

# Human Kallikrein 4: Quantitative Study in Tissues and Evidence for Its Secretion into Biological Fluids

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**Background:** Human kallikrein 4 (hK4) is a proteolytic enzyme belonging to the tissue kallikrein family of serine proteases. Previous tissue expression studies have demonstrated highest *KLK4* mRNA expression in prostatic tissue, but there has been only limited evidence for the presence of hK4 protein in prostate and other tissues and in corresponding biological secretions. **Methods:** To investigate the concentrations of hK4 in tissues and biological fluids, we developed a new hK4-specific sandwich-type immunoassay using a monoclonal antibody as the capture reagent.

**Results:** The assay has a detection limit of 0.02  $\mu\text{g/L}$  and <0.1% cross-reactivity toward any of the other 14 human kallikreins. Twelve of 40 tissue extracts prepared from various human tissues contained detectable hK4 concentrations (0.68–7143 ng/g of total protein), with healthy prostate tissue containing the highest amount of hK4. Examination of 16 malignant and 18 benign prostate tissues revealed no significant differences in hK4 protein content, and the tissues contained a wide range of values (benign, <0.02 to 801 ng/g; malignant, <0.02 to 824 ng/g). Among the biological fluids tested, seminal plasma and urine contained widely varying amounts of

hK4; concentrations in 54 urine samples were <0.02 to 2.6  $\mu\text{g/L}$ , whereas concentrations in 58 seminal plasma samples were 0.2–202  $\mu\text{g/L}$ . Affinity purification of hK4 from seminal plasma and subsequent mass spectrometry demonstrated the secreted nature of hK4 in seminal plasma.

**Conclusions:** hK4 is found primarily in prostate tissue and is secreted in seminal plasma. Its value as a novel prostatic biomarker needs to be defined further.

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Serine proteases are the most intensely studied group of enzymes in biology (1), reflecting their diverse physiologic functions in digestion, coagulation, immune regulation, fibrinolysis, reproduction, and development (2). The activity of most serine proteases is governed by one or more regulatory mechanisms through transcriptional control, by translation into zymogen forms needing subsequent activation at their sites of action, and through complex formation with protease inhibitors. Deregulated, abnormal serine protease activity can lead to disorders as diverse as asthma (3), clotting abnormalities (4), emphysema (5), and cancer (6,7). Interestingly, closely related serine proteases appear to contribute to the pathogenesis of various malignancies. Among these cancer-associated proteases, those belonging to the matrix metalloprotease family are the best known (8), although others belonging to the human kallikrein family have also recently been the focus of attention (9).

The human kallikrein 4 gene (*KLK4*)<sup>5</sup> is a member of the human kallikrein family. The expression of this gene,

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Received February 22, 2005; accepted May 27, 2005.

Previously published online at DOI: 10.1373/clinchem.2005.049692

<sup>5</sup> Nonstandard abbreviations: *KLK* and *hK*, human kallikrein gene and protein, respectively; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; MS, mass spectrometry; BSA, bovine serum albumin; and ALP, alkaline phosphatase.

discovered independently by two groups (10–12), was initially deemed to be restricted to human prostate based on Northern blotting data (10). However, subsequent reverse transcription-PCR studies demonstrated that *KLK4* mRNA is also expressed in other tissues, although at much lower concentrations than in the prostate (11, 13). Up-regulation of *KLK4* expression was shown to be induced by androgens and progestins in the androgen receptor-positive prostate cancer cell line LNCaP (10), although later studies have demonstrated of estrogenic up-regulation to a similar extent in this cell line (14). In addition, increased *KLK4* mRNA expression has also been reported in the BT-474 breast cancer cell line (11) and in the KLE endometrial cancer cell line (15) in response to androgen and progesterone and to estrogen and progesterone, respectively. *KLK4* mRNA overexpression was shown to occur in ovarian cancer tissues (16), and it was shown to be an independent indicator of unfavorable prognostic outcome in well- and moderately differentiated ovarian cancers (17). At the protein level, hK4 production in ovarian cancer was recently associated with paclitaxel resistance (18). Evidence for the overexpression of *KLK4* mRNA in prostate cancer has recently been presented from data obtained in tissue microarray studies (19). However, the function of *KLK4* in either malignant ovarian or in healthy prostate tissues remains to be determined.

All of the above studies have examined total *KLK4* mRNA expression. It is now clear, however, that the *KLK4* gene gives rise to at least 8 different mRNA forms through alternative splicing and/or an alternative transcription start site (20) and that these transcripts are expected to give rise to at least 7 different protein moieties, most of which are truncated forms lacking protease function. A semiquantitative study, using the number of PCR cycles needed to amplify a transcript as the measure of relative *KLK4* mRNA abundance, compared the relative amounts of 2 *KLK4* transcripts that are predicted to encode the secreted and intracellular forms of hK4; the study suggested that, at least in prostate tissue, the *KLK4* mRNA encoding for intracellular hK4, with a transcription start site downstream of the secreted form, is the most abundant *KLK4* mRNA species (14).

In contrast to the multiple studies examining *KLK4* at the mRNA level, only 3 published studies have investigated the *KLK4* gene product (the hK4 protein). One report described intracellular hK4 protein concentrations in response to steroid hormone stimulation of endometrial cancer cell lines, using an hK4-specific antipeptide antibody and Western blotting (15). The apparent size of the protein (38–40 kDa) was higher than that predicted for pro-hK4 (29 kDa), and this difference was suggested to be attributable to the extent of glycosylation of hK4. Another study by our group measured total hK4 concentrations in various biological fluids and prostate tissue extracts by means of a sandwich-type immunoassay using mouse and rabbit polyclonal antibodies raised against

full-length hK4 (21). Although hK4 concentrations were highest in prostate tissues among the tissues examined, the actual values were unexpectedly low (based on mRNA abundance) in both prostate tissue and biological secretions. Although this study demonstrated absence of cross-reactivity to other kallikreins, it could not exclude the possibility that poor detection of immunoreactive hK4 may have been attributable to low efficiency of the assay for recognizing hK4 in its native conformation and/or inefficient or absent hK4 translation and secretion. The third study (19) focused on the intracellular form of hK4. Recent concerns raised over the lack of specificity of the antibody used to study intracellular hK4 and the discrepancy between its predicted molecular mass (22 kDa) and that of the protein identified as hK4 (45 kDa), which has an unexpected nuclear localization (22), call for further investigations.

The physiologic function of hK4 has not yet been ascertained. Although its murine and porcine counterparts play a role in maturation of the dental enamel with stage-specific production in ameloblasts and odontoblasts (23–25), studies in this area in humans have not been reported. Drawing conclusions on possible functional similarities between murine or porcine EMSP1 proteins and human hK4 is hampered by the fact that the former have glycosylation patterns, molecular forms, and propeptides distinct from human hK4 (26). Recent findings, however, have excluded *KLK4* as a candidate gene in amelogenesis imperfecta (27, 28) but suggested possible involvement of *KLK4* mutations as a cause of the recessive form of hypomaturation amelogenesis imperfecta (29).

Our initial studies used an hK4 assay that lacked the sensitivity to unequivocally demonstrate that this kallikrein is secreted in biological fluids (21). Given the lack of consensus as to the nature and concentration of hK4 both in tissues and body fluids, we wished to reexamine these issues by first developing a specific and sensitive sandwich-type enzyme-linked immunofluorometric assay based on a monoclonal capture antibody with which to measure hK4 in serum, seminal plasma, urine, and other biological fluids and in a panel of nonneoplastic tissues.

