Clinical Chemistry 49:5
746–751 (2003)

Proteomics and
Protein Markers

Purification of Human Kallikrein 6 from Biological Fluids and Identification of its Complex with α_1-Antichymotrypsin

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Background: **Human kallikrein 6 (hK6) is significantly increased in serum in many patients with ovarian cancer and may have a role in amyloid precursor processing and Alzheimer disease. The forms of hK6 in biological fluids are poorly characterized.**

Methods: **hK6 protein was immunoaffinity-purified and positively identified by Western blotting, N-terminal sequencing, and mass spectrometry. hK6 in cerebrospinal fluid (CSF), milk, ascites, and serum was size-fractionated by chromatography and then measured by a highly sensitive and specific immunoassay. Hybrid assays were performed to detect the possible interactions between hK6 and proteinase inhibitors in CSF, milk, ascites fluid, and serum.**

Results: **N-Terminal sequencing identified hK6 in the proform in both CSF and milk. hK6 exists in two forms in milk and ascites fluid: a free form with a molecular mass of ~25 kDa and a higher molecular mass form. Hybrid sandwich assays (capture antibody for hK6 and detection antibody for inhibitors), utilizing a panel of known serine protease inhibitors, indicated that α_1-antichymotrypsin forms a complex with hK6 in milk and ascites fluid. Only the free form of hK6 was detected in CSF and serum.**

Conclusions: **hK6 exists mainly as a proenzyme in milk and CSF. A fraction of this enzyme is partially complexed with α_1-antichymotrypsin in milk and ascites fluid of ovarian cancer patients.**

The kallikrein gene family consists of 15 genes localized tandemly on chromosome 19q13.4 *(1, 2)*. All of the genes encode for secreted serine proteases with molecular masses of ~30 kDa. Kallikreins show significant homology at both the DNA and amino acid level *(3, 4)*. The human kallikrein 6 (*KLK6*)[3] gene, which encodes for hK6 protein, was cloned independently by three groups. The cDNA of this gene was isolated by Anisowicz et al. *(5)*, who used a differential display technique from primary and metastatic breast cancer cell lines, and was named protease M. They showed that protease M is down-regulated in metastatic breast cancer cell lines but is strongly expressed, at the mRNA level, in some primary breast cancer cell lines and in ovarian cancer tissues and cell lines *(5)*. The same gene was also cloned from a cDNA library prepared from a human colon adenocarcinoma cell line (COLO 201) and was named neurosin *(6)*. Neurosin was found to be highly expressed in the brain. Finally, the same cDNA was cloned from Alzheimer disease brain by PCR amplification *(7)*. The gene was named zyme, and it was found to be expressed predominantly in the brain, kidney, and salivary glands.

The *KLK6* gene spans 10.5 kb of genomic sequence on chromosome 19q13.4 *(8)*. The gene consists of seven exons, the first two of which are untranslated *(8)*. *KLK6* cDNA is 1526 nucleotides in length and contains 245 bp of 5′-untranslated sequence, 732 bp of coding sequence, and 549 bp of 3′-untranslated region *(5)*. Fine mapping of the area indicates that *KLK6* lies between the *KLK5* and *KLK7* genes *(9)*.

hK6 is recognized as a serine protease because the amino acid residues known to be crucial for substrate binding, specificity, and catalysis of the serine proteases

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Received November 21, 2002; accepted February 14, 2003.

[3] Nonstandard abbreviations: *KLK*, human kallikrein (gene); hK, human kallikrein (protein); PSA, prostate-specific antigen; CSF, cerebrospinal fluid; and SDS, sodium dodecyl sulfate.

(including serine, histidine, and aspartic acid) are highly conserved *(5–8)*. The *KLK6* gene encodes for a trypsin-like serine protease 244 amino acids in length, of which 16 amino acids constitute the signal peptide and 5 amino acids (Glu-Glu-Gln-Asn-Lys) constitute the activation peptide. This resembles a propeptide with a trypsin-susceptible cleavage site after Lys (Lys ↓ Leu) to convert hK6 to the active enzyme of 223 amino acids *(1, 7)*.

