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A New Method for the Determination of Human Urinary Kallikrein Activity using an Immunoadsorption Technique[1)]

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A new method for the determination of urinary kallikrein activity was developed. A polystyrene bead coated with anti-human urinary kallikrein antibody was used, with prolyl-phenylalanyl-arginyl-4-methylcoumaryl-7-amide as a substrate. The proposed method is simpler, more accurate, and more specific to urinary kallikrein than the conventional method. Coefficients of variation for within-day precision and for day-to-day precision were 9.1% ($n=20$) and 14.4% ($n=7$), respectively, and recovery was $102\pm2.5\%$ ($n=10$). The enzyme activities in urine from patients with renal failure and hypertension were lower than those in urine from normal subjects, but the activity in urine from patients with nephrotic syndrome was higher.

Keywords——human urinary kallikrein; anti-kallikrein antibody; kallikrein synthetic substrate; renal failure; hypertension

It has been reported that urinary kallikrein activity decreases in patients with essential hypertension[2)] and glomerulonephritis,[3)] and increases in patients with Bartter's syndrome[4)] and primary aldosteronism.[5)] However, a specific assay of urinary kallikrein activity has not so far been established. Various enzymes exist in the urine of healthy humans, such as esterases, peptidases, proteases and amylases,[6–9)] but the determination of urinary enzymes is not very useful in diagnostic analysis because the activities are generally very low, and there are many substances in urine causing interference.

In particular, urinary kallikrein activity cannot be accurately detected by conventional methods using *N*-α-tosyl arginine methylester[10)] or *N*-α-benzoyl arginine ethylester[11)] as substrates, or by bioassay.[12)] As substrates of glandular kallikreins, prolyl-phenylalanyl-arginyl and valyl-leucyl-arginyl derivatives are available.[13)] However, these substrates are not specific for the determination of urinary kallikrein activity because various peptidases, esterases and proteases in urine may also hydrolyze them. Therefore, in order to determine the true urinary kallikrein activity, it is very important to establish a method for specific extraction or specific trapping of the enzyme.

In this paper, we will describe a new method for the determination of urinary kallikrein by the use of anti-human urinary kallikrein antibody coated on polystyrene as a trapping step, and with prolyl-phenylalanyl-arginyl-4-methylcoumaryl-7-amide as a substrate.

Materials and Methods

Materials——Prolyl-phenylalanyl-arginyl-4-methyl coumaryl-7-amide (Pro–Phe–Arg–MCA; Protein Research Foundation, Osaka), polystyrene beads (Ichiko Co. Ltd.), and bovine serum albumin (BSA; Wako Pure Chemical Industries, Ltd.) were purchased. Other reagents were of analytical reagent grade.

Preparation of Anti-human Urinary Kallikrein Antibody——Human urinary kallikrein was purified according to the previous paper.[14)] The purification procedures consisted of silica gel adsorption, diethylaminoethyl (DEAE)-

cellulose column chromatography, Sephadex G-100 gel filtration and immunoadsorption column chromatography. The homogeneity of human urinary kallikrein was confirmed by disc electrophoresis and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis; the purified enzyme showed a single protein band. The purified enzyme also exhibited a single precipitin line against anti-human urinary kallikrein antibody on immunoelectrophoresis. Purified enzyme (0.5 mg) was mixed with an equal volume of complete Freund's adjuvant and injection of enzyme was repeated three times at two-week intervals. One week after the last injection, the rabbit was bled. Ammonium sulfate was added to the antiserum to bring it to 33% saturation. The precipitate was dissolved in a small amount of distilled water and dialyzed against 20 mM potassium phosphate buffer (pH 8.0). The antibody was purified on a column of DE-32 cellulose, according to the method of Peterson and Sorber.[15]

Preparation of Antibody-coated Polystyrene Beads——Anti-human urinary kallikrein antibody was coated on polystyrene beads according to the method of Kato *et al.*[16] The polystyrene beads (3.2 mm, 1000 pieces) were washed with a detergent (Scat-20 X), and then incubated in 150 ml of anti-human urinary kallikrein antibody (1.0 mg/ml) dissolved in 0.25 M sodium phosphate buffer (pH 7.5) at 37 °C for 30 min, and allowed to stand at 4 °C overnight. The beads were then washed with 10 mM sodium phosphate buffer (pH 7.5) and next with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$ and 0.1% BSA (Buffer A). The anti-human urinary kallikrein antibody-coated polystyrene beads were stored in Buffer A at 4 °C; these beads can be used for at least one year.