## Materials and Methods

### RECOMBINANT hK4

**Production.** A pro-hK4-expressing *Pichia pastoris* KM71 yeast clone (D4-G) was used for hK4 production. This clone had been stably transformed with the pPICZ9 vector of the *P. pastoris* yeast expression reagent set (Invitrogen) and contained the hK4 zymogen-coding cDNA sequence encoding amino acids 27–254 (numbering based on GenBank accession no. NP\_004908). The clone was streaked on minimal dextrose agar plates (13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, 20 g/L dextrose, and 15 g/L agar) and grown at 30 °C for 3 days. A single colony was transferred to minimal dextrose medium and grown in a 30 °C shaking incubator overnight. The culture was transferred to BMGY medium [10 g/L yeast extract,

20 g/L peptone, 100 mmol/L potassium phosphate (pH 6.0), 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, and 10 mL/L glycerol] and grown to an  $A_{600} \geq 2$ . Cells were recovered by centrifugation and resuspended in BMMY medium [10 g/L yeast extract, 20 g/L peptone, 100 mmol/L potassium phosphate (pH 6.0), 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, and 5 mL/L methanol] to one-fifth of the original volume and grown for 5 days, with medium daily supplementation with methanol to a final concentration of 10 mL/L. The supernatant was recovered from the culture by centrifugation. To test for the presence of recombinant hK4, the supernatant was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of the protein samples to a Hybond-C membrane (GE Healthcare). Subsequently, the membrane was probed with hK4-specific rabbit antiserum (diluted 1:500 in Tris-buffered saline containing Tween 20) raised against hK4 (21). After extensive washes with Tris-buffered saline–Tween 20 and application of a horseradish peroxidase–labeled goat anti-mouse IgG (GE Healthcare), fluorescence was detected by use of SuperSignal<sup>®</sup> West Pico chemiluminescent substrate (GE Healthcare).

*Purification.* Recombinant pro-hK4 was purified from the medium by anion-exchange and reversed-phase chromatography. Before purification, supernatant from the yeast culture was concentrated by use of a positive pressure ultrafiltration cell concentrator (Millipore). The first step of purification was performed on a DEAE FF Sepharose Column (GE Healthcare) connected to an automated Amersham Pharmacia  $\Delta$ KTA FPLC Chromatography System (Amersham) at a flow rate of 2.5 mL/min. The hK4 was eluted with a linear gradient (0%–100%) of elution buffer (10 mmol/L Tris-HCl–1 mol/L KCl, pH. 8.0). The hK4-containing fractions (17%–28% of elution buffer) were then pooled, and trifluoroacetic acid (TFA) was added to a final concentration of 10 mL/L. This sample was then further purified by use of a C<sub>4</sub> reversed-phase column (Vydac) connected to an Agilent series 1100 HPLC chromatography system with 1 mL/L TFA in deionized water as the running buffer and 1 mL/L TFA in acetonitrile as the elution buffer. The protein was eluted with a linear gradient of 10%–30% acetonitrile for 10 min and then 30%–80% acetonitrile for 50 min, at a flow rate of 1.0 mL/min. Purified hK4 was collected at 50%–55% acetonitrile, and its purity was assessed on an SDS-PAGE gel stained with SimplyBlue<sup>™</sup> SafeStain according to the manufacturer's instructions (Invitrogen). Nitrogen gas was bubbled through the fractions at a pressure of 15 psi to remove the acetonitrile, and the identity of the purified protein was verified by Western blotting as described above. The total protein concentration of the purified sample was determined by the bicinchoninic acid method, as described below. The identity of hK4 was confirmed by tandem mass spectrometry (MS/MS) and matrix-assisted laser desorption/ionization time-of-flight MS as de-

scribed elsewhere (30) and by N-terminal sequencing using Edman degradation (31). Our MS protocol included reduction, alkylation, and trypsin digestion before MS-MS analysis. For details, see Luo et al. (30).

#### ANTI-hK4 RABBIT POLYCLONAL ANTIBODIES

Purified recombinant hK4 protein (100  $\mu$ g) was diluted 1:1 in complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (subsequent injections) and was injected subcutaneously into female New Zealand White rabbits every 3 weeks for a total of 6 injections per animal. Blood samples taken from the animals 2 weeks after every injection were tested for immunoreactivity by a previously described immunofluorometric method (21) with the pre-immune rabbit serum used as the negative control.

#### ANTI-hK4 MOUSE MONOCLONAL ANTIBODIES

*Preparation.* BALB/c mice were immunized with purified pro-hK4 and tested for immunoreactivity against recombinant pro-hK4 in the same way as the immunized rabbits (described above). On completion of the immunization schedule, the mouse with the highest immunoreactivity against pro-hK4 was sacrificed to obtain its spleen. Using standard hybridoma technology (32), we generated hybridomas by fusing splenocytes with murine myeloma cells.

*Screening.* We prescreened supernatants from hybridoma cultures for positive clones by coating 96-well polystyrene plates overnight with 100 ng/well of sheep anti-mouse antibody (Jackson ImmunoResearch) and adding hybridoma supernatants at 1:10 dilution in a bovine serum albumin (BSA) solution [60 g/L BSA, 50 mmol/L Tris (pH 7.80), 0.5 g/L sodium azide]. After 1 h of incubation, the plates were washed, and 10 ng of biotinylated, purified pro-hK4 protein was added to each well. Binding of biotinylated hK4 was detected by addition of 5 ng of streptavidin–alkaline phosphatase (ALP) conjugate per well. ALP activity was detected by time-resolved fluorometry as described elsewhere (33). We then rescreened supernatants from positive clones for best compatibility with respect to the rabbit polyclonal serum, using the same method we used to optimize the polyclonal antibody–based assay (21). The 5 best clones were then tested for IgG isotype, after which limiting dilutions of the clones were prepared. These clones were expanded in flasks in CD-1 (serum-free) medium (Invitrogen) supplemented with 200 mmol/L glutamine.

*Purification.* Monoclonal anti-hK4 antibodies were purified by use of an Affi-Gel<sup>®</sup> Protein A MAPS II reagent set (Bio-Rad) according to the manufacturer's instructions. Purified antibodies were dialyzed overnight against 8 g/L sodium bicarbonate.

#### hK4-SPECIFIC IMMUNOFUOROMETRIC ASSAY

We added 500 ng of anti-hK4 monoclonal antibody (clone designation 9D4.2D12) diluted in coating buffer (50 mmol/L Tris buffer, pH 7.80) to each well of a 96-well white polystyrene microtiter plate and incubated the plate overnight at room temperature. The plate was then washed 3 times with the wash buffer (9 g/L NaCl and 0.5 mL/L Tween 20 in 10 mmol/L Tris buffer, pH 7.40), after which 50  $\mu$ L of the pro-hK4 calibrators or samples and 50  $\mu$ L of assay buffer A [50 mmol/L Tris buffer (pH 7.80) containing 60 g/L BSA, 5 mL/L Tween 20, 100 mg/L goat  $\gamma$ -immunoglobulins, 20 mg/L mouse IgG, and 1 g/L normal bovine IgG] were added to each well. The plate was incubated for 2 h with shaking, followed by 6 washes. The anti-hK4 rabbit serum (100  $\mu$ L), diluted 1:3000 in assay buffer A, was then pipetted into each well and incubated for 1 h with shaking. The plate was washed 6 times, and 100  $\mu$ L of the ALP-conjugated heavy- and light chain-specific goat anti-rabbit IgG (Jackson Immuno-Research), diluted 1:3000 in assay buffer B [50 mmol/L Tris buffer (pH 7.80) containing 60 g/L BSA, 5 mL/L Tween 20, 500 mmol/L KCl, 100 mg/L goat  $\gamma$ -immunoglobulins, 20 mg/L mouse IgG, and 1 g/L normal bovine IgG] was added to each well and incubated for 45 min with shaking. This was followed by 6 washes as before. The ensuing ALP activity was detected as described previously (21). This optimal assay configuration was obtained after testing for the optimal monoclonal antibody, dilutions and diluents for monoclonal and polyclonal antibodies, and incubation times for each step of the procedure. Assay conditions, including cross-reactivity to all other kallikreins, recovery, within- and between-run imprecision, and detection limit, were determined similarly to earlier protocols (21). We further assessed assay linearity by measuring serial dilutions of a prostate tissue extract in a solution containing 60 g/L BSA.

