

Purification and Characterization of Human Kallikrein 11, a Candidate Prostate and Ovarian Cancer Biomarker, from Seminal Plasma

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Abstract Purpose: Preliminary data suggest that hK11 is a novel serum biomarker for prostate and ovarian cancer. To examine the enzymatic characteristics of hK11, we purified and functionally characterized native hK11 from seminal plasma.

Experimental Design: hK11 was purified from seminal plasma by immunoaffinity chromatography and characterized by kinetic analysis, electrophoresis, Western blots, and mass spectrometry.

Results: hK11 is present in seminal plasma at concentrations ranging from 2 to 37 $\mu\text{g/mL}$. Using immunoaffinity chromatography and reverse-phase high-performance liquid chromatography, we purified hK11 to homogeneity. In seminal plasma, hK11 is present as a free enzyme of ~ 40 kDa. About 40% of hK11 is enzymatically active, whereas the rest is inactivated by internal cleavage after Arg¹⁵⁶ (Genbank accession no. AF164623), which generates two peptides of ~ 20 kDa, connected by internal disulfide bonds. Purified hK11 possesses trypsin-like activity and cleaves synthetic peptides after arginine but not lysine residues. It does not cleave chymotrypsin substrates. Antithrombin, α_1 -antichymotrypsin, α_2 -antiplasmin, and α_1 -antitrypsin have no effect on hK11 activity and do not form complexes with hK11 *in vitro*. The strongest inhibitor, APMSF, completely inhibited hK11 activity at a concentration of 2.5 mmol/L. Aprotinin and an hK11-specific monoclonal antibody inhibited hK11 activity up to 40%. Plasmin is a strong candidate for cleaving hK11 at Arg¹⁵⁶.

Conclusion: This is the first report on purification and characterization of native hK11. We speculate that hK11, along with other kallikreins, proteases, and inhibitors, participates in a cascade enzymatic pathway responsible for semen liquefaction after ejaculation.

Human tissue kallikreins are a group of secreted serine proteases, encoded by homologous genes tandemly localized on chromosome 19q13.4 (1). The genes are designated as *KLK1* to *KLK15* and the proteins as hK1 to hK15 (2). Human tissue kallikrein 11 (hK11) is a member of this family. The *KLK11* gene was initially identified by PCR amplification of hippocampus cDNA with primers derived from conserved regions of serine proteases. Because its encoded protein is similar to trypsin and because this gene was found to be highly expressed in the prostate, it was originally named trypsin-like serine protease and hippostasin (hippocampus and prostate trypsin; ref. 3). In our efforts to characterize the human tissue kallikrein

gene locus, we cloned a novel gene that was found to be identical to trypsin-like serine protease/hippostasin (4). The trypsin-like serine protease/hippostasin gene is now known as *KLK11* (2).

The *KLK11* gene spans about 5.3 kb of genomic DNA and consists of six exons and five introns (4). *KLK11* has three tissue-specific splice variants. The brain type is highly expressed in the brain and prostate and encodes for a typical serine protease of 250 amino acids (termed isoform 1). The prostate type is mainly expressed in the prostate and encodes for a protein that has 31 additional amino acids at the NH₂ terminus of the brain type (termed isoform 2; ref. 5). The variant type is also expressed in the prostate, and its encoded protein shares the same NH₂ terminus as the brain type. However, it contains a 25-amino-acid insertion in the catalytic domain (isoform 3; ref. 6).

The highest expression of hK11 is found in prostatic tissue. However, the gene is also expressed in a variety of other tissues, such as stomach, trachea, and skin. hK11 is present in many biological fluids, including milk of lactating women, amniotic fluid, serum, and seminal plasma (7). The presence of hK11 in diverse tissues and biological fluids suggests that it may have different physiologic functions in different tissues. Recombinant hK11 (including isoforms 1-3) has been expressed and found to cleave peptide bonds of synthetic peptides after arginine residues (5, 6). However, its physiologic substrates still remain elusive. Preliminary data suggest that hK11 is a

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novel biomarker for prostate and ovarian cancer. In prostate cancer, expression of the prostate type *KLK11* mRNA is increased (8). Over 60% of prostate cancer patients have been found to have elevated serum hK11 levels, suggesting a potential diagnostic value (7). In addition, the ratio between serum hK11 and total prostate-specific antigen (PSA) seems to help discriminate prostate cancer from benign prostatic hyperplasia (9). In ovarian cancer, the *KLK11* gene is overexpressed, and this overexpression seems to be associated with poor prognosis (10). At the protein level, higher hK11 levels in ovarian cancer tissue extracts broadcasts a favorable prognosis (11). Serum hK11 is also elevated in >70% of ovarian cancer patients (7). These data suggest that hK11 is a promising new biomarker for prostate and ovarian cancer diagnosis and prognosis.

Seminal plasma levels of hK11 are relatively high (10-15 $\mu\text{g}/\text{mL}$). We here describe purification of hK11 from seminal plasma and characterization of its structure and enzymatic activity.

Materials and Methods

Seminal plasma. Seminal plasma samples were obtained from the Andrology Laboratory at Mount Sinai Hospital. Our protocol has been approved by the Mount Sinai Hospital Ethics Committee.

ELISA assay for hK11. The concentration of hK11 in seminal plasma was measured with a sandwich-type immunoassay (7). In brief, an hK11-specific monoclonal antibody (clone 18-1; developed in-house) was first immobilized on a 96-well white polystyrene plate by incubating overnight 500 ng/100 μL /well in a coating buffer [50 mmol/L Tris, 0.05% sodium azide (pH 7.8)]. After washing three times with washing buffer [50 mmol/L Tris, 150 mmol/L NaCl, 0.05% Tween 20 (pH 7.8)], 100 μL of seminal plasma (diluted 1,000-fold) or standards were pipetted into each well. After a 1-hour incubation, the plate was washed six times. Subsequently, 100 μL of rabbit anti-hK11 polyclonal antibody (developed in-house) diluted 1,000-fold in assay buffer [50 mmol/L Tris, 6% bovine serum albumin, 10% goat IgG, 2% mouse IgG, 1% bovine IgG, 0.5 mol/L KCl, 0.05% sodium azide (pH 7.8)] was added and incubated for 1 hour. After the plate was incubated and washed as above, alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted 3,000-fold in assay buffer, was added and incubated for 1 hour. After washing as above, the alkaline phosphatase substrate difluoridyl phosphate was added and incubated for 10 minutes followed by addition of a developing solution containing Tb^{3+} , EDTA, and NaOH, as described elsewhere (12). Finally, fluorescence was measured with the CyberFluor time-resolved fluorometer (12).

