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Development of an immunofluorometric assay for human kallikrein 15 (KLK15) and identification of KLK15 in tissues and biological fluids

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Abstract

Background: Human kallikrein 15 (KLK15) may have some utility as a prostate, ovarian, and breast cancer biomarker, based on previous studies, which examined mRNA levels of *KLK15*. The aim of this study was to develop analytical technology for human kallikrein 15, including recombinant protein, specific antibodies, and a sensitive and specific ELISA immunoassay. The assay was then used to examine levels of KLK15 in tissues and biological fluids.

Methods: We produced human, recombinant pro-KLK15 in HEK 293 cells. Recombinant KLK15 was purified with various chromatographic steps and used to immunize rabbits and mice for production of KLK15 polyclonal antibodies. We used these antibodies to develop a highly sensitive and specific KLK15 immunoassay and to study KLK15 expression in various tissues and biological fluids.

Results: Large amounts of pure, recombinant KLK15 have been produced and characterized. KLK15 mouse and rabbit polyclonal antibodies have been employed for development of a KLK15 immunoassay. This assay has a lower detection limit of 0.05 μg/L, and no cross-reactivity with any of the other fourteen kallikreins. Using this assay, KLK15 was detected in prostate, colon, and thyroid tissues, as well as in breast milk and seminal plasma.

Conclusions: The KLK15 reagents developed here will allow for analysis of KLK15 protein expression levels in tissues and biological fluids, both normal and cancerous. This will expand upon previously characterized tissue *KLK15* mRNA expression studies which suggested that KLK15 might be useful as a biomarker for breast, ovarian, and prostate cancer. KLK15 is another serine protease that is produced in prostate and other tissues and is secreted in seminal plasma and other fluids. Its physiological function needs to be further elucidated.

Keywords: Kallikrein; KLK15; Immunoassay; Tissue expression; Serine proteases

Introduction

The human tissue kallikrein family, located on chromosome 19q13.4, consists of 15 genes, all predicted or shown to encode secreted serine proteases [1,2]. Kallikrein 15 (*KLK15*, *prostinogen*, *ACO protease*) is the most recently cloned member of the kallikrein gene family and maps between the two classical kallikreins, *KLK1* and *KLK3*, on the kallikrein locus [3]. mRNA studies indicate that *KLK15* is highly expressed in the thyroid, salivary, and adrenal glands, prostate, and colon ([1], and our unpublished observations). *KLK15* has also been found to be up-regulated by steroid hormones in the prostate cancer cell line LNCaP [3].

The function of the protein encoded by *KLK15* (KLK15) is currently unknown. However, KLK15 is predicted to be a secreted protein [1]. Preliminary functional studies indicate that KLK15 is a trypsin-like serine protease, preferring to cleave after arginine and/or lysine [4,5]. KLK15 has also been shown to cleave and activate pro-PSA (KLK3) into active PSA [3], indicating that perhaps KLK15 may be involved in an enzymatic cascade within the prostate [6].

Many kallikreins have been found to be useful cancer biomarkers (reviewed in [7,8]). KLK3 (PSA) and KLK2 have

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proven useful in diagnosing and monitoring prostate cancer patients [9,10]. mRNA studies indicate that *KLK15* may also have some utility as a cancer biomarker. *KLK15* has been shown to be up-regulated in cancerous versus non-cancerous prostate tissues, as well as in more aggressive prostate tumors, at the mRNA level [3,11], indicating that it may be useful for distinguishing between more or less aggressive forms of prostate cancer. *KLK15* may also serve as an unfavorable marker for ovarian cancer as it was found to be up-regulated in cancerous versus benign ovarian tumors [12]. *KLK15* was found to be a predictor of reduced progression-free and overall survival for ovarian cancer [12]. For breast cancer, however, mRNA studies suggest that *KLK15* may serve as a predictor of longer progression-free and overall survival [13].

Thus far, the clinical utility of *KLK15* as a cancer biomarker has been studied only at the mRNA level, and KLK15 protein levels in tissues and biological fluids have not been examined. This is due to the lack of reagents for monitoring KLK15 protein levels. The physiological function of KLK15 is currently unknown, and PSA is the only proposed KLK15 substrate [4]. To further elucidate this protein's role as a biomarker and to study its physiological role, well-characterized, recombinant protein is required. Recombinant KLK15 can also be used for production of specific monoclonal and polyclonal antibodies, for development of a KLK15 immunoassay, for enzyme kinetic studies and KLK15 substrate analysis.