#### TISSUE PREPARATIONS

*Normal tissue panel.* Using the hK4-specific immunoassay described above, we assessed the relative amounts of hK4 in the following tissues: adrenal gland, axillary lymph node, bone, breast, colon, endometrium, esophagus, fallopian tube, kidney, liver, lung, mesenteric lymph node, skeletal muscle, ovary, pancreas, prostate, seminal vesicle, skin, small intestine, spleen, stomach, testis, thyroid, trachea, ureter, uterus, salivary gland, pharyngeal tonsil, placenta, cervix, cerebellum, frontal lobe cortex, hippocampus, medulla, midbrain, occipital cortex, pituitary, pons, temporal lobe cortex, and spinal cord. This panel was prepared by extraction of tissue cytosols from 0.2 g of snap-frozen tissues as described previously (34).

*Benign and malignant prostate tissues.* Eighteen benign and 16 malignant prostate tissues were obtained from men who had undergone radical retropubic prostatectomy for prostatic adenocarcinoma at the Evangelismos Hospital in Athens, Greece. Tissue samples underwent histologic

assessment and dissection to obtain cancerous and non-cancerous (benign) parts of the prostate. These tissue sets included 8 matched pairs in which malignant and benign parts of the same prostate were obtained. Use of these samples for research was approved by the research ethics committee of Evangelismos Hospital. Samples were stored in liquid nitrogen and shipped on dry ice to Mount Sinai Hospital in Toronto, where they were pulverized in liquid nitrogen. Cytosolic extracts of the pulverized tissues were prepared the same way as for the healthy control tissues and stored at  $-80^{\circ}\text{C}$  until use.

*Total protein measurement.* Total protein concentrations were assessed in tissue extracts by the bicinchoninic acid total protein assay as described by the manufacturer (Pierce), with BSA dilutions used as calibrators.

#### BIOLOGICAL FLUIDS

Various biological fluids (cerebrospinal fluid, breast milk, amniotic fluid, seminal plasma, ascites fluid from ovarian cancer patients, urine, and sera from males and females without known disease) had been submitted previously for routine biochemical testing and were stored at  $-80^{\circ}\text{C}$ .

*Recovery of hK4 in serum.* We pooled 250- $\mu$ L portions from 10 male or female serum samples and separated 200  $\mu$ L of this pool a silica-based TSK-GEL gel filtration column at a flow rate of 0.5 mL/min as described elsewhere (35); 0.5-mL fractions were collected in a Bio-Rad 2110 automatic fraction collector. To determine the molecular masses range of the collected fractions, a gel-filtration calibrator (Bio-Rad) was fractionated in a separate run. To control for the variability of total protein content, 500  $\mu$ L of a 120 g/L BSA solution was added to these fractions. As a control, 500  $\mu$ L of the unfractionated serum pool was treated similarly. The fractions, the unfractionated control, and the 60 g/L BSA solution were then mixed with the 20  $\mu$ g/L assay calibrator at a 3:1 ratio and incubated at room temperature for 30 min. These preparations were then assayed for hK4 by the hK4-specific ELISA to test for the recovery of the exogenous hK4. This experiment was repeated with hK4 labeled with  $^{125}\text{I}$ , using Iodobeads<sup>®</sup> as described by the manufacturer (Pierce).

*Screening of seminal plasma and urine for hK4 content.* Samples were centrifuged at 23 000g for 10 min before assay. For initial screening, the hK4 content in 50  $\mu$ L of each undiluted seminal plasma sample was measured by the hK4-specific assay described above. Samples testing positive for hK4 were then rescreened at 1:10 and 1:100 dilutions in the 60 g/L BSA solution.

*Fractionation by HPLC.* A 100- $\mu$ L seminal plasma sample with relatively high hK4 content (as determined by the hK4-specific immunoassay) and another with relatively low hK4 content were separated on a silica-based TSK-GEL gel filtration column [60  $\times$  0.75 (i.d.) cm], as de-

scribed elsewhere (35), with a running buffer of 100 mmol/L  $\text{NaH}_2\text{PO}_4$ –100 mmol/L  $\text{Na}_2\text{SO}_4$  (pH 6.8) at a flow rate of 0.5 mL/min. The column was calibrated with 670-, 158-, 44-, 17-, and 1.3-kDa molecular mass calibrators (Bio-Rad) in a separate run. Gel-filtration fractions were subsequently analyzed with the hK4-specific immunoassay, and fluorescence was plotted against fraction number. This fractionation procedure was repeated for a 250- $\mu\text{L}$  urine sample containing a high hK4 concentration [concentrated 10-fold by use of a Centricon YM 10 concentrator (Amicon) before HPLC] as well as for 250  $\mu\text{L}$  of a prostate tissue extract with relatively high (50  $\mu\text{g}/\text{L}$ ) hK4 content. To the tissue and biological fluid fractions we added 0.5 mL of 120 g/L BSA to equalize total protein content, and fractions were assayed for hK4 with the newly developed immunoassay and for other prostate-specific kallikreins (hK2, hK3, and hK11) by the respective immunoassays described elsewhere (35–37).

**Precipitation of hK4 from seminal plasma.** Seminal plasma samples were pooled and deprived of hK11 by elution from an immunoaffinity column containing immobilized hK11-specific antibody. The seminal plasma samples were incubated with soybean trypsin inhibitor–agarose beads (Sigma) overnight at 4 °C. The beads were then washed sequentially with 20 mmol/L Tris buffer containing 150 mmol/L NaCl and 1 mL/L Triton X-100. Bound proteins were then eluted with 0.2 mol/L glycine (pH 2.5). Proteins from eluted fractions were precipitated with trichloroacetic acid–acetone, the pellet was resuspended in water, and the suspension was subjected to MS analysis.

## Results

### RECOMBINANT hK4

*P. pastoris* yeast supernatants reached maximum secreted hK4 content after 4 days of induction with 10 mL/L methanol. As shown in Fig. 1A, after anion-exchange and reversed-phase chromatography, 2 distinct bands were visible on an SDS-PAGE gel stained with SimplyBlue SafeStain: a major band at 28–29 kDa and another band at ~33 kDa. MS confirmed the identities of both bands as hK4. Deglycosylation experiments revealed that the discrepancy between the size of the pro-hK4 species determined on the basis of their electrophoretic migration and predicted size based on amino acid sequence was entirely attributable to glycosylation: a single band at ~25 kDa was visible on the gel after the sample underwent deglycosylation with PNGase F (Fig. 1B). Western blotting of pro-hK4 with an anti-hK4 rabbit polyclonal antibody confirmed the identity of both bands as hK4. N-Terminal sequencing data further confirmed the production of the pro-form of hK4 (Gly-Ser-Cys-Ser-Gln-Ile-Ile-Asn-Gly-Glu) with 5 additional amino acids (activation peptide) at the  $\text{NH}_2$  terminus (underlined), suggesting the absence of autoactivation.