Immunoaffinity purification of hK11 from seminal plasma. Immunoaffinity beads were prepared by coupling an hK11-specific monoclonal antibody (clone 18-1) to agarose beads using the Affi-Gel Hz Immunoaffinity kit (Bio-Rad Labs, Hercules, CA), following the manufacturer's recommendations. To purify hK11, 25 mL of pooled seminal plasma samples (average hK11 concentration was 15 $\mu\text{g}/\text{mL}$) were first diluted 2-fold in 20 mmol/L Tris buffer, 0.15 mol/L NaCl (pH 7.5) and mixed with 1 mL of immunoaffinity beads at 4°C overnight. The agarose beads were then packed in Econo chromatography columns (Bio-Rad Labs) and sequentially washed with 10 bed volumes of 20 mmol/L Tris, 150 mmol/L NaCl (pH 7.5), and 20 mmol/L Tris, 500 mmol/L NaCl, 0.1% Triton X-100 (pH 7.5). hK11 was eluted with 5 bed volumes of 0.2 mol/L glycine-HCl (pH 2.5). Fractions of 1 mL were collected and immediately neutralized with 0.5 mol/L NaHCO_3 . Fractions containing hK11 were identified by ELISA.

Reverse-phase high-performance liquid chromatography. After immunoaffinity purification, hK11 was further purified to homogeneity with reverse-phase high-performance liquid chromatography. The eluted fractions containing hK11 from the immunoaffinity purification were

supplemented with trifluoroacetic acid (final concentration 1%) and then loaded on a Vydac C4 column equilibrated with 0.1% trifluoroacetic acid in water. A linear gradient of 10% to 90% (1%/min) acetonitrile with 0.1% trifluoroacetic acid was then done. The peaks containing hK11 were collected and pooled, and acetonitrile was evaporated by nitrogen gas. Total protein concentration of the purified hK11 was determined with a bicinchoninic acid-based method using the Bicinchoninic Acid Protein Assay kit purchased from Pierce Biotechnology, Inc. (Rockford, IL).

SDS-PAGE and Western blot analysis. SDS-PAGE was done with NuPAGE precast 4% to 12% Bis-Tris polyacrylamide gels and a Novex mini-gel electrophoresis system (Invitrogen, Carlsbad, CA). For reducing conditions, DTT at a final concentration of 100 mmol/L was added to the sample along with loading buffer. For nonreducing conditions, no DTT was added. Electrophoresis was then done as recommended by the manufacturer. For Western blot analysis, proteins were transferred to Hybond-C membranes (Amersham Biosciences, Piscataway, NJ). The membranes were then blocked with 5% nonfat dry milk in TBS-T [20 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween 20 (pH 7.6)] for 2 hours at room temperature. Subsequently, they were incubated with rabbit anti-hK11 polyclonal antibody (diluted 2,000-fold in 1% nonfat dry milk TBS-T) for 1 hour at room temperature. After the membranes were washed thrice for 15 minutes in TBS-T, they were incubated in alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; final concentration of 0.5 mg/L in 1% nonfat dry milk in TBS-T) for 30 minutes. Finally, after the membranes were washed again as above, a dioxetane-based substrate was added (Pierce Chemical Co., Rockford, IL), and chemiluminescence emission was captured on X-ray film.

NH_2 -terminal sequencing. Purified hK11 was separated on SDS-PAGE under reducing conditions and then transferred to polyvinylidene difluoride membranes. The membranes were stained with Coomassie blue G-250, and the bands were excised. NH_2 -terminal sequencing was done with an Applied Biosystems ABI 492 sequencer (Foster City, CA).

Enzymatic analysis of hK11. The enzymatic activity of purified hK11 was measured with fluorogenic synthetic peptides coupled to 7-amino-4-methylcoumarin (AMC), purchased from Bachem Bioscience (King of Prussia, PA). They included Val-Pro-Arg-AMC, Phe-Ser-Arg-AMC, Pro-Phe-Arg-AMC (PFR-AMC), Gln-Gly-Arg-AMC, Gly-Pro-Arg-AMC, Leu-Arg-Arg-AMC, Gln-Ala-Arg-AMC, Leu-Gly-Arg-AMC, Leu-Lys-Arg-AMC, Val-Leu-Lys-AMC, Gly-Pro-Lys-AMC, Glu-Lys-Lys-AMC, Gly-Lys-Lys-AMC, Leu-Leu-Val-Thr-AMC, and Ala-Ala-Pro-Phe-AMC. The reaction was set-up in a microtiter plate at a volume of 100 μL as follows: 100 ng (25 nmol/L) purified hK11, 0.4 mmol/L substrates, 20 mmol/L Tris, 150 mmol/L NaCl, 0.01% Tween 20 (pH 7.5). The microtiter plate was then incubated in the Wallac Victor Fluorometer (Perkin-Elmer, Wellesley, MA) at 37°C. Fluorescence was measured with wavelengths set at 355 nm for excitation and 460 nm for emission. Enzyme-free reactions were used as negative controls. All experiments were done in triplicate. A standard curve with known concentrations of AMC was used to calculate the rate of product formation. The Michaelis-Menten constants were calculated by nonlinear regression analysis using the Enzyme Kinetics Module 1.1 (Sigma Plot, SSPS, Chicago, IL). More details are given elsewhere (13).

Cation-exchange chromatography. To separate the cleaved hK11 from intact hK11, cation-exchange chromatography was done. In brief, CM-Sepharose beads (Pharmacia, Piscataway, NJ) packed in an Econo Chromatography column (Bio-Rad Labs) were first activated with 1 mol/L KCl [prepared in 10 mmol/L MES buffer (pH 7.5)], then equilibrated in 10 mmol/L MES buffer (pH 7.5). hK11 was absorbed on the CM-Sepharose beads by passing through the purified hK11, diluted in 10 mmol/L MES buffer (pH 7.5). The beads were then washed with 10 mmol/L MES buffer (pH 7.5), and hK11 was eluted with a step gradient of KCl ranging from 50 to 300 mmol/L. Subsequently, the fractions were separated on SDS-PAGE and stained with silver. The

fractions containing the cleaved hK11 were concentrated with an Amicon Ultra centrifugal filter device (Millipore Corp., Bedford, MA) and used for enzymatic assays.

