Clinical Chemistry 47:2
237–246 (2001)

Enzymes and Protein Markers

Immunofluorometric Assay of Human Kallikrein 10 and Its Identification in Biological Fluids and Tissues

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Background: **The human kallikrein 10 gene [*KLK10*, also known as normal epithelial cell-specific 1 gene (*NES1*)] is a member of the human kallikrein gene family. The *KLK10* gene encodes for a secreted serine protease (hK10). We hypothesize that hK10 is secreted into various biological fluids and that its concentration changes in some disease states. The aim of this study was to develop a sensitive and specific immunoassay for hK10.**

Methods: **Recombinant hK10 protein was produced and purified using a *Pichia pastoris* yeast expression system. The protein was used as an immunogen to generate mouse and rabbit polyclonal anti-hK10 antisera. A sandwich-type immunofluorometric assay was then developed using these antibodies.**

Results: **The hK10 immunoassay has a detection limit of 0.05 μg/L. The assay is specific for hK10 and has no detectable cross-reactivity with other homologous kallikrein proteins, such as prostate-specific antigen (hK3), human glandular kallikrein 2 (hK2), and human kallikrein 6 (hK6). The assay was linear from 0 to 20 μg/L with within- and between-run CVs <10%. hK10 is expressed in many tissues, including the salivary glands, skin, and colon and is also detectable in biological fluids, including breast milk, seminal plasma, cerebrospinal fluid, amniotic fluid, and serum.**

Conclusions: **We report development of the first immunofluorometric assay for hK10 and describe the distribution of hK10 in biological fluids and tissue extracts. This assay can be used to examine the value of hK10 as a disease biomarker.**

The human kallikrein gene family is a group of genes that are clustered on chromosome 19q13.3-q13.4 and share significant homologies at both the nucleotide and amino acid levels *(1–5)*. Originally, this gene family was thought to contain only three members: *KLK1*,[5] which encodes for human kallikrein 1 (hK1), also known as pancreatic/renal kallikrein; *KLK2*, which encodes for hK2; and *KLK3*, which encodes for hK3, also known as prostate-specific antigen (PSA). More recently, new genes have been identified in the same chromosomal region and are now considered to be members of the kallikrein gene family *(6)*. These new kallikrein genes were originally given various empirical names. An international group of investigators has recently agreed on new human kallikrein gene nomenclature *(7)*. This gene family now contains at least 15 genes, which are designated *KLK1–KLK15*; their encoded proteins are designated hK1–hK15.

The normal epithelial cell-specific 1 (*NES1*) gene is one of these newly identified genes. With the new nomenclature, which will be used throughout this report, *NES1* is designated as *KLK10* and the encoded protein as hK10. *KLK10* was isolated with subtractive hybridization, by virtue of its down-regulation in radiation-transformed

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Received August 23, 2000; accepted November 13, 2000.

[5] Nonstandard abbreviations: *KLK*, kallikrein gene; hK, human kallikrein protein; PSA, prostate-specific antigen; *NES1*, normal epithelial cell-specific 1 gene; CSF, cerebrospinal fluid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and MS-MS, tandem mass spectrometry.

breast epithelial cells *(8)*. *KLK10* resides on chromosome 19q13.3-q13.4, spans ~5.5 kb of genomic DNA sequence, and contains six exons (one untranslated) and five introns *(9)*. *KLK10* encodes for a secreted serine protease whose amino acid sequence has 35–40% identity and 50–55% similarity with other members of the human kallikrein gene family, including PSA. The physiological function of *KLK10* is still not clear. Because the *KLK10* gene is down-regulated in breast cancer cell lines, it is considered to be involved in the regulation of normal cell growth. Further experimental evidence suggests that *KLK10* may encode for a tumor suppressor gene. When the *KLK10* gene was transfected into the tumorigenic breast cancer cell line MDA-MB-231, its anchorage-independent growth was reduced, and when this cell line was inoculated into nude mice, tumor formation was significantly decreased *(10)*.

Because *KLK10* encodes for a secreted serine protease and its expression appears to be altered in some disease states, including breast and prostate cancer *(8, 10, 11)*, we hypothesized that hK10 protein may be secreted into various biological fluids and that its concentration might change during disease initiation and progression. However, no suitable method for measuring hK10 with high sensitivity and specificity has been described to date. Consequently, the concentrations of this protein in biological fluids and tissue extracts have not been reported. In this report, we describe the first immunoassay for hK10 protein, which is suitable for measuring hK10 with high sensitivity and specificity. With this method, we were able to quantify hK10 in serum, breast milk, seminal plasma, cerebrospinal fluid (CSF), and amniotic fluid as well as in various tissue extracts. This newly developed immunoassay is suitable for examining the possible value of hK10 as a biomarker in various human diseases, including cancer.

Materials and Methods

PRODUCTION AND PURIFICATION OF RECOMBINANT hK10

Cloning of KLK10 cDNA into the yeast expression vector. To produce recombinant hK10, the Easyselect™ *Pichia pastoris* yeast expression system (Invitrogen) was used. Two primers were designed to amplify the *KLK10* cDNA sequence encoding for amino acids 43–276 [the numbering of amino acids of hK10 is reported in Ref. *(8)*]: forward primer, 5′-AACGACGAATTCTTGGACCCCG-AAGCCT-3′; reverse primer, 5′-CGTAGAATTCGGAT-CAGTTGGA-3′. Human ovarian cDNA was used as template. PCR was carried out in a 20-μL reaction mixture, containing 1 μL of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 100 ng of the primers, and 2.5 U of pfu DNA polymerase (Stratagene) on a Perkin-Elmer 9600 thermal cycler. The PCR conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR product was then cloned into the yeast expression vector pPIZαA using standard molecular biology techniques *(12)*.

