

Immunofluorometric Assay of Human Kallikrein 6 (Zyme/Protease M/Neurosin) and Preliminary Clinical Applications

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Background: The human kallikrein gene family has contributed the best prostatic biomarkers currently available, including prostate-specific antigen (PSA) and human glandular kallikrein 2 (hK2). Recently, new members of the human kallikrein gene family have been identified. One new member is the KLK6 gene, encoding for human kallikrein 6 (hK6), which is also known as zyme/protease M/neurosin. In this paper, we describe development of antibodies and a sensitive immunofluorometric procedure for hK6 protein.

Methods: Recombinant hK6 protein was used as immunogen to develop polyclonal antibodies in rabbits and mice. These antibodies were used to develop a sandwich-type time-resolved immunofluorometric procedure for hK6.

Results: The newly developed hK6 immunofluorometric assay has a detection limit of 0.5 µg/L and upper concentration range of 200 µg/L. The assay is highly specific (no detectable cross-reactivity from PSA and hK2) and was used to quantify hK6 protein in various biologic fluids. Highest concentrations of hK6 were found in milk of lactating women, cerebral spinal fluid, nipple aspirate fluid, and breast cyst fluid. hK6 was also detected in male and female serum, in the majority of seminal plasmas and in a small fraction of amniotic fluids and breast tumor cytosols. hK6 was not detectable in urine. Chromatographic studies indicated that hK6 is present in these biologic fluids in its free, 30-kDa form.

Conclusions: This is the first reported sensitive immunofluorometric procedure for quantifying hK6 protein. hK6 is a secreted proteolytic enzyme that is found at high levels in cerebrospinal fluid and all breast secretions. This assay will facilitate further studies to examine the possible application of hK6 in diagnostics, including cancer and neurodegenerative disorders. Copyright © 2000 The Canadian Society of Clinical Chemists

KEY WORDS: kallikreins; immunoassay; time-resolved

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fluorescence; zyme; protease M; neurosin; milk; cerebrospinal fluid.

Introduction

The human kallikrein gene family was, until recently, thought to consist of only three genes: pancreatic/renal kallikrein (KLK1, encoding for hK1 protein), human glandular kallikrein 2 (KLK2, encoding for hK2 protein), and human kallikrein 3 (KLK3, encoding for hK3 protein or prostate specific antigen [PSA]). This gene family has contributed two excellent biomarkers (PSA and hK2) that are currently used for diagnosis and monitoring of prostate cancer (1–6).

More recently, new members of the human kallikrein gene family have been discovered. This gene family now contains 14 genes that are all encoding for serine proteases, show significant homologies at both the DNA and the amino acid level, and are all localized in tandem on human chromosome 19q13.3-q13.4. Recent developments on this gene family have been reviewed (1).

The KLK6 gene (encoding for human kallikrein 6 [hK6]) has been cloned independently by three groups of investigators and was previously given the names zyme (7), protease M (8) and neurosin (9). The gene encodes for a trypsin-like serine protease of 244 amino acids in length, of which sixteen amino acids constitute the signal peptide and five amino acids the activation peptide. The mature enzyme consists of 223 amino acids. The KLK6 gene and the encoded hK6 protein share significant homologies with PSA and other kallikreins and the enzyme is predicted to be a secreted protein. Little *et al.* (7) have demonstrated that this enzyme has amyloidogenic potential in the brain and may play a role in

the development and progression of Alzheimer's disease. Anisowicz *et al.* have cloned the gene by the method of differential display, based on the significant differences in expression between normal and malignant breast cells and they postulated that this gene is dramatically down-regulated in aggressive forms of breast cancer (8). The same gene was cloned by Yamashiro *et al.* from the human colon adenocarcinoma cell line COLO 201 (9).