Effect of serine protease inhibitors and anti-hK11 monoclonal antibody 18-1 on hK11 enzymatic activity. Antithrombin, α_1 -antichymotrypsin, α_2 -antiplasmin, and α_1 -antitrypsin were purchased from Calbiochem (San Diego, CA). Benzamidine, aprotinin, and APMSF were purchased from Sigma (St. Louis, MO). One hundred nanograms of purified hK11 were incubated with antithrombin/ α_1 -antichymotrypsin/ α_2 -antiplasmin/ α_1 -antitrypsin in molar ratios ranging from 1:1 to 1:10, respectively, in a reaction mixture containing 20 mmol/L Tris, 150 mmol/L NaCl in a final volume of 20 μ L. These mixtures were incubated at 37°C for 2 hours, and the hK11 enzymatic activity was measured with the PFR-AMC substrate as described above. For benzamidine, aprotinin, and APMSF, they were first incubated with 100 ng of hK11 at concentrations ranging from 0.25 to 2.5 μ mol/L, in a 100 μ L reaction mixture containing 20 mmol/L Tris, 150 mmol/L NaCl, 0.01% Tween 20 (pH 7.5), for 30 minutes at room temperature. PFR-AMC was then added at a final concentration of 0.4 mmol/L, and hK11 enzymatic activity was measured as described earlier. To examine the effect of hK11 monoclonal antibody clone 18-1 on hK11 enzymatic activity, 125 nmol/L of 18-1 were added in a reaction mixture containing 25 nmol/L purified hK11, 20 mmol/L Tris, 150 mmol/L NaCl, 0.01% Tween 20 (pH 7.5). After a 1-hour incubation at room temperature, PFR-AMC was added at a final concentration of 0.4 mmol/L. The plate was then incubated at 37°C, and fluorescence was measured as described above.

Isolation of enzymes with soybean trypsin inhibitor agarose beads from hK11-depleted seminal plasma. Soybean trypsin inhibitor beads were purchased from Sigma. One milliliter of beads was incubated with hK11-depleted seminal plasma (flow-through fraction from hK11 immunoaffinity purification) at 4°C, overnight. The beads were then packed in an Econo chromatography column. Column washing and elution were done following procedures described for the hK11 immunoaffinity purification. Twenty microliters of eluate from each fraction were then incubated at 37°C, overnight, with 100 ng of recombinant hK11, produced in our laboratory in Chinese hamster ovarian cells. The reaction mixture was then separated on SDS-PAGE under reducing and nonreducing conditions and transferred to Hybond-C membranes. Western blot analysis was done as described above.

Cleavage of hK11 by plasmin and hK2. Active plasmin was purchased from Sigma. Active hK2 was a gift from Dr. Stephen Mikolajczyk (Hybritech Division, Beckman-Coulter). To cleave recombinant hK11, 1 μ g of plasmin and 0.5 μ g of hK2 were incubated with 100 ng of hK11 in a 20 μ L reaction mixture containing 20 mmol/L Tris, 150 mmol/L NaCl (pH 7.5), at 37°C, overnight. The reaction mixtures were subjected to Western blot analysis as described above.

Results

Concentration of hK11 in seminal plasma. The concentration of hK11 in 42 seminal plasmas was measured with an hK11-specific immunoassay. hK11 concentration ranged from 2 to 37 μ g/mL, with a mean of 15 μ g/mL and a median of 11 μ g/mL.

Purification of hK11 from seminal plasma. hK11 was purified to homogeneity with immunoaffinity chromatography followed by reverse-phase high-performance liquid chromatography. The recovery rates of these two steps were about 95% and 85%, respectively (data not shown). When purified hK11 was separated on SDS-PAGE under nonreducing conditions and stained with Coomassie blue, it appeared as one band with a 40-kDa molecular mass. When SDS-PAGE was done under reducing conditions, in addition to the 40-kDa band, there was another band of about 20 kDa (Fig. 1). The intensity of the 20-

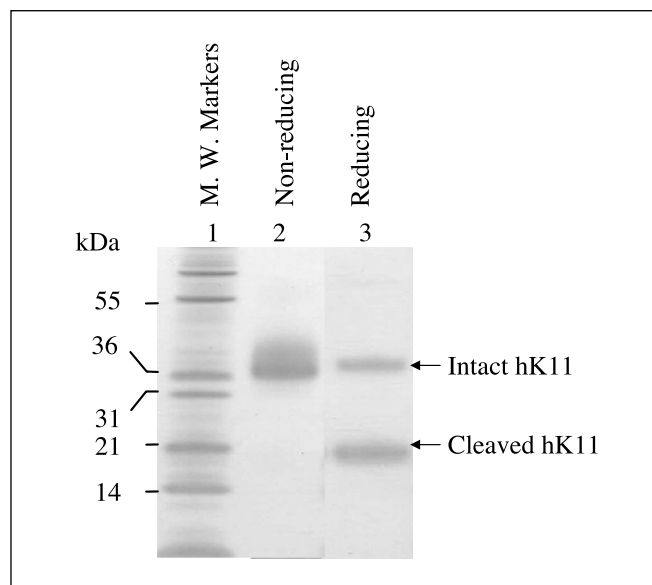


Fig. 1. Purified hK11 from seminal plasma separated by SDS-PAGE under nonreducing (lane 2) and reducing (lane 3) conditions and stained with Coomassie blue. Lane 1, molecular weight markers. Intact and cleaved hK11 forms are indicated by arrows. For comments, see Results.

kDa band was about 2-fold higher than the 40-kDa one. To verify their identity, these two bands were excised from the gel and subjected to in-gel trypsin digestion and tandem mass spectrometry. The results confirmed that both bands were hK11 because multiple peptides matched the sequence of hK11 protein (data not shown). These data indicate that in seminal plasma, intact hK11 is present as a 40-kDa enzyme. About 60% of hK11 is cleaved, generating two peptides with approximately the same molecular mass (~20 kDa). These two peptides are held together by internal disulfide bonds (Fig. 2). Under nonreducing conditions, the cleaved hK11 comigrates with intact hK11 as a 40-kDa protein.