Production of hK10 in yeast. pPIZαA vector containing the *KLK10* cDNA was introduced into the yeast strain X-33, and a stable clone was selected following the manufacturer's instructions. hK10 was produced by growing the stable yeast clone in a medium containing 10 g/L yeast extract, 20 g/L peptone, 100 mmol/L potassium phosphate (pH 6.0), 13.4 g/L yeast nitrogen base, 40 mg/L biotin, and 5 mL/L methanol in a 30 °C shaking incubator (250 rpm) for 5 days. The cells were then spun down, and the supernatant was collected.

Purification of hK10 with cation-exchange and reversed-phase chromatography. The recombinant hK10 protein was purified from the yeast culture supernatant by cation-exchange chromatography using CM-Sepharose fast flow (Pharmacia) and reversed-phase liquid chromatography using a C_4 column (0.45 × 5 cm; Vydac). The presence of hK10 in various fractions was identified with Western blotting using an anti-hK10 peptide antibody. In brief, the CM-Sepharose beads previously activated with 1 mol/L KCl were equilibrated in 10 mmol/L MES buffer (pH 6.5). The yeast culture supernatant was first adsorbed on CM-Sepharose beads by incubation at 4 °C overnight under agitation. The beads were then washed with 10 mmol/L MES buffer (pH 6.5), and hK10 was eluted with 300 mmol/L KCl in 10 mmol/L MES buffer (pH 6.5). Trifluoroacetic acid as ion-pairing agent was added into this eluate (final concentration, 10 mL/L), which was then loaded on a C_4 column equilibrated with 1 mL/L trifluoroacetic acid in water. A linear gradient (1%/min) of acetonitrile from 15% to 50% in 1 mL/L trifluoroacetic acid was then performed. hK10 eluted at 42% acetonitrile. The fraction containing hK10 was then immediately evaporated on a SpeedVac (Savant Instrument). The purified material was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Blue to assess its purity and its molecular mass. The protein concentration of the purified hK10 was determined by the bicinchoninic acid method, which uses bovine serum albumin as a calibrator (Pierce Chemical).

IDENTIFICATION OF hK10 BY MASS SPECTROMETRY

Proteolytic digestion. Polyacrylamide gels were stained with Coomassie G-250 for visualization, and selected bands were subsequently excised and destained with 300 mL/L acetonitrile in 100 mmol/L ammonium bicarbonate. Each band was then reduced (10 mmol/L dithiothreitol in 50 mmol/L ammonium bicarbonate, pH 8.3) and alkylated (55 mmol/L iodoacetamide in 50 mmol/L ammonium bicarbonate, pH 8.3) before overnight trypsin digestion (Promega). Peptide fragments were then extracted with 50 mL/L acetic acid, evaporated to dryness on a Savant concentrator, and reconstituted in 10 μL of a

solution of methanol-water-acetic acid (500:495:5 by volume).

Mass spectrometry. All nanoelectrospray mass spectrometry experiments were conducted on a Q-Star (PE/Sciex) hybrid quadrupole/time-of-flight instrument, for high resolution and online tandem mass spectrometry (MS-MS) experiments *(13)*. Conventional mass spectra were obtained by operating the quadrupole in a radiofrequency-only mode while a pusher electrode was pulsed (frequency ~7 kHz) to transfer all ions to the time-of-flight analyzer. MS-MS experiments on tryptic peptides identified in survey scans were conducted using a nanoelectrospray source. Precursor ions were selected by the first quadrupole while a pusher electrode was pulsed (frequency ~7 kHz) to transfer fragment ions formed in the radiofrequency-only quadrupole cell to the time-of-flight analyzer. Mass spectral resolution was typically 9000–10 000. A scan duration of 1 and 2 s was set for conventional and MS-MS mass spectral acquisition, respectively. Collisional activation was performed using nitrogen collision gas with typically a 30-V offset between the DC voltage of the entrance quadrupole and the radiofrequency-only quadrupole cell. Data were acquired and processed using LC Tune and Biomultiview programs from PE/Sciex.

PRODUCTION OF POLYCLONAL ANTIBODIES AGAINST hK10

The purified recombinant hK10 was used as an immunogen to immunize rabbits and mice. hK10 (100 μg) was injected subcutaneously into female Balb/c mice and New Zealand White rabbits. The protein was diluted 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the subsequent injections. Injections were repeated six times at 3-week intervals. Blood was drawn from the animals and tested for antibody generation. To test for production of anti-hK10 polyclonal antibodies, we used the following immunoassay. Sheep anti-mouse or goat anti-rabbit IgG (Jackson ImmunoResearch) was immobilized on 96-well white ELISA plates. The mouse/rabbit serum was then applied to the plates in different dilutions ranging from 1:500 to 1:50 000. Biotinylated recombinant hK10 was then added (5–10 ng/well). Finally, alkaline phosphatase-conjugated streptavidin was added, and the alkaline phosphatase activity was detected with time-resolved fluorescence (for more details, see below).

IMMUNOFLUOROMETRIC ASSAY FOR hK10

Standard assay procedure. White polystyrene microtiter plates were coated with sheep anti-mouse IgG, Fc fragment-specific antibody (Jackson ImmunoResearch) by overnight incubation of 100 μL of coating antibody solution (containing 500 ng of antibody diluted in 50 mmol/L Tris buffer, pH 7.80) in each well. The plates were then washed six times with the washing buffer (9 g/L NaCl and 0.5 g/L Tween 20 in 10 mmol/L Tris buffer, pH 7.40). Mouse anti-hK10 antiserum was diluted 5000-fold in a general diluent [60 g/L bovine serum albumin, 50 mmol/L Tris (pH 7.80), and 0.5 g/L sodium azide], and 100 μL was applied to each well. After a 1-h incubation, the plates were washed six times with washing buffer.