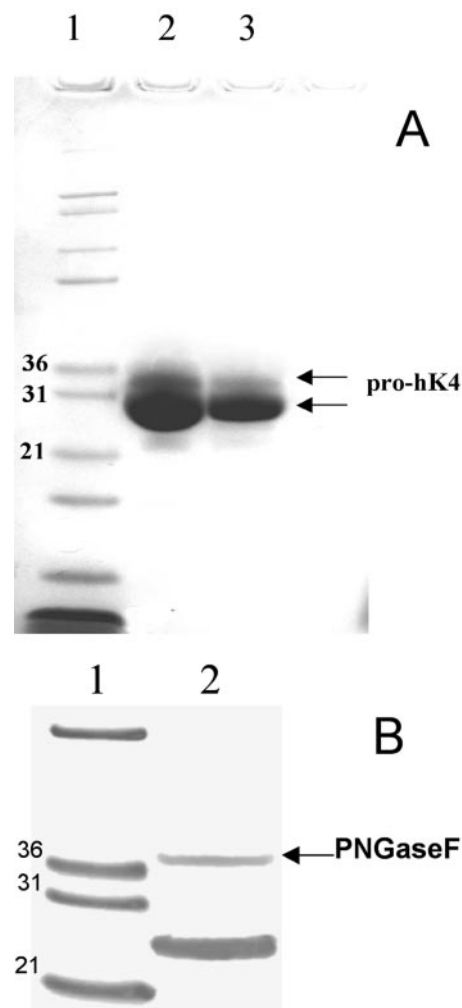


Fig. 1. Recombinant hK4 protein.

(A), purified recombinant pro-hK4 as visualized on an SDS-PAGE gel by SimplyBlue SafeStain. Lane 1, molecular marker; lanes 2 and 3, recombinant hK4. (B), deglycosylation of recombinant pro-hK4 by PNGase F. Lane 1, molecular marker; lane 2, hK4 deglycosylated by PNGase F.

### hK4-SPECIFIC IMMUNOASSAY

The hK4-specific immunoassay developed here uses a monoclonal antibody for hK4 capture that recognizes both the zymogen and mature forms of recombinant hK4 made by the *P. pastoris* expression system, as well as mammalian recombinant hK4 zymogen obtained from transfection of CHO cells (data not shown). When any of the other 14 human kallikrein proteins (recombinant) were used in large excess (1000  $\mu\text{g}/\text{L}$ ), the cross-reactivity of the hK4 assay was <0.1%, indicating good specificity. Dilutions of recombinant hK4 protein were used as the calibrators for the assay at 0, 0.05, 0.2, 2, 5, and 20  $\mu\text{g}/\text{L}$ . The detection limit of the assay, defined as the hK4 concentration that corresponds to 2 SD above the 0 calibrator signal, was 0.02  $\mu\text{g}/\text{L}$ . The within- and between-run imprecision (CV) was <10%. Within the range of the calibrators, the assay demonstrated excellent linearity, as demonstrated by serial dilutions of various samples in 60 g/L BSA solution (data not shown).

**hK4 IN TISSUES**

*Healthy tissues.* Among the 40 adult human tissues tested, 12 contained hK4 concentrations above the assay detection limit (range, 0.68–7143 ng/g of total protein). The hK4 concentrations (adjusted for total protein content) in a panel of healthy tissues are shown in Fig. 2. In this series, concentrations were the highest in prostatic, endometrial, and breast tissues, which is in agreement with previously reported mRNA data.

*Prostate extracts.* The distributions of hK4 concentrations in benign and malignant prostate tissues are shown in Table 1. hK4 in the benign tissue extracts ranged from below the detection limit to 801 ng/g (median, 63 ng/g). The hK4 content in malignant tissue extracts ranged from below the detection limit to 824 ng/g (median, 29 ng/g). The difference between benign and malignant prostate tissues with respect to hK4 content was not statistically significant, nor were hK4 concentrations significantly different between the 8 pairs of matched (benign/malignant) prostate tissues. To further demonstrate the specificity of the assay and to establish the molecular mass of the immunoreactive species, we performed gel-filtration liquid chromatography of an extract from a healthy

**Table 1. hK4 concentrations in benign and malignant prostate tissues.<sup>a</sup>**

Tissue type	hK4, ng/g				
	Percentile			Mean	Range
	25th	50th	75th		
Benign (n = 18)	6.3	63	308	92	<0.02 to 801
Malignant (n = 16)	8.0	29	69	169	<0.02 to 824

<sup>a</sup> Differences were not statistically significant between benign and malignant tissues.

prostate tissue with a relatively high hK4 concentration (7143 ng/g). As shown in Fig. 3, hK4 concentrations peaked in fraction 39, corresponding to a molecular mass of 30 kDa, based on separation of the gel filtration calibrators. Furthermore, the elution volume was different from those of hK11, hK2, and hK3, 3 other prostate-specific kallikreins assessed in the same fractions with their respective immunoassays. Added to the fact that hK4 concentrations in these tissues did not correlate with hK3 (prostate-specific antigen) concentrations (data not shown), our results confirm that the newly developed hK4 immunoassay can specifically detect hK4 in prostate

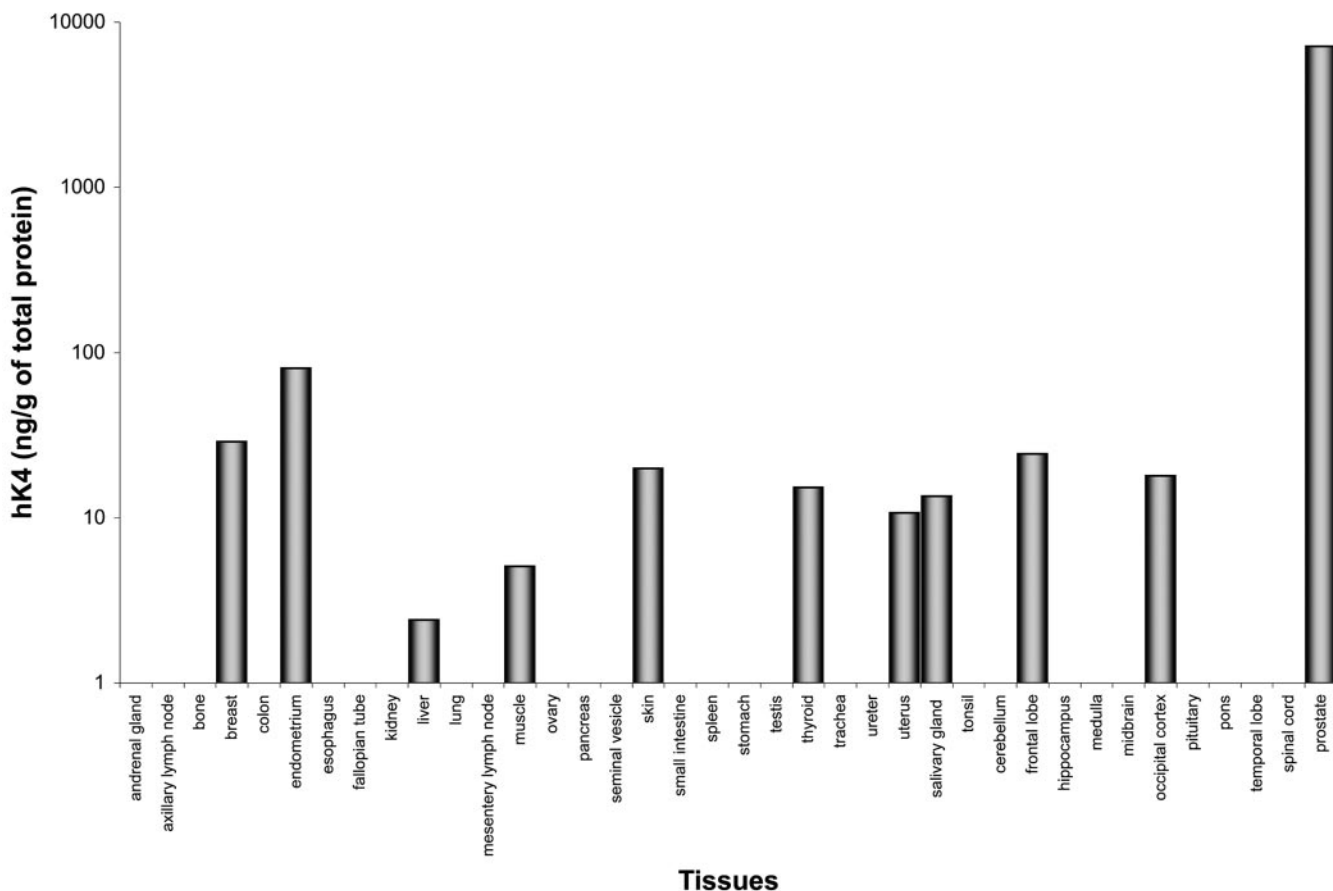


Fig. 2. hK4 content of healthy human tissues (ng/g of total protein). The y axis is logarithmic. Note that the highest concentration is in the prostate.