NH₂-terminal sequencing. To determine whether the purified hK11 from seminal plasma is an active enzyme and to determine its internal cleavage site, the 40- and 20-kDa bands were subjected to NH₂-terminal sequencing. The NH₂ terminus of the 40-kDa band was I-I-K-G-F-E. This sequence matches exactly with amino acids 22 to 27 of brain-type and prostate-type hK11 (Genbank accession no. AF164623). By homology comparison with other human tissue kallikreins, active hK11 was predicted to start from amino acid 22 (isoleucine; ref. 1). Our results confirm this prediction and show that hK11 is present in seminal plasma as an active enzyme and not as a proform. NH₂-terminal sequencing of the 20-kDa band showed a mixture: I(L)-I(P)-K(H)-G(T)-F(L)-E(R). These sequences match perfectly with amino acids 22 to 27 and 157 to 162 of hK11. These two peptides are derived from active hK11, and the cleavage site is between amino acids 156 and 157 (R↓L). Because amino acid 156 is an arginine residue, we speculated that hK11 is internally cleaved at this position by an enzyme with trypsin-like activity.

Western blot analysis of seminal plasma. To determine whether seminal plasma contains forms of hK11 that were not captured by affinity chromatography (e.g., hK11 bound to proteinase inhibitors), we did Western blot analysis on eight randomly selected seminal plasma samples with rabbit

anti-hK11 polyclonal antibodies. The results are shown in Fig. 3. Under nonreducing conditions, two major bands with approximate molecular masses of 40 and 30 kDa were detected. Under reducing conditions, there are three major bands with approximate molecular masses of 40, 30, and 20 kDa. To determine whether the 30-kDa band is a cleaved hK11 fragment missed by our immunoaffinity column, hK11-depleted seminal plasma (hK11 immunoaffinity purification flow-through fraction) was subjected to cation-exchange chromatography separation. Mass spectrometry and Western blot analysis (with PSA-specific antibodies) showed that the 30-kDa band represented PSA (data not shown), cross-reacting with our rabbit hK11 polyclonal antibody. PSA is present at extremely high levels in seminal plasma (1-2 mg/mL). These results show that the seminal plasma-purified hK11 most likely represents all hK11 forms in this fluid. The absence of higher molecular weight bands suggests that hK11-inhibitor complexes are not major constituents of seminal plasma.

To further verify the relative molecular masses of hK11, PSA, hK2, and hK4 in seminal plasma, we separated seminal plasma on a gel filtration column and analyzed the fractions by ELISA assays. The results are shown in Fig. 4. The molecular masses are as follows: hK11 (~40 kDa) > hK4 (~33 kDa) > hK2, PSA (~30 kDa).

Enzymatic activity of the purified hK11. The enzymatic activity of seminal plasma-purified hK11 was examined with a panel of synthetic fluorogenic peptides. We found that hK11 cleaves most efficiently the peptide PFR-AMC followed by Val-Pro-Arg-AMC, Phe-Ser-Arg-AMC, Gln-Ala-Arg-AMC, and Gly-Pro-Arg-AMC (Table 1). No cleavage was observed towards the peptides Gln-Gly-Arg-AMC, Leu-Arg-Arg-AMC, Leu-Gly-Arg-

AMC, Leu-Lys-Arg-AMC, Val-Leu-Lys-AMC, Gly-Pro-Lys-AMC, Glu-Lys-Lys-AMC, and Gly-Lys-Lys-AMC. These results show that hK11 preferentially cleaves peptide bonds after arginine but not after lysine residues. hK11 showed no enzymatic activity on chymotrypsin substrates, including Leu-Leu-Val-Thr-AMC and Ala-Ala-Pro-Phe-AMC. The optimal pH for the hK11 enzymatic activity is 7.5, as determined by PFR-AMC (data not shown).

Enzymatic activity of cleaved hK11. Serine proteases are synthesized as single-chain polypeptides. During the process of activation, they are cleaved at the NH₂ terminus or internally. In the latter case, the resulting peptides form subunits of the active enzymes. Some examples include thrombin and protein C. To determine whether the cleavage of hK11 at Arg¹⁵⁶/Leu¹⁵⁷ results in activation or inactivation of the enzyme, we examined the enzymatic activity of cleaved hK11. Cleaved hK11 was separated from total hK11 with cation-exchange chromatography (Fig. 5A). The enzymatic activity of cleaved hK11 was compared with that of total hK11 with the PFR-AMC substrate. As Fig. 5B shows, the enzymatic activity of cleaved hK11 is clearly decreased compared with that of total hK11. These results suggest that cleavage of hK11 at Arg¹⁵⁶/Leu¹⁵⁷ leads to at least partial inactivation.

Inhibition of hK11 enzymatic activity by serine protease inhibitors and monoclonal antibody 18-1. The effect of some common serine protease inhibitors on hK11 enzymatic activity was examined. Antithrombin, α₁-antichymotrypsin, α₂-antiplasmin, and α₁-antitrypsin have no effect on hK11 enzymatic activity, even at a molar ratio of 10:1. No complex formation was observed by SDS-PAGE or Western blots even at a ratio of 50:1 (data not shown). The strongest inhibitor of hK11 activity is APMSF. This inhibitor completely inhibits