We have recently molecularly characterized the KLK6 gene and demonstrated by reverse transcription-polymerase chain reaction that it is expressed in various tissues. The transcription of this gene is regulated by steroid hormones in breast carcinoma cell lines (10). However, despite the knowledge of the precise structure of this gene, the encoded protein has not as yet been detected in tissues or biologic fluids. We hypothesize that this secreted protein may have applications as a biomarker for cancer, neurologic or other chronic diseases. In order for these possibilities to be examined, highly sensitive and specific immunologic assays for this enzyme must be developed. In this paper, we describe the development of the first sandwich-type immunofluorometric assay for hK6 and demonstrate its presence in various human tissue extracts and biologic fluids.

Materials and methods

Diflunisal phosphate (DFP) was synthesized in our laboratory (diflunisal, obtained from Sigma Chemical Co., St. Louis, MO, USA). The stock solution of DFP was 0.01 mol/L in 0.1 mol/L NaOH. DFP stock solutions are stable at 4 °C for at least 6 months. Alkaline phosphatase-labeled goat antirabbit IgG and sheep antimouse immunoglobulin G (Fc fragment-specific) were obtained from Jackson Immunoresearch (West Grove, PA, USA). Working solutions of labeled goat antirabbit IgG-alkaline phosphatase were prepared by diluting the stock solution 3,000-fold in the assay buffer (described below). White, opaque 12-well polystyrene microtiter strips were obtained from Dynatech Labs (Alexandria, VA, USA). The substrate buffer was a Tris buffer (0.1 mol/L, pH 9.1) containing 0.1 mol of NaCl and 1 mmol of $MgCl_2/L$. The substrate working solution (DFP, 1 mmol/L in substrate buffer) was prepared just before use by diluting the DFP stock solution 10-fold in the substrate buffer. The wash solution was prepared by dissolving 9 g of NaCl and 0.5 g of polyoxyethylenesorbitan monolaurate (Tween 20) in 1 L of a 10 mmol/L Tris buffer, pH 7.4. The developing solution contained 1 mol of Tris base, 0.4 mol of NaOH, 2 mmol of $TbCl_3$, and 3 mmol of EDTA/L (no pH adjustment). The assay buffer A was a 50 mmol/L Tris buffer, pH 7.4, containing 60 g of bovine serum albumin, 0.5 g of sodium azide, 100 mL of normal goat serum, 25 mL of normal mouse serum, 5 g of bovine IgG, and 0.5 g of Tween 20/L. The assay buffer B was the same as assay buffer A except that mouse serum was omitted.

CLINICAL SAMPLES

Several clinical samples were used to examine the presence of hK6. These included serum and urine samples from male and female individuals (healthy blood donors), breast cyst fluids obtained by needle aspiration, breast tumor cytosolic extracts, prepared as described previously (11), amniotic fluids, milks from lactating women, seminal plasmas, nipple aspirate fluids, and cerebrospinal fluids. In addition, we tested a panel of human tissue cytosolic extracts, prepared as previously described (11). To establish optimal measuring conditions, all samples were tested at various dilutions. Our procedures are in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 1983.

All tissues and fluid samples were stored at $-80^{\circ}C$ until use.

INSTRUMENTATION

A time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada) was used to measure Tb^{3+} fluorescence in white microtiter wells. This procedure has been described in detail elsewhere (12, 13).

PROCEDURES

Production and purification of recombinant hK6 protein

Human 293 cells transfected with a plasmid containing the 1.4-kb hK6 cDNA were subjected to selection by growth in G418 (400 mg/L) for 3 weeks, after which time stable transformants were isolated. One clone generated identifiable amounts of hK6 protein in the culture medium. This cell line was cultured and the tissue culture supernatant was collected and concentrated by using Centricon ultrafiltration devices (Millipore, Waltham, MA, USA). Purification of hK6 from the concentrated cell culture supernatants was achieved by reversed-phase high-performance liquid chromatography (HPLC; C-8, Aquapore RP-300, 0.45×25 cm, Applied Biosystems, Foster City, CA, USA) by using a linear gradient of 0.1% trifluoroacetic acid/acetonitrile. Generally, the gradient increased at a rate of 1% acetonitrile/min. Fractions containing hK6 were located by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, collected, lyophilized, and stored at $-20^{\circ}C$ (7).