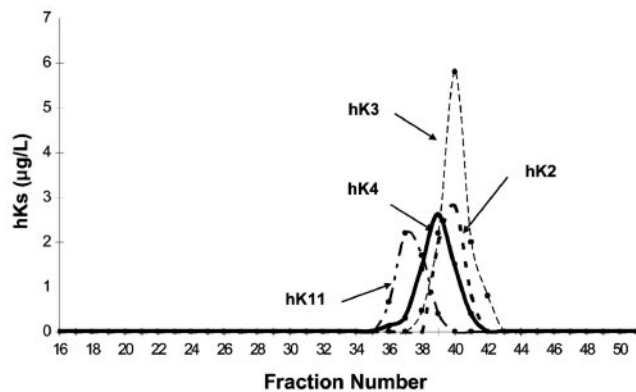


Fig. 3. Gel-filtration fractionation of human kallikreins (*hKs*) from healthy prostate tissue.

The relative concentrations of the 4 human kallikreins measured by ELISA have no quantitative meaning because the extract was diluted to bring assays within range, as follows: hK2, 1:100; hK3, 1:10 000; hK11, 1:10. Note the distinct peak for each kallikrein, suggesting no cross-reactivity.

tissues without cross-reactivity from other kallikreins (hK2, hK3, and hK11) present at much higher concentrations.

#### hK4 IN BIOLOGICAL FLUIDS

**Seminal plasma.** As shown in Table 2, all seminal plasma samples contained measurable amounts of hK4; most samples (68%) had  $<5.0 \mu\text{g/L}$ . No sample had an hK4 value between 20 and  $35 \mu\text{g/L}$ , whereas 4 samples had relatively high hK4 concentrations ( $>100 \mu\text{g/L}$ ). As shown in Fig. 4, gel-filtration HPLC fractionation of 2 seminal plasma samples with high ( $150 \mu\text{g/L}$ ) and low ( $0.1 \mu\text{g/L}$ ) hK4 content and measurement of hK4 in the fractions revealed a peak present in the high-hK4 sample but not in the low-hK4 sample. Moreover, the peak was unique to hK4 in that it did not coincide with the peaks measured for other kallikreins (hK2, hK11, and hK3) known to be present in seminal plasma at concentrations much higher than that of hK4. MS results on affinity-precipitated serine proteases from seminal plasma identified 2 peptide fragments (FTEWIEK and APCGQVGVPGVYTN) close to the COOH terminus that were unique to hK4, further confirming the presence of hK4 in seminal plasma.

**Urine.** In the 54 urine samples tested for hK4, 2 had concentrations below the detection limit of the assay; the others had values ranging from  $0.02$  to  $2.6 \mu\text{g/L}$  and 50%

**Table 2. hK4 concentrations in biological fluids.<sup>a</sup>**

Fluid	hK4, $\mu\text{g/L}$				
	Percentile			Mean	Range
	25th	50th	75th		
Seminal plasma (n = 58)	1.4	3.2	7.6	18.3	0.05–202
Urine (n = 54)	0.2	0.2	0.4	0.3	$<0.02$ to 2.6

<sup>a</sup> For concentrations in other fluids, see the text.

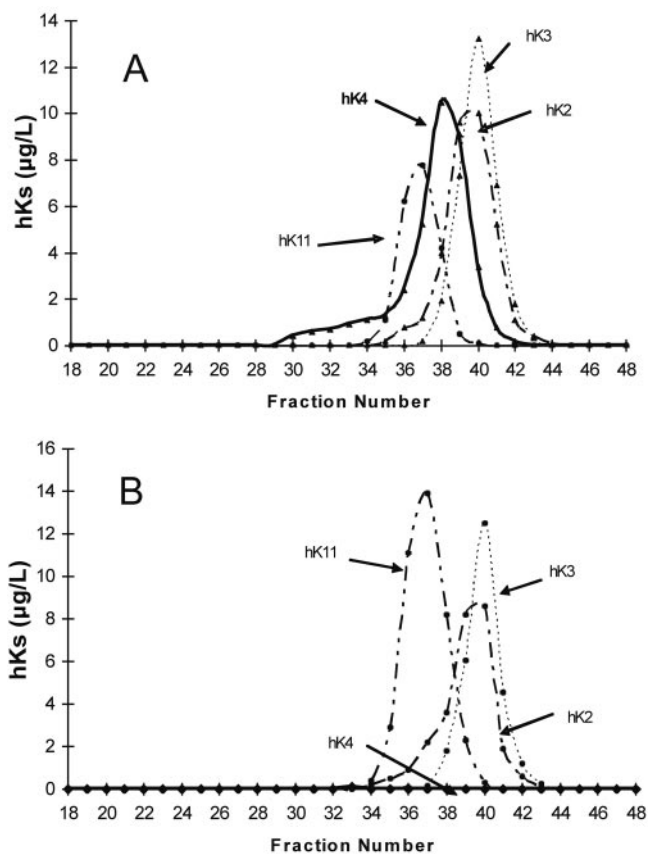


Fig. 4. Gel-filtration fractionation of seminal plasma samples.

(A), seminal plasma sample with  $150 \mu\text{g/L}$  (high) hK4; (B), seminal plasma sample with  $0.1 \mu\text{g/L}$  (low) hK4. Also shown are hK2, hK3, and hK11 concentrations in the same fractions as quantified by their respective assays. To assess hK2, hK3, and hK11, fractions were further diluted 1:100, 1:10 000, and 1:100, respectively. Note the distinct peak for each kallikrein, as in Fig. 3, and the absence of hK4 in the hK4-negative seminal plasma (B).

had hK4 values  $<0.2 \mu\text{g/L}$  (Table 2). Gel-filtration fractionation of a urine sample with the highest hK4 content gave a single peak appeared at fraction 40 (molecular mass  $\sim 30 \text{ kDa}$ ) that coincided with the peak obtained by fractionation of the recombinant hK4 protein calibrator used in our assay, suggesting that the molecular mass of hK4 in urine is similar to that of intact recombinant hK4 ( $\sim 30 \text{ kDa}$ ; data not shown).

**All other fluids.** Similar analysis of breast milk (n = 6), amniotic fluid (n = 6), and saliva (n = 6) for hK4 concentrations demonstrated trace amounts ( $<0.05 \mu\text{g/L}$ ) of hK4, whereas normal male and female sera (n = 10) and cerebrospinal fluid samples (n = 6) had undetectable concentrations of hK4 (data not shown).

**Recovery of hK4 in serum.** For this experiment, we first fractionated a serum sample by gel-filtration chromatography into 50 fractions and then added a constant amount of hK4 to each fraction. We also added hK4 to unfractionated serum and a BSA solution. We then assayed each fraction for hK4 by the ELISA to investigate possible

hK4-binding proteins. As shown in Fig. 5, recovery of hK4 was lowest in fractions 23 and 24. These fractions corresponded to molecular masses of 630–700 kDa (based on the gel-filtration calibrators). Moreover, recovery in these fractions was comparable to the recovery in unfractionated serum. When we defined the recovery of the hK4 calibrator in 60 g/L BSA solution as 100%, the recovery of hK4 in fractions 23 and 24 was 60%, similar to the recovery obtained in unfractionated serum supplemented with hK4. We hypothesized that the low recovery in serum was attributable to binding of hK4 to high-molecular-mass (650–700 kDa) serum proteins. Addition of radiolabeled hK4 to serum fractions and subsequent separation on an SDS-PAGE gel under reducing conditions revealed that hK4 formed an SDS-stable, covalent complex with high-molecular-mass serum proteins (Fig. 5). On the basis of the molecular masses and binding to serine proteases, we suggest that the major binding protein of hK4 in serum is  $\alpha_2$ -macroglobulin.