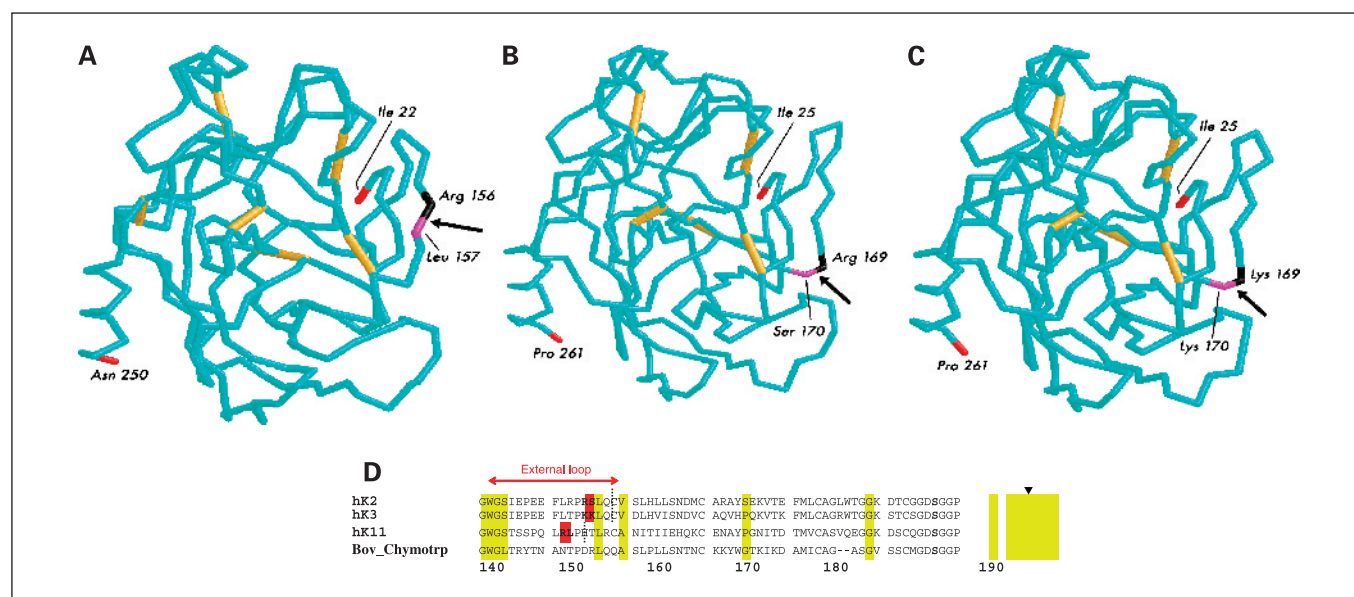


Fig. 2. Cleavage sites for inactivation of hK11, hK2, and hK3. Computationally predicted structures of the active forms of human kallikrein proteins hK11 (A), hK2 (B), and hK3 (C). The cleavage site shown in this study for deactivation of hK11 is located in an exposed unordered loop region. Similar cleavage sites for deactivation have been experimentally demonstrated to exist in hK2 and hK3 and are both located in the same loop structure. In all cases, cleavage sites are indicated by an arrow with the residue upstream of the cleavage site colored in black and the downstream residue colored in purple. The disulfide bonds are shown in yellow color. The NH₂ and COOH termini of the protein have been labeled and are colored in red. D, an aligned segment of hK11, hK2, and hK3, along with the orthologous region of bovine chymotrypsin. The cleavage sites in the inactivation loop are indicated with hashed lines, and the flanking residues are shaded in red. The catalytic serine is indicated on the right side of the alignment by an arrow. Residues in yellow are conserved in all aligned sequences. The surface loop that contains the cleavage sites is indicated by an arrow above the sequences. Numbering of the alignment is according to the bovine chymotrypsin sequence.

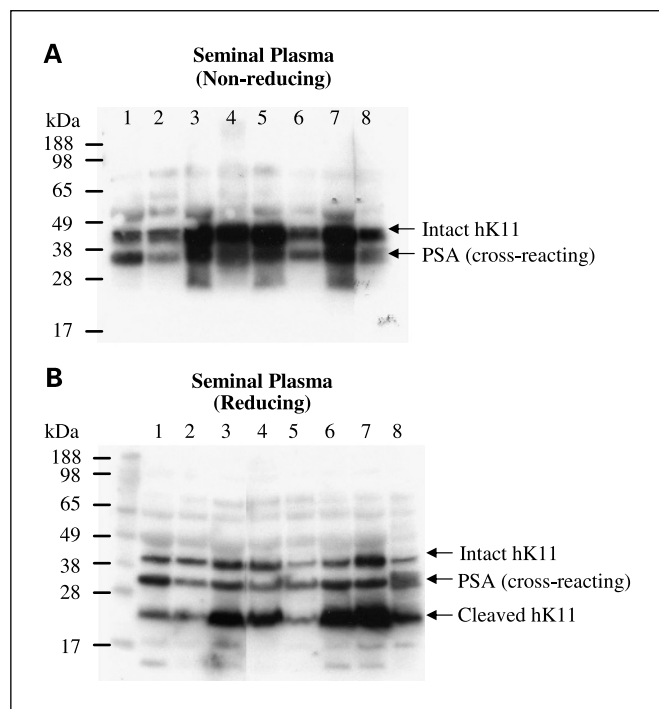


Fig. 3. Western blot analysis of eight seminal plasma samples with a polyclonal rabbit anti-hK11 antibody. SDS-PAGE done under nonreducing (A) or reducing (B) conditions. Intact and cleaved hK11 and the cross-reacting hK3 (PSA) are shown by arrows.

hK11 enzymatic activity at a concentration of 2.5 $\mu\text{mol/L}$, whereas 2.5 $\mu\text{mol/L}$ aprotinin and benzamidine could only inhibit hK11 activity up to 30% to 40%. An hK11-specific monoclonal antibody (clone 18-1) inhibited hK11 enzymatic activity up to 40% at 5-fold molar excess.

Identification of the enzyme that cleaves hK11. hK11 is cleaved at Arg¹⁵⁶/Leu¹⁵⁷ by an enzyme with trypsin-like enzymatic activity. We hypothesized that if this enzyme is present in seminal plasma, then addition of exogenous hK11 to hK11-depleted seminal plasma should generate a band with the same characteristics as cleaved hK11. We produced recombinant hK11 in Chinese hamster ovarian cells. Recombinant hK11 was purified with ion-exchange followed by reverse-phase high-performance liquid chromatography. NH₂-terminal sequencing revealed that the enzyme represents the proform of hK11 with little or no enzymatic activity. When recombinant hK11 was mixed with hK11-depleted seminal plasma (hK11 immunoaffinity purification flow-through fraction), we found that it was cleaved and generated a 20-kDa band with the same characteristics as the cleaved native hK11 (Fig. 6). These results show that the enzyme responsible for hK11 cleavage is present in seminal plasma. Further experiments showed that this cleavage could be inhibited by soybean trypsin inhibitor (data not shown). Therefore, to identify the enzyme that cleaves hK11, we used soybean trypsin inhibitor-immobilized agarose as an affinity column. Stepwise elution revealed that certain fractions (mainly fraction 2) were able to cleave recombinant hK11 (Fig. 7). When proteins in fraction 2 were separated on SDS-PAGE, we identified distinct bands, compared with other fractions (Fig. 7). These bands were excised from the gel and

then subjected to in-gel digestion with trypsin and tandem mass spectrometric analysis (LC/MS/MS). The two major proteins identified were plasmin and galectin 3 binding protein. Trace amounts of hK2, hK3 (PSA), and epitheliasin (TMPRSS2) were also isolated. Among these proteins, plasmin, hK2, and epitheliasin have trypsin-like enzymatic activity. Because active plasmin is commercially available and active hK2 was provided to us, we further examined their ability to cleave hK11 *in vitro*. Our results are presented in Fig. 8. Plasmin cleaves hK11 and generates a 20-kDa band, which is detected only when DTT is present. hK2 also cleaves recombinant hK11 and generates a band of ~ 20 kDa. However, this band is detected under both reducing and nonreducing conditions. We concluded that although both of these enzymes can cleave hK11, only the band generated by plasmin has the same characteristics as the cleaved, native hK11. hK2 likely cleaves hK11 not only after Arg¹⁵⁶ but also at other sites.