We have expressed and characterized recombinant KLK15 using a mammalian expression system and used this recombinant protein to produce KLK15 polyclonal antibodies. Using our rabbit polyclonal antibody in combination with a KLK15 monoclonal antibody (obtained as a pre-release reagent from R&D Systems Inc.), we have developed a novel KLK15 "sandwich-type" immunoassay. We used this assay to analyze KLK15 expression in various human tissues and biological fluids. KLK15 is found primarily in prostate, colon, and thyroid tissues. KLK15 is also found in seminal plasma, confirming that it is a secreted protein.

Materials and methods

Cloning of KLK15 into a mammalian expression vector

KLK15 mRNA was obtained from LNCaP prostate cancer cells (purchased from ATCC), by Trizol (Invitrogen) extraction, as per the manufacturer's instructions. *KLK15* mRNA was reversed transcribed into first strand cDNA using superscript first strand synthesis (Invitrogen). *KLK15* cDNA (NM_017509) was amplified by PCR using the forward primer 5′-cacccaggatggtgacaagttg-3′ and reverse primer 5′-gtcacttcctcttcatggtttccc-3′. PCR was performed in a 25 μL reaction mixture containing 15 ng cDNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 100 ng of primers and 2.5 U of *pfu* turbo DNA polymerase (Stratagene). The PCR conditions were 94°C for 2 min, followed by 94°C for 1 min, 66°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR product was subsequently cloned into the pcDNA 3.1-v5-HIS-Topo vector (Invitrogen), using the manufacturer's recommended method. The correct sequence of the above construct was confirmed by sequencing.

Production of KLK15 in human embryonic kidney (HEK 293) cells

HEK 293 cells were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS). The *KLK15*-pcDNA3.1 construct was introduced into the HEK 293 cells using Fugene 6 transfection agent (Roche), as per the manufacturer's recommendations. pcDNA3.1 is neomycin (G418) resistant, and 48 h following transfection, 30 μg/mL G418 was added to the medium, as a positive selection agent. Massive cell death occurred within 2 weeks of G418 addition. Viable clones were visualized under the light microscope, marked, and picked via pipetting. Stable clones were picked and grown to confluency in DMEM containing 10% FBS and 15 μg/mL G418. KLK15 expression was monitored by Western blotting, using a rabbit polyclonal antibody developed in our laboratory against recombinant KLK15 protein produced in *E. coli*. The clone which produced the highest levels of KLK15 (clone C4) was characterized further. Once confluent in serum containing medium, C4 cells were grown in serum free CD CHO (BD Biosciences) medium containing 15 μg/mL G418, for 10 days, after which the cells were pelleted by centrifugation and the supernatant was retained for purification.

KLK15 purification from HEK 293 cells using cation exchange and reversed phase chromatography

Recombinant KLK15 was purified from HEK 293 cell culture supernatant using two stages of chromatography. Firstly, cation exchange chromatography was performed using an SP sepharose fast flow column (5 mL; GE Healthcare) and, secondly, reversed phase chromatography was performed using a C_4 column (Vydac). Briefly, SP sepharose beads previously activated with 1 M NaCl were equilibrated with 50 mM sodium acetate (pH 5.2). C4 cell supernatant was pumped through the SP sepharose column at a flow rate of 1.0 mL/min to allow for protein binding to the beads. The beads were then washed with 50 mM sodium acetate (pH 5.2). KLK15 was eluted using a step gradient starting with a linear gradient from 0 to 200 mM NaCl elution over 20 min followed by constant 200 mM NaCl over 20 min. This step was followed by a second linear gradient from 200 mM to 1 M NaCl over 40 min. KLK15 was eluted around 400 mM NaCl. Trifluoroacetic acid, as an ion-pairing agent, was added to this eluate (final concentration 10 mL/L), which was then loaded on a C_4 column equilibrated with 1 mL/L trifluoroacetic acid in water. A step gradient increasing from 28 to 40% in 1% increments over 80 min of acetonitrile in 1 mL/L trifluoroacetic acid was then performed. The fraction containing KLK15 was concentrated by evaporation of the acetonitrile. The purified material was

