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Enzymes and Protein Markers

Development of an Ultrasensitive Immunoassay for Human Glandular Kallikrein with No Cross-Reactivity from Prostate-specific Antigen

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Background: **Studies demonstrating that human glandular kallikrein (hK2) is increased in prostate cancer patients have prompted speculation that this marker may of use in addition to prostate-specific antigen (PSA).**

Methods: **An ultrasensitive hK2 sandwich immunoassay was developed, and its detection limit, cross-reactivity, analytical recovery, precision, and linearity of dilution were evaluated. hK2 was measured in seminal plasma and sera from healthy males, females, and prostatectomized patients.**

Results: **Our assay has an excellent detection limit (6 ng/L) and precision (>90%). Recovery studies indicated that hK2 binds to serum protease inhibitors. All sera from healthy males had measurable hK2 concentrations (median, 402 ng/L). Almost all female sera had undetectable hK2. Serum hK2 and PSA in males correlated positively (r = 0.44), but hK2 was present at concentrations ~2.5-fold lower than PSA. The PSA/hK2 ratio in male sera was 0.1–34, with a median of 2.6. In seminal plasma, this ratio was 100–500. More than 94% of immunoreactive hK2 in serum was in the free form (~30 kDa); traces of hK2 complexed to α_1-antichymotrypsin were present.**

Conclusions: **The limit of detection of the method for hK2 measurement described here (~20-fold lower than any other reported assay for hK2) allows the generation of new clinical information. When combined with a previously described method for PSA measurement that has no cross-reactivity from hK2, this methods allows the relative proportions of hK2 and PSA in biological fluids to be measured.**

Prostate cancer is the most frequently diagnosed cancer in North American males *(1)*. Prostate-specific antigen (PSA or hK3),[3] a serine protease belonging to the family of proteins known as kallikreins *(2)*, has emerged as the most useful marker for prostate cancer *(3)*. However, assays for serum PSA alone cannot always distinguish between prostate cancer and some nonmalignant prostatic pathologies, such as benign prostatic hyperplasia *(4)*. Therefore, there exists a continuing need for additional prostatic tumor markers.

Recently, interest has emerged in a second member of the kallikrein family, human glandular kallikrein (hK2), which has an 80% amino acid sequence identity with PSA *(5)*. Like PSA, hK2 is expressed predominantly in prostatic tissue and is up-regulated in response to androgenic stimulation *(6)*. It recently was reported that the function of hK2 is to proteolytically activate PSA following its secretion into the ductal system of the prostate gland *(7–9)*. Thus, hK2 appears to play a physiological role in the regulation of PSA activity.

Recent studies have reported hK2 overexpression in prostatic tumors *(10)*. Furthermore, like PSA *(11)*, hK2 is increased in the sera of prostate cancer patients *(12)*. These findings demonstrate the potential of hK2 as an additional marker of prostate cancer. The recent development of recombinant hK2 and hK2-specific antibodies has generated interest in immunoassays for hK2. The purpose

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[3] Nonstandard abbreviations: PSA, prostate-specific antigen; hK2, human glandular kallikrein or human kallikrein 2; BSA, bovine serum albumin; A2M, α_2-macroglobulin; and ACT, α_1-antichymotrypsin.

of this study was to design an ultrasensitive immunoassay for hK2 with minimal cross-reactivity from PSA.

Materials and Methods

ANTIBODIES

All antibodies used were mouse monoclonal antibodies. The capture antibody used in the hK2 assay (encoded G586) was supplied by Hybritech Inc., San Diego, CA and was raised against recombinant hK2. The capture antibody for the PSA immunoassay, coded 8301, was obtained from Diagnostic Systems Laboratories. The same detection antibody (coded 8311; Diagnostic Systems Laboratories) was used in both the hK2 and the PSA assays.

WESTERN BLOT ANALYSIS

Western blots were performed to confirm antibody specificity. All necessary equipment for Western blot analysis was obtained from Novex. Recombinant hK2 (Hybritech) and PSA purified from seminal plasma (a gift from Dr. Tom Stamey, Stanford University, Stanford, CA) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions on 4–12% Tris-glycine polyacrylamide gels. Separated proteins were transferred electrophoretically to nitrocellulose membranes. After the membranes were blocked overnight at 4 °C with 50 g/L nonfat dry milk in Tris-buffered saline-Tween buffer (20 mmol/L Tris-HCl, pH 7.6, 137 mmol/L NaCl, 1.0 g/L Tween 20,), the membranes were cut into strips and incubated with the PSA and hK2 antibodies, followed by horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham). Biotinylated molecular mass markers were visualized by reacting with streptavidin-horseradish peroxidase, simultaneously with the secondary antibody. The blots were incubated for 1 min with enhanced chemiluminescence reagents (ECL; Amersham) as specified by the manufacturer, and exposed to x-ray film for detection of immunoreactive protein bands.

CALIBRATORS

Calibration solutions for both hK2 and PSA immunoassays were prepared in a 50 mmol/L Tris buffer, pH 7.80, containing 60 g/L of bovine serum albumin (BSA). PSA calibrators at concentrations of 0, 1, 5, 20, 100, 500, and 2000 ng/L were prepared using highly purified PSA isolated from seminal plasma. The hK2 calibrators were prepared using recombinant hK2 at concentrations of 0, 5, 20, 100, 500, and 2000 ng/L. The recombinant hK2 was a gift from Dr. R. Wolfert, Hybritech Inc., San Diego, CA. The concentration of purified hK2 was assigned by total protein analysis.