We have recently developed a highly sensitive and specific immunofluorometric assay for measuring hK6 protein in serum and biological fluids, with no cross-reactivity from other kallikreins *(10)*. Our preliminary studies indicated that the hK6 concentration in serum is significantly increased in a large proportion of patients with ovarian cancer and that hK6 may constitute a biomarker for the diagnosis and monitoring of ovarian carcinoma *(11)*.

There are different mechanisms for controlling serine protease activity to avoid any unwanted protein degradation and to allow spatial and temporal regulation of proteolytic activity. One mechanism involves producing enzymes in the form of inactive precursors (proenzymes), which can be activated, when necessary, by cleavage of an N-terminal peptide. Another mechanism includes binding of enzymes to general or specific inhibitors. hK3 [better known as prostate-specific antigen (PSA)] has been shown to form complexes with many extracellular protease inhibitors, such as α_1-antichymotrypsin, α_2-macroglobulin, and α_1-antitrypsin, and human glandular kallikrein 2 (hK2) was found to bind to α_1-antichymotrypsin, α_2-antiplasmin, antithrombin III, plasminogen activator inhibitor-1, and α_2-macroglobulin *(12–20)*. These complexes have important clinical applications for differential diagnosis of prostatic diseases *(21, 22)*. Other kallikreins were also found to interact with specific proteinase inhibitors *(23)*.

In this study, we isolated pure hK6 from various biological fluids, showed that it is present mainly in these fluids as a proform, and identified a novel complex of hK6 with the proteinase inhibitor α_1-antichymotrypsin.

Materials and Methods

BIOLOGICAL FLUIDS

Human breast milk was obtained from nursing women 7–10 days postdelivery at Mount Sinai Hospital (Toronto, Canada). Cerebrospinal fluid (CSF) and serum were left-over samples obtained from the routine laboratory of Mount Sinai Hospital (Toronto, Canada). Ascites fluid was obtained from advanced ovarian cancer patients during therapeutic removal of the fluid. Our procedures were approved by the Institutional Review Board of Mount Sinai Hospital. Our procedures were approved by the Institutional Review Board of Mount Sinai Hospital. Informed consent was obtained.

IMMUNOAFFINITY PURIFICATION OF hK6

hK6 was purified from CSF and human breast milk with use of the Affi-Gel HZ Immunoaffinity Kit (Bio-Rad). We coupled 2 mg of clone E24 protein A affinity-purified hK6 monoclonal antibody to 1 mL of Affi-Gel HZ hydrazide gel in 0.1 mol/L sodium acetate containing 0.15 mol/L NaCl (pH 5.5). We then incubated 20 mL of sample overnight with the E24 antibody-coupled beads. After thorough washing with phosphate-buffered saline, hK6 was eluted with 0.1 mol/L acetic acid, and 1-mL fractions were collected into tubes containing 1 mL of 0.5 mol/L sodium bicarbonate to neutralize the acid.

WESTERN BLOT ANALYSIS

We denatured 20 μL of sample, containing 5–50 ng of protein, by heating in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis sample buffer [50 mmol/L Tris-HCl (pH 6.8), 0.1 mmol/L dithiothreitol, 20 g/L SDS, 1 g/L bromphenol blue, 100 g/L glycerol] at 75 °C for 10 min and run on a 4–12% polyacrylamide/SDS gel (Invitrogen). Proteins were transferred by electroblotting to nitrocellulose membranes. Rabbit anti-hK6 antibody (diluted 1:2000) was used as a probe in Western blotting.

CHARACTERIZATION OF hK6 FROM CSF BY MASS SPECTROMETRY

Polyacrylamide gels were stained with Coomassie G-250, 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 (50 mmol/L iodoacetamide in 50 mmol/L ammonium bicarbonate, pH 8.3) before overnight trypsin digestion. 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).

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 experiments. Conventional mass spectra were obtained by operating the quadrupole in a radiofrequency-only mode while a pusher electrode was pulsed at a frequency of ~7 kHz to transfer all ions to the time-of-flight analyzer. Tandem mass spectrometry experiments on trypsin-digested peptides identified in survey scans were conducted with 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 tandem mass spectrometry mass spectral acquisition, respectively. Collisional activation was

performed with 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.