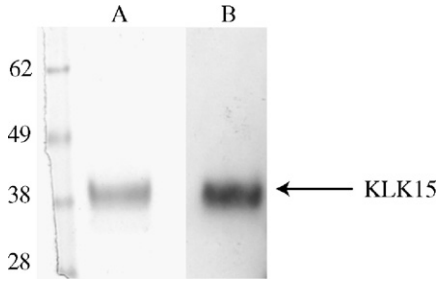

Fig. 1. Coomassie stained SDS-PAGE and a Western blot showing recombinant KLK15 protein produced by HEK 293 cells. Purified, recombinant KLK15 produced in HEK 293 cells was resolved by SDS-PAGE, and the protein was either (A) stained with Coomassie blue or (B) transferred to nitrocellulose for Western blotting with a KLK15 polyclonal antibody (raised against *E. coli* KLK15, produced in our laboratory). KLK15 appears as a diffuse band of approximately 38 kDa. On the left panel are molecular mass standards in kDa.

separated by SDS-PAGE and stained with Coomassie blue to assess its purity and molecular mass (Fig. 1A). Bands stained with Coomassie blue from this purified sample were subjected to electrospray ionization tandem mass spectrometry, as previously described [14], and N-terminal sequencing analysis to confirm their identity as KLK15 and their N-terminal sequence as the proform of KLK15. Both of these methods have been described in detail elsewhere [14].

Production of KLK15 specific polyclonal antibodies

Purified, recombinant KLK15 produced in HEK 293 cells was used as an immunogen to immunize BALB/c mice and New Zealand white rabbits. KLK15 (100 μg) was injected subcutaneously into mice and rabbits. The protein was diluted 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the subsequent injections. Injections were repeated six times at 3-week intervals. Blood was drawn from the animals and tested for antibody generation. To test for production of anti-KLK15 polyclonal antibodies, we used the following assay. Fifty nanograms of purified KLK15 protein diluted in 50 mmol/L Tris buffer, pH 7.80, was immobilized on 96-well white ELISA plates and incubated overnight. The rabbit serum (immune and non-immune) was then applied to the plates in different dilutions ranging from 1:10,000 to 1:1,000,000. After 1 h incubation, the plates were washed 6 times in washing buffer (9 g/L NaCl, 0.5 g/L Tween 20 in 10 mmol/L Tris buffer, pH 7.40). 100 μL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific (Jackson ImmunoResearch), diluted 1:5000 in 6% BSA was added to each well and incubated for 45 min. The plates were then washed as above. Diflunisal phosphate [100 μL of a 1 mmol/L solution in substrate buffer (0.1 mol/L Tris pH 9.1), 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$] was added to each well and incubated for 10 min. Developing solution (100 μL, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Noridion). The calibration and data reduction were performed automatically, as described in detail elsewhere [15].

Blood was also drawn from the animals and tested for antibody generation by Western blotting. Blood was diluted 1:3000 in 1% milk in TBST (25 mM Tris–HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20) for Western blotting.

Production and purification of recombinant KLK15 from E. coli

Briefly, *KLK15* cDNA was obtained from LNCaP prostate cancer cells as above and was amplified by PCR, using the forward primer 5′-cacccaggatggtgacaagttg-3′ and reverse primer 5′-gtcacttcctcttcatggtttccc-3′, using the conditions used in the above section. The PCR product was cloned into pET 200 *E. coli* expression vector (Invitrogen), using the manufacturer's recommended method. The correct sequences of the above constructs were confirmed by an automated DNA sequencer.

E. coli BL21 cells were transformed with the KLK15-pET200 expression plasmid, and KLK15 expression was induced with 0.5 mM IPTG for 5 h. Inclusion bodies were recovered by centrifuging the *E. coli* cells for 20 min at 5000×*g* and resuspending them in 50 mM NaH_2PO_4, 300 mM NaCl, and pH 8 containing 1 mg/mL lysozyme. Resuspended cells were kept on ice for 30 min, following which RNAseA and DNAseI were added. The cell lysate was then subjected to three freeze–thaw cycles and centrifuged at 20,000×*g* for 40 min to pellet the inclusion bodies. Inclusion bodies were resuspended in 100 mM NaH_2PO_4, 10 mM Tris–HCl, 8 M urea, pH 8.0, and incubated at room temperature for 45 min with rotation. The cell lysate was then centrifuged at 10,000×*g* for 20 min, and the supernatants were retained for KLK15 purification. KLK15 containing supernatant was purified as described in detail elsewhere [16].