hK2 IMMUNOASSAY

A two-step sandwich ELISA was used for hK2 analysis. The hK2 capture antibody was immobilized onto polystyrene microtitration wells (Dynatech Laboratories) at a concentration of 300 ng per 100-μL well in coating buffer (50 mmol/L Tris, pH 7.8). This incubation was performed overnight. Samples were applied undiluted at a volume of 100 μL simultaneously with 50 μL of assay buffer (50 mmol/L Tris, pH 7.8, 100 mL/L goat serum, 60 g/L BSA, 50 mL/L mouse serum, 10 g/L bovine immunoglobulin, 5 g/L Tween 20, 500 mmol/L KCl) and incubated at room temperature for 1 h. After the wells were washed with wash buffer (150 mmol/L NaCl, 50 mmol/L Tris, 1 mmol/L NaN_3, 0.5 g/L Tween 20), they were incubated with 100 μL of biotinylated detection antibody diluted to a concentration of 500 ng per 100-μL well in assay buffer for 1 h. The wells were washed, and alkaline phosphatase-labeled streptavidin (Jackson Immunoresearch) diluted to a concentration of 5 ng per 100-μL well in a 60 g/L BSA solution was added for 15 min. Diflunisal phosphate (10 mmol/L stock in 10 mmol/L NaOH, prepared in house) diluted 10-fold in substrate buffer (100 mmol/L Tris, pH 9.1, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 7.5 mmol/L NaN_3) was added for 10 min following washing. Developing solution (1 mol/L Tris, 400 mmol/L NaOH, 3 mmol/L EDTA, 2 mmol/L Tb^{3+}), was then added to the diflunisal phosphate solution, and the resulting fluorescence was measured on a Cyberfluor 615 Immunoanalyzer (Nordion International). More details on these time-resolved immunofluorometric procedures can be found elsewhere *(13, 14)*.

PSA IMMUNOASSAY

PSA measurements were performed with an ELISA-type assay described in detail elsewhere *(14)*. The procedure and reagents were identical to the hK2 assay with the following modifications. The coating antibody for the PSA assay was coded 8301. This assay was a one-step sandwich assay in which 50 μL of biotinylated antibody 8311, diluted to a concentration of 250 ng per 100-μL well, was incubated simultaneously with 100 μL of sample. The detection limit of this assay was determined previously to be 1 ng/L *(14)*.

DETECTION LIMIT

The detection limit of the hK2 immunoassay was determined by analyzing 12 replicates of the zero hK2 calibrator. The hK2 concentration that corresponded to the fluorescence of the zero calibrator plus 2 SD was determined to be the detection limit of the assay.

CROSS-REACTIVITY

To check for PSA cross-reactivity in the hK2 assay, the hK2 assay was performed with PSA diluted to 1, 5, 20, 100, 500, 2000, 10 000, and 100 000 ng/L in a 60 g/L BSA solution. In addition, various concentrations of PSA were incubated simultaneously with a 100-fold lower concentration of hK2 to test for negative cross-reactivity (defined as the ability of PSA to interfere with the hK2 measurement).

ANALYTICAL RECOVERY

To evaluate the recovery of hK2, small volumes of purified recombinant hK2 or sera containing a known amount of hK2 were added to various sample matrices to a final concentration of 100 and 2000 ng/L. The matrices used were three female serum samples, three male serum samples, and a 60 g/L BSA solution (as a control). Recovery of hK2 was measured in quadruplicate 30 min and 24 h after the addition of hK2.

LINEARITY OF DILUTION

The hK2 assay was evaluated for linearity at a range of 5–2000 ng/L by assaying, in quadruplicate, various specimens that had been serially diluted in a 60 g/L BSA solution. Male serum samples with endogenous hK2 concentrations, in addition to male and female serum samples that had been supplemented with a known concentration of recombinant hK2 to give a final concentration ~2000 ng/L, were diluted 2-, 4-, 8-, 16-, and 32-fold and analyzed for hK2.

PRECISION

The within-run and day-to-day assay precision was evaluated by analyzing 12 replicates of three male serum samples with hK2 concentrations of 75, 150, and 300 ng/L and one female serum sample supplemented with serum containing a known amount of hK2 to a final concentration of 1000 ng/L.

ANALYSIS OF SERUM SAMPLES

hK2 in serum. Serum samples from 61 males without prostate cancer, ages 51–88 years, and 33 unselected females were obtained from the clinical laboratories of Mount Sinai Hospital, Toronto, Canada. Each sample was analyzed in duplicate for hK2 and PSA.

Characterization of hK2 in serum by gel filtration. hK2 and PSA from two male serum samples were analyzed by gel filtration on a Hewlett-Packard 1100 HPLC system (Hewlett-Packard). A TSK-GEL silica-based column (TosoHaas) was used, and the mobile phase consisted of 100 mmol/L Na_2SO_4 and 100 mmol/L NaH_2PO_4, pH 6.8. The isocratic runs were maintained at a flow rate of 0.5 mL/min. Column calibration was achieved with a molecular mass calibration solution containing thyroglobulin (670 kDa), IgG (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.4 kDa; Bio-Rad Laboratories). Fractions were collected with the Pharmacia FRAC-100 fraction collector and analyzed in duplicate with both the hK2 and PSA immunoassays.

hK2 concentrations in serum of prostatectomized patients. hK2 and PSA were measured in serial sera of six patients with histologically confirmed prostate cancer who were treated with radical prostatectomy. Four of the patients showed clinical signs of cancer relapse, whereas two remained relapse free.

Seminal plasma. Seminal plasma samples were obtained from subjects undergoing investigations for infertility. These samples were diluted with a 60 g/L BSA solution and then analyzed for both hK2 and PSA.

Results

ANTIBODY SPECIFICITY

Western blotting (Fig. 1) demonstrated that the hK2-specific capture antibody (Hybritech) used in the hK2 assay recognizes hK2. This antibody previously was confirmed to have no cross-reactivity with recombinant PSA (data not shown). The coating antibody for the PSA immunoassay (coded 8301) was specific for PSA and showed no detectable cross-reactivity with hK2. The detection antibody (coded 8311), used for both the hK2 and the PSA immunoassays, reacted strongly with both hK2 and PSA.