N-terminal sequencing

N-Terminal sequencing was performed with use of Edman degradation. Proteins to be sequenced were transferred by electroblotting to a polyvinylidene difluoride membrane and visualized with Coomassie Blue stain. The bands were excised and applied to the sequencer.

Fractionation of biological fluids by size-exclusion HPLC

To determine the molecular masses of the proteins detected in biological fluids and to identify different forms of hK6 in biological fluids, we fractionated various samples with gel-filtration chromatography, as described elsewhere *(24)*. We used a mobile phase of 0.1 mol/L Na_2SO_4–0.1 mol/L NaH_2PO_4, pH 6.80. The flow rate was 0.4 mL/min. The gel filtration column was a Bio-Sil SEC-250 column [600 × 75 mm (i.d.); Bio-Rad]. The fractions were collected and analyzed for hK6 by an immunofluorometric assay, as described below. We injected 500 μL of sample for each experiment.

Immunofluorometric assay for hK6

We used a monoclonal-polyclonal hK6 ELISA with high sensitivity and specificity *(10)*. Briefly, the assay was as follows: white polystyrene microtiter plates were coated with monoclonal anti-hK6 antibody 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). hK6 calibrators or samples were then pipetted into each well (25 μL/well; diluted 1:2 in bovine serum albumin) and incubated for 2 h with shaking; the plates were then washed six times with washing buffer. Subsequently, 100 μL of rabbit anti-hK6 antiserum diluted 500-fold in buffer A (containing 60 g/L bovine serum albumin, 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 1 h; plates were then washed as described earlier. Finally, 100 μL of alkaline phosphatase-conjugated goat anti-rabbit IgG, H+L chain-specific (Jackson ImmunoResearch), diluted 3000-fold in buffer A, was added to each well and incubated for 45 min; plates were then washed as above.

Diflunisal phosphate (100 μL of a 1 mmol/L solution) in substrate buffer [0.1 mol/L Tris (pH 9.1), containing 0.1 mol/L NaCl and 1 mmol/L $MgCl_2$] was added to each well and incubated for 10 min. Developing solution (100 μL, containing 1 mol/L Tris base, 0.4 mol/L NaOH, 2 mmol/L $TbCl_3$, and 3 mmol/L EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion). The calibration and data reduction were performed automatically, as described elsewhere *(25)*. The assay measures hK6 in the range of 0.5–200 μg/L with imprecision (CV) <10%. More details can be found elsewhere *(25)*.

hK6 hybrid assays

Hybrid immunoassays were performed as above. The hK6 monoclonal antibody was used as the capture antibody; the rabbit polyclonal hK6 detection antibody was replaced with rabbit antibodies against each of six common serine protease inhibitors (α_2-macroglobulin, α_1-antitrypsin, α_1-antichymotrypsin, C1 esterase inhibitor, α_2-antiplasmin, and antithrombin III) at a 1:2000 dilution. Other steps were identical to those of the hK6 assay described above.

Results

Purification of hK6 from biological fluids

hK6 was immunoaffinity-purified from CSF and milk with use of a solid-phase monoclonal antibody. The starting hK6 concentration in these fluids was ~2 mg/L. Using the same method, we further immunopurified (as a control) recombinant hK6 produced by a HEK 293 stable cell line (approximate concentration, 6 mg/L), developed as described elsewhere *(7)*. The final yield was ~50%. Western blotting was performed to confirm the identity of the purified protein. The immunoaffinity-purified protein from CSF, milk, and mammalian cell culture migrated at the expected molecular mass of ~30 kDa (Fig. 1A). Some minor contaminating proteins were also seen on Coomassie staining (Fig. 1B). The immunoreactive bands in lanes 2 and 3 of Fig. 1A likely represent fragmented hK6, which is known to have autoactivation and autocatalytic activity *(26, 27)*.