Treatment of recombinant KLK15 with peptide: N-glycosidase F (PNGase F)

KLK15 was treated with PNGase F, according to the manufacturer's recommended method (New England Biolabs). Briefly, 10 μg of purified KLK15 was incubated 1:1 with denaturation buffer for 10 min at 100°C. 10× G7 buffer and NP-40 (1/10) were then added to the reaction mixture, along with 1 μL of PNGase F, and the reaction was incubated at 37°C for 1 h. Samples were stored at −20°C until needed.

Development of a KLK15 immunoassay

A monoclonal antibody against full length, human recombinant KLK15 (produced in the murine myeloma cell line NSO) clone 820, was obtained from R&D Systems (as a pre-release reagent). White polystyrene microtiter plates were coated with the monoclonal antibody (100 μL of coating antibody solution containing 250 ng of antibody diluted in 50 mmol/L Tris buffer, pH 7.80 in each well) by incubation overnight at room temperature. The plates were then washed

two times with washing buffer (9 g/L NaCl and 0.5 g/L Tween 20 in 10 mmol/L Tris buffer pH 7.40). KLK15 calibrators or samples were then pipetted into each well (50 μL/well along with 50 μL of assay buffer (60 g/L BSA, 50 mmol/L Tris (pH 7.80), 0.5 g/L sodium azide, 25 mL/L normal mouse serum, 100 mL/L normal goat serum, and 10 g/L bovine IgG)) and incubated for 1 h at room temperature with shaking. The plates were then washed with wash buffer six times. KLK15 rabbit polyclonal antibody was then diluted 3000-fold in assay buffer, and 100 μL was added to each well. After 1 h incubation at room temperature with shaking, the plates were washed six times in wash buffer. 100 μL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc fragment specific), diluted 5000-fold in assay buffer, was then added to each well and incubated for 45 min, and plates were washed as above. Diflusinal phosphate was then added, and the assay was completed as described above.

Human tissue cytosolic extracts and biological fluids

Human tissue cytosolic extracts were prepared as follows: various frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Extraction buffer (1 mL, containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 g/L NP-40 surfactant, 1 mmol/L phenylmethylsulfonyl fluoride, 1 g/L aprotonin, and 1 g/L leupeptin)) was added to the tissue powders, and the mixture was incubated on ice for 30 min with repeated shaking and vortex-mixing every 10 min. Mixtures were then centrifuged at 14,000×*g* at 4°C for 30 min. The supernatants (cytosolic extracts) were then collected.

The biological fluids were leftovers of samples submitted for routine biochemical testing. All tissue extracts and biological fluids were stored at −80°C until use.

Fractionation of seminal plasma with size exclusion HPLC

Two hundred microliters of recombinant KLK15 (500 μg/L diluted in 6% BSA) and 300 μL of seminal plasma (diluted 1:1 in 0.1 M NaH_2PO_4, 0.1 M Na_2SO_4 pH 6.5) were fractionated using gel filtration chromatography, as described elsewhere [17]. The fractions were collected and analyzed for the presence of KLK15 and KLK3 using KLK15 and KLK3 [18] immunoassays, respectively.

Results

Recombinant KLK15 produced in human embryonic kidney (HEK 293) cells

cDNA encoding the proform of *KLK15* was cloned into the mammalian expression vector pcDNA3.1-v5-His-topo; the construct was designed so that pro-KLK15 would be secreted into the cell culture supernatant. A stable cell line expressing pro-KLK15 was created, and pro-KLK15 was purified from the cell culture supernatant by successive chromatographic steps. Purified pro-KLK15 was resolved by SDS-PAGE and stained with Coomassie blue, as shown in Fig. 1A. A Western blot of purified pro-KLK15 was performed using a KLK15 rabbit polyclonal antibody, produced previously in our laboratory (raised against *E. coli*-produced KLK15) (Fig. 1B). pro-KLK15 produced by HEK 293 cells appears as a diffuse band of approximately 38 kDa molecular mass. The 38 kDa band was confirmed as KLK15 by electrospray ionization tandem mass spectrometry. A total of ten peptides were identified, providing coverage of the entire KLK15 protein (Table 1). Edman degradation performed on the 38 kDa band revealed the N-terminal sequence of recombinant KLK15, D G D K L L, matching the first five amino acids of the proform of KLK15, as previously reported [3].