CALIBRATION CURVE AND DETECTION LIMIT

A typical calibration curve for the proposed hK2 assay is shown in Fig. 2. The analytical detection limit of the hK2 assay was estimated as the dose that was equivalent to the fluorescence of the mean of 12 replicates of the zero calibrator plus 2 SD. The hK2 concentration that corresponded to this signal was calculated to be 6 ng/L.

CROSS-REACTIVITY

The hK2 assay exhibited no detectable positive PSA cross-reactivity up to a PSA concentration of 2000 ng/L

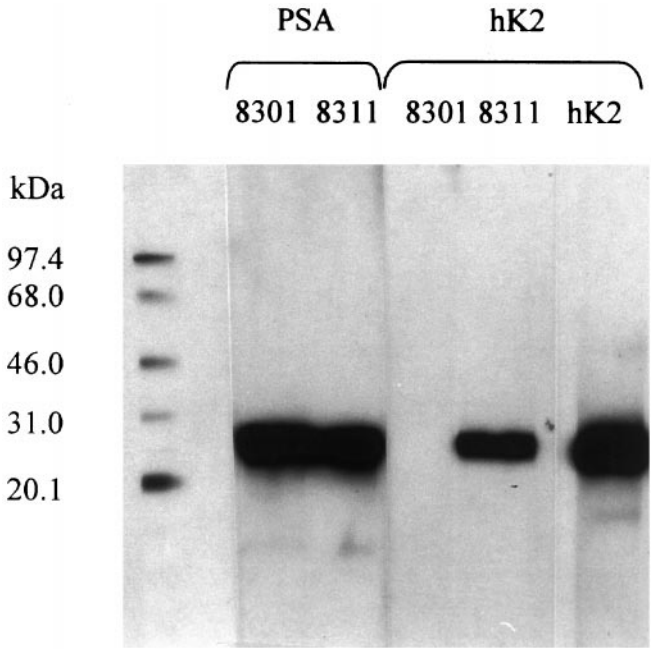

Fig. 1. Western blot analysis to confirm antibody specificity.

The PSA capture antibody (*8301*) reacted with PSA, but not hK2. The PSA/hK2 detection antibody (*8311*) reacted with PSA and hK2. The hK2 capture antibody recognized hK2. The masses of the molecular standards are shown in kDa on the *left*. Two lanes contain PSA antigen (*PSA*), and three lanes contain recombinant hK2 (*hK2*).

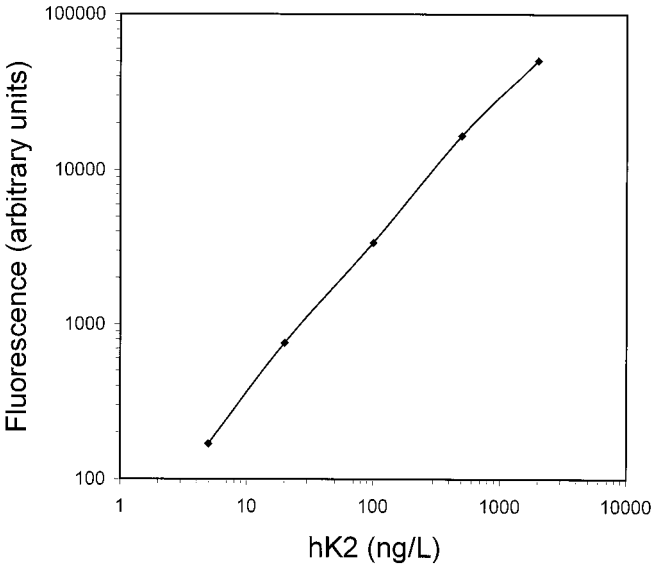

Fig. 2. Calibration curve for the hK2 immunoassay.

The background signal obtained with the zero calibrator was ~550 arbitrary fluorescence units and was subtracted from all other measurements.

PSA (Table 1). The cross-reactivity did not exceed 0.2% up to a concentration of 100 000 ng/L PSA. No negative cross-reactivity was apparent when PSA was present in a mixture with hK2 but at a 100-fold higher concentration. Similarly, the PSA assay described previously *(14)* had no detectable cross-reactivity from hK2 even at hK2 concentrations as high as 100 000 ng/L (data not shown).

ANALYTICAL RECOVERY

To evaluate the recovery of hK2 in serum, we added recombinant hK2 diluted in a 60 g/L BSA solution to human sera and BSA (for control purposes) to final concentrations of 200 and 1000 ng/L. The recoveries are listed in Table 2. As expected, the recovery of hK2 from the BSA solution was complete, at 97–110% recovery. The recovery of hK2 from female sera was 25–36% after 30 min and dropped to 13–26% after 24 h. Similar results were obtained for male sera, with 24–31% and 22–35% recovery after 30 min and 24 h, respectively. The low recovery may reflect the time-dependent binding of hK2 to serum proteins, forming complexes that are not measurable with this assay. hK2 from a male serum sample that contained high hK2 (~25 000 ng/L) was also added to pools of male and female sera. The recovery for these pools was 70–104%.

Table 1. Cross-reactivity of the hK2 assay with PSA.

Actual hK2 concentration,[a] ng/L	Actual PSA concentration,[a] ng/L	hK2 detected,[b] ng/L	Cross-reactivity with PSA, %
0	0	0	0
0	1	0	0
0	5	0	0
0	20	0	0
0	100	0	0
0	500	0	0
0	2000	5	0.25
0	10 000	20	0.20
0	100 000	195	0.20
20	2000	21	0.05
100	10 000	101	0.01

[a] PSA and hK2 were diluted in 60 g/L BSA to the final concentrations shown.
[b] Each mixture was measured using the proposed hK2 immunoassay.

LINEARITY OF DILUTION

Serum samples with a hK2 concentration of 150-2000 ng/L (the highest concentration in the calibration curve) were serially diluted with a 60 g/L BSA solution and measured. The assay showed good linearity: near-linear dilution curves were obtained with all samples tested (Fig. 3). The measured concentrations from the dilution were 80–120% of the expected value, based on the concentration of the undiluted hK2 .

