogen, catalogue no. K4900-01). The construct was used to transform TOP10 *E. coli* cells, and positive clones were selected on ampicillin agar plates. Then plasmid DNA was isolated and used for DNA sequencing.

Production of hK9 using CHO cells CHO cells were transfected with the pcDNA3.1/KLK9 plasmid using PolyFect transfection reagent and were subjected to selection by growth in the presence of 0.4 μg/ml geneticin (Invitrogen) for 3 weeks. CHO cells stably transformed with full-length *KLK9* cDNA were cultured (37°C, 5% CO_2) to 80% confluence in 75-cm^2 polystyrene flasks with 25 ml of F12 media containing 10% fetal calf serum and 0.4 μg/ml geneticin. The cells were then detached using trypsin/EDTA, suspended in 75 ml of the serum-containing medium and were divided into three 175-cm^2 flasks. After growth for 1 day, the medium in each flask was replaced with 50 ml of CHO serum-free media (Invitrogen) containing 0.4 μg/ml of geneticin. After an additional 7 days the culture supernatant was recovered and the presence of hK9 was probed using the hK9-specific rabbit antibody raised against *E. coli* protein, as described above.

Purification of mammalian hK9 using FPLC and HPLC Recombinant hK9 was purified from the stably transformed CHO cell culture supernatant using FPLC (AKTA FPLC Chromatography System; GE Healthcare, Piscataway, NJ, USA). The supernatant was concentrated by passing it through an ultrafiltration membrane (Millipore 5000 MWCO) and applied to a HiTrap CM Sepharose FF cation-exchange column (5-ml bed volume; GE Healthcare) equilibrated with running buffer (50 mM sodium acetate, pH 5.3). Impurities were removed by repeated washing of the column with running buffer. hK9 was eluted using a linear gradient of elution buffer (0–1 M NaCl) at a flow rate of 3 ml/min. hK9 was eluted at approximately 300 mM NaCl. The fractions enriched with hK9 were diluted in 0.1% trifluoroacetic acid and loaded onto a C4 column (1-ml bed volume; Vydac, Hesperia, CA, USA) for additional purification using reversed-phase HPLC. A multi-step gradient of 10–80% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min was performed. hK9 eluted at approximately 40% acetonitrile. Acetonitrile from the fractions containing hK9 was evaporated using a SpeedVac apparatus (Savant Instrument Inc., Farmingdale, NY, USA). The identity of the purified protein was confirmed using Western blotting and mass spectrometry.

Identification of hK9 by mass spectrometry

hK9 produced in *E. coli* prior to and after purification, as well as purified mammalian hK9 produced in CHO cells, was analyzed by mass spectrometry. Protein samples were resolved by SDS-PAGE, the corresponding bands were excised from the gel, digested by trypsin and analyzed by nanoelectrospray tandem mass spectroscopy as detailed elsewhere (Luo et al., 2003c).

N-Terminal sequencing

N-Terminal sequencing was performed using the Edman degradation method. Following SDS-PAGE, hK9 was electroblotted onto a polyvinylidene difluoride membrane and stained with Coomassie Blue. The band was excised and used for sequencing.

Preparation of human tissue cytosolic extracts

Samples of 200 μg of various frozen (-80°C) normal human tissues (adrenal, bone, kidney, colon, liver, lung, muscle, pancreas, prostate, seminal vesicle, skin, spleen, testis, thyroid, stomach, small intestine, trachea, esophagus, breast, endometrium, fallopian tube, ovary, uterus, auxiliary lymph nodes, mesentery lymph nodes, salivary gland and spinal cord) were pulverized on dry ice to a fine powder. Then 2 ml of extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 surfactant, 1 mM phenylmethylsulfonyl fluoride, 1 g/l aprotinin and 1 g/l leupeptin; pH 8.0) was added to the tissue powder. The mixture was incubated for 30 min on ice with repeated shaking and vortex-mixing every 10 min. Mixtures were then centrifuged at 22 000 *g* at 4°C for 30 min and the supernatants (cytosolic extracts) were collected. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce) using known amounts of bovine serum albumin (BSA) as standards. The total amount of protein for each of the tissue cytosolic extracts was adjusted to 1 mg/ml. Samples of 10 μg of each extract were resolved by SDS-PAGE and analyzed by Western blotting using a rabbit antibody against hK9.

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