Glycosylation status of recombinant KLK15

The diffuse appearance of KLK15, visualized by Coomassie staining, is characteristic of a glycosylated protein. The larger than expected size of KLK15, 38 kDa (predicted to be 30 kDa), also suggests glycosylation of KLK15 produced by HEK 293 cells. KLK15 has two predicted glycosylation sites at amino acids 171 and 232 [3].

To assess the glycosylation status of KLK15 produced in HEK 293 cells, we treated KLK15 with the deglycosylation enzyme PNGase F. Fig. 2 shows the results of PNGase F treatment. Before PNGase F treatment, the multi-molecular mass bands, of approximately 38 kDa, are present when stained with Coomassie blue (left panel), and they are immunoreactive when immunoblotted with a KLK15 polyclonal antibody (right panel). Following treatment with PNGase F, the larger multi-molecular mass bands are no longer present, and instead, a single lower

Table 1
Peptides identified by mass spectrometry analysis of recombinant *KLK15* produced by HEK 293 cells

KLK15 form	# of peptides	Peptides identified	Location [a]
Mammalian (38 kDa)	10	LLEGDECAPHSQPWQVALYER	22–42
		FNCGASLISPHWVLSAAHCQSR	45–66
		VRLGEHNLR	70–78
		LGEHNLRK	72–79
		RDGPEQLR	80–87
		DGPEQLR	81–87
		LNPQVRPAVLPTR	118–130
		CPHPGEACVVSGWGLVSHNEPGTAGSPR	131–158
		LTNTMVCAGAEGR	188–200
		VCHYLEWIR	242–250

[a] Amino acid numbering based on Genbank accession # NM_017509.

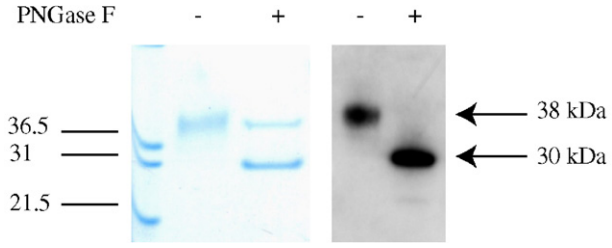

Fig. 2. Coomassie stained SDS-PAGE and a Western blot showing recombinant KLK15 before and after treatment with PNGase F to assess glycosylation status of the protein. Ten micrograms of purified KLK15 produced in HEK 293 cells was treated with PNGase F. The treated protein was separated by SDS-PAGE alongside untreated protein. One gel was stained with Coomassie blue (left panel), and the other was transferred to nitrocellulose for Western blotting (right panel). The 38 kDa band is seen on the Coomassie stained gel and by Western blotting with our rabbit polyclonal antibody (raised against KLK15 produced in *E. coli*), in the untreated protein. A single, 30 kDa band can be seen in the lanes containing the PNGase F treated protein, by both Coomassie staining and Western blotting. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

molecular mass band of 30 kDa is present. This single band is visible when stained with Coomassie blue and immunoreactive when immunoblotted with the KLK15 polyclonal antibody. The shift in molecular mass indicates that KLK15 is glycosylated.

