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ACTIVE SITE RADIOIMMUNOASSAY FOR HUMAN UROKALLIKREIN AND DEMONSTRATION BY RADIOIMMUNOASSAY OF A LATENT FORM OF THE ENZYME[1]

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A radioimmunoassay specific for the active site in urokallikrein has been developed with a monospecific antibody that neutralizes the enzymatic activities of urokallikrein and a radioligand purified so as to maintain the active site. In order to favor the involvement of the antibody with high affinity for the active site in the competition between urokallikrein in biologic fluids and radiolabeled urokallikrein, the radioligand was separated from denatured radiolabeled urokallikrein by affinity chromatography for the active site. The concentration of specific IgG used in the assay bound approximately 80% of the radioligand, which was displaced in a dose-related fashion by 0.2 to 2.5 ng of the unlabeled urokallikrein. When random urine samples from 21 healthy volunteers were assessed for endogenous urokallikrein by both active site radioimmunoassay and kinin generation, there was a linear relationship between the results of the two assays with a correlation coefficient of 0.89. A combined correlation plot of the results of the bioassay and the active site radioimmunoassay for the 21 urine samples before and after trypsin activation gave a linear regression line with a correlation coefficient of 0.91. The finding that trypsin activation increased the urokallikrein concentration of urine to a similar extent in both the radioimmunoassay and the bioassay means that latent urokallikrein was not detected until its active site was uncovered.

Human urinary kallikrein (urokallikrein), a kininogenase (E.C.3.4.21.8), generates kinin polypeptides from plasma α-2-globulin substrates, kininogens (1). Neither the cellular source nor the function of urokallikrein has been conclusively defined, although reduction in the mean urinary excretion of alkaline arginine esterolytic activity, considered to be urokallikrein, has been associated with essential hypertension (2–4). Studies of urokallikrein excretion in physiologic and pathologic circumstances are difficult to interpret when esterolytic assays are used because of the presence of nonurokallikrein esterases that cleave the same synthetic substrates (5, 6). The observation that trypsin treatment of crude urine or a partially purified fraction of urine increased the esterolytic activity for substrates containing arginine suggested the existence of a latent form of urokallikrein (7), thereby further complicating any attempts to relate excretion of one form of the enzyme to a particular clinical situation.

The characterization of a monospecific anti-human urokallikrein antibody in terms of its capacity to neutralize the active site of the enzyme (8) afforded the opportunity to develop an active site-specific radioimmunoassay by using a radioligand prepared and isolated so as to maintain its active site. Crude urine, before and after incubation with trypsin, was analyzed for endogenous and latent urokallikrein with both the radioimmunoassay and a kinin-generating assay; the correlation of the two assays established that the radioimmunoassay was specific for the active site.

MATERIALS AND METHODS

Materials. Synthetic bradykinin triacetate and Bolton-Hunter reagent (New England Nuclear Corp., Boston, Mass.); lyophilized trypsin (Worthington Biochemical Corp., Freehold, N. J.); Sephadex G-25 (medium), Blue Dextran 2000, and activated CH-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.); atropine sulfate (Vega Biochemicals, Tucson, Ariz.); aprotinin (Boeringer-Mannheim Biochemicals, Indianapolis, Ind.); and an IgG fraction of goat anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, Pa.) were obtained from the sources indicated.

Preparation and labeling of urokallikrein. Human urokallikrein for use in the radioimmunoassay was purified from concentrated fresh pooled human urine by aprotinin-CH-Sepharose 4B affinity chromatography modified for a batch procedure and by Sephadex G-100 gel filtration (9). Forty milliliters of 100-fold concentrated urine were incubated at 23°C for 2 hr with 40 ml settled volume of aprotinin-CH-Sepharose 4B containing approximately 1 μmole aprotinin/ml of gel. After a further 18-hr incubation at 4°C, the affinity gel was washed in a Buchner coarse fritted glass funnel with six 250-ml volumes of 0.1 M $NaHCO_3$, pH 7.8, 0.5 M NaCl until the OD_{280} of the effluent was 0.01. The gel was then washed with three 200-ml volumes of 0.05 M Tris-HCl, pH 6.5, 0.5 M NaCl until the OD_{280} again returned to baseline. The urokallikrein was then eluted from the affinity gel with 250 ml of 0.1 M Na acetate-acetic acid buffer, pH 3.2, 1.0 M NaCl, and collected in 50 ml of 2.0 M Tris-HCl, pH 8.5. The urokallikrein was further purified by gel filtration as described, and the final preparation was homogeneous by alkaline disc gel electrophoresis, by sodium dodecyl