PRECISION

To evaluate assay precision, 12 replicates of four serum samples with hK2 concentrations of 75, 150, 300, and 1000 ng/L were analyzed. The imprecision (CV) did not exceed 6% (within-run) or 9% (between-run) for any of the replicates.

ANALYSIS OF SERUM SAMPLES

PSA and hK2 were measured in sera from 61 males without prostate cancer (defined as an absence of clinical symptoms and serum PSA <4 μg/L, except one patient with total PSA of 4.8 μg/L) and 33 females; the values for males are listed in Table 3. The PSA concentration in male serum was 10–4770 ng/L, with a mean of 1223 ng/L and a median of 1020 ng/L. All samples had detectable hK2. The hK2 concentration in male serum was 21–3536 ng/L and generally appeared to be ~2.5-fold less than the PSA concentration. The mean hK2 value was 515 ng/L, and the median was 402 ng/L. A positive correlation was observed between the hK2 and PSA concentrations in male serum (r = 0.44; Fig. 4). The PSA/hK2 ratios in the 61 sera were 0.1–34, with a median of 2.6 (Table 3). Correlation between total PSA or hK2 and patient age indicated that both biochemical markers increase slightly with age. Linear regression gave the following equations: PSA (ng/L) = 22 (age, years) − 322, r = 0.26, P = 0.04; hK2 (ng/L) = 7.7 (age, years) − 21, r = 0.16, P = 0.21 (not statistically significant).

Thirty-one of 33 female sera contained hK2 concentrations below the detection limit of the assay (<6 ng/L). Two sera had hK2 concentrations of 40 and 60 ng/L. The PSA concentrations in these two sera were also the highest among the female samples, with PSA concentrations of 11 and 61 ng/L, respectively. Ten female sera had PSA concentrations between 1 and 7 ng/L, whereas PSA was undetectable in the other female samples.

The hK2 and PSA concentrations in five seminal

Table 2. Analytical recovery of hK2.

			30-min incubation		24-h incubation	
Sample matrix	Initial hK2,[a] ng/L	hK2 added,[b] ng/L	hK2 recovered, ng/L	Recovery, %	hK2 recovered, ng/L	Recovery, %
BSA, 60 g/L	0	200	203	101	197	99
		1000	966	97	1115	110
Female serum	<6	200	71	36	25	13
		1000	349	35	244	24
Female serum	<6	200	49	25	42	21
		1000	327	33	242	24
Female serum	7	200	56	28	48	24
		1000	301	30	261	26
Male serum	80	200	55	28	69	35
		1000	279	28	231	23
Male serum	22	200	53	27	52	26
		1000	275	28	296	30
Male serum	54	200	62	31	43	22
		1000	244	24	248	25

[a] Concentration before the addition of hK2.
[b] Recombinant hK2 was added to various samples to the final concentration shown.

plasma samples diluted in a 60 g/L BSA solution were determined. The PSA concentration was 0.1×10^9 to 2.3×10^9 ng/L (median, 0.8×10^9 ng/L); the hK2 concentration was 0.8×10^6 to 1.4×10^7 ng/L (median, 6×10^6 ng/L). The concentration of hK2 in seminal plasma was ~100- to 500-fold lower than the PSA concentration.

Characterization of hK2 in serum by gel filtration

The elution profiles of two male sera from prostate cancer patients (with very high PSA and hK2 concentrations) for PSA and hK2 fractionated by HPLC are shown in Figs. 5 and 6, respectively. The first serum sample (male serum 1) had a total PSA concentration of 2 000 000 ng/L and a hK2 concentration of 25 000 ng/L (PSA/hK2 ratio = 80). The second serum sample (male serum 2) had a total PSA concentration of 1 100 00 ng/L and a hK2 concentration of 200 000 ng/L (PSA/hK2 ratio = 5.5). Three clearly distinguishable hK2 peaks were present in both samples (Fig. 6), corresponding to molecular masses of ~700 kDa (first peak), ~100 kDa (second peak), and ~30 kDa (third peak). The first two peaks, based on their molecular masses, likely correspond to hK2 complexed to α_2-macroglobulin (A2M) and α_1-antichymotrypsin (ACT), respectively. The third peak is uncomplexed (free) hK2 and accounts for the vast majority of serum immunoreactive hK2 (>95% of total immunoreactivity; see Fig. 6, A and C).

As expected, fractionation of PSA produced two major peaks, which represent PSA complexed to ACT (~100 kDa, first peak) and uncomplexed PSA (~33 kDa, second peak). PSA-ACT represents the major PSA immunoreactive form (Fig. 5).

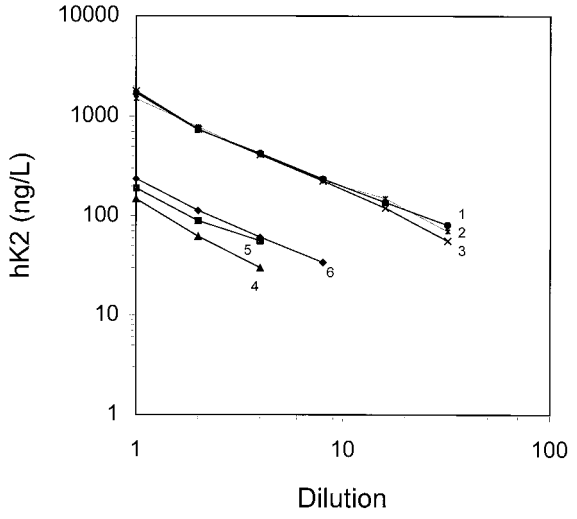

Fig. 3. Dilution curves for hK2 in human sera.

The diluted fluids were a 60 g/L BSA solution (control; *1*), hK2-supplemented male serum (*2*), hK2-supplemented female serum (*3*), and male sera (*4–6*). The diluent used was a 60 g/L BSA solution. Supplementation was with recombinant hK2.