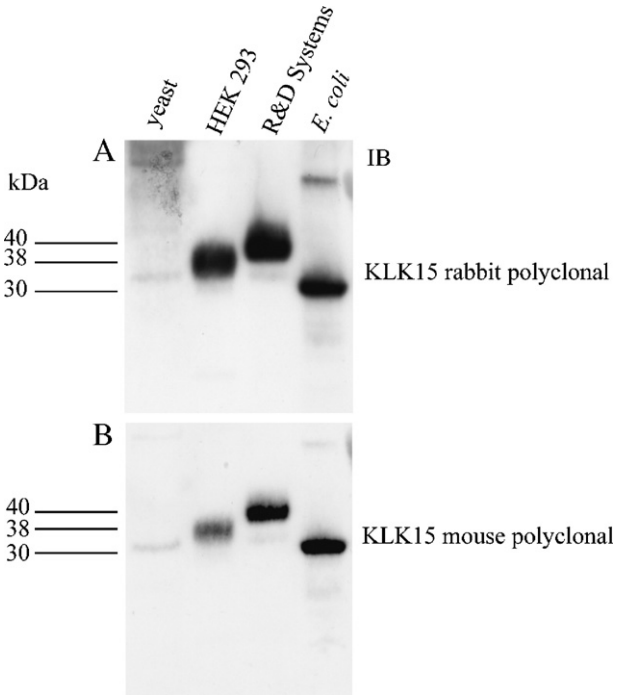

Fig. 3. Western blots show that KLK15 mouse and rabbit polyclonal antibodies recognize yeast, HEK 293, R&D Systems Inc., and *E. coli*-produced recombinant KLK15 protein. Recombinant KLK15 produced in yeast cells, HEK 293 cells, by R&D Systems Inc, and in *E. coli* cells was resolved by SDS-PAGE and transferred to nitrocellulose for Western blotting. (A) Western blotting with our rabbit polyclonal antibody raised against HEK 293 produced KLK15 protein, (B) Western blotting with a mouse polyclonal antibody raised against HEK 293 produced KLK15 protein. Note that both polyclonal antibodies recognize KLK15 produced in yeast (33 kDa), mammalian cells (38 kDa (HEK 293), 40 kDa (R&D Systems)), and *E. coli* (30 kDa). Both antibodies were used at a dilution of 1:3000.

Production of KLK15 antibodies

Recombinant KLK15 produced by HEK 293 cells was used as an immuogen in rabbits and mice for production of polyclonal antibodies. Fig. 3 shows the results of Western blotting using our polyclonal rabbit (Fig. 3A) and mouse (Fig. 3B) antibodies. Recombinant KLK15 proteins produced by us in *E. coli*, *P. pastoris*, and HEK 293 cells, as well as recombinant KLK15 purchased from R&D Systems (produced in murine NSO cells), were resolved by SDS-PAGE and blotted with each of the above antibodies. The results shown in Fig. 3 indicate that both polyclonal antibodies recognize all four forms of KLK15, but with less efficiency in the case of yeast protein.

KLK15 specific immunoassay

The KLK15 immunoassay was developed and optimized as described in the Materials and methods section. Pure, recombinant KLK15 was diluted in 6% bovine serum albumin (BSA) to produce six assay calibrators of the following concentrations: 0, 0.05, 0.2, 1.0, 5.0, 20.0 μg/L. The lower detection limit of this assay was 0.05 μg/L, and a typical calibration curve is shown in Fig. 4. Cross-reactivity was assessed against the other fourteen human kallikreins, each at a concentration of 1000 μg/L (all produced in our laboratory). Our immunoassay shows 0.04% and 0.2% cross-reactivity with recombinant KLK15 and KLK9 respectively and no detectable cross-reactivity with the other human kallikreins.

KLK15 in tissue extracts and biological fluids

Tissue extracts

Three adult tissue extracts colon, prostate, and thyroid contained small amounts of KLK15, 36, 3.3, and 8.0 ng/g respectively. The other tissue extracts (adrenal, axillary lymph node, bone, breast, endometrium, esophagus, fallopian tube, kidney, liver, lung, mesentery lymph node, muscle, ovary, pancreas, seminal vesicle, skin, small intestine, spleen, stomach, testis, trachea, ureter, uterus, and salivary gland) contained no KLK15, or perhaps KLK15 levels below the detection limit of the immunoassay.

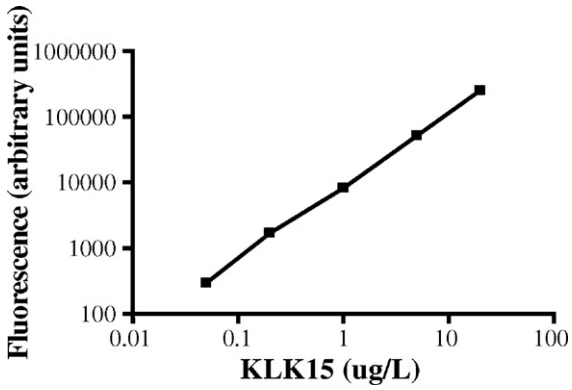

Fig. 4. A typical calibration curve for the KLK15 immunoassay, showing the lower detection limit of the immunoassay as 0.05 μg/L.