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sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[4] with and without reduction, and by amino terminal analysis (9). The concentration of the purified urokallikrein was determined by Folin assay using a standard curve developed with a reference urokallikrein preparation quantitated on the basis of its amino acid content (9). A single preparation of this purified urokallikrein, designated "urokallikrein standard", was divided into aliquots, stored at −70°C, and used to prepare all standard curves in the radioimmunoassay.

One millicurie of [^{125}I] monoiodo-Bolton-Hunter reagent (10) was incubated at 4°C with 15 to 20 μg of purified urokallikrein in a volume of 30 μl in 0.1 M potassium phosphate buffer, pH 7.7, for 15 min with frequent mixing and then incubated for 18 hr at 4°C. The reaction mixture was applied to a 10-ml Sephadex G-25 column equilibrated in 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl containing 0.25% gelatin and filtered at a flow rate of 20 ml/hr; 1-ml fractions were collected. The ^{125}I-urokallikrein present in the fraction at the exclusion volume of the Sephadex G-25 column containing 14×10^6 cpm/μg (12.7 μCi/μg) was directly applied to a 6-ml column of aprotinin-CH-Sepharose 4B equilibrated at room temperature with 0.1 M $NaHCO_3$, pH 7.8, 0.5 M NaCl. The flow rate was 1 ml/min and the effluent was repeatedly reapplied for 30 min. The column was then washed with 200 ml of cold equilibration buffer, and the ^{125}I-urokallikrein was eluted with cold 0.1 M Na acetate-acetic acid buffer, pH 3.2, 1.0 M NaCl. Two-milliliter fractions were collected in tubes containing 100 μl of 2.0 M Tris-HCl, pH 8.5, containing 2% gelatin. This procedure yielded ^{125}I-urokallikrein with a specific activity of approximately 6×10^5 cpm/μg (0.5 μCi/μg), and this radioligand was divided into aliquots and stored at −70°C.

Performance of the radioimmunoassay. The monospecific anti-urokallikrein IgG used for the radioimmunoassay inhibited both the kinin-generating and esterolytic functions of purified urokallikrein in a substrate dose-dependent fashion (8), thereby indicating specificity for determinants at the active site of the enzyme. The radioimmunoassay was performed in 0.1 M Na borate buffer, pH 8.5, 0.1 M NaCl containing 0.25% gelatin. Seventy-five microliter portions of a 1/400 dilution of anti-urokallikrein IgG were mixed with crude urine samples or with urokallikrein standard in either buffer or urokallikrein-free urine; the final volume of each reaction mixture was adjusted to 100 μl with buffer. After the addition of 5 μl of ^{125}I-urokallikrein containing approximately 2000 cpm, the samples were incubated at 4°C for 18 hr. Then 5 μl of normal rabbit serum diluted 1/20 or 1/30 and 100 μl of IgG fraction of goat anti-rabbit IgG diluted 1/2 were added to each tube. After incubation at 4°C for 24 hr, the tubes were centrifuged at 4°C at 23,000 × G for 15 min and the supernatants were removed. The precipitates were resuspended in 100 μl of borate buffer and centrifuged again. The precipitates and the pooled supernatants from each tube were counted in a Searle Analytic Model 1185 gamma-counter for 5 min to yield bound and free counts, respectively.

The assay was calibrated with varying dilutions of urokallikrein standard, each prepared in 10 μl of urokallikrein-free urine. Urokallikrein-free urine was prepared by treating 10 ml of freshly voided urine with 1.7 mg insolubilized trypsin for 20 min at room temperature, sedimenting the insolubilized trypsin at 250 × G for 4 min, and passing the treated urine over aprotinin-CH-Sepharose 4B at pH 8.0. More than 97% of the endogenous and trypsin-inducible kinin-generating activity was removed from the urine by this procedure. The urokallikrein-free urine was divided into aliquots and stored at −70°C.