Standard Assay Procedure——An anti-human urinary kallikrein antibody-coated polystyrene bead was incubated with 5 μl of urine in 0.15 ml of Buffer A at 4 °C overnight (about 16 h) with shaking, and then the bead was washed twice with 1.5 ml of Buffer A. The activity of kallikrein on the bead was assayed. The bead was preincubated in 0.2 ml of 0.1 M sodium phosphate buffer (pH 8.0) containing 0.3% BSA and 0.15 M NaCl, at 37 °C for 5 min, and then the enzyme reaction was started by the addition of 10 μl of 10 mM Pro–Phe–Arg–MCA dissolved in dimethyl sulfoxide. The enzyme mixture was incubated at 37 °C for 60 min with shaking, then the enzyme reaction was terminated by the addition of 2.5 ml of 0.1 M acetate buffer (pH 4.5). The amount of released 7-amide-4-methylcoumarin (AMC) was measured by spectrofluorophotometry at 380 nm for excitation and 440 nm for emission. Under the above conditions, 1 unit of kallikrein activity was defined as the amount that liberated 1 μmol AMC per min.

Conventional Method——The amidolytic activity of kallikrein towards Pro–Phe–Arg–MCA was determined according to the method of Kato *et al.*[17] with minor modifications. A 5 μl urine sample was used without extraction of the enzyme, the enzyme reaction was performed according to the standard assay procedure.

Creatinine Determination——First-time urine samples were collected in the morning from healthy subjects and from patients with various diseases. The urinary creatinine was determined by using the Bonsness and Taussky method.[18]

Results

Fundamental Investigation of Conditions for the Proposed Method

The immunoreaction of kallikrein with immobilized antibody reached a plateau within 16 h. Therefore, the urine sample was incubated with the immunoadsorbent overnight to extract the enzyme. Kinetic parameters of the adsorbed enzyme on polystyrene beads were also investigated in comparison with those of free kallikrein. The K_m and V_{max} values of urinary kallikrein on the bead towards Pro–Phe–Arg–MCA were determined to be 120 μM and 7.9 μmol/mg, respectively. This K_m value is very similar to that of free kallikrein (110 μM), but the V_{max} is 80% of that of free kallikrein (10.0 μmol/mg). The activity of kallikrein on beads was linearly related to the amount of enzyme and the enzymatic reaction time.

Under the standard assay conditions, the calibration curve was linear up to 16 units/l, passing through the origin, as shown in Fig. 1.

Precision and Recovery of the Proposed Method

By the use of urine from healthy males (300 mU/g of creatinine), coefficients of variation for within-day and for day-to-day precision were 9.1% ($n=20$) and 14.4% ($n=7$), respectively. The recovery from a urine sample to which 5 units/l of purified kallikrein had been added was examined, and was found to be $102 \pm 2.5\%$ ($n=10$).

Correlation between the Results of the Proposed Method and the Conventional Method

The urinary kallikrein activity in urine from various patients was determined by the proposed method and the conventional method. As shown in Fig. 2, the correlation between

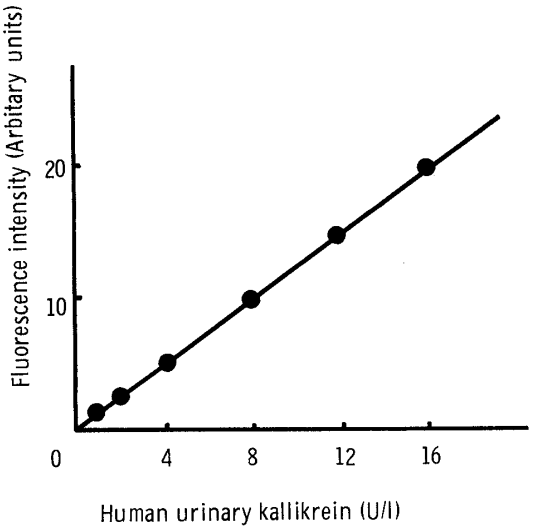

Fig. 1. Calibration Curve for Urinary Kallikrein obtained by the Proposed Method

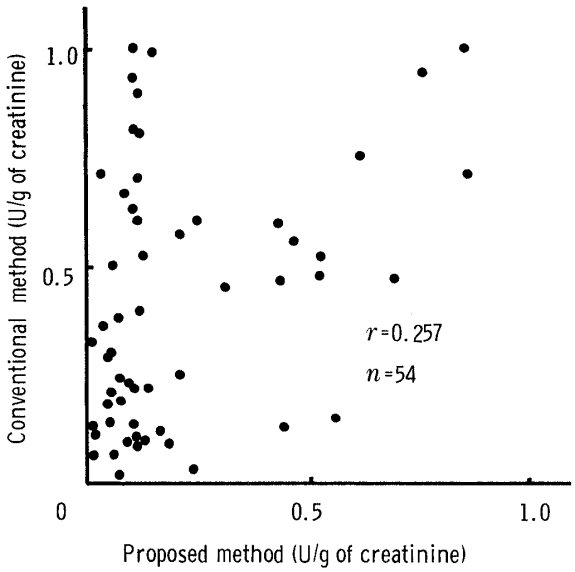

Fig. 2. Correlation between Urinary Kallikrein Activity determined by the Proposed Method and the Conventional Method