Biological fluids

In a panel of biological fluids (six samples each), KLK15 was undetectable in urine, cerebral spinal fluid, follicular fluid, amniotic fluid, and ascites of ovarian cancer patients. Of six breast milk samples tested, four were KLK15 positive (0.95, 7.8, 13.6, and 43 μg/L). Of twelve breast cancer cytosols, two were KLK15 positive (0.08 and 0.17 μg/L). Four saliva samples were examined, of which one was positive (0.11 μg/L). A panel of eighty-four seminal plasma samples was tested, seventy-nine of which were KLK15 positive, ranging from 0.12 to 16 μg/L.

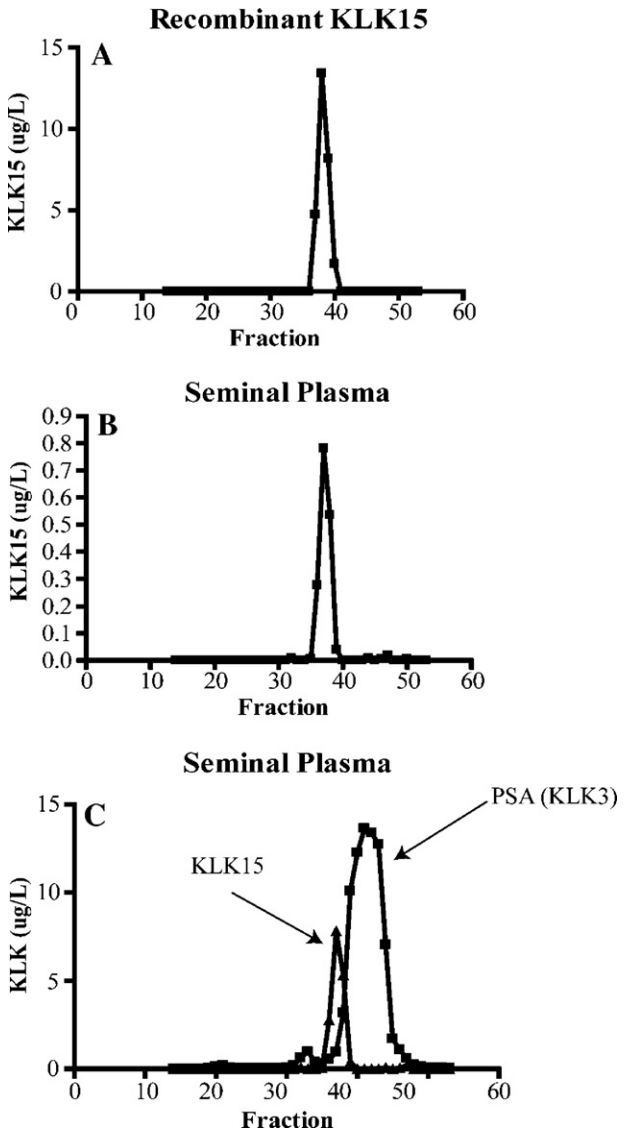

Fig. 5. Graphs showing elution time for recombinant KLK15 and endogenous KLK15 from seminal plasma samples by gel filtration chromatography. Samples of recombinant KLK15 or seminal plasma were separated by size exclusion chromatography using a gel filtration column. Fractions were collected and analyzed by ELISA for KLK15. Panel A shows that recombinant KLK15 elutes around fraction 39, corresponding to a 38 kDa protein. Panel B shows that endogenous KLK15 in seminal plasma also elutes around fraction 39, corresponding to a 38 kDa protein. Panel C shows that endogenous KLK3 (PSA) elutes around fraction 42, corresponding to a 32 kDa protein. Note that fractions were diluted 10^6-fold for KLK3 measurement, and KLK15 values were multiplied by 10 (panel C only), for clearer representation.