An antibody titration curve, developed by using varying doses of anti-urokallikrein IgG and a fixed amount of ^{125}I-urokallikrein, was dose-related between 1/100 and 1/1600 dilutions of anti-urokallikrein. To prepare a reference curve, 0, 1, 2, 5, or 10 μl of urokallikrein standard (0.445 ng/μl) were used in the assay, and the ratios of bound to free counts were plotted as a function of the concentration of urokallikrein standard. The amount of ^{125}I-urokallikrein used was chosen such that approximately 80% of the bindable counts were precipitated by the 1/400 dilution of anti-urokallikrein IgG used for the radioimmunoassay. Crude urine samples were assayed in 5-, 10-, and 20-μl volumes and bound:free count ratios were used to determine urokallikrein concentrations from the standard curve.

Preparation of samples for assay. Random urine samples, collected from healthy laboratory personnel, were immediately placed at 4°C and were assayed on the day of collection. Each urine sample was tested for urokallikrein by both radioimmunoassay and bioassay before and after trypsin treatment.

Trypsin-CH-Sepharose 4B was prepared by using activated CH-Sepharose 4B by a modification of the manufacturer's recommended procedure. One gram of the activated-CH-Sepharose 4B was washed in a coarse Buchner fritted glass funnel with 200 ml of ice-cold 1 mM HCl, followed by 100 ml of 0.1 M potassium phosphate, pH 5.8. The gel was then transferred to a solution of 20 ml of the same buffer containing 357 mg of trypsin. The mixture was shaken for 4 hr at room temperature and for 2 to 18 hr at 4°C. The pH of 5.8 was chosen to favor attachment of the trypsin at its amino terminus to the CH-Sepharose 4B (11). The unreacted trypsin was separated from the trypsin-CH-Sepharose 4B on a Buchner funnel, and the gel was treated with ethanolamine and washed with buffers as directed. Approximately 50% of the trypsin bound to the gel, as assessed by Folin assay of the pooled washes. The trypsin-CH-Sepharose 4B was suspended in 10 to 12 ml of 0.1 M Na acetate-acetic acid buffer, pH 4.0, 1.0 M NaCl and stored at 4°C.

Urine samples were treated with trypsin by mixing 35 μl settled volume of insolubilized trypsin with 1 ml of cold urine and incubating at room temperature for 20 min, during which time the tubes were shaken every 5 min. The tubes were then centrifuged at 250 × G for 4 min, and the supernatants were removed and stored at 4°C until assayed.

The enzymatic activity of urokallikrein was assessed by its ability to generate kinin from heat-inactivated plasma. Two to 200-μl samples of urine, with or without exposure to insolubilized trypsin, were incubated with 0.3 ml of heat-inactivated plasma (5, 12) for 5 min at 37°C. The mixtures were immediately assayed for formed kinin on a guinea pig terminal ileum segment suspended in Tyrode's buffer (13) made 0.5 μM in atropine. The kinin generated was quantitated by comparison with the contractile response of the ileum to standards of synthetic bradykinin.

RESULTS

The calibration curve for the urokallikrein radioimmunoassay in which bound:free cpm are plotted against the input of unlabeled urokallikrein standard is shown in Figure 1. A dose-response curve was obtained in the range of 0.2 to 2.5 ng. When one set of standards was prepared in urokallikrein-free urine and another set in borate buffer, similar curves were obtained. Because this assay was to be applied to the determination of urokallikrein in unfractionated urine samples, urokallikrein-free urine was routinely used in the preparation of the standards,

[4] Abbreviations used in this paper: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