Discussion

We purified hK11 from seminal plasma and studied its structure and enzymatic activity. Seminal plasma hK11 concentration averages 15 $\mu\text{g/mL}$. Among the biological fluids tested, seminal plasma contains the highest amount of hK11. Many members of the human tissue kallikrein family are found in seminal plasma (1). Previously, the most abundant seminal plasma tissue kallikreins were thought to be hK3 and hK2. hK3 concentration averages 1,000 $\mu\text{g/mL}$, whereas hK2 is about 6 $\mu\text{g/mL}$ (14, 15). Other tissue kallikreins (hK4, hK5, hK6, hK10, and hK13) are present in seminal plasma at levels below 1 $\mu\text{g/mL}$. Thus, hK11 is the second most abundant tissue kallikrein in seminal plasma, after hK3 (PSA).

hK11 is present in seminal plasma as a free (unbound) active enzyme of about 40 kDa molecular mass, and it is a glycosylated protein. Four hypothetical N-glycosylation sites are found in the protein sequence (Asn⁹⁸, Asn¹⁶⁵, Asn¹⁸¹, and Asn²¹⁰). In terms of molecular mass, hK11 is larger than hK4, and hK4 is larger than hK2 and hK3, as shown by gel

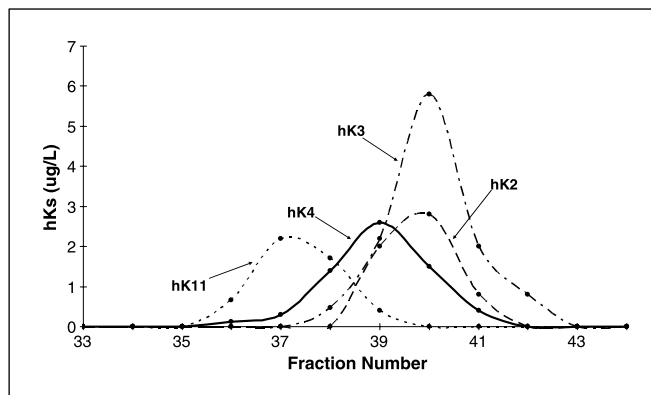


Fig. 4. Separation of seminal plasma by gel filtration chromatography and measurement of human kallikreins hK11, hK4, hK3 (PSA), and hK2 by ELISA assays. The elution profiles of hK11 (~ 40 kDa), hK4 (~ 33 kDa), hK2, and hK3 (~ 30 kDa) have no quantitative meaning because seminal plasma was diluted to bring kallikrein measurements within the ELISA range. For discussion, see text.

Table 1. Substrate specificity of hK11 as determined by synthetic fluorogenic peptides

Substrate*	K_m (mmol/L) [†]	K_{cat} (min ⁻¹)	K_{cat}/K_m (mmol/L ⁻¹ min ⁻¹)
PFR-AMC	0.53 ± 0.04	1.86	3.52
VPR-AMC	0.28 ± 0.04	0.45	1.60
FSR-AMC	0.44 ± 0.06	0.39	0.89
QAR-AMC	0.27 ± 0.02	0.23	0.85
GPR-AMC	1.18 ± 0.03	0.21	0.18

Abbreviations: VPR-AMC, Val-Pro-Arg-AMC; FSR-AMC, Phe-Ser-Arg-AMC; QAR-AMC, Gln-Ala-Arg-AMC; GPR-AMC, Gly-Pro-Arg-AMC.

*Amino acids are shown with single letter codes.

[†] ± SD.

filtration chromatography, followed by ELISA analysis (Fig. 4). Apparently, the molecular mass differences are due to glycosylation because the lengths of the peptide moieties of active hK11, hK4, hK3, and hK2 are 229, 224, 237, and 237, respectively (1).

About 60% of seminal plasma hK11 is internally cleaved. The cleavage occurs after Arg¹⁵⁶. The resulting two peptides are about 20 kDa each and are held together by internal disulfide bonds (Fig. 2). The cleavage of active hK11 at this

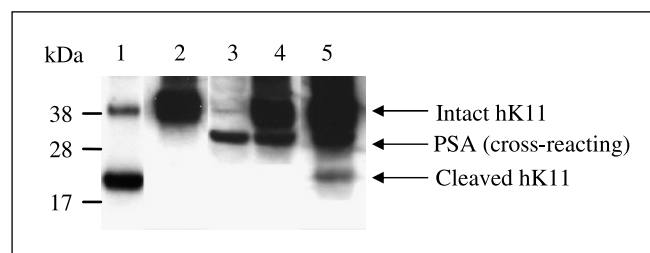


Fig. 6. Cleavage of recombinant hK11 by hK11-depleted seminal plasma, as shown by Western blot analysis. Proteins in lanes 1 to 3 were separated under reducing conditions. Lane 1, purified native hK11; lane 2, recombinant hK11 alone, lane 3, hK11-depleted seminal plasma; Lanes 4 and 5, recombinant hK11 mixed with native hK11-depleted seminal plasma, under nonreducing and reducing conditions, respectively. Note generation of cleaved hK11 in lane 5.