Table 3. PSA and hK2 in male sera from 61 non-prostate cancer patients.

	PSA, ng/L	hK2, ng/L	PSA/hK2 ratio[a]
Percentile			
0	10	21	0.1[b]
5	43	45	0.5
25	430	136	1.4
50	1020	402	2.6
75	1720	650	4.8
95	3210	1192	15.6
100	4770	3536	34
Mean	1223	515	
SD	1022	573	

[a] One sample had undetectable hK2.
[b] For each patient specimen, the ratio of PSA to hK2 was calculated, and the percentiles of the frequency distribution of this ratio are presented.

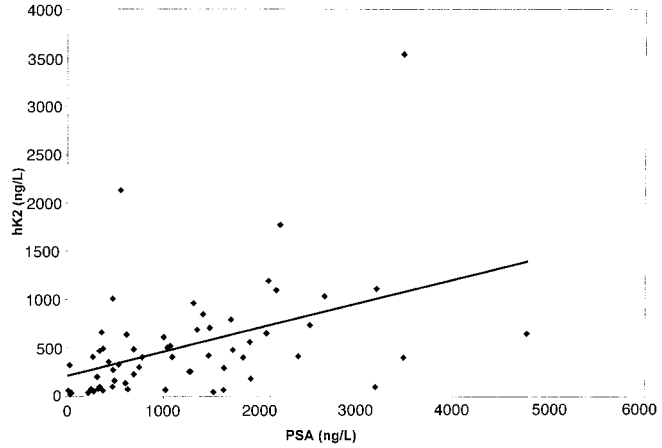

Fig. 4. Correlation of PSA and hK2 for 61 male sera.

A positive correlation was observed between PSA and hK2 in male sera. Linear regression: hK2 (ng/L) = 0.25 PSA (ng/L) + 211; $r = 0.44$; $P = 0.0004$.

hK2 concentrations in prostatectomized patients

Serial serum specimens from six prostatectomized males were analyzed for PSA and hK2. The PSA and hK2 concentrations over time in four prostatectomized males who showed clinical signs of prostate cancer relapse are shown in Fig. 7. The change in hK2 concentration over the course of disease progression was proportional to that of PSA. PSA and hK2 were undetectable in all sera from the two prostatectomized patients who remained relapse-free over the monitoring period of ~5 years. The approximate PSA/hK2 ratios in the four patients of Fig. 7 were 6, 23, 15, and 3.

Discussion

PSA currently is considered to be the best available prostate tumor marker and is widely used for prostate cancer diagnosis and monitoring. The major disadvantage of PSA in clinical practice is its lack of specificity for prostate cancer. Serum PSA is increased not only in

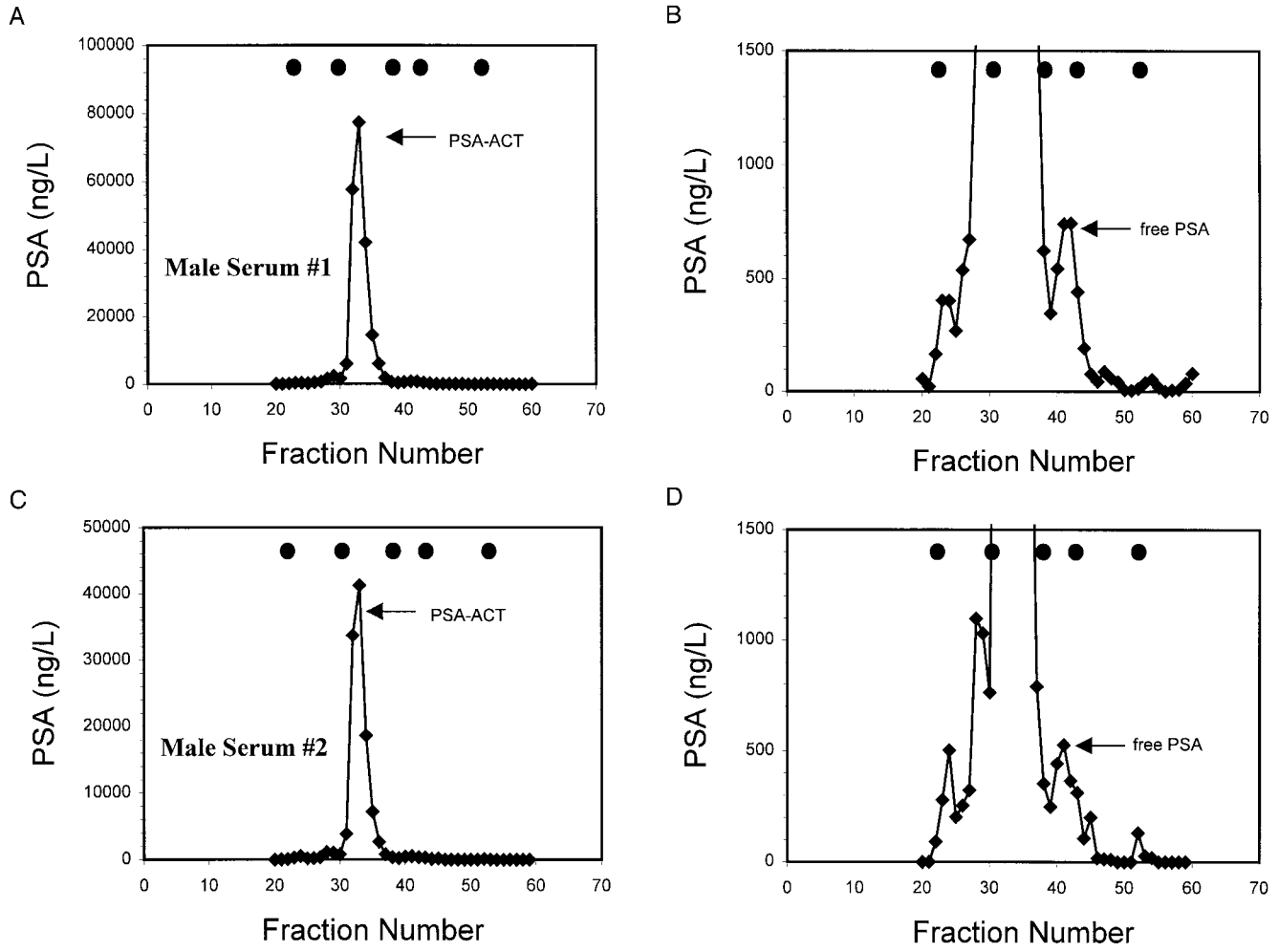

Fig. 5. PSA fractionation of two male sera by HPLC and analysis with the PSA immunoassay.