N-terminal sequencing and mass spectrometry

The N-terminal sequence of hK6 purified from HEK 293 cells, milk and CSF was found to be Glu-Glu-Gln-Asn-Lys-Leu-Val-His-Gly, which is identical to the deduced N-terminal sequence of the pro (inactive) form of the protein. The five amino acids of the activation peptide are underlined *(1)*. Mass spectrometric analysis of tryptic digests of the immunopurified protein and sequencing of at least six selected fragments further confirmed the identify of the protein as hK6. More specifically, we detected the following peptides of hK6 (numbers in parentheses represent positions of peptides in hK6 sequence reported in GenBank accession no. NM_002774): YTNWIQK (233–239), ESSQEQSSVVR (81–91), EKPGVYTNVCR (222–232), LSELIQPLPLER (118–129), YTNWIQKTIQAK (233–244), and KPNLQVFLGKHNLR (66–78).

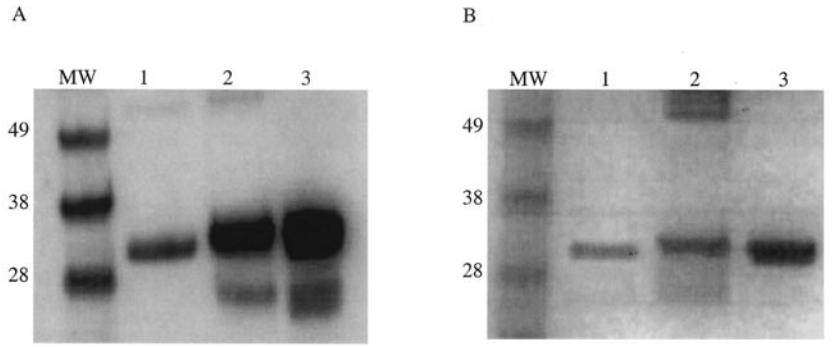

Fig. 1. Western blot analysis (*A*) and Coomassie-stained SDS–polyacrylamide gel (*B*) of immunoaffinity-purified hK6 from CSF (*lane 1*), milk (*lane 2*), and HEK293 mammalian cell culture supernatants (*lane 3*).

The hK6 band is located at ~30 kDa. *MW*, molecular mass markers (in kDa). For a discussion, see the text.

SIZE FRACTIONATION OF hK6 BY SIZE-EXCLUSION HPLC

To determine the molecular mass of the protein detected in biological fluids by hK6 ELISAs, samples were fractionated on a gel-filtration column. The presence of hK6 in various fractions was then assessed by an immunofluorometric assay (ELISA).

HPLC separation was performed on four biological fluids—milk, CSF, serum, and ascites fluid—from ovarian cancer patients. In all four fluids, the major immunoreactive peak eluted around fraction 42, corresponding to a molecular mass of ~30 kDa (Fig. 2). Sera from healthy individuals and ovarian cancer patients with increased hK6 concentrations and CSF showed only this single peak with no higher molecular mass complexes. These results indicate that the protein detected by the hK6 immunoassay is a single species with a molecular mass of ~30 kDa, which is consistent with the predicted molecular mass of free (noncomplexed) hK6. In milk and ascites fluid, a second peak was seen around fraction 33, corresponding to a molecular mass of ~100 kDa. We hypothesized that this peak represents hK6 bound to a proteinase inhibitor.

hK6 from milk was immunoaffinity-purified and then size-fractionated (Fig. 3). We were able to immunoaffinity-purify both the free (~30 kDa) and the bound (~100 kDa) forms of hK6. Similar data were obtained with ascites fluid.