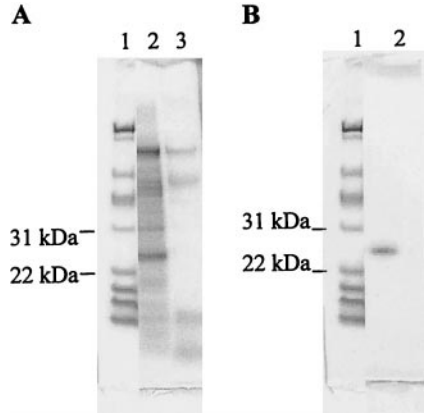

Fig. 1. Production of recombinant hK10 protein with the *P. pastoris* yeast expression system.

The proteins were separated on SDS-PAGE and stained with Coomassie Blue. (*A*), *lane 1*, molecular mass markers; *lane 2*, culture supernatant obtained from a yeast clone transfected with pPIZαA vector containing *KLK10* cDNA; *lane 3*, culture supernatant obtained from a yeast clone transfected with empty pPIZαA vector. (*B*), purified recombinant hK10 from the yeast expression system. *Lane 1*, molecular mass markers; *lane 2*, purified recombinant hK10. Purification was achieved with cation-exchange followed by reversed-phase chromatography. The *intense band* between 31 and 22 kDa in *lane 2* represents hK10.

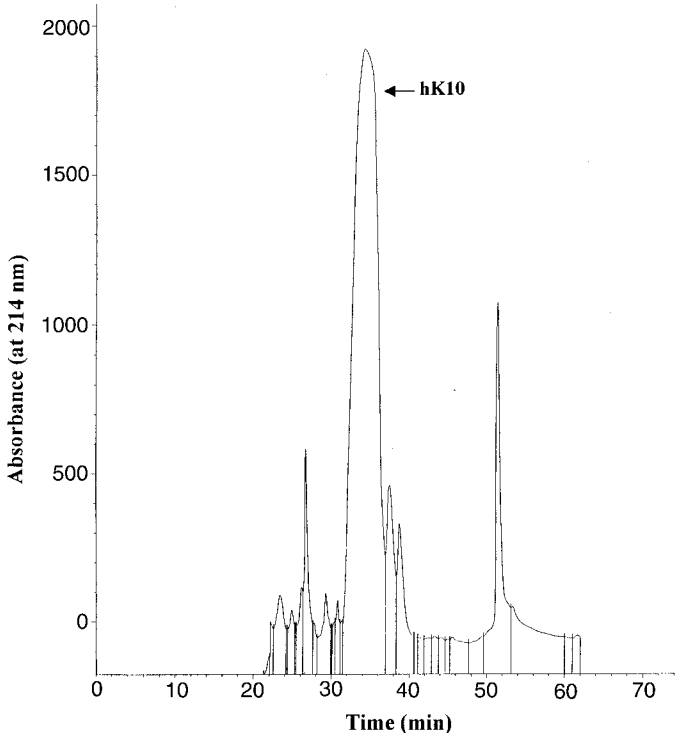

Fig. 2. Typical chromatogram showing the purification of recombinant hK10 from yeast culture supernatant with reversed-phase HPLC.

Recombinant hK10 elutes at ~35 min (41–42% acetonitrile). For more details, see the text.

