Prostatic Inhibin-like Peptide Quantified in Urine of Prostatic Cancer Patients by Enzyme-Linked Immunosorbent Assay

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This is a highly specific enzyme-linked immunosorbent assay (ELISA) for measuring prostatic inhibin-like peptide (PIP) in urine, in which we use penicillinase (EC 3.5.2.6) conjugated with PIP and, as solid phase, a polystyrene microtiter plate. We used this ELISA to measure PIP in 24-h urine specimens from men with prostatic cancer (PCa) and from age-matched controls. For prostatic cancer patients the mean ± SEM urinary PIP of 36.1 ± 5 μg/24 h was significantly (P < 0.001) lower than the mean of 127.1 ± 9 μg/24 h for the age-matched controls. PIP values for 30 samples measured by both ELISA and RIA correlated well (r = 0.985). We could detect as little as 1.56 ng of PIP in a sample. Analytical recovery of added PIP ranged from 91% to 104%. Mean CVs were 8.9% within-assay and 12.7% between-assay. We believe that this ELISA will be useful in assessing the status of PIP in men with normal and diseased prostates and in examining the function of the hypothalamus–pituitary–prostatic axis.

Additional Keyphrases: seminal plasma · penicillinase · radioimmunoassay compared · reference interval · tumor marker

The high incidence of prostatic carcinoma in elderly men has motivated intensified study of this gland during the last two decades. Although prostatic cancer (PCa) ranks third behind lung and colorectal cancers as a cause of death from cancer (1), relatively little is known about its etiology.4 Unfortunately, two-thirds of the men with PCa already have metastases by the time their primary tumor is diagnosed (2, 3).

In our laboratory (4), the prostate has been demonstrated to be a rich source of prostatic inhibin-like peptide (PIP), a peptide involved in suppressing the synthesis and release of follitropin (follicle-stimulating hormone), from the pituitary (5). The prostatic origin of PIP (6) and its biosynthesis by both rat (7) and human (8) prostate has been demonstrated, as has the presence of specific receptors for PIP on rat (9) as well as human (10) prostatic tissue. Further, this prostate-specific peptide (PIP), the amino acid sequence of which is known (11), has been isolated and purified from human seminal plasma in our laboratory (12). Immunocytochemical-localization studies of PIP in normal, benign, and malignant prostates and metastatic lesions support its use as a histological marker for detection of prostatic origin of tumor (13, 14). An important tool for diagnosis and treatment of PCa has been the measurement of such biological markers. The development of immunological assays such as enzyme-linked immunosorbent assay (ELISA) and RIA for the measurement of biological markers has increased our ability to detect small changes in these components in several biological fluids.

Here we report the development of a specific ELISA to measure PIP in urine more conveniently than by RIA. Using this ELISA, we estimated PIP in urine specimens from PCa patients and in age-matched controls.

Materials and Methods

Antigen: We used PIP isolated and purified to homogeneity from human seminal plasma at our laboratory (12) as reference standard and for conjugate preparation.

Antiserum: Antibodies to PIP were raised in rabbits by active immunization. In vivo and in vitro neutralization experiments were carried out as described by Sheth et al. (15). Details regarding the high specificity of this antiserum have been published elsewhere (15).

Enzyme and substrate: Penicillinase (EC 3.5.2.6; from Bacillus cereus; M, 28,000; specific activity 67 MU per gram of protein) and the substrate, penicillin V (M, 387), were from Hindustan Antibiotics Ltd., Pune, India.

Chemicals: All chemicals were of analytical grade. Glutaraldehyde, 250 mL/L, was from E. Merck, Darmstadt, F.R.G. Sephadex G-75 was from Pharmacia, Uppsala, Sweden. Soluble starch was from Reanal, Budapest, Hungary. Tween 20 surfactant, bovine serum albumin, and silicone were from Sigma Chemical Co., St. Louis, MO.

Apparatus: Disposable, U-shaped polystyrene microtiter plates were purchased from NUNC Laboratories, Roskilde, Denmark. Absorbance was measured with a micro-ELISA Autoreader (Dynatech Laboratories Inc., Alexandria, VA).

Buffers and Reagents

Buffer A: 0.1 mol/L phosphate-buffered isotonic saline, pH 7.2 (PBS).

Buffer B: 50 mmol/L carbonate–bicarbonate buffer, pH 9.6 (coating buffer).

Buffer C: 0.2 mol/L phosphate buffer, pH 7.0 (enzyme assay buffer).

Buffer D: PBS containing 1 g of bovine serum albumin per liter (radioimmunoassay buffer).

Buffer E: 0.1 mol/L phosphate buffer, pH 7.0 (elution buffer).

Washing solution: 0.15 mol/L sodium chloride containing 0.5 mL of Tween 20 per liter (saline–Tween 20).

Iodine reagent: 80 mmol of iodine per liter in a 3.2 mol/L solution of potassium iodide.

Starch–iodine reagent: 30 mL of starch solution (hydrolyzed starch, 20 g/L), 190 mL of buffer C, and 0.25 mL of iodine reagent.