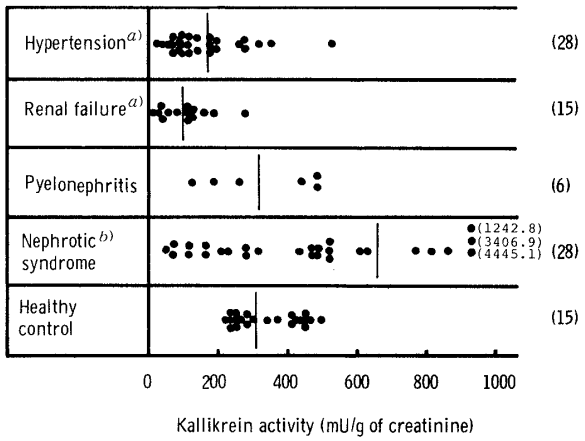

Fig. 3. Kallikrein Activity in Urine determined by Using Anti-human Urinary Kallikrein Antibody-coated Polystyrene Beads

Bars represent the mean values of urinary kallikrein activity. Figures in parenthesis indicate the number of patients.

a) Indicate a statistically significant ($p<0.01$) difference between normal subjects and patients.

b) ($p<0.1$).

the values is poor, and the correlation coefficient is 0.257.

Kallikrein Activity in Urine from Normal Subjects and Patients with Diseases

Using the proposed method, the activity of urinary kallikrein was measured in normal subjects and in patients with hypertension, renal failure, pyelonephritis and nephrotic syndrome. The results are shown in Fig. 3. Kallikrein activiries in urine from patients with renal failure (mean $\pm$ SD; 105.5 ± 71.6 mU/g creatinine) and hypertension (170.9 ± 124.1) were statistically lower than that in urine from normal subjects (339.0 ± 92.1), but the activity in urine from nephrotic syndrome patients (672.6 ± 940.4) was somewhat higher than that in urine from normal subjects. There is no correlation between the urinary protein concentration and urinary kallikrein activity.

Discussion

Various methods for the determination of kallikrein have been reported.[13,19-28] Among the methods available, photometric assays using *N*-α-tosyl arginine methylester[10] and *N*-α-benzoyl arginine ethylester[11] as substrates are most often used, but have poor specificity for kallikrein. Recently, various tripeptides, Bz-prolyl-phenylalanyl-arginyl-*p*-nitroanilide, D-prolyl-phenylalanyl-arginyl-*p*-nitroanilide, valyl-leucyl-arginyl-*p*-nitroanilide, prolyl-phenylalanyl-arginyl-α-naphthylester (Pro–Phe–Arg–NE) and Pro–Phe–Arg–MCA, have been developed for use as kallikrein substrates, but these are not specific for kallikrein and are hydrolyzed by other proteases and esterases. Therefore, in order to determine the true kallikrein activity, the extraction of kallikrein from body fluid containing various enzymes is necessary.

In the present work, we combined the immunological trapping method with the use of a sensitive synthetic substrate, Pro–Phe–Arg–MCA, and developed a new method for the determination of kallikrein activity in urine. As a trapping step for kallikrein, a kallikrein antibody-coated polystyrene bead was used. V_{max} of kallikrein on the solid phase was somewhat smaller than that of the free enzymes. This may be due to steric hindrance or conformational change of the enzyme depending on the support, and such phenomena are generally observed in immobilized enzymes. However, the affinity of the enzymes for the substrate, K_m, was not changed.

No significant correlation was found between the values of urinary kallikrein activity determined by the proposed method and the conventional method. This discrepancy may be explained by the facts that there are various enzymes in urine, such as proteases, peptidases and esterases which can hydrolyze the synthetic substrate, that there are some inhibitors[29] which are reversible, and that the behavior of urinary kallikrein is different from that of other urinary enzymes. In recent years, it has been reported that a large amount of prokallikrein exists in human urine.[30] This observation is thought to be important, but the determination of physiological active-type kallikrein will certainly be useful in diagnostic tests.

Pro–Phe–Arg–NE is a substrate developed by Hitomi *et al.*[31] and is used in the field of kallikrein activity staining and determination of urinary kallikrein activity. However, this substrate has the disadvantage of higher autohydrolyzing rate than other synthetic substrates. In our preliminary studies, urinary kallikrein activities towards Pro–Phe–Arg–NE was unstable and its sensitivity to kallikrein was lower than that of Pro–Phe–Arg–MCA. Therefore, we have used the substrate Pro–Phe–Arg–MCA in the present study. Other commercially available substrates were insensitive in this proposed method, and they necessitate a femtomole concentration of enzyme. Antibody-coated beads were very stable and could be used for at least one year.

Urinary kallikrein excretion has been reported to decrease in patients with essential hypertension and to increase in patients with primary aldosteronism. However, the situation is still unclear, and we are planning further studies on the relationship between urinary kallikrein activity and diseases.

References and Notes

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