The peaks corresponding to PSA-ACT (~100 kDa) and free PSA (~30 kDa) are indicated by *arrows*. In *B* and *D*, the *y-axis* was expanded to visualize the minor free-PSA peak at fractions 41–42. The elution volumes of molecular mass calibrators of 670, 158, 44, 17, and 1.4 kDa are indicated by ●.

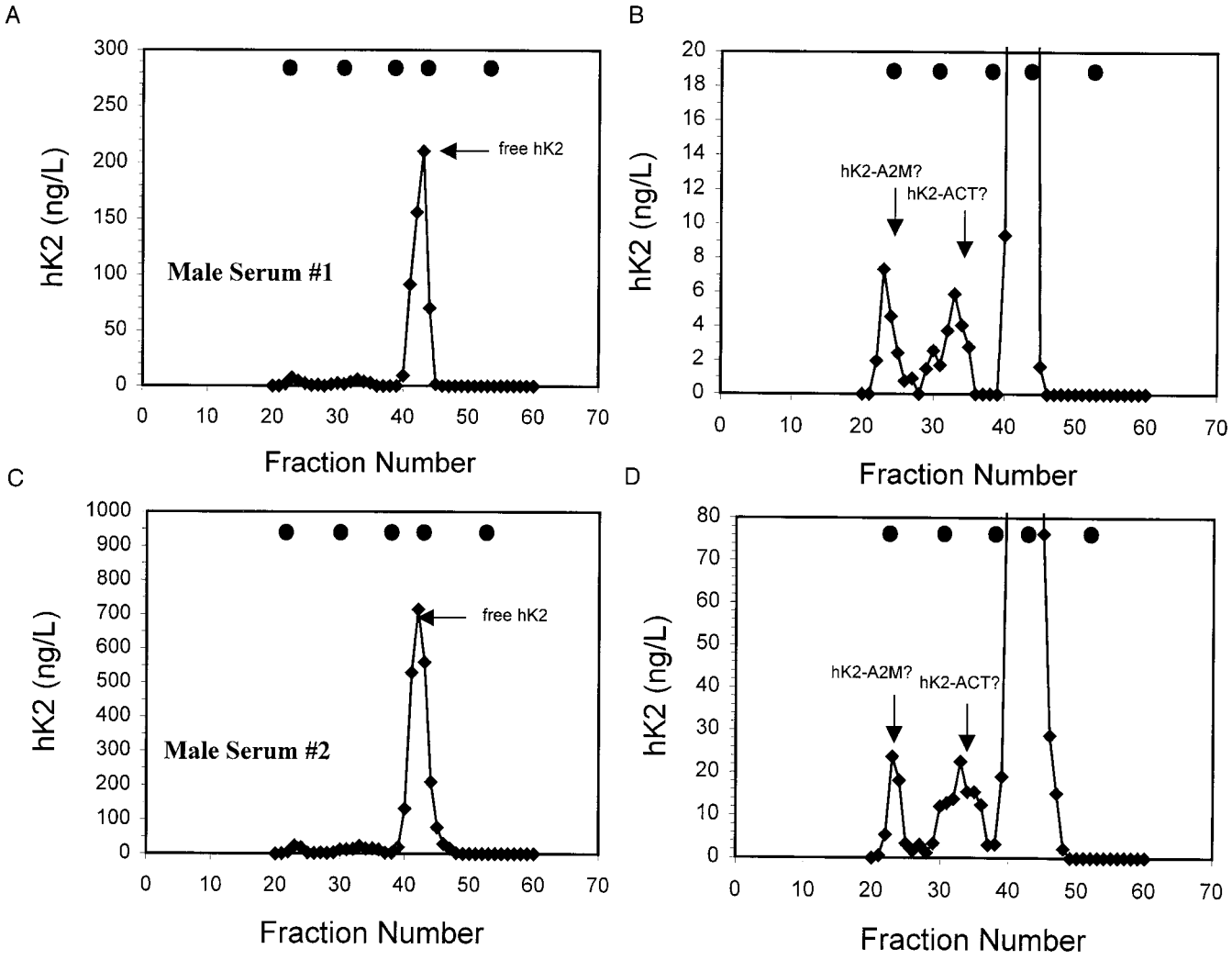

Fig. 6. hK2 fractionation of two male sera of Fig. 5 by HPLC and analysis with the hK2 immunoassay.