position leads to inactivation. Because we did not find hK11 complexes with protease inhibitors in seminal plasma and *in vitro*, it seems that the major mechanism regulating hK11 enzymatic activity in seminal plasma is through internal cleavage. Internal cleavage is a common mechanism of human tissue kallikrein enzyme inactivation. Around 30% of hK3 in seminal plasma is cleaved between Lys¹⁴⁵ and Lys¹⁴⁶ (16). For hK2, many cleavage sites have been identified, including Arg¹⁰¹, Arg¹⁴⁵, and Arg²²⁶ (17, 18). One of these sites is localized in the same loop as the cleavage site of PSA and hK11 (Fig. 2). This susceptible area lies outside the core of the enzyme, and it is accessible for cleavage. hK6 is able to self cleave after Arg⁷⁶ (19). However, unlike hK11, the kallikreins hK2, hK3, and hK6 form complexes *in vivo* and *in vitro* with many protease inhibitors, such as antithrombin, α_1 -antichymotrypsin, protein C inhibitor, and α_1 -antitrypsin (16–21).

hK11 preferentially cleaves peptide bonds after arginine residues, the most optimal substrate being PFR-AMC (Table 1). This result is consistent with previous reports obtained with recombinant hK11 (5, 6). To search for candidate physiologic substrates for hK11, we examined the cleavage of a panel of recombinant proteins, including hK3, hK5, plasminogen, kininogen, and collagen type I to IV. These proteins have been previously shown to be cleaved by some tissue kallikreins (19).³ To examine whether hK11 can also cleave these proteins, we incubated purified hK11 with these proteins at equal molar ratio at 37°C in a buffer containing 50 mmol/L Tris, 0.15 mol/L NaCl (pH 7.5), for up to 24 hours. However, we did not observe any cleavage of these proteins by hK11. It seems that hK11 is not a broadly specific serine protease; rather, it may prefer cleavage of specific substrates. Although hK11 is also present in many biological fluids (7), considering its relatively high amounts in seminal plasma, its major physiologic substrates are most likely to be present in this fluid. Candidates include proteins involved in semen liquefaction. It is known that hK2 and hK3 cleave seminogelin I and II and fibronectin, the major components of seminal clot (22–25). We further examined the cleavage of these three proteins by hK11. Similarly, we incubated purified hK11 with these proteins at equal molar

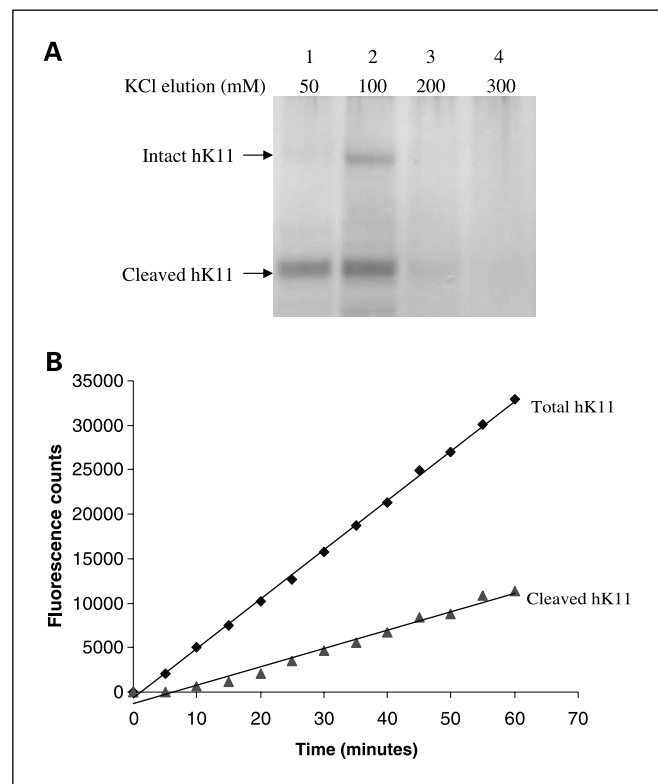


Fig. 5. Separation of cleaved hK11 from total hK11 by cation-exchange chromatography (A) and measurement of its enzymatic activity (B). For more details, see text. Lane 1, 50 mmol/L KCl elution; lane 2, 100 mmol/L KCl elution; lane 3, 200 mmol/L KCl elution; lane 4, 300 mmol/L KCl elution. B, enzymatic activity of cleaved hK11 (fraction of lane 1) compared to that of total hK11 (fraction of lane 2).

³ Unpublished data.

ratio at 37°C in a buffer containing 50 mmol/L Tris, 0.15 mol/L NaCl, 0.2 mol/L urea, 0.1 mmol/L EDTA (pH 7.5), for up to 24 hours. However, no cleavage was observed (data not shown).

In an effort to identify the enzyme that cleaves and inactivates hK11, we used soybean trypsin inhibitor-immobilized agarose for affinity purification. Three serine proteases with trypsin-like enzymatic activity were identified by tandem mass spectrometry, including plasmin, hK2, and epitheliasin. In subsequent experiments, we showed that although hK2 could cleave hK11 at Arg¹⁵⁶ to generate a band with the same size as the cleaved native hK11, hK2 also cleaved additional peptide bonds. Plasmin cleaved hK11, generating two 20-kDa peptides that are separated only under reducing conditions, similar to the cleaved, native hK11. Plasmin seems to cleave hK11 only once and at Arg¹⁵⁶. Therefore, plasmin is a good candidate for cleavage of hK11 under physiologic conditions. Plasmin is a component of the blood clotting system. Its major physiologic substrates are fibrinogen and fibrin (26, 27). However, the physiologic roles of plasmin are quite diverse (28). It may function in processes where cell movement is essential, such as breakdown of the follicular wall for ovulation, angiogenesis, and keratinocyte accumulation after wound healing (29–31). In addition, it is implicated with tumor cell invasion and metastasis and proteolytic activation/destruction of other proteins (28, 32, 33). The presence of plasmin in seminal plasma has been previously reported (34, 35), but its role in this fluid was obscure. It has been suggested that it is involved in the activation of transforming growth factor- β 1 in seminal plasma (36) and that it is associated with sperm motility