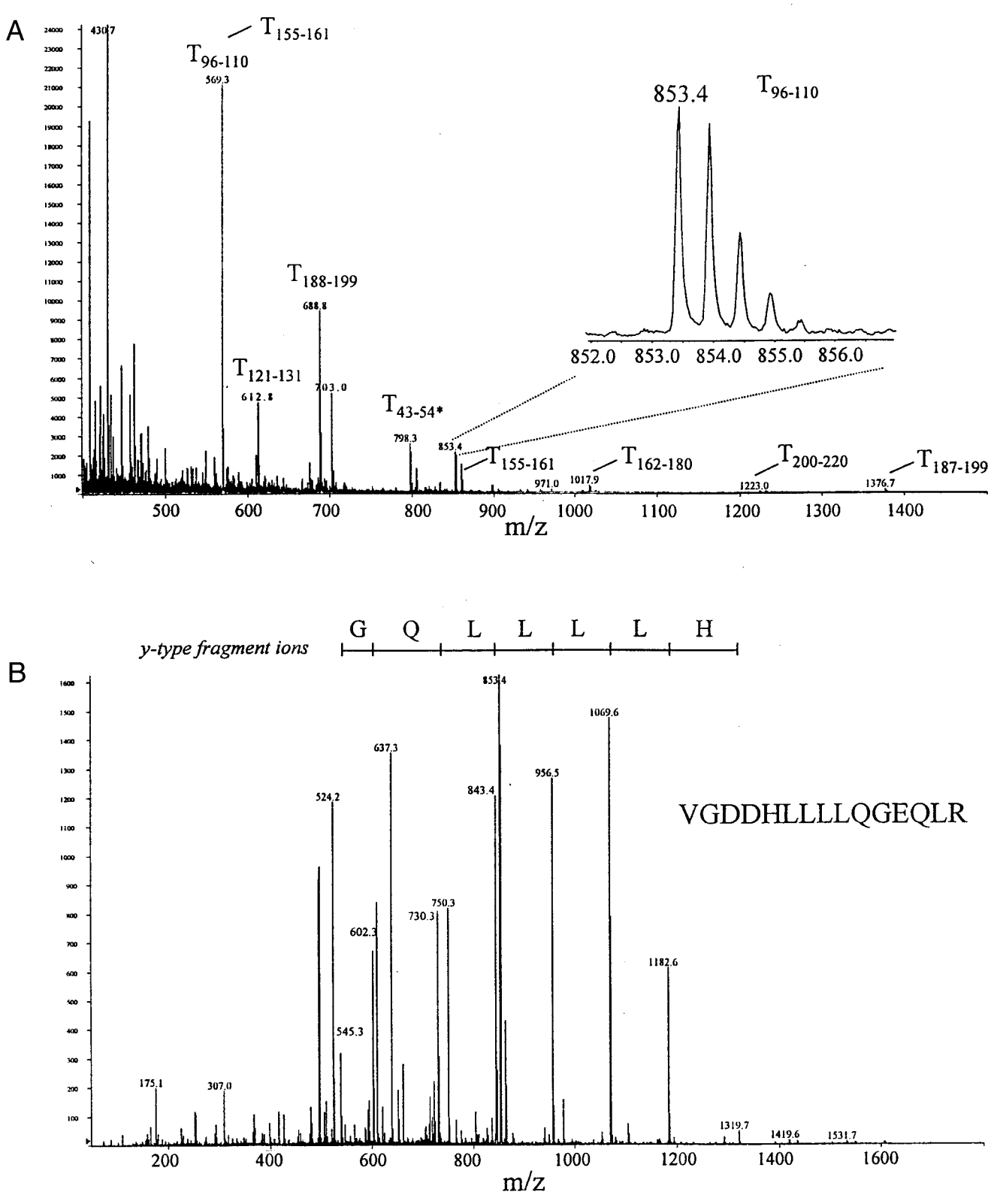

Fig. 3. Mass spectral characterization of hK10 and its tryptic peptides isolated from the band in Fig. 1B, lane 2.

(*A*), nanoelectrospray mass spectrum of gel-extracted peptides. Product ion spectrum of precursor *m/z* 853.4 using a collision energy of 30 V offset, with nitrogen as the target gas. (*B*), *y-type fragment ions* refers to cleavage of peptide bonds with charge retention on the C-terminal end *(16)*. Amino acids are annotated using single-letter code. The peptide sequence VGDDHLLLLQGEQLR represents amino acids 96–110 of the hK10 sequence, as reported by Liu et al. *(8)*.

hK10 calibrators or samples were pipetted into each well (100 μL/well) and incubated for 1 h with shaking; the plates were then washed with washing buffer six times. Subsequently, 100 μL of rabbit anti-hK10 antiserum diluted 5000-fold in buffer A (containing the components of the general diluent plus 25 mL/L normal mouse serum, 100 mL/L normal goat serum, and 10 g/L bovine IgG) was applied to each well and incubated for 30 min; plates were then washed as above. Finally, 100 μL/well of alkaline phosphatase-conjugated goat anti-rabbit IgG, Fc fragment-specific (Jackson ImmunoResearch), diluted 3000-fold in buffer A was added to each well and incubated for 30 min, and plates were washed as above.

Diflunisal phosphate [100 μL of a 1 mmol/L solution in substrate buffer (0.1 mol/L Tris (pH 9.1), 0.1 mol/L NaCl, and 1 mmol/L $MgCl_2$] was added to each well and incubated for 10 min. Developing solution (100 μL, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mol/L EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion). The calibration and data reduction were performed automatically, as described in detail elsewhere *(14)*.

Determination of the sensitivity of the hK10 immunoassay. Recombinant hK10 was used to generate the calibration curve. hK10 calibrators were prepared by diluting the purified recombinant hK10 in the general diluent. These calibrators were used to define the detection limit of the assay.

Determination of the specificity of the hK10 immunoassay. Milk and seminal plasma samples and recombinant hK10 were used to determine the specificity of the developed immunoassay. These samples were first measured by the standard assay procedure described above. The mouse and rabbit anti-hK10 antisera were then successively replaced with sera from the same animals, obtained before immunization (preimmune sera). The samples were then measured again, and the fluorescence counts were compared with the counts obtained by the standard assay. The cross-reactivities of other homologous proteins were investigated using purified recombinant PSA, hK2, and hK6 (available in-house). Recombinant hK10 (0.025–2 ng), PSA (20 ng to 1 μg), hK2 (2–10 ng) and hK6 (11–55 ng) were used as samples and were measured with the standard procedure described above; their fluorescence counts were then compared (all specified amounts refer to mass of analyte per assay).

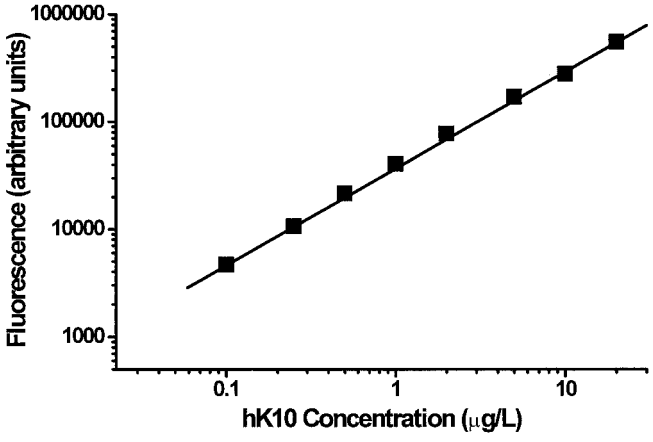

Fig. 4. Typical calibration curve for the hK10 immunoassay.

The background fluorescence (zero calibrator) has been subtracted from all measurements. The dynamic range of the assay is 0.05–20 μg/L.

Linearity of the hK10 immunoassay. To determine the linearity of the hK10 immunoassay, various clinical samples were serially diluted 2-, 4-, 8-, 16-, and 32-fold in general diluent, and their hK10 concentrations were measured with the standard assay.