Three peaks are visible, with corresponding molecular masses of ~700 kDa (*hK2-A2M?*), ~100 kDa (*hK2-ACT?*), and ~30 kDa (*free hK2*). In *B* and *D*, the *y-axis* was expanded to visualize the minor peaks at fractions around 22 and 33. More than 95% of hK2 immunoreactivity represents free hK2. The elution volumes of molecular mass calibrators of 670, 158, 44, 17, and 1.4 kDa are indicated by ●.

prostate cancer patients, but also in those with prostatitis or benign prostatic hyperplasia *(4)*. These increases complicate the interpretation of serum PSA measurements and may lead to unnecessary biopsies. Therefore, it is not surprising that a continuing interest exists in improving the diagnostic capabilities of PSA. This can be accomplished by the use of molecular forms of PSA to increase its specificity for prostate cancer detection *(15)* or by the supplementation of PSA testing with additional prostatic markers.

hK2, also known as glandular kallikrein, and human kallikrein 3 (hK3, also known as PSA) are the products of androgen-regulated genes that are expressed primarily in the human prostate gland *(16)*. It recently was reported that hK2 converts pro-PSA, an inactive zymogen, to mature, enzymatically active PSA *(7–9)*, thus establishing a physiological connection between hK2 and PSA. PSA and hK2 have high sequence homology (80% identity at the amino acid level) and the same molecular mass (~30 kDa). It is challenging to ascertain whether purified preparations of PSA or hK2 from seminal plasma (which contains relatively large amounts of both proteins) are devoid of cross-contamination. Thus, only recombinant preparations are reliable reagents for antibody production, standardization, and cross-reactivity studies. Recombinant hK2 has been produced by a number of groups, and monoclonal antibodies have been raised *(17–19)*. Surprisingly, many investigators have found that it is possible to generate monoclonal antibodies against hK2 that have little or no cross-reactivity with PSA, opening the possibility for development of assays that are highly specific for hK2 [Ref. *(20)* and see below]. Furthermore, investigators have found that many PSA assays already reported in the literature do not cross-react with hK2; an assay developed in our laboratory is one example *(14)*.

Recently, many investigators have begun to focus on

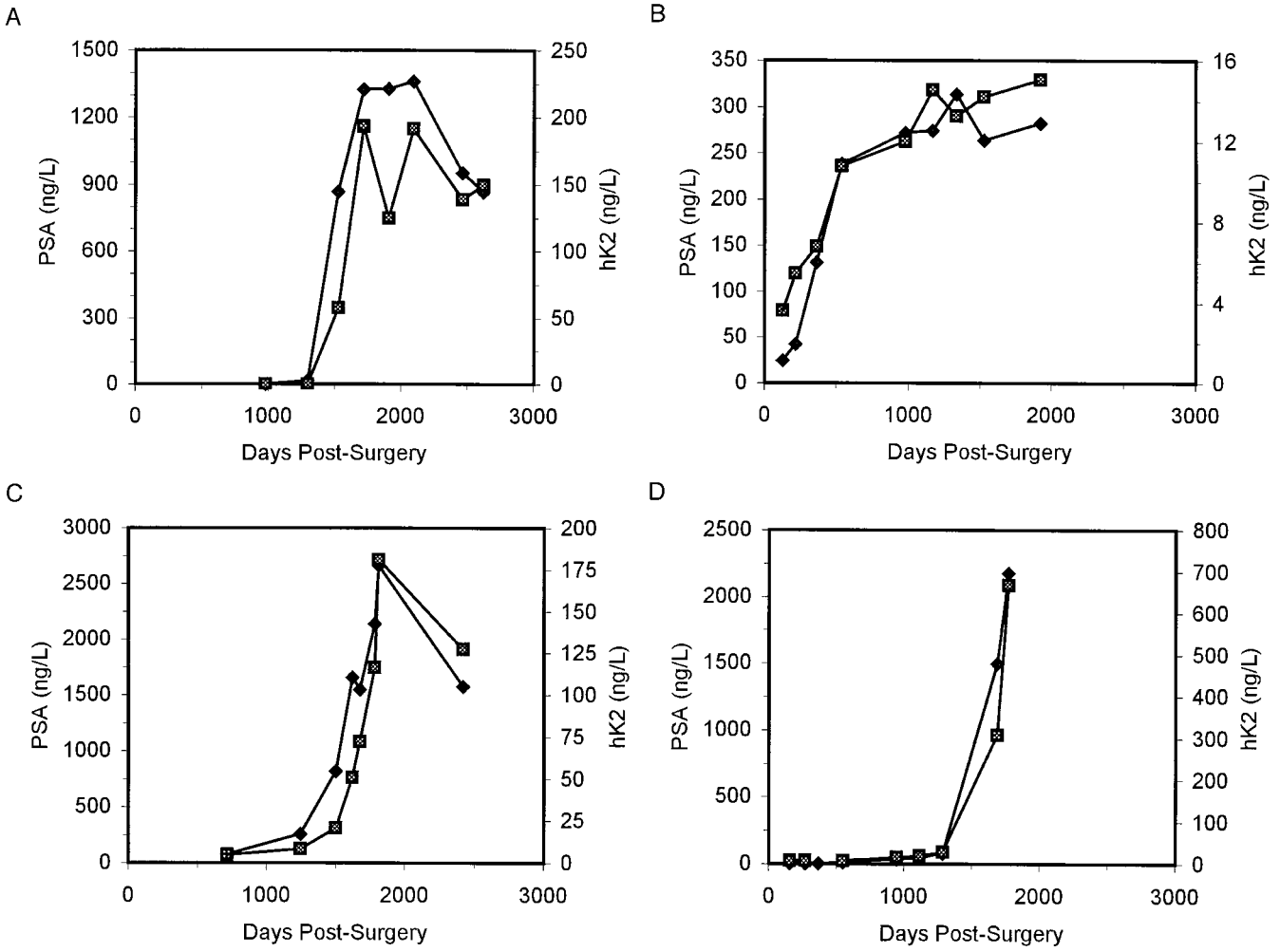

Fig. 7. PSA and hK2 in prostatectomized prostate cancer patients.

Changes in serum PSA and hK2 over time in four prostate cancer patients who underwent radical prostatectomy and then relapsed. ◆, PSA; ▨, hK2.

hK2 as a potential prostatic marker that could have utility, alone or in combination with PSA, for the diagnosis and monitoring of prostatic disease. Assays for hK2 that do not cross-react with PSA have already been reported by others, and preliminary clinical data have been published *(12, 21, 22)*. The method reported here is an important extension of the previous studies, primarily because of its improved detection limit (6 ng/L); this assay thus can provide clinical insight not easily obtainable with previously developed assays, which have detection limits of ~100 ng/L.