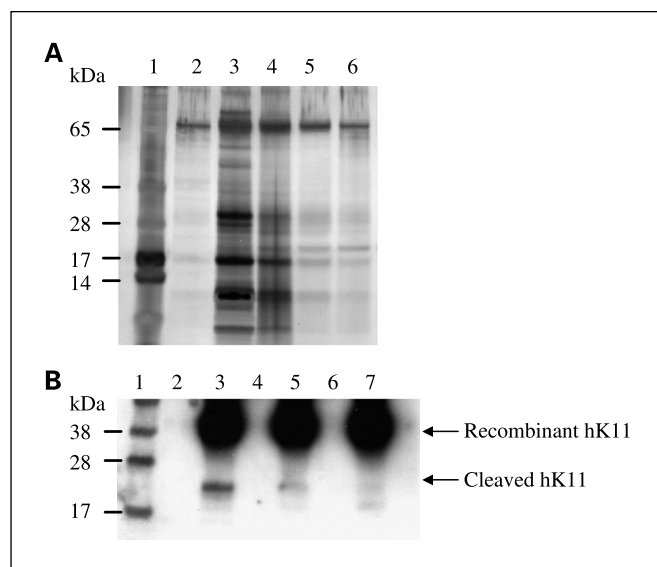


Fig. 7. Isolation of serine proteases from hK11-depleted seminal plasma with soybean trypsin inhibitor agarose beads (*A*) and their ability to cleave recombinant hK11 (*B*). *A*, lane 1, molecular weight markers; lanes 2 to 6, fractions 1 to 5 of the column eluates (silver staining). *B*, cleavage of recombinant hK11 by proteins isolated with soybean trypsin inhibitor beads (Western blot analysis). SDS-PAGE was done under reducing conditions. Lane 1, molecular weight markers; lane 2, fraction 2 alone; lane 3, fraction 2 mixed with recombinant hK11; lane 4, fraction 3 alone; lane 5, fraction 3 mixed with recombinant hK11; lane 6, fraction 4 alone; lane 7, fraction 4 mixed with recombinant hK11. Fractions 1 and 5 did not proteolyze recombinant hK11 (data not shown).

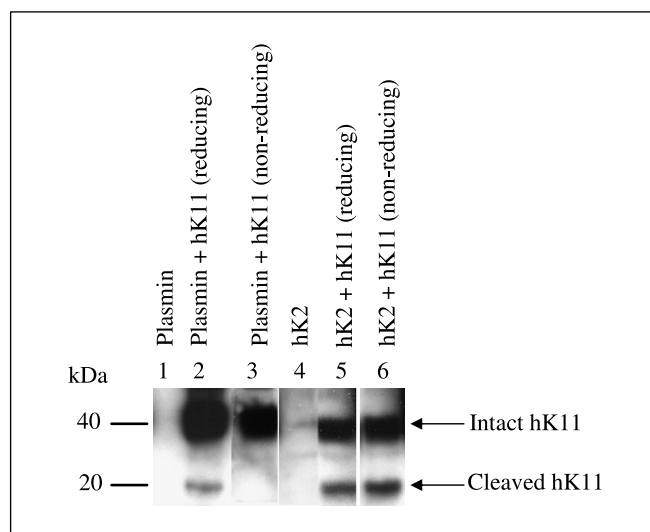


Fig. 8. Cleavage of recombinant hK11 (produced in CHO cells) with purified plasmin and hK2, as shown by Western blot analysis. Identities of lanes 1 to 6 are shown on top. For more discussion, see text.

(37). A potent plasmin inhibitor can inhibit semen liquefaction (38). Furthermore, the plasmin-plasminogen activator system has been reported to crosstalk with hK2 and hK3 (39, 40). In this work, we failed to show complete cleavage of native hK11 by plasmin. We speculate that this finding may be due to the following reasons. (*a*) Lack of an essential cofactor. It is known that most of the enzymatic activities of plasmin are also catalyzed by other related enzymes, such as trypsin, or enzymes of the coagulation system (28). It is possible that plasmin, hK11, and other proteins may participate a cascade pathway that is involved in semen liquefaction. (*b*) Inhibitory effect of cleaved hK11. In our experiments, the hK11 used is a mixture of intact (active) hK11 and internally cleaved (inactive) hK11. To investigate the cleavage of hK11 by plasmin, we used both recombinant pro-hK11 produced in the Chinese hamster ovarian cells and purified native hK11. In both experiments, we only observed partial cleavage of hK11 by plasmin (Fig. 8).⁴ When pro-hK11 is used, the scissile bond of Arg¹⁵⁶/Leu¹⁵⁷ may not be easily accessible by plasmin, due to conformational differences between the pro and active hK11. On the other hand, when purified native hK11 is used, it is a mixture of intact (active) and internally cleaved (inactive) hK11. The cleaved hK11 may exert an inhibitory effect on the cleavage of active hK11 by plasmin. We attempted to completely separate intact hK11 from cleaved hK11 with many methods, such as ion exchange and affinity columns (benzamidine, aprotinin, and soybean trypsin inhibitor). However, none of these methods proved to be successful (data not shown). In all the seminal plasma samples tested, we noticed that the amount of cleaved hK11 is roughly proportional to the amount of intact hK11 (Fig. 2). It seems that there is homeostatic regulation of the ratio of active/cleaved hK11. Collectively, although our data on the cleavage of hK11 by plasmin are not conclusive,

⁴ Unpublished data.

they suggest that plasmin is a candidate physiological inactivator of hK11; further investigations are warranted.

Another candidate enzyme that could cleave hK11 is epitheliasin (also termed TMPRSS2), a transmembrane serine protease (41, 42). The enzymatic activity of its protease domain has not as yet been directly determined. Epitheliasin is predicted to have trypsin-like enzymatic activity, based on the amino acids occupying the substrate-binding pocket (43). Epitheliasin is mainly expressed in epithelial cells of the prostate, kidney, lung, and colon (44). The relevance of this protease to seminal plasma has not been established. Although we identified trace amounts of this enzyme in our column elution, whether it is able to cleave hK11 needs to be further examined.

In summary, we here report purification and characterization of native hK11 from seminal plasma. Our results show

that hK11 in seminal plasma is either enzymatically active or is internally cleaved and inactivated. Most likely, hK11, along with other seminal plasma kallikreins, such as hK2, hK3, hK4, hK5, hK6, and hK15, and other proteolytic enzymes and their inhibitors participate in cascade enzymatic pathways that lead to semen liquefaction and increased sperm motility. The players participating in this complex system are first activated and then inactivated by internal cleavage or by binding to inhibitors. The operational details of these enzymatic cascade pathways warrant further investigations. Previously, we speculated on the existence of such a kallikrein proteolytic cascade pathway and its involvement in tumor progression and metastasis (45). More recently, many others have started delineating the details of this pathway as it relates to skin physiology and pathobiology (46–48).

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