Development of polyclonal antibodies against hK6

Purified recombinant hK6 protein was used to immunize rabbits and mice by using standard procedures (14). The rabbit and mice antisera were used for the development of the immunofluorometric assay without further purification.

Coating of microtiter plates with sheep antimouse immunoglobulin

We coated white polystyrene microtiter wells by incubating overnight 500 ng/100 μ L per well of the coating antibody diluted in a 50 mmol/L Tris buffer, pH 7.8. The wells were then washed six times with the wash solution and blocked for 1 h with 200 μ L/well of the blocking solution (10 g/L bovine serum albumin in 50 mmol/L Tris, pH 7.8). After another six washes, the wells were ready to use.

hK6 calibration

hK6 calibrators of 0, 1, 5, 20, 50, and 200 μ g/L were prepared by diluting recombinant purified hK6 protein in a 50 mmol/L Tris buffer, pH 7.8, containing 60 g of bovine serum albumin and 0.5 g sodium azide/L.

hK6 assay

Calibrators or samples (100 μ L) were pipetted into the microtiter wells and 50 μ L of the polyclonal mouse anti-hK6 antiserum, diluted 5,000-fold in assay buffer B, were added. The wells were then incubated with shaking at room temperature for 2 h and washed six times. To each well, we then added 100 μ L of rabbit anti-hK6 antibody, diluted 1,000-fold in assay buffer A, incubated for 30 min as described above, and then washed six times. To each well, we then added 100 μ L of a goat antirabbit immunoglobulin, conjugated to alkaline phosphatase, diluted 3,000-fold in assay buffer A and incubated for 30 min, as described above. The wells were then washed six times; we then added 100 μ L of 1 mmol/L DFP working substrate solution and incubated for 10 min, as described above. We added 100 μ L of developing solution to each well, mixed by mechanical shaking for 1 min and measured the fluorescence with the time-resolved fluorometer. The calibration and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

HPLC

We have fractionated various biologic fluids on a gel filtration column, using the procedures described elsewhere (15,16). HPLC fractions were collected and analyzed for hK6 with the developed immunofluorometric assay.

Results

ASSAY OPTIMIZATION

We used two polyclonal antibodies against recombinant hK6 protein, one developed in mice and one developed in rabbits. The chosen assay configuration (indirect coating of the wells with a sheep antimouse antibody and detection of the immuno-

complex with a goat antirabbit immunoglobulin, conjugated to alkaline phosphatase) demonstrated good sensitivity (see below) without the need for any purification or conjugation of the primary antibodies. We have further carefully optimized the amounts of antibodies used, the diluents, and incubation times of the various assay steps. Optimal conditions were selected based on the lowest achievable detection limit and best assay linearity and dynamic range. The final conditions are described in the experimental section.

CALIBRATION CURVE, DETECTION LIMIT, AND PRECISION

A typical calibration curve of the proposed hK6 assay is shown in Figure 1. The detection limit, defined as the concentration of hK6 corresponding to the fluorescence of the zero calibrator plus 2 SDs, is ≤ 0.5 μ g/L. Within-run and between-run precision was assessed at various hK6 concentrations between 2 and 50 μ g/L and with various clinical samples. In all cases, the coefficients of variation (CVs) were between 2% and 9%, consistent with the precision of typical microtiter plate-based immunoassays.

SPECIFICITY

We have detected hK6 protein in various biologic fluids (Table 1). To ensure that our immunofluorometric assay measures hK6 with high sensitivity and specificity, we have separated in a gel filtration column three biologic fluids with relatively high hK6 concentration, namely one human milk from a lactating woman, one cerebrospinal fluid and one serum sample from an ovarian cancer patient who was found to have high levels of this biomarker in serum. The results are shown in Figure 2. In all three biologic fluids tested, we detect a single immunoreactive species of a molecular mass of ~ 30 kDa, which is consistent with the molecular mass of hK6 protein. We did not detect any higher molecular weight complexes, suggesting that hK6 is present in these biologic fluids in its free form. Other serum proteinases (e.g., PSA) are present in serum and other fluids mostly bound to proteinase inhibitors (17,18). We have further tested for cross-reactivity by the homologous proteins PSA and hK2. PSA and hK2, up to the maximum concentrations tested, 10,000 μ g/L and 1,000 μ g/L, respectively, did not produce any measurable hK6 assay signal.