**Discussion**

The lack of consensus on the nature and concentration of hK4 in bodily fluids and tissues prompted us to examine hK4 concentrations in biological fluids to ascertain whether this protein is indeed secreted. The aims of our study also included the development of a specific and sensitive sandwich-type, enzyme-linked immunofluorometric assay based on a monoclonal capture antibody to minimize the background signal and improve the sensitivity of our first-generation, double polyclonal antibody-based hK4 assay (21). We produced hK4 proprotein without any tag or additional protein sequence to ensure conformational similarity to native hK4. As with recombinant hK4 protein produced previously (21), hyperglycosylation of the recombinant protein was not observed, and its size of ~33 kDa was consistent with the presence of a single N-glycosylation site. Our monoclonal-polyclonal immunoassay configuration surpassed the sensitivity of our previous polyclonal-polyclonal assay and had a

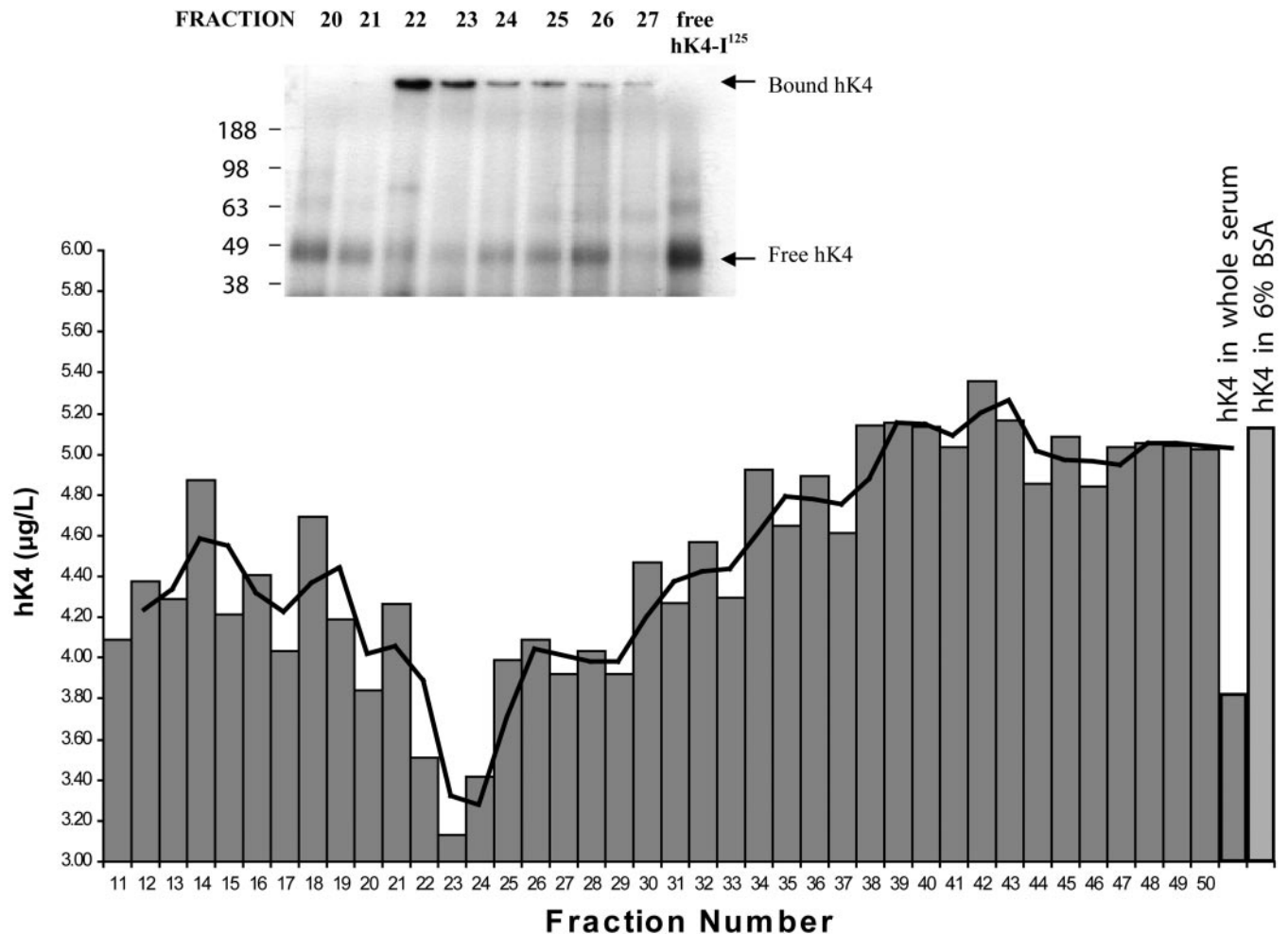


Fig. 5. Recovery of hK4 in serum previously fractionated by gel-filtration chromatography. hK4 concentrations by ELISA are shown after 5 µg/L hK4 was added after fractionation to serum fractions 11–50. The last 2 columns indicate 5 µg/L added to whole (unfractionated) serum and to 60 g/L BSA solution, respectively. (Inset), autoradiography of serum fractions 20–27 supplemented with <sup>125</sup>I-labeled hK4. Note the high-molecular-mass hK4 complex in fractions 23 and 24. For additional information, see the text.



lower background signal, making possible the reliable detection of even lower concentrations of hK4 (down to 0.02  $\mu\text{g}/\text{L}$ ). This assay also recognized enzymatically active (and presumably correctly folded) recombinant hK4 (data not shown), which strongly suggested that the assay should also be able to detect native hK4 in fluids and tissues.

In the adult normal tissue extract panel, the assay detected highest hK4 expression in healthy prostate, with concentrations exceeding those found in endometrium, breast, skin, and brain tissues by 100-fold. The relative concentrations of hK4 detected in these tissues correlated well with previous mRNA data (11, 38–40) and further support the nearly prostate-specific expression of hK4, similarly to hK2 and hK3. Comparatively, the concentrations of the 5 documented kallikreins in seminal plasma and prostatic tissue extracts were as follows: hK3  $\gg$  hK2, hK11  $>$  hK4, hK5.

Although we analyzed several sera from both males and females for hK4, we were not able to detect native hK4 above the detection limit (0.02  $\mu\text{g}/\text{L}$ ) in any of these specimens (data not shown). Recovery experiments indicated that once added to serum, only 39%–58% of hK4 remained immunoreactive with the ELISA. From the results obtained by fractionation of serum by gel-filtration chromatography and subsequent addition of recombinant radiolabeled or nonlabeled hK4, it is apparent that hK4 forms a covalent complex in serum with a very high molecular-mass protein ( $>600$  kDa; Fig. 5). It is known that serine protease inhibitors in serum, among them  $\alpha_2$ -macroglobulin, bind kallikreins such as hK2, hK3, hK5, and hK13 (41–45); therefore, hK4 in serum likely forms a complex with  $\alpha_2$ -macroglobulin. This notion is supported by the fact that  $\alpha_2$ -macroglobulin binds many other kallikreins (hK2, hK3, and others) and because of its large size, renders them immunologically undetectable (46, 47) unless the complex is exposed to a high (nonphysiologic) pH before assaying (48). Complex formation with this inhibitor leads to relatively fast clearance from the circulation by the liver via binding of  $\alpha_2$ -macroglobulin to  $\alpha_2$ -macroglobulin-receptor/LDL receptor-related protein (49). Thus, detection of low amounts of hK4 protein in serum becomes more difficult with formation of this complex. In contrast to findings in serum, similar hK4 complexes were not observed in seminal plasma (data not shown), although  $\alpha_2$ -macroglobulin is known to be present in this fluid (50). One explanation may be that the amount of  $\alpha_2$ -macroglobulin varies widely, ranging from 1 to 1000  $\mu\text{g}/\text{L}$  in seminal plasma (51), and that this is only a fraction of the protein found in serum, where concentrations range from 1.5 to 5 g/L in healthy individuals (52). Complexing of hK4 with serum serine proteinase inhibitors, leading to nonimmunoreactive complexes, may be another explanation for the lack of serum immunoreactivity of hK4.