HUMAN TISSUE CYTOSOLIC EXTRACTS AND BIOLOGICAL FLUIDS

Human tissue cytosolic extracts were prepared as follows: Various frozen human tissues (0.2 g) were pulverized on dry ice to fine powders. Extraction buffer [1 mL, containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 10 g/L NP-40 surfactant, 1 mmol/L phenylmethylsulfonyl fluoride, 1 g/L aprotinin, 1 g/L leupeptin] was added to the tissue powders, and the mixture was incubated on ice for 30 min with repeated shaking and vortex-mixing every 10 min. Mixtures were then centrifuged at 14 000 rpm at 4 °C for 30 min. The supernatants (cytosolic extracts) were then collected. The biological fluids were leftovers of samples submitted for routine biochemical testing. All tissue cytosolic extracts and biological fluids were stored at −80 °C until use.

RECOVERY

Recombinant hK10 was added to the general diluent, serum, CSF, breast milk, seminal plasma, and amniotic fluid at different concentrations and measured with the developed hK10 immunoassay. Recoveries were then calculated after subtraction of the endogenous concentrations.

FRACTIONATION OF BIOLOGICAL FLUIDS WITH SIZE-EXCLUSION HPLC

Serum, milk, and seminal plasma were fractionated on a silica-based gel filtration column essentially as described elsewhere *(15)*. The fractions were collected and analyzed for hK10 with the developed immunoassay.

Results

PRODUCTION AND PURIFICATION OF hK10 RECOMBINANT PROTEIN

hK10 is a secreted serine protease. Hydrophobicity and structural homology analysis suggested that the active form of hK10 was predicted to start from amino acid 43

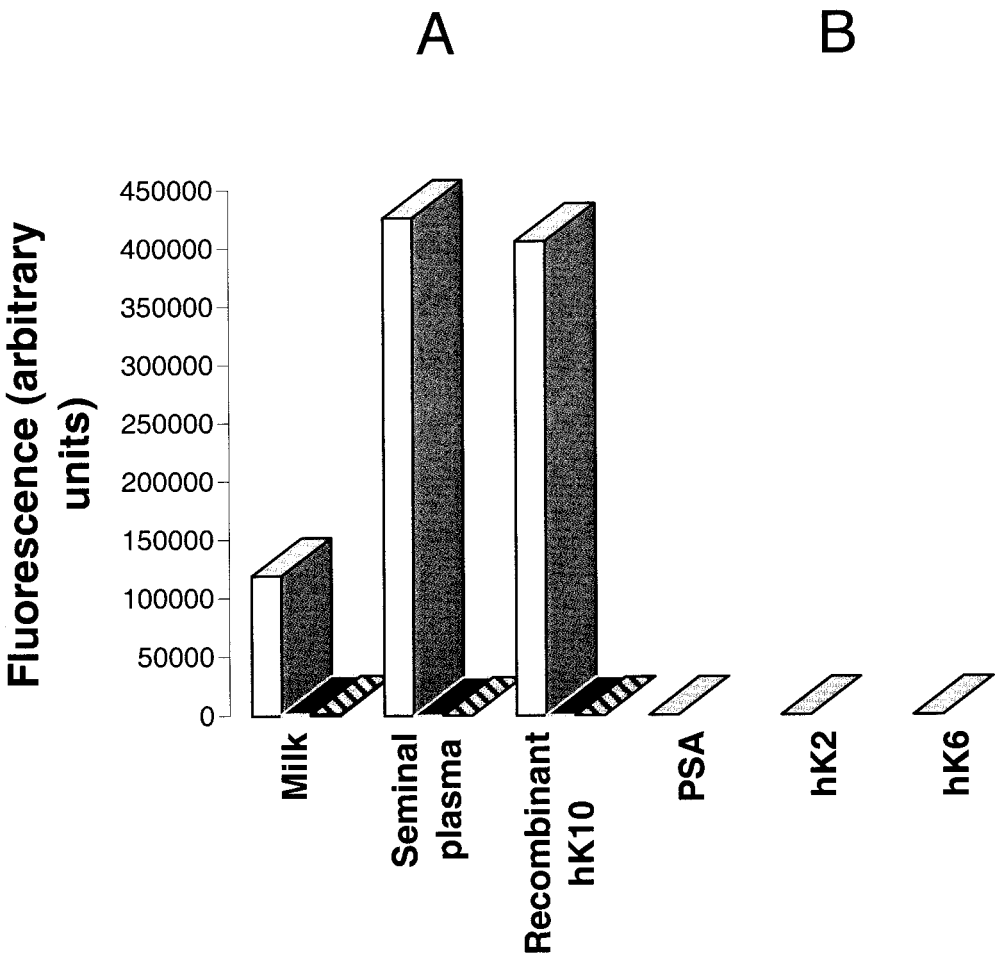

Fig. 5. Bar diagrams showing the specificity of the hK10 immunoassay (*A*) and the cross-reactivity between hK10 and PSA, hK2, and hK6 (*B*).
For details of this experiment, see the text. (*A*), *open columns*, mouse and rabbit anti-hK10 antisera; *filled columns*, preimmune mouse serum plus rabbit anti-hK10 antiserum; *hatched columns*, mouse anti-hK10 antiserum plus preimmune rabbit serum. Note the absence of immunoreactivity when the hK10 antisera are replaced with preimmune sera. (*B*), cross-reactivity of PSA, hK10, and hK6 in the standard hK10 immunoassay. No detectable cross-reactivity was found (for more details, see text).