hK6 IN BIOLOGIC FLUIDS AND TISSUE EXTRACTS

To obtain preliminary information on the presence of hK6 in biologic fluids, we analyzed various clinical samples, as shown in Table 1. The highest concentration of hK6 was found in milk of lactating women, followed by cerebrospinal fluid, nipple aspirate fluid, and breast cyst fluid. We also detected hK6 in male and female serum samples, in the majority of seminal plasmas and in a relatively

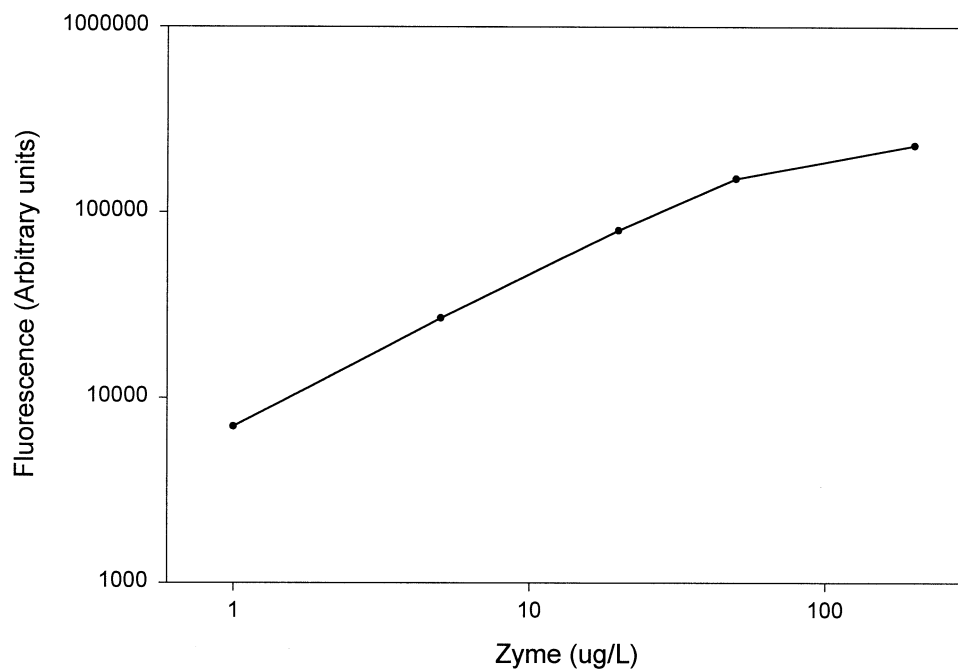


Figure 1 — Calibration curve of the newly developed hK6 zyme protein assay. The fluorescence of the zero standard (~18,000 arbitrary fluorescence units) was subtracted from all other measurements.

small percentage of amniotic fluids and breast tumor cytosolic extracts. We could not detect hK6 protein in urine.

We have also tested a number of human tissue cytosolic extracts. The highest concentration of hK6 was detected in the salivary glands, followed by lung, colon, fallopian tube, placenta, breast, pituitary, and kidney. The following tissues tested negative: skin, spleen, bone, thyroid, heart, ureter, liver, muscle, endometrium, testis, pancreas, seminal vesicle, ovary, adrenals, and prostate (Figure 3).