Of the biological fluids examined (serum, amniotic fluid, breast milk, cerebrospinal fluid, urine, and seminal

plasma), the only ones in which hK4 was detected were urine and seminal plasma, with by far the highest hK4 concentrations in seminal plasma (Table 2). Indeed, concentrations in fluids other than urine were either borderline detectable or undetectable. These results agree with previous findings (21). The specificity of our assay for hK4 was confirmed by fractionation of high- vs low-hK4-containing seminal plasma samples (Fig. 4) and demonstration that hK4 is immunologically distinct from hK3, hK2, and hK11, which are found at much higher concentrations in seminal plasma. The simultaneous presence of at least 4 kallikreins in seminal plasma, as well as other serine proteases such as trypsin, supports the idea of an enzymatic cascade (53) reminiscent of the coagulation cascade, in which hK4 present in low amounts takes part in the activation of pro-hK11 or pro-hK2, and pro-hK2 can then amplify its own activity by autoactivation; in turn, hK2 and/or hK4 can activate the highly abundant pro-hK3 (pro-prostate-specific antigen), leading to cleavage of the seminogelin proteins and dissolution of the seminal clot. There is already evidence indicating that hK4 can activate pro-hK3 (54). In most seminal plasma samples, the amounts of hK4 were quite low, but occasional samples had very high hK4 concentrations for reasons that are not as yet understood. Confirmation of the presence of hK4 in seminal plasma by MS unequivocally established that hK4, like other kallikreins, is secreted. Although other investigators were quick to dismiss the secreted form of hK4 as physiologically irrelevant based on its low mRNA concentrations relative to mRNA coding for the intracellular form of hK4 (14, 19), the surprising finding that intracellular hK4, predicted to be 22 kDa, was a 45-kDa nuclear protein made the existence of intracellular hK4 protein debatable. This is particularly the case because the nonglycosylated nature of nuclear proteins excludes posttranslational modification to account for the size discrepancy. In addition, the use of an anti-peptide antibody whose target sequence has no amino acids in common with intracellular hK4 makes this claim even more controversial (22). Our findings of hK4 in seminal plasma point to a possible physiologic role of hK4 that is in accord with its secreted nature, with the well-established degradative roles of its porcine and murine homolog (23, 25, 26, 55), and with the extracellular nature of its enzymatic substrates (56).

In summary, we demonstrated predominant hK4 production in the prostate and the presence of hK4 in seminal plasma and urine, in support of its secreted nature. Our hK4-specific immunoassay and autoradiograms demonstrated covalent binding of hK4 to a high-molecular-mass serum protein, most likely  $\alpha_2$ -macroglobulin. Our data warrant further investigations on the possible role of hK4 as a serum marker for detection and monitoring of prostate cancer. Furthermore, the role of hK4 as a member of a cascade enzymatic pathway operating in the prostate and seminal plasma should be further investigated.

## References

1. Walker B, Lynas JF. Strategies for the inhibition of serine proteases. *Cell Mol Life Sci* 2001;58:596–624.
2. Rawlings ND, Barrett AJ. Evolutionary families of peptidases. *Biochem J* 1993;290:205–18.
3. Schwartz LB. Cellular inflammation in asthma: neutral proteases of mast cells. *Am Rev Respir Dis* 1992;145:S18–21.
4. Ratnoff OD. The molecular basis of hereditary clotting disorders. *Prog Hemost Thromb* 1972;1:39–74.
5. Kanazawa H, Kurihara N, Hirata K, Fujimoto S, Takeda T. The role of free radicals and neutrophil elastase in development of pulmonary emphysema. *Intern Med* 1992;31:857–60.
6. DeClerck YA, Mercurio AM, Stack MS, Chapman HA, Zutter MM, Muschel RJ, et al. Proteases, extracellular matrix, and cancer: a workshop of the path B study section. *Am J Pathol* 2004;164:1131–9.
7. DeClerck YA, Imren S, Montgomery AM, Mueller BM, Reisfeld RA, Laug WE. Proteases and protease inhibitors in tumor progression. *Adv Exp Med Biol* 1997;425:89–97.
8. Freije JM, Balbin M, Pendas AM, Sanchez LM, Puente XS, Lopez-Otin C. Matrix metalloproteinases and tumor progression. *Adv Exp Med Biol* 2003;532:91–107.
9. Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. *Nat Rev Cancer* 2004;4:876–90.
10. Nelson PS, Gan L, Ferguson C, Moss P, Gelinis R, Hood L, et al. Molecular cloning and characterization of prostase, an androgen-regulated serine protease with prostate-restricted expression. *Proc Natl Acad Sci U S A* 1999;96:3114–9.
11. Yousef GM, Obiezu CV, Luo LY, Black MH, Diamandis EP. Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated. *Cancer Res* 1999;59:4252–6.
12. Yousef GM, Luo LY, Diamandis EP. Identification of novel human kallikrein-like genes on chromosome 19q13.3-q13.4. *Anticancer Res* 1999;19:2843–52.
13. Stephenson SA, Verity K, Ashworth LK, Clements JA. Localization of a new prostate-specific antigen-related serine protease gene, KLK4, is evidence for an expanded human kallikrein gene family cluster on chromosome 19q13.3-13.4. *J Biol Chem* 1999;274:23210–4.
14. Korkmaz KS, Korkmaz CG, Pretlow TG, Saatcioglu F. Distinctly different gene structure of KLK4/KLK-L1/prostase/ARM1 compared with other members of the kallikrein family: intracellular localization, alternative cDNA forms, and regulation by multiple hormones. *DNA Cell Biol* 2001;20:435–45.
15. Myers SA, Clements JA. Kallikrein 4 (KLK4), a new member of the human kallikrein gene family is up-regulated by estrogen and progesterone in the human endometrial cancer cell line, KLE. *J Clin Endocrinol Metab* 2001;86:2323–6.
16. Dong Y, Kaushal A, Bui L, Chu S, Fuller PJ, Nicklin J, et al. Human kallikrein 4 (KLK4) is highly expressed in serous ovarian carcinomas. *Clin Cancer Res* 2001;7:2363–71.
17. Obiezu CV, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S, et al. Higher human kallikrein gene 4 (KLK4) expression indicates poor prognosis of ovarian cancer patients. *Clin Cancer Res* 2001;7:2380–6.
18. Xi Z, Kaern J, Davidson B, Klock TI, Risberg B, Trope C, et al. Kallikrein 4 is associated with paclitaxel resistance in ovarian cancer. *Gynecol Oncol* 2004;94:80–5.
19. Xi Z, Klock TI, Korkmaz K, Kurys P, Elbi C, Risberg B, et al. Kallikrein 4 is a predominantly nuclear protein and is overexpressed in prostate cancer. *Cancer Res* 2004;64:2365–70.
20. Kurlender L, Borgono C, Michael IP, Obiezu CV, Yousef G, Diamandis E. A survey of alternative transcripts of human tissue kallikrein genes. *Ciophys Acta* 2005;1755:1–14.
21. Obiezu CV, Soosaipillai A, Jung K, Stephan C, Scorilas A, Howarth DH, et al. Detection of human kallikrein 4 in healthy and cancerous prostatic tissues by immunofluorometry and immunohistochemistry. *Clin Chem* 2002;48:1232–40.
22. Simmer JP, Bartlett JD. Kallikrein 4 is a secreted protein. *Cancer Res* 2004;64:8481–2.
23. Hu JC, Sun X, Zhang C, Liu S, Bartlett JD, Simmer JP. Enamelysin and kallikrein-4 mRNA expression in developing mouse molars. *Eur J Oral Sci* 2002;110:307–15.
24. Nagano T, Oida S, Ando H, Gomi K, Arai T, Fukae M. Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. *J Dent Res* 2003;82:982–6.
25. Hu JC, Zhang C, Sun X, Yang Y, Cao X, Ryu O, et al. Characterization of the mouse and human PRSS17 genes, their relationship to other serine proteases, and the expression of PRSS17 in developing mouse incisors. *Gene* 2000;251:1–8.
26. Ryu O, Hu JC, Yamakoshi Y, Villemain JL, Cao X, Zhang C, et al. Porcine kallikrein-4 activation, glycosylation, activity, and expression in prokaryotic and eukaryotic hosts. *Eur J Oral Sci* 2002;110:358–65.
27. Hart TC, Hart PS, Gorry MC, Michalec MD, Ryu OH, Uygur C, et al. Novel ENAM mutation responsible for autosomal recessive amelogenesis imperfecta and localized enamel defects. *J Med Genet* 2003;40:900–6.
28. Hart PS, Wright JT, Savage M, Kang G, Bensen JT, Gorry MC, et al. Exclusion of candidate genes in two families with autosomal dominant hypocalcified amelogenesis imperfecta. *Eur J Oral Sci* 2003;111:326–31.
29. Hart PS, Hart TC, Michalec MD, Ryu OH, Simmons D, Hong S, et al. Mutation in kallikrein 4 causes autosomal recessive hypomaturation amelogenesis imperfecta. *J Med Genet* 2004;41:545–9.
30. Luo LY, Grass L, Howarth DJ, Thibault P, Ong H, Diamandis EP. Immunofluorometric assay of human kallikrein 10 and its identification in biological fluids and tissues. *Clin Chem* 2001;47:237–46.
31. Edman P. Sequence determination. *Mol Biol Biochem Biophys* 1970;8:211–55.
32. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975;256:495–7.
33. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. *Anal Chem* 1992;64:342–6.
34. Borgono CA, Grass L, Soosaipillai A, Yousef GM, Petraki CD, Howarth DH, et al. Human kallikrein 14: a new potential biomarker for ovarian and breast cancer. *Cancer Res* 2003;63:9032–41.
35. Yu H, Diamandis EP. Ultrasensitive time-resolved immunofluorometric assay of prostate-specific antigen in serum and preliminary clinical studies. *Clin Chem* 1993;39:2108–14.
36. Black MH, Magklara A, Obiezu CV, Melegos DN, Diamandis EP. Development of an ultrasensitive immunoassay for human glandular kallikrein with no cross-reactivity from prostate-specific antigen. *Clin Chem* 1999;45:790–9.
37. Diamandis EP, Okui A, Mitsui S, Luo LY, Soosaipillai A, Grass L, et al. Human kallikrein 11: a new biomarker of prostate and ovarian carcinoma. *Cancer Res* 2002;62:295–300.
38. Gan L, Lee I, Smith R, Argonza-Barrett R, Lei H, McCuaig J, et al. Sequencing and expression analysis of the serine protease gene cluster located in chromosome 19q13 region. *Gene* 2000;257:119–30.
39. Day CH, Fanger GR, Retter MW, Hylander BL, Penetrante RB, Houghton RL, et al. Characterization of KLK4 expression and detection of KLK4-specific antibody in prostate cancer patient sera. *Oncogene* 2002;21:7114–20.
40. Komatsu N, Takata M, Otsuki N, Toyama T, Ohka R, Takehara K, et al. Expression and localization of tissue kallikrein mRNAs in