The hK2 assay developed here displays minimal cross-reactivity with PSA (≤0.2%; Table 1), enabling hK2 to be measured accurately in the presence of a large excess of PSA. Because of the absence of cross-reactivity in both assays, this hK2 assay and the previously described ultrasensitive PSA assay *(14)* provide reliable tools for monitoring independently, and with very high sensitivity, the concentrations of PSA and hK2 in biological fluids.

The recovery of recombinant hK2 added to female and male sera after a 30-min or 24-h incubation is low (<35%) and is similar to the recovery of PSA in serum *(14)*. Low recovery was also reported by Piironen et al. *(21)* and may be the result of the binding and masking of hK2 by A2M, as is also suggested by the data of Mikolajczyk et al. *(17)*. However, when hK2 from a male serum sample with a high concentration of hK2 was added to sera from healthy males and females, the recovery was much improved (70–104%), which also is in accord with the results reported for hK2 by Piironen et al. *(21)* and those reported for PSA *(14)*. These data suggest that like PSA *(15, 23)*, hK2 binds to proteinase inhibitors in serum. Free hK2 may represent a fraction that cannot bind to serum proteinase inhibitors. Similarly to PSA, free hK2 may represent either a proenzyme or an enzymatically inactive molecular form.

Studies of healthy male and female sera with previously described hK2 assays have concluded that male and female sera have similar values, generally <350 ng/L *(12)*. Piironen et al. *(21)*, were unable to measure hK2 in 57% of the male sera. The data from these two studies should be regarded as unreliable because the hK2 assays

used did not possess the low detection limits required to accurately measure hK2 in healthy male or female serum. We have concluded that hK2 in serum is much higher in men compared with women (Table 3). All 61 males without prostatic disease had detectable hK2, with a median concentration of 402 ng/L. In women, 31 of 33 serum samples had undetectable (<6 ng/L) hK2 concentrations, and the two hK2-positive samples also had detectable PSA. The ratio of PSA to hK2 in male serum was quite variable, with a median of 2.6, which is much higher than the ratio reported earlier *(21)*. On average, hK2 is present in male serum at concentrations ~2.5-fold than PSA. The variability of the PSA/hK2 ratio among different serum samples should be examined for possible clinical value. We are now investigating if the PSA/hK2 ratio or free PSA/hK2 ratio has any value in discriminating between benign prostatic hyperplasia and prostate cancer. Preliminary investigations by others and our unpublished data suggest that this may be true *(24)*.

The correlation between hK2 and PSA in serum is good ($r = 0.44$), in accordance with the findings of others *(21)* but in some disagreement with the data of Charlesworth et al. *(22)*, who found a weaker correlation.

Gel filtration of two male sera with high hK2 concentrations revealed that >95% of the total hK2 immunoreactivity elutes as free hK2 at a molecular mass of ~30 kDa. This finding is very different for PSA, for which most immunoreactivity elutes as a PSA-ACT complex at a molecular mass of ~100 kDa (Figs. 5 and 6). Similar conclusions for hK2 serum immunoreactivity were reported by Piironen et al. *(21)*. The improved detection limit of our assay allowed the detection of another two minor peaks with molecular masses of ~700 kDa and 100 kDa (Fig. 6). We speculate that these peaks represent hK2 complexed to A2M and ACT, respectively. However, we do not know the relative response of our assay to free hK2, hK2-A2M, and hK2-ACT complexes, and therefore can draw no conclusions related to the actual ratios of these hK2 subfractions in serum. We have, however, indications from our recovery experiments that once hK2 is added to serum, its immunoreactivity is substantially reduced, presumably because of the formation of such complexes. Finlay et al. *(12)* reported that their hK2 assay was skewed, detecting free hK2 with a 3.5-fold higher efficiency that hK2-ACT. The formation of complexes between hK2 and ACT is less likely to occur than between PSA and ACT, mainly because of the suggested trypsin-like enzymatic activity of hK2 *(18)*.

It has been reported that the hK2 mRNA in the prostate represents ~10–50% of that of PSA mRNA *(24)*. In the present study, analysis of five seminal plasma specimens revealed that PSA is present at a concentration 100- to 500-fold higher than that of hK2. Our data on this agree with the data presented previously *(12)*. Whether this finding is because of much lower production of hK2 in the prostate cells in comparison with PSA or to the degradation, inactivation, or binding of hK2 in seminal plasma requires further investigation.

Previous studies have reported increased hK2 in the serum of patients with prostate cancer *(12, 21)*. These findings were verified in our study with a small number of prostate cancer patients. Because the current major application of PSA is monitoring prostatectomized patients, we studied the hK2 concentrations of six patients who had serial serum samples collected over many years postprostatectomy, an application not examined in previous reports of hK2 detection methods. In the two patients who did not relapse over 5 years of monitoring, both PSA and hK2 remained undetectable in all serum samples collected. The remaining four patients suffered a relapse, with PSA rising over time, as shown in Fig. 7. Remarkably, hK2 correlates very well with PSA; thus, they have utility for monitoring. However, it appears from this limited data that there is no advantage of hK2 over PSA because for the former, the concentrations are lower by a factor of 3- to 23-fold. The variable PSA/hK2 ratios in postprostatectomy patients was also seen in healthy subjects (Table 3) and may have clinical importance.

In conclusion, we have presented a new method that can measure hK2 accurately in the presence of a large excess of PSA. This method, in combination with a method that can measure PSA in the presence of a large excess of hK2, will allow for the measurement of either hK2 or PSA in clinical samples that contain both analytes. Because our method has a detection limit that is ~20-fold lower than previously reported assays, hK2 concentrations in the serum of healthy males could be determined. Furthermore, it is evident here that the major immunoreactive fraction in serum is free hK2. There is a significant correlation between serum hK2 and PSA, the hK2 concentration being approximately 2.5-fold lower than that of PSA. More clinical studies will be necessary to establish if the simultaneous measurement of PSA and its subfractions and hK2 in serum has any advantage over PSA measurements alone.

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