Starch–iodine–penicillin reagent: 20 mL of starch solution, 100 mL of buffer C, 0.2 mL of iodine reagent, and 18.24 mg of penicillin V (0.39 mmol/L).

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4Nonstandard abbreviations: PCa, prostatic cancer; PIP, prostatic inhibin-like peptide; ELISA, enzyme-linked immunosorbent assay; and PBS, phosphate-buffered isotonic saline.

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Methods

All glassware was siliconized with a 30 g/L silicone suspension.

Preparation of PIP—penicillinase conjugate: We conjugated PIP with penicillinase by the method of Avramaes (16), using glutaraldehyde as the linking agent. We placed 2 mg of PIP and 2 mg of penicillinase in a siliconized vial and diluted the mixture to 1.5 mL with buffer E. After slowly adding glutaraldehyde (42 mL/L, diluted from stock) to the vial, with constant mixing, we left the vial at room temperature for 3 h, with occasional mixing. We then dia lyzed the mixture against buffer E and chromato graphed the clear dialyze on a 1.2 × 55 cm column of Sephadex G-75, eluting with buffer E at a flow rate of 15 mL/h. We collected 1-mL fractions and monitored each fraction for enzyme activity by the rapid decolorization assay of Ghosh and Borkar (17), mixing 20 μL of each fraction with 1 mL of the starch–iodine–penicillin solution. Those fractions containing peak enzyme activity were pooled, preserved with phenol (10 mL/L) and bovine serum albumin (10 g/L), and stored at 4–8 °C in a siliconized container.

Preparation of immunosorbents: Antiserum was diluted in buffer B to a predetermined dilution and 0.2 mL of the diluted antiserum was then dispensed into each well of the ELISA plate. To coat the plates, we incubated them at 2–8 °C for 12–16 h, then rinsed them three times with the washing solution.

Preparation of standard: The stock standard solution of PIP was serially diluted in buffer D to give concentrations ranging from 1.56 to 100 ng per 0.1 mL.

Selection of optimum dilution of conjugate and antibody: We selected the optimum dilution of conjugate and antibody to be used in the assay on the basis of "checkerboard" titration as follows. After coating the wells of the microtiter plate with various dilutions (1:2000–1:16 000) of the antibody, we added various dilutions (1:50–1:1600) of conjugate in the absence of the PIP standard (i.e., the zero-concentration sample), to determine specific binding, and in the presence of excess standard, to determine nonspecific binding, and incubated for 24 h at 37 °C. Bound enzyme activity was determined by the ELISA described below. The combination of antibody and conjugate dilutions that gave (a) the greatest absorbance difference between the specific and nonspecific binding wells and (b) the lowest nonspecific binding of the conjugate was used in the subsequent assays.

Further, we measured the absorbance of the zero-concentration samples and the nonspecific-binding samples after incubating with reagent from 5 to 45 min, to determine the optimum duration of incubation before the enzyme reaction was stopped.

After deriving optimum conditions for the ELISA, we prepared a standard curve for PIP, covering the range from 1.56 ng to 100 ng per well.

ELISA procedure: To perform the assay, add to the wells of a microtiter-ELISA plate, coated with a predetermined dilution of antiserum, 0.1 mL of assay standard or unknown sample or excess standard (e.g., 200 ng of PIP per well) or sample buffer D, and 0.1 mL of a predetermined dilution of conjugate. Incubate the plate for 2 h at 37 °C, decant the contents of the wells, and rinse the plate with washing solution. To measure the penicillinase activity in each well, add 0.25 mL of starch–iodine–penicillin reagent to each well, incubate for 30 min at room temperature, and measure the absorbance of each well at 620 nm.

Samples: We obtained 21 24-h urine specimens from men (ages 50–90 years) with histologically proven Grade III/IV carcinoma of the prostate. We obtained 24-h urine specimens from 20 healthy men (ages 45–85 years) as control samples. The specimens were kept at 4 °C during the period of collection, without any preservatives. Aliquots of the specimens were stored at −20 °C until they could be analyzed for PIP by ELISA and (or) RIA (18). Just before analysis, we diluted the samples with buffer D. We determined the creatinine content of the samples by the method of Bonsnes and Taussky (19). To evaluate the statistical significance of the data we used Student's t-test. All the samples were analyzed in a single assay and were re-assayed twice to confirm the analysis.

Results

Figure 1 depicts the chromatographic elution profile of PIP—penicillinase conjugate on a 1.2 × 55 cm column of Sephadex G-75. The time required for decolorization of the blue starch–iodine–penicillin reagent is inversely proportional to the penicillinase enzyme activity (17), so we plotted decolorization time vs the fraction number. The active fractions containing the peak enzyme activity were pooled, preserved with phenol (10 mL/L) and bovine serum albumin (10 g/L), and stored at 4–8 °C.

Next we determined the enzyme activity of the conjugate at different dilutions, finding it to be proportional to the amount of conjugate added to the reaction mixture.

Further, on the basis of the checkerboard titration, we routinely used optimum dilutions of 1:200 and 1:8000 for the conjugate and antibody, respectively, in the ELISA.

We also determined 30 min to be the optimum time for the enzyme reaction in the ELISA to reach completion.