[the segment 1–42 represents the signal peptide (33 amino acids) and the activation peptide (9 amino acids)] *(8)*. The cDNA encoding for this active form of hK10 was cloned into a *P. pastoris* yeast expression system. The 26-kDa recombinant hK10 protein was produced and secreted into the yeast culture supernatant (Fig. 1A), and then purified with cation-exchange and reversed-phase chromatography (Fig. 1B and Fig. 2). The sequence of this recombinant protein was verified by mass spectrometry (Fig. 3). The nanoelectrospray mass spectrum of the tryptic peptides extracted from the SDS-PAGE band is presented in Fig. 3A, and shows several doubly and triply protonated molecules ($[M+H]^{2+}$ and $[M+H]^{3+}$) at *m/z* 550-1400. The calculated masses derived from the *m/z* values were compared with the list of tryptic peptide masses predicted from the hK10 protein sequence. Tryptic peptides corresponding to a segment of the protein are indicated as subscripts in Fig. 3A. Confirmation of the sequence assignment was achieved using MS-MS on selected precursor ions observed in Fig. 3A. For example, the product ion spectrum of the doubly protonated ion *m/z* 853.4 (Fig. 3B) shows a series of well defined y-type fragment ions corresponding to amide bond cleavage with charge retention on the C-terminal peptide segment *(16)*. The partial sequence VGDDHLLLLQGEQLR was identified from the *m/z* spacing between adjacent fragment ions and precisely matched that expected for the T96–110 peptide of hK10.

SENSITIVITY, SPECIFICITY, LINEARITY, AND PRECISION OF THE hK10 IMMUNOFLUOROMETRIC ASSAY

Sensitivity. A typical calibration curve for the hK10 immunofluorometric procedure is shown in Fig. 4. The detection limit, defined as the concentration corresponding to the fluorescence of the zero calibrator plus 2 SD, was 0.05 μg/L.

Specificity. To confirm that our immunoassay measures hK10 specifically, we replaced mouse and rabbit anti-hK10 antisera with preimmune mouse and rabbit sera,

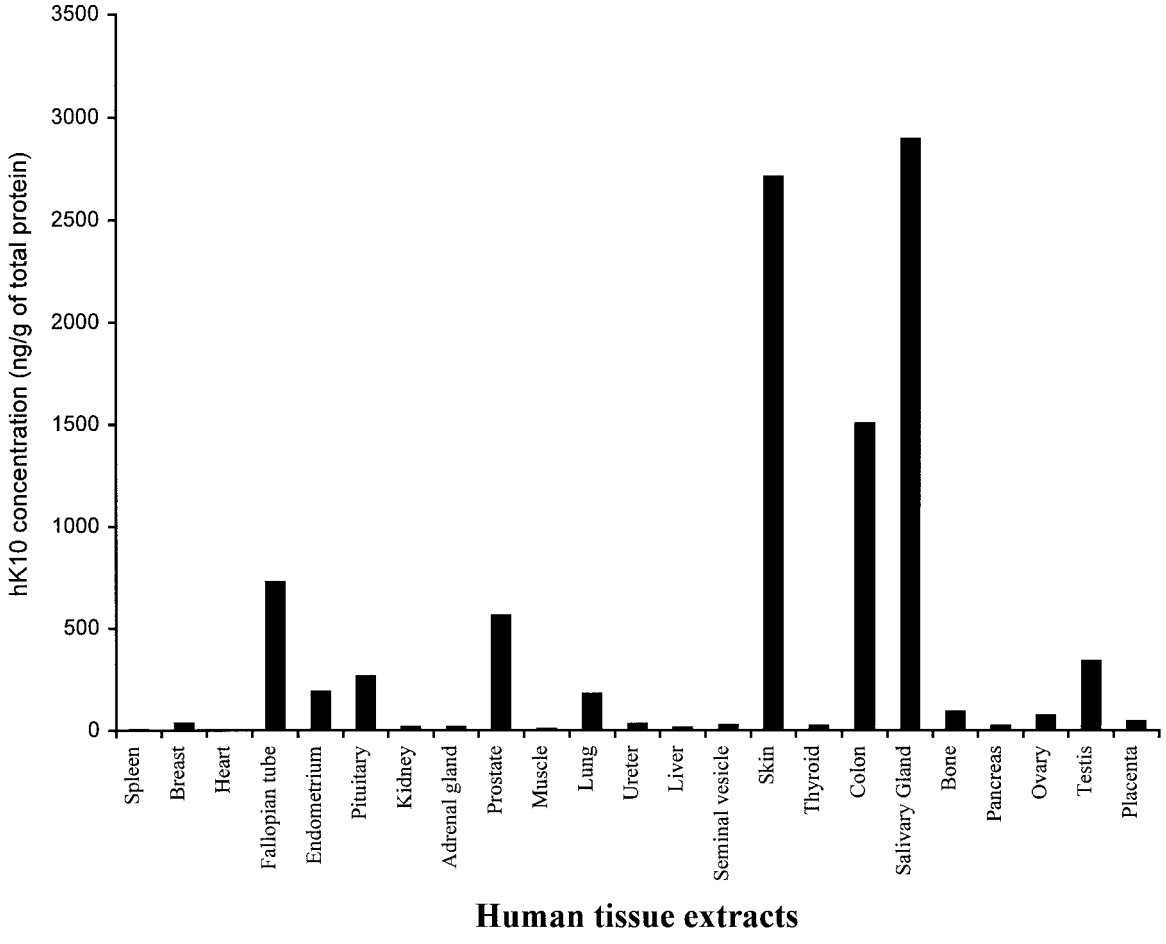

Fig. 6. Concentration of hK10 (ng of hK10 per g of total protein) in cytosolic extracts from various human tissues.

respectively. Milk and seminal plasma samples and recombinant hK10 were measured. With the standard procedure, these samples all produced relatively high counts (>100 000 arbitrary fluorescence units). However, when either mouse or rabbit anti-hK10 polyclonal antibodies were replaced with preimmune sera, the fluorescence counts of these samples were reduced almost to zero (Fig. 5). Because hK10 is a member of the human kallikrein family, it shares significant amino acid sequence homology with other members. To further demonstrate that there is no interference from these homologous proteins, the cross-reactivities of hK10, PSA, hK2, and hK6 were examined. As shown in Fig. 5, when recombinant PSA, hK2, and hK6 were measured with the developed standard hK10 assay, they produced no counts even when they were present at amounts 5–500 times higher than hK10. These results suggest that our immunoassay can efficiently discriminate hK10 from other homologous proteins and that it measures hK10 with high specificity.