Discussion

The human kallikrein gene family was, until recently, thought to contain only 3 genes. One of these

genes, KLK3, encodes for prostate-specific antigen, which is currently the best tumor marker available and is used widely for diagnosis and monitoring of prostatic carcinoma (2–4). More recently, another member of the human kallikrein gene family, KLK2, encoding for hK2 protein, has found applications as an additional prostatic biomarker (5,6). More recent developments have indicated that the human kallikrein gene family is larger than originally thought. The recently constructed detailed human kallikrein gene locus indicates that it contains at least 14 genes that are tandemly aligned on chromosomal locus 19q13.3-q13.4. All 14 genes in that locus share significant homologies at both the DNA and protein level, and they all encode for serine proteases with either trypsin-like or chymotrypsin-like activity.

TABLE 1
Analysis of hK6 Protein in Various Fluids

Sample	hK6 ($\mu\text{g/L}$)			No. Positivity Rate (%)	
	Range	Mean (SD)	Median		
Milk ^a	398–7,638	2,588 (1,607)	2,531	20	100
Cerebrospinal fluid (CSF)	41–2,053	605 (485)	525	21	100
NAF (normal) ^b	—	914	—	1 (pool)	100
NAF (cancer) ^c	—	737	—	1 (pool)	100
Breast cyst fluid	34–97	74 (25)	84	5 (pools)	100
Male serum	2.0–12.6	6.9 (2.6)	6.7	18	100
Female serum	0–8.1	4.1 (2.0)	4.4	18	100
Seminal plasma	0–17.7	6.8 (5.5)	5.0	16	81
Amniotic fluid	0–9.5	1.1 (2.2)	0	21	33
Breast tumor cytosols	0–33	2.1 (7.0)	0	36	17
Urine	0	0	0	10	0

^aFrom lactating women.

^bNipple aspirate fluid.

^cNAF obtained from patients with breast cancer.

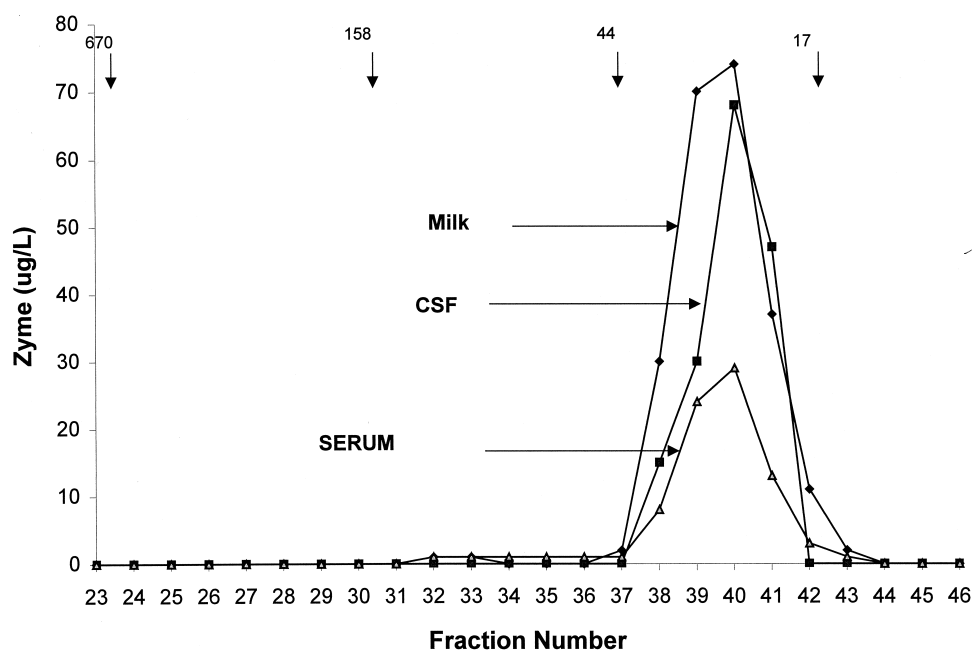


Figure 2 — HPLC separation of three biologic fluids and analysis of all fractions with the developed hK6 zyme immunoassay. In all three fluids, we detect a single immunoreactive peak around fractions 38 to 42, corresponding to a molecular mass of ~30 kDa. The column was calibrated with molecular weight standards (shown on top with arrows; masses are in kDa). The milk sample was diluted 10 times before injection into the HPLC column. For more details on the HPLC procedure, see Refs. 15,16.