Standard curve and sensitivity. Figure 2 shows a graphical representation of logit-log transformation of a composite calibration curve (n = 6) for PIP. The standard curve is linear over the range of 1.56 to 25 ng. Sensitivity was determined as the smallest amount of unlabeled protein that gave a result significantly different from zero at the 95% confidence limit. The detection limit was 15.6 ng/mL, corresponding to a sensitivity of 1.56 ng of urinary PIP per well.

Specificity: Antiserum used in the ELISA has been tested
for cross-reactivity with 1 and 2 μg of several peptides of pituitary (follitropin, lutropin, prolactin), placental (placental lactogen, chorionadotropin), and fetal (carcinoembryonic antigen, α-fetoprotein) origin (15). We obtained evidence for specificity of the present assay indirectly, by parallelism studies. Using the present ELISA, we analyzed urine samples diluted 1:2, 1:4, and 1:8 for PIP and compared the response curve thus obtained with that for standard PIP. We found that the curve for the sample dilutions paralleled that for the standard PIP, their slopes being −1.90 and −1.85, respectively.

**Accuracy:** Accuracy was assessed as reflected in analytical recovery of PIP added to urine samples. Various amounts of standard PIP, 3.12 ng to 25 ng, were added to 100 μL of twofold-diluted urine sample. Thus in each case, we assayed 100 μL of urine of known PIP concentration containing 3.12 ng to 25 ng of added PIP. Analytical recoveries of the added PIP ranged from 91% to 104%.

**Precision:** We measured within-assay precision by assaying six times each within a single assay three urine samples having different concentrations of PIP. We estimated between-assay precision by assaying four urine samples six separate times. As Table 1 shows, the within-assay CV was 8.9%, the between-assay CV 12.7%.

**PIP in 24-h urine:** We observed that the volumes and creatinine contents of 24-h urine specimens from the prostatic-cancer patients did not differ significantly from those of the control subjects. As depicted in Figure 3, the mean (± SEM) concentration of PIP (36.1 ± 5 μg/24 h) as determined by the present ELISA was significantly lower (P <0.001) in the urine of prostatic-cancer patients as compared with that in the age-matched controls (127.1 ± 9 μg/24 h). PIP values for the control subjects ranged from 58.2 to 210 μg/24 h. All of the PCa patients had urinary PIP values lower than the mean value for the control men. Also 17 of the 21 PCa patients (81%) had urinary PIP values lower than the lowest value, 58.2 μg/24 h, observed for the controls.

**Comparison of ELISA with EIA:** We analyzed 30 samples for PIP by both the present method and a well-established RIA (18). Figure 4 illustrates the high correlation (r = 0.985) between results by the two methods. We calculated the standard error of the correlation coefficient to be 0.006, which is highly significant (P <0.001).

**Discussion**

We report here the first ELISA for PIP in human urine. Using it, we found significant hypoexcretion of PIP in PCa patients as compared with age-matched controls. Earlier studies in our laboratory on PIP concentrations in sera of men with PCa have demonstrated them to be increased in these patients as compared with control men (15). This contrast for serum and urine is intriguing. Studies on the clearance rate of PIP and on the secretory activity of the prostatic cells with respect to PIP, in normal and abnormal conditions of the prostate, may help explain it. The volumes.
and creatinine contents of the urine specimens from the control men and PCa patients were similar, implying that these factors do not play any role in the finding of low PIP concentrations in urine of PCa patients.

Our ELISA for urinary PIP provides an alternative technique for measuring PIP that does not involve precipitation, agglutination, or radioactivity. This assay can be completed within 4 h. It has good accuracy and precision. The high specificity of the antiserum we used was unaltered in the present assay, as judged by the good correlation \((r = 0.985)\) between estimates by RIA and the present ELISA. Non-interference of urinary constituents is evident from the satisfactory analytical recovery of added PIP and the parallelism between the standard curve and a sample-dilution curve. Our method is highly precise for samples with various concentrations of PIP, and the detection limit is comparable with that for RIA. We chose to use the enzyme penicillinase as label because of its high turnover number (1 200 000), low molecular mass (28 000 Da), easy availability, and reasonable cost (currently, $2.00 per gram) (20). Further, its assay is simple, sensitive, and reproducible. All the characteristics mentioned above make the present assay method suitable for reliable quantification of normal and pathological PIP concentration in urine.

The discovery of prostatic acid phosphatase, prostate specific antigen, and, later, PIP has raised much hope for early detection of PCs at a potentially curable stage. It may be noted that, like prostatic acid phosphatase and prostate specific antigen, PIP is a tissue-type-specific (6) and cell-specific protein for the prostate and is of cytoplasmic origin (13). This property has important clinical significance, because changing patterns of PIP concentrations in urine possibly are correlated with the degree of prostatic disease activity. The use of PIP as a specific histological marker for PCs and, in case of metastasis, as a marker for excluding or including the prostatic origin of tumor has been demonstrated (13, 14). The present ELISA for PIP thus can be a useful tool in studies of the normal or diseased prostate, and so may aid in elucidating the significance of PIP in the pathophysiology of the prostate and in examining the function of the hypothalamus–pituitary–prostate axis.

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References