Linearity. To assess the linearity of this assay, various samples were diluted serially and hK10 was measured. There is excellent dilution linearity with this assay.

Precision. Within- and between-run precision was assessed with various hK10 calibrators and clinical samples. In all cases, the CVs were 2–9%, consistent with the precision of typical microtiter plate-based immunoassays (data not shown).

DISTRIBUTION OF hK10 IN VARIOUS HUMAN TISSUE EXTRACTS AND BIOLOGICAL FLUIDS

The distribution of hK10 in various human tissue extracts and biological fluids was investigated with the developed hK10 immunoassay. As shown in Fig. 6, hK10 is present in

Table 1. Analysis of hK10 in various biological fluids.

Biological fluid	hK10 concentration, μg/L			No. of samples tested	Positivity rate, %
	Range	Mean (SD)	Median		
Breast milk	0.1–32.3	6.8 (9.2)	2.4	27	100
Seminal Plasma	3.3–23.6	10.5 (5.8)	8.9	24	100
Amniotic fluid	0.4–20.9	11.6 (5.6)	11.9	23	100
Male serum	0.3–1.2	0.7 (0.2)	0.7	29	100
Female serum	0.1–1.3	0.6 (0.3)	0.6	28	100
CSF	0.0–1.2	0.4 (0.3)	0.3	19	95

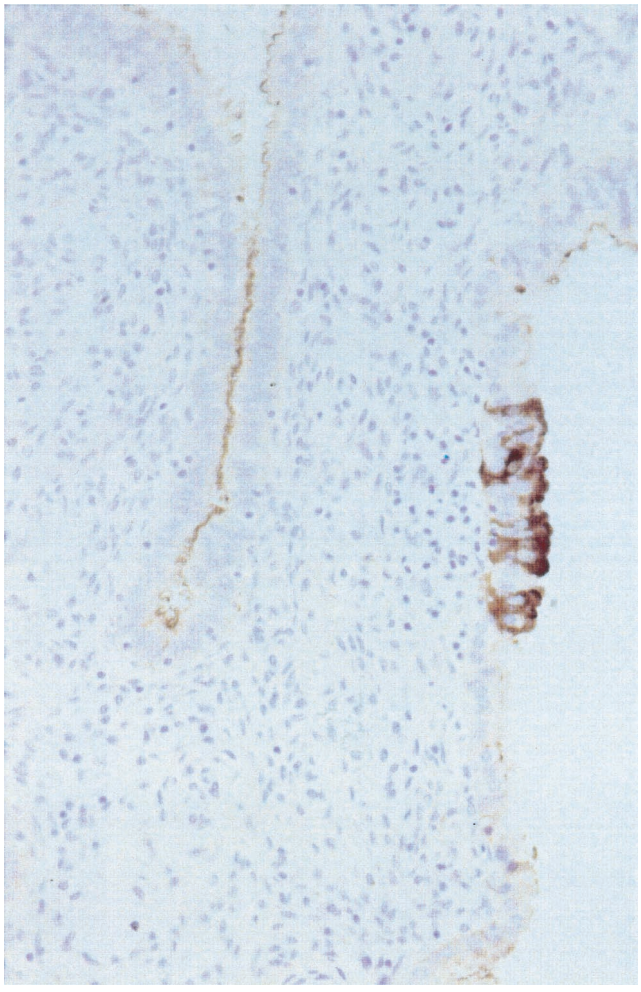

Fig. 7. Immunohistochemical staining of hK10 in fallopian tube (formalin-fixed paraffin-embedded tissue).

There is positivity (*dark gray*) on the luminal surface of the epithelial lining cells of the fallopian tube with focal intense positivity within the cytoplasm of some of the cells (original magnification, ×400).

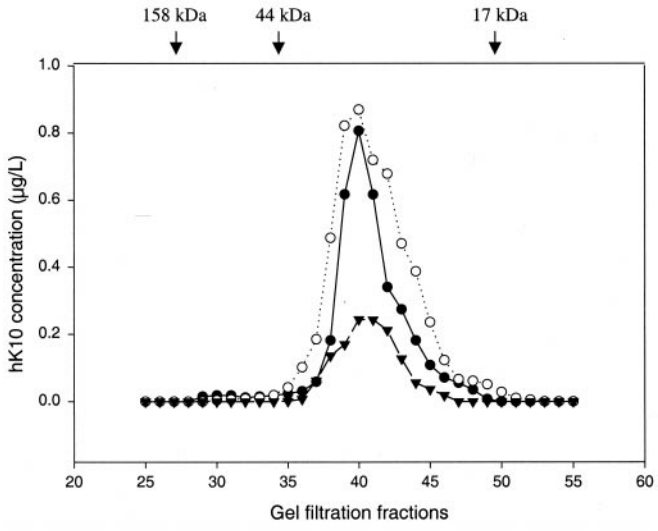

Fig. 8. Fractionation of milk (○), seminal plasma (●), and serum (▼) with size-exclusion HPLC.

The presence of hK10 in different fractions was measured with the hK10 immunoassay. A single peak (*fraction 40*) was detected in all three samples. Molecular mass standards were also separated on the same column, and their corresponding fractions are shown at the *top* of the chromatogram. The immunoreactive peak elutes at ~30 kDa in all three clinical samples.

many tissues, such as salivary gland, skin, colon, fallopian tube, prostate, testis, pituitary, endometrium, and lung. hK10 is also detectable in various biological fluids, including milk, seminal plasma, serum, CSF, and amniotic fluid (Table 1).