These new developments have recently been reviewed (1).

We do not as yet have available reagents or methodologies for measuring the newly discovered kallikrein molecules. It is possible that at least some of these serine proteinases have applications as disease biomarkers. To examine these possibilities, highly sensitive and specific immunologic procedures need to be developed. The experience with prostate specific antigen has indicated that only when specific and sensitive immunoassays became available, PSA testing has gained widespread use (4). In this paper, we describe development of polyclonal antibodies and an immunofluorometric procedure suitable for quantifying hK6 protein in biologic fluids and tissue extracts. Because a rich natural source of hK6 protein is not known, we have chosen to use recombinant hK6 protein for the development of polyclonal rabbit and mice antibodies. This recombinant protein ensures high purity without any contaminating proteins. The chosen assay configuration does not need any further purification or conjugation of the primary antibodies used, and it is thus a convenient method for developing sensitive immunofluorometric procedures. The same principle has been adopted previously for measuring the p53 tumor suppressor in biologic fluids (11).

The developed immunoassay for hK6 protein demonstrates good sensitivity and dynamic range (Figure 1). We have further verified that this assay detects a single immunoreactive band in the biologic fluids examined. In serum, this proteinase is present in its free form, similarly to our observations with

hK2 measurements (19). However, this is in contrast to the situation with PSA, which is known to be present in serum mainly bound to α_1 -antichymotrypsin (17,18).

The survey of a relatively large number of biologic fluids has indicated that hK6 protein is present at relatively high concentrations in milk of lactating women and other breast secretions, including nipple aspirate fluid and breast cyst fluid (Table 1). Previously, we have demonstrated presence of other kallikreins, including PSA and hK2, in these biologic fluids (20–27). We have further detected large amounts of hK6 protein in cerebrospinal fluid, which are consistent with the observation that hK6 is expressed at high levels in brain tissue (7). We have further demonstrated presence of hK6 in male and female sera and seminal plasmas and in a small percentage of amniotic fluids and breast tumor cytosols. Previously, we have demonstrated presence of PSA and hK2 in these biologic fluids as well (20–27). It is interesting to note that although seminal plasma contains extremely high levels of PSA and hK2 (2,6), our assay detected very small amounts of hK6 in this biologic fluid (Table 1). This further demonstrates that the homologous proteins PSA and hK2 do not have any major cross-reactivity with the developed hK6 assay.

The assay developed here represents the first method for detecting hK6 protein in biologic fluids. Our results further demonstrate that hK6 is a secreted protein, as predicted by its deduced amino acid sequence (10). We did not establish if hK6 is secreted as an active enzyme or as a pro-form.