- human epidermis and appendages. *J Invest Dermatol* 2003;121:542–9.
41. Christensson A, Laurell CB, Lilja H. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur J Biochem* 1990;194:755–63.
  42. Kapadia C, Chang A, Sotiropoulou G, Yousef GM, Grass L, Soosaipillai A, et al. Human kallikrein 13: production and purification of recombinant protein and monoclonal and polyclonal antibodies, and development of a sensitive and specific immunofluorometric assay. *Clin Chem* 2003;49:77–86.
  43. Lilja H. Structure, function, and regulation of the enzyme activity of prostate-specific antigen. *World J Urol* 1993;11:188–91.
  44. Yousef GM, Kapadia C, Polymeris ME, Borgono C, Hutchinson S, Wasney GA, et al. The human kallikrein protein 5 (hK5) is enzymatically active, glycosylated and forms complexes with two protease inhibitors in ovarian cancer fluids. *Biochim Biophys Acta* 2003;1628:88–96.
  45. Mikolajczyk SD, Millar LS, Kumar A, Saedi MS. Human glandular kallikrein, hK2, shows arginine-restricted specificity and forms complexes with plasma protease inhibitors. *Prostate* 1998;34:44–50.
  46. Kioukia-Fougia N, Christofidis I, Strantzalis N. Physicochemical conditions affecting the formation/stability of serum complexes and the determination of prostate-specific antigen (PSA). *Anticancer Res* 1999;19:3315–20.
  47. Leinonen J, Zhang WM, Stenman UH. Complex formation between PSA isoenzymes and protease inhibitors. *J Urol* 1996;155:1099–103.
  48. Zhang WM, Finne P, Leinonen J, Vesalainen S, Nordling S, Rannikko S, et al. Characterization and immunological determination of the complex between prostate-specific antigen and  $\alpha_2$ -macroglobulin. *Clin Chem* 1998;44:2471–9.
  49. Birkenmeier G, Struck F, Gebhardt R. Clearance mechanism of prostate specific antigen and its complexes with  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antichymotrypsin. *J Urol* 1999;162:897–901.
  50. Birkenmeier G, Usbeck E, Schafer A, Otto A, Glander HJ. Prostate-specific antigen triggers transformation of seminal  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M) and its binding to  $\alpha_2$ -macroglobulin receptor/low-density lipoprotein receptor-related protein ( $\alpha_2$ -M-R/LRP) on human spermatozoa. *Prostate* 1998;36:219–25.
  51. Kramer MD, Simon MM, Tilgen W, Naher H, Justus CW, Petzoldt D.  $\alpha_2$ -Macroglobulin and  $\alpha_2$ -macroglobulin/proteinase complexes in human seminal fluid. *Fertil Steril* 1992;57:417–21.
  52. Ritchie RF, Palomaki GE, Neveux LM, Navolotskaia O. Reference distributions for  $\alpha_2$ -macroglobulin: a comparison of a large cohort to the world's literature. *J Clin Lab Anal* 2004;18:148–52.
  53. Yousef GM, Diamandis EP. Human tissue kallikreins: a new enzymatic cascade pathway? *Biol Chem* 2002;383:1045–57.
  54. Takayama TK, McMullen BA, Nelson PS, Matsumura M, Fujikawa K. Characterization of hK4 (prostase), a prostate-specific serine protease: Activation of the precursor of prostate specific antigen (pro-PSA) and single-chain urokinase-type plasminogen activator and degradation of prostatic acid phosphatase. *Biochemistry* 2001;40:15341–8.
  55. Simmer JP, Hu JC. Expression, structure, and function of enamel proteinases. *Connect Tissue Res* 2002;43:441–9.
  56. Matsumura M, Bhatt AS, Andress D, Clegg N, Takayama TK, Craik CS, et al. Substrates of the prostate-specific serine protease prostase/KLK4 defined by positional-scanning peptide libraries. *Prostate* 2005;62:1–13.