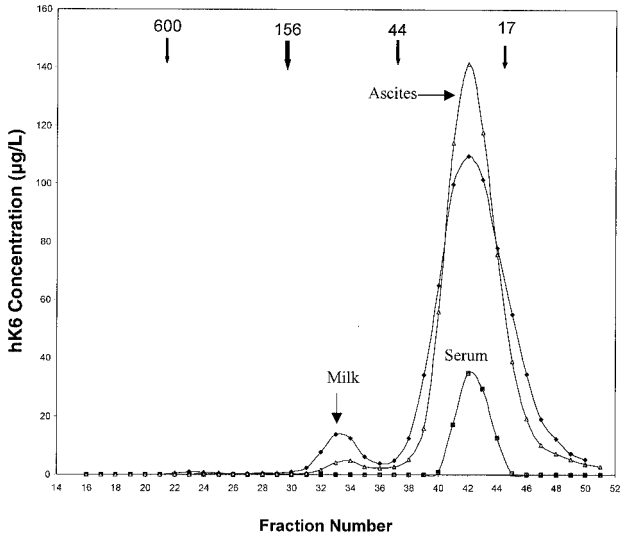

Fig. 2. Size-exclusion HPLC fractionation of hK6 from three biological fluids (milk, serum, and ascites fluid).

The serum shown was from an ovarian cancer patient with a hK6 concentration of 200 µg/L (reference values, <5 µg/L). The concentrations of unfractionated hK6 in the milk of a lactating woman and ascites fluid from an ovarian cancer patient were 800 and 1200 µg/L, respectively. In all fluids, there is a major immunoreactive peak around fraction 42, corresponding to a molecular mass of ~30 kDa. In the milk and ascites fluid, a second peak is seen around fraction 33–34, corresponding to a molecular mass of ~100 kDa. The column was calibrated with molecular mass calibrators (shown at *top* with *arrows*; masses are in kDa).

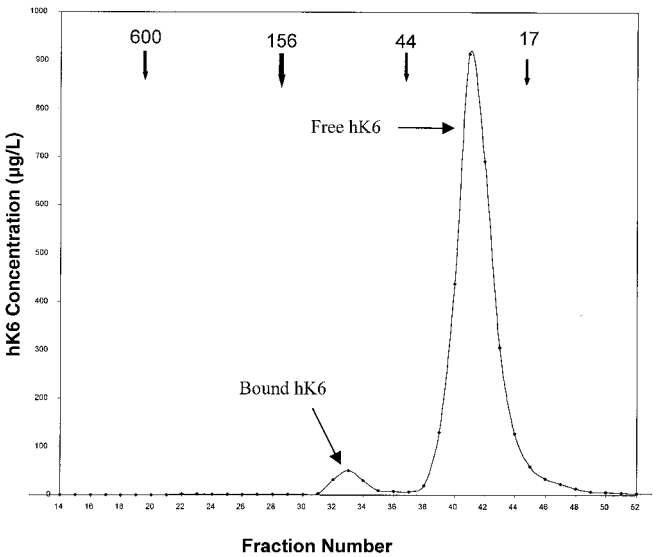

Fig. 3. Size-exclusion HPLC fractionation of the bound and free forms of hK6 in milk.

The milk was immunoaffinity-purified before injection on the HPLC column. The HPLC fractions were analyzed by the hK6 ELISA. There is an immunoreactive peak around fraction 42, corresponding to a molecular mass of ~30 kDa. A second peak is seen around fraction 33, corresponding to a molecular mass of ~100 kDa. The column was calibrated with molecular mass calibrators (shown at *top* with *arrows*; masses in kDa).

INTERACTION OF hK6 WITH PROTEINASE INHIBITORS

HPLC fractionation suggested that hK6 could be bound to a proteinase inhibitor in milk and ascites fluid. We examined the possible interaction of hK6 with serine proteinase inhibitors by performing different hybrid assays using anti-hK6 monoclonal antibody as a capture antibody and a polyclonal rabbit antibody against each of six common proteinase inhibitors as the detection antibody. HPLC fractions from milk and ascites were used in these analyses.

Data obtained from the hK6/α_1-antichymotrypsin hybrid assay for HPLC-fractionated milk are shown in Fig. 4. The milk was immunoaffinity-purified before injection into the HPLC column. A single immunoreactive peak was observed at fraction 33 when we used polyclonal rabbit α_1-antichymotrypsin as the detection antibody. This signal was much higher than that of a negative control plate (using anti-thyroxine monoclonal antibody as an irrelevant capture antibody). Similar results were obtained for HPLC-fractionated purified hK6 from ascites fluid (data not shown). Hybrid assays of fractionated milk using polyclonal rabbit antibodies against α_2-macroglobulin, α_1-antitrypsin, C1 esterase inhibitor, α_2-antiplasmin, and antithrombin III did not reveal any immunoreactive peaks (data not shown).