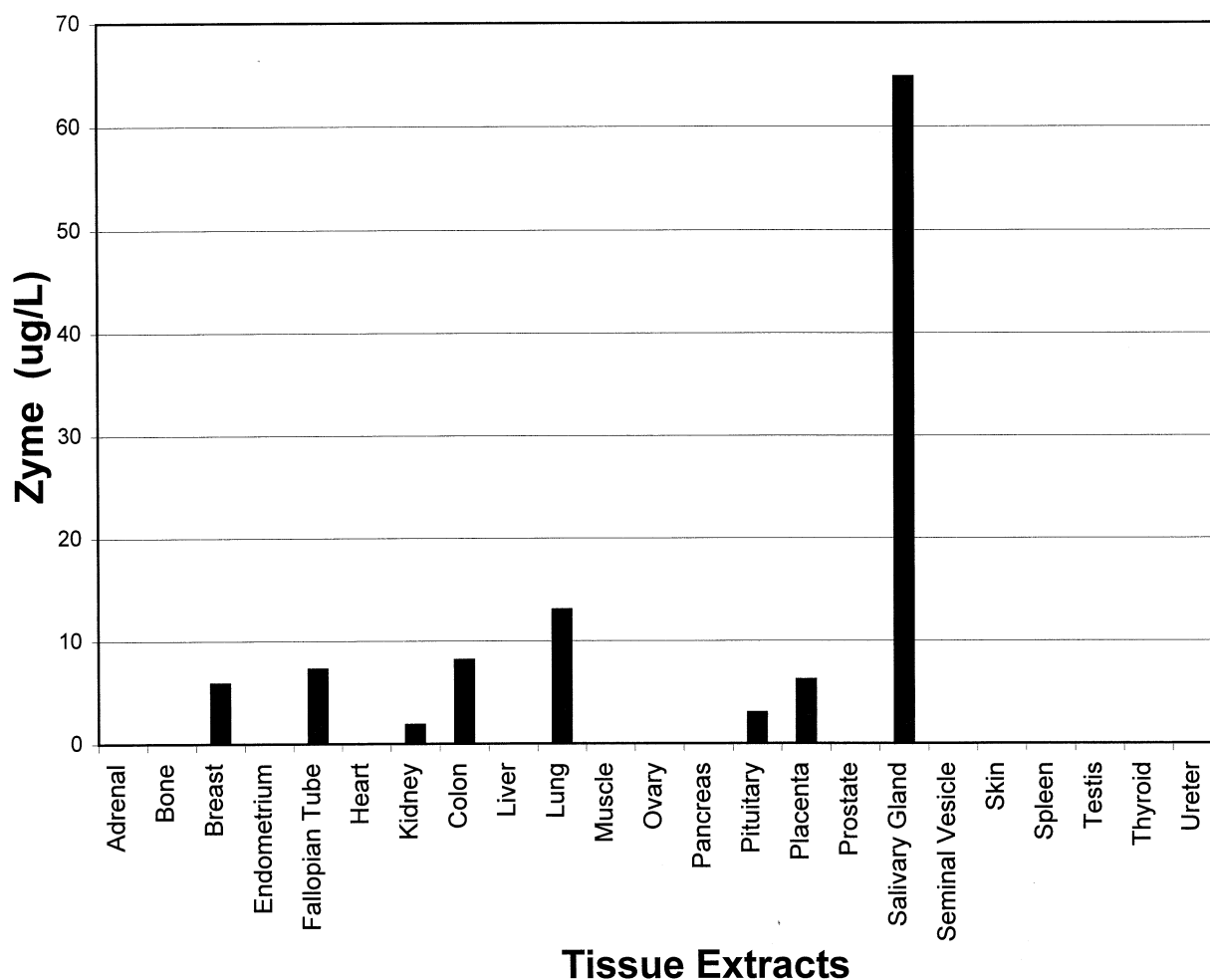


Figure 3 — Analysis of various human tissue cytosolic extracts for hK6 zyme protein. For more discussion, see text.

The newly developed immunofluorometric assay for hK6 may find applications toward the possible diagnostic/prognostic value of this biomarker in various human diseases. More specifically, we are interested to examine if the levels of hK6 in cerebrospinal fluid are associated with Alzheimer's or other degenerative neurologic diseases, as preliminarily suggested by Little *et al.* (7). We are further interested to examine if hK6 concentration in serum or other biologic fluids is altered in various malignancies, with mechanisms similar to those operating for PSA and hK2. These future studies will establish if hK6 is an additional clinical biomarker of the kallikrein gene family.

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References

1. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV. The new human kallikrein gene family: implications in carcinogenesis. *Trends Endocrinol Metab* 2000; **11**: 54–60.
2. Diamandis EP. Prostate specific antigen—its usefulness in clinical medicine. *Trends Endocrinol Metab* 1999; **25**: 14–16.
3. McCormack RT, Rittenhouse HG, Finlay JA, Sokoloff RL, Wang TJ, Wolfert RL, Lilja H, Oesterling JE. Molecular forms of prostate-specific antigen and the human kallikrein gene family: a new era. *Urology* 1995; **45**: 729–44.
4. Chu TM. Prostate-specific antigen and early detection of prostate cancer. *Tumor Biol* 1997; **18**: 123–34.
5. Stenman U-H. New ultrasensitive assays facilitate studies on the role of human glandular kallikrein (hK2) as a marker for prostatic disease. *Clin Chem* 1999; **45**: 753–4.
6. Rittenhouse HG, Finlay JA, Mikolajczyk SD, Partin AW. Human kallikrein 2 (hK2) and prostate-specific antigen (PSA): Two closely related, but distinct, kallikreins in the prostate. *Crit Rev Clin Lab Sci* 1998; **35**: 275–368.
7. Little SP, Dixon EP, Norris F, *et al.* Zyme, a novel and potentially amyloidogenic enzyme cDNA isolated from