Using the rabbit anti-hK10 antiserum, we immunohistochemically localized hK10 in the epithelial cells of fallopian tube (Fig. 7). hK10 is present in the cytoplasm of epithelial cells lining the lumen.

We found that, with the exception of CSF (recovery, 88–100%), recovery from various biological fluids was incomplete: 50–80% in serum, 0–77% in milk, 64–100% in seminal plasma, and 18–53% in amniotic fluid.

FRACTIONATION OF BIOLOGICAL FLUIDS WITH SIZE-EXCLUSION HPLC

To determine the molecular mass of the protein detected in the biological fluids, three samples were fractionated with gel filtration, including milk, seminal plasma, and serum. The presence of hK10 in various fractions was measured with the developed immunoassay. When the fluorescence counts were plotted against the different fractions, a single peak (fraction 40), which corresponds to ~30 kDa, was detected (Fig. 8). No higher molecular mass complexes were found. These results indicate that the protein detected with the hK10 immunoassay is a single species, with a molecular mass of 30 kDa, which is consistent with the molecular mass of free hK10.

Discussion

The *KLK10* gene was initially named *NES1* and was cloned by subtractive hybridization between a tumorigenic and a nontumorigenic breast cell line *(8)*. We recently delineated the genomic organization of this gene and mapped it to the same chromosomal locus as other human kallikreins *(9)*. On the basis of these data and several other similarities between NES1 and other human kallikreins [reviewed in Ref. *(6)*], we have classified this gene as a new member of the human kallikrein gene family. Recently, uniform nomenclature for the expanded human kallikrein gene family has been developed, and *NES1* was renamed as *KLK10 (7)*.

Although hK10 was predicted to be a secreted protein *(8)*, no methods currently exist for detecting the protein with high sensitivity and specificity. We have thus undertaken the task of developing such a method, based on noncompetitive immunoassay principles. For detection, we used time-resolved fluorometry to achieve high sensitivity *(14)*. Because there is no known natural source of large amounts of hK10, we expressed it in *P. pastoris*. We then purified the protein to homogeneity by combining

ion-exchange and reversed-phase chromatography. This technology ensures complete absence of homologous proteins and other contaminants in the final preparation. The recombinant hK10 protein was positively identified by mass spectrometry. However, we did not attempt to characterize the enzymatic activity of this protein. It appears that our protein has a smaller molecular mass than native hK10 *(8)*, probably because of differences in glycosylation.

The developed assay is highly sensitive, detecting hK10 at concentrations of 0.05 μg/L or higher. Furthermore, this assay appears to be very specific: we did not observe any detectable cross-reactivities with the highly homologous kallikrein proteins PSA (hK3), hK2, and hK6. In addition, substitution of our antibodies with preimmune sera from the same animals completely abolished the signal. Size-exclusion HPLC further indicated that the three biological fluids tested (serum, milk, and seminal plasma) contain only one immunoreactive peak of the expected molecular mass (~30 kDa). These data suggest that our immunoassay detects the free fraction of hK10 in these biological fluids. We cannot exclude the possibility that hK10, like PSA, may be partially complexed to proteinase inhibitors *(17–20)*.

We detected hK10 in various tissue extracts but predominantly in salivary glands, skin, and colon. We further identified relatively high concentrations of hK10 in many biological fluids, including milk of lactating women, seminal plasma, amniotic fluid, male and female serum, and CSF (Table 1). Because hK10 is a secreted protein, we speculate that the concentration of this biomarker in serum may change during initiation and progression of various diseases, including cancer. The availability of the highly sensitive hK10 immunoassay will facilitate such studies in the near future. Currently, there is no report in the literature associating expression of hK10 with any human disease with the exception of a report on *KLK10* down-regulation in breast cancer cell lines *(8)* and an abstract describing down-regulation of *KLK10* mRNA in aggressive forms of prostate cancer *(11)*.

Immunohistochemical localization of hK10 in the fallopian tube indicated that this antigen is produced by the luminal epithelial cells lining the fallopian tube. The immunoreactivity was detected in the cytoplasm and lumen, further suggesting that this protease is secreted. The biological function of this protease in the fallopian tube and other glandular tissues merits further investigation. Furthermore, the presence of high concentrations of hK10 in milk, seminal plasma, amniotic fluid, and CSF suggests that this protein is actively secreted by epithelial cells of the breast, male reproductive organs, the fetoplacental unit, and brain cells. More studies will be necessary to precisely localize the production of hK10 in these tissues.

The recoveries of hK10 from the biological fluids tested were incomplete, with the exception of CSF. In serum, the recovery was 50–82%, a situation similar to the recovery of PSA *(15)*. In seminal plasma, the recovery was 64–100%. The lowest recoveries were seen with milk and amniotic fluid. We speculate that the low recoveries could be attributable either to sequestration of hK10 by proteinase inhibitors (e.g., α_2-macroglobulin), as happens with PSA *(21)*, or to uptake of hK10 in lipids present in milk and amniotic fluid.

In conclusion, we describe here the first highly sensitive and specific immunofluorometric assay for measuring hK10 in biological fluids and tissue extracts. This assay will facilitate further studies on this interesting serine protease, to examine the possibility that hK10 may represent a new biomarker of human disease, similarly to other members of this family, which have already found important applications in prostate cancer diagnostics (PSA and hK2). These studies are now in progress.

This work was supported by a grant from Diagnostic Systems Laboratories to E. P. Diamandis. We thank Tammy-Lynn Tremblay (IBS) for technical assistance in the processing of gel-isolated samples.

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