Discussion

The kallikrein family is a cluster of 15 serine protease genes on chromosome 19q13.4 *(1, 2)*. Among the ~150 serine proteases within the human genome, this family represents the largest serine protease cluster. All members of this family are predicted to be secreted proteases *(1, 2)*.

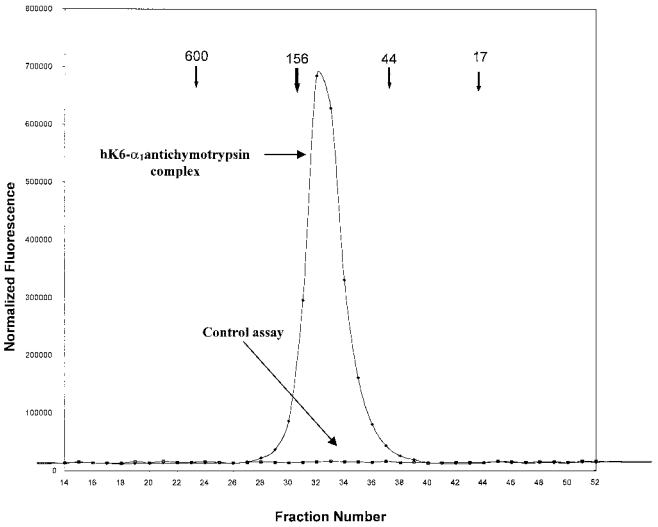

Fig. 4. Results of the hK6/α_1-antichymotrypsin hybrid ELISA for HPLC-fractionated milk.

Milk was immunoaffinity-purified before injection on the HPLC column. A single immunoreactive peak is seen around fraction 33 (~100 kDa), corresponding to hK6 bound to α_1-antichymotrypsin. The *control assay* (coating with an anti-thyroxine monoclonal antibody) shows no immunoreactive peaks for the milk fractions. Molecular mass calibrators were also separated on the same column, and their corresponding elution profile is shown at the *top* of the chromatogram.

We have previously shown that hK6 is a secreted serine protease found in many biological fluids and tissues, including CSF, breast nipple aspirate fluid, breast cyst fluid, male and female serum, seminal plasma, amniotic fluid, and breast and ovarian cancer tissues extracts *(10)*. Regulation of the proteolytic activity of this enzyme is indicated by its secretion as a proform, requiring activation, as well as by interaction with inhibitors. It has been demonstrated that hK6 has autocatalytic activity and that it can activate and inactivate itself by cleavage of the propeptide and by internal cleavage, respectively *(26, 27)*.

In this work, we have undertaken to immunopurify and characterize hK6 from various biological fluids. Our data suggest that hK6 is present in biological fluids mainly as a proform that contains the five amino acids of the activation peptide at the N-terminal end. Previously, Okui et al. *(28)* characterized the forms of hK6 in CSF and concluded that the protein is present in this fluid in its proform, in agreement with our data. In this report we show for the first time that hK6 is present in two forms in milk and ascites fluid from ovarian cancer patients: a 30-kDa free form and an ~100-kDa bound form. We characterized the bound form of hK6 by hybrid ELISAs and found that it is a complex of hK6 with the proteinase inhibitor α_1-antichymotrypsin. It has already been reported that PSA (hK3) and hK2, two other members of this family, form complexes with various proteinase inhibitors, including α_1-antichymotrypsin *(12, 13, 20, 22)*. To this end, hK6 behaves in a similar fashion. It is likely, similarly to PSA and hK2, that the complexes of hK6 with the proteinase inhibitors represent active forms that have been inactivated through this mechanism.

Many literature reports have focused on the clinical value of free and bound PSA and hK2 forms for differential diagnosis of prostate cancer *(21, 22)*. It is possible that the molecular forms of hK6 may, in the future, have similar applications.

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