- Alzheimer's disease brain. *J Biol Chem* 1997; **272**: 25135–42.
8. Anisowicz A, Sotiropoulou G, Stenman G, Mok SC, Sager R. A novel protease homolog differentially expressed in breast and ovarian cancer. *Mol Med* 1996; **2**: 624–36.
 9. Yamashiro K, Tsuruoiko N, Kodama S, *et al.* Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. *Biochim Biophys Acta* 1997; **1350**: 11–4.
 10. Yousef GM, Luo LY, Scherer SW, Sotiropoulou G, Diamandis EP. Molecular characterization of zyme/ protease M/neurosin, a hormonally-regulated kallikrein-like serine protease. *Genomics* 1999; **62**: 251–9.
 11. Hassapoglidou S, Diamandis EP, Sutherland DJA. Quantification of p53 protein in tumor cell lines, breast tissue extracts and serum with time-resolved immunofluorometry. *Oncogene* 1993; **8**: 1501–9.
 12. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. *Anal Chem* 1992; **64**: 342–6.
 13. Ferguson RA, Yu H, Kalyvas M, Zammit S, Diamandis EP. Ultrasensitive detection of prostate specific antigen by a time-resolved immunofluorometric assay and the Immulite® Immunochemiluminescent third generation assay: potential applications in prostate and breast cancers. *Clin Chem* 1996; **42**: 675–84.
 14. Campbell AM. Production and purification of antibodies. In: Diamandis EP, Christopoulos TK, Eds. *Immunoassay*. Pp 95–115. San Diego, CA: Academic Press, 1996.
 15. Yu H, Diamandis EP. Ultrasensitive time-resolved immuno-fluorometric assay of prostate specific antigen in serum and preliminary clinical studies. *Clin Chem* 1993; **39**: 2108–14.
 16. Diamandis EP, Nadkarni S, Bhaumik B, *et al.* Immunofluorometric assay of pepsinogen C and preliminary clinical applications. *Clin Chem* 1997; **43**: 1365–71.
 17. Stenman U-H, Leinonen J, Alfthan H, *et al.* A complex between prostate-specific antigen and alpha 1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. *Cancer Res* 1991; **51**: 222–6.
 18. 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.
 19. 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.
 20. Yu H, Diamandis EP. Prostate specific antigen in milk of lactating women. *Clin Chem* 1995; **41**: 54–8.
 21. Sauter ER, Daly M, Linahan K, *et al.* Prostate specific antigen levels in nipple aspirate fluid correlate with breast cancer risk. *Cancer Epidemiol Biomarkers Prevent* 1996; **5**: 967–70.
 22. Diamandis EP, Yu H, Lopez-Otin C. Prostate specific antigen—a new constituent of breast cyst fluid. *Breast Cancer Res Treat* 1996; **38**: 259–64.
 23. Black MH, Magklara A, Obiezu C, *et al.* Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumors and in normal breast secretions. *Br J Cancer* 2000; **82**: 361–7.
 24. 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**: 790–799.
 25. Yu H, Diamandis EP. Prostate specific antigen immunoreactivity in amniotic fluid. *Clin Chem* 1995; **41**: 204–210.
 26. Black MH, Diamandis EP. The diagnostic and prognostic utility of prostate specific antigen for diseases of the breast. *Breast Cancer Res Treat* 2000; **59**: 1–14.