Size fractionation of KLK15 by size exclusion HPLC

To determine the molecular mass of endogenous KLK15, as determined by the KLK15 immunoassay, samples were fractionated, according to size, using a gel filtration column. Fractions were then analyzed by our ELISA.

Gel filtration chromatography was performed for recombinant KLK15 and seminal plasma. In both cases, one immunoreactive peak eluted around fraction 39, corresponding to a molecular mass of approximately 38 kDa (Figs. 5A, B). Eluted seminal plasma fractions were also subjected to analysis by a KLK3 (PSA) immunoassay to rule out cross-reactivity due to the very high levels of KLK3 in seminal plasma. *KLK3* eluted as a single, distinct peak around fraction 42, corresponding to a molecular mass of approximately 32 kDa (Fig. 5C).

Discussion

Human kallikrein 15 although cloned in 2001 [3] has received little attention, thus far particularly at the protein level. This can be attributed to the lack of suitable reagents. We developed tools useful for characterizing the tissue expression of this protein and for determining whether KLK15 has clinical utility as a biomarker for prostate and/or ovarian cancer, as suggested by mRNA studies [3,11,12]. Here we describe, for the first time, production and characterization of recombinant KLK15 protein, KLK15 specific antibodies, and a sensitive and specific KLK15 immunoassay. Our mammalian expression system (HEK 293 cells) ensured correct protein folding and post-translational modifications. HEK 293 cells were specifically used to create a stable cell line, primarily because of their high transfection efficiency, allowing for production of large amounts of secreted, recombinant KLK15. Recombinant KLK15 produced by HEK 293 cells is glycosylated. KLK15 has two potential glycosylation sites, and glycosylation was confirmed by treatment of KLK15 with PNGase F.

Using recombinant KLK15, we developed mouse and rabbit polyclonal antibodies, able to recognize, on Western blots, KLK15 proteins produced from a variety of sources such as yeast, mammalian cells, and *E. coli*. We have employed our rabbit polyclonal antibody in conjunction with a mouse monoclonal antibody (obtained as a pre-release reagent from R&D Systems Inc.) to develop a sandwich-type KLK15 immunoassay. This assay is highly specific and sensitive, showing minimal or no cross-reactivity with the other fourteen human kallikreins.

We analyzed KLK15 protein expression levels in various human tissues, and biological fluids, using this immunoassay. Based on previous mRNA findings, we expected KLK15 to be expressed primarily in the prostate, colon, thyroid, salivary, and adrenal glands [3] (and our unpublished observations). Using normal, adult tissue cytosolic extracts, we found relatively low levels of KLK15 in the prostate, colon, and thyroid (3.3, 36, and 8.0 ng/g respectively) and undetectable levels in all other tissues analyzed. We speculate that the low levels of KLK15 expression in tissues may be due to degradation [6].

KLK15 expression was also examined in a panel of human biological fluids. KLK15 was found predominantly in seminal

plasma, which is expected, given that KLK15 is predicted to be secreted [3], and has been shown previously to be expressed in the prostate at the mRNA level [3,11], and in this study, at the protein level. Size exclusion HPLC indicates that seminal plasma contains an immunoreactive peak of 38 kDa. This molecular mass suggests that endogenous KLK15 is glycosylated, similarly to recombinant KLK15 produced in HEK 293 cells, and that our immunoassay detects free, endogenous KLK15, not KLK15 in complex with protease inhibitors. However, it is possible that KLK15 is partially complexed with inhibitors, similarly to other kallikreins, such as KLK3, KLK6, and KLK5 [19–23], and that these complexes are not recognized by our immunoassay. Size exclusion HPLC revealed an immunoreactive peak of 32 kDa molecular mass, when analyzed for KLK3 (PSA) (Fig. 5C), confirming that PSA and KLK15 are distinct proteins in seminal plasma.

This study confirms, for the first time, that KLK15 is a glycosylated protein of approximately 38 kDa in mass, produced in the prostate and secreted into seminal plasma. Low levels of KLK15 were also detected in colon and thyroid tissues, and relatively high levels of KLK15 were found in some human breast milks. Our technology will aid in the further characterization of KLK15 protein expression, delineation of KLK15's physiological function, and its role as a potential cancer biomarker.

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