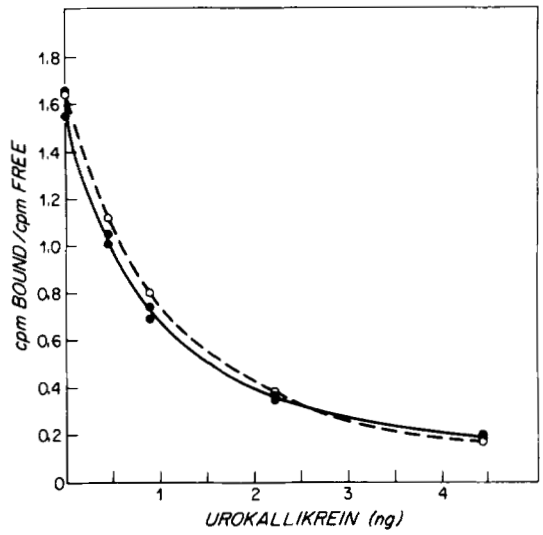

Figure 1. Calibration curves for the urokallikrein radioimmunoassay in which the ratios of bound to free cpm are plotted against the input of unlabeled urokallikrein standard. The calibration was carried out in the presence (●) or absence (○) of urokallikrein-free urine.

which were run in duplicate each time the assay was performed. The mean percent variation from the mean of each pair of duplicate values was 3.4% for 50 pairs in 10 standard curves. In the absence of added unlabeled urokallikrein, the anti-urokallikrein IgG bound approximately 80% of the labeled ligand, whereas normal rabbit IgG bound less than 3%.

The reproducibility of the measurement of urokallikrein in fresh urine from a single donor was demonstrated by use of duplicate samples, doubled volumes, and the addition of known amounts of urokallikrein standard to fixed urine sample volumes (Table I). The urokallikrein in ng/sample was the same for the duplicates and was double that amount when the volume of the sample tested was also doubled. When the data obtained with increasing volumes of urine for each of 41 donors were normalized by being expressed as ng/ml, the standard deviation of the normalized values for each donor varied from the mean by an average of 7.6% with a range of 0 to 32%. When four different urine samples were fortified with increasing amounts of urokallikrein standard, as depicted for one subject in Table I, the urokallikrein recovered for the four subjects was between 88 and 107% of that expected by summation of the endogenous and added urokallikrein for the sample, with a mean of 98%.

When human urine was treated with insolubilized trypsin for 20 min at room temperature and centrifuged to remove the trypsin, the kinin-generating capacity of the urine increased by 36 to 423%, with a mean of 162%. Treatment of the urokallikrein standard with trypsin did not change its kinin-generating activity, and buffer incubated with insolubilized trypsin neither acquired kinin-generating capacity nor behaved differently from untreated buffer in the radioimmunoassay.

The relationship between the radioimmunoassay of urokallikrein and the bioassay of kinin-generating activity for 21 fresh random urine samples with and without trypsin treatment is depicted in Figure 2. There is a linear correlation (r = 0.91) for the two assays and the calculated regression line intersects the Y axis at a point close to the origin, 0.149. The fact that this relationship is linear through the origin indicates that the two assays are comparable in their measurement of active urokallikrein, endogenous, and trypsin activated. When a sample of urokallikrein standard containing 445 ng/ml was subjected to bioassay, the kinin-generating activity was 24 μg kinin/ml, a value consistent with the linear correlation of the two assays in urine.

The mean value obtained for endogenous urokallikrein by the radioimmunoassay in 21 random urine samples was 67 ng/ml, with a range of 21 to 215 ng/ml. For trypsin-treated urine samples, the mean urokallikrein concentration was 112 ng/ml, with a range of 38 to 450 ng/ml. When the latent urokallikrein, the difference between the endogenous and the trypsin-treated values, was plotted for the two assays, a linear correlation was again found, with the correlation coefficient r = 0.82 (Fig. 3). The fact that the line intersects the Y axis above the origin suggests that there is a small threshold before the radioimmunoassay recognizes the latent urokallikrein uncovered by trypsin treatment.

DISCUSSION

A radioimmunoassay specific for the active site in urokallikrein has been developed with a monospecific antibody that neutralizes the enzymatic activities of urokallikrein and a radioligand purified so as to maintain the active site. Monovalent Fab fragments of the IgG fraction of the rabbit anti-urokallikrein used in the radioimmunoassay had been shown to neutralize the kinin-generating and esterolytic functions of purified

TABLE I

Radioimmunoassay for urokallikrein in varying amounts of a single urine and in urine with added urokallikrein

Volume Assayed	Urokallikrein Added	Urokallikrein Found	Urokallikrein Expected (ng)	% Recovery
μl	*ng*	*ng*	*ng*	
10		0.34		
10		0.34		
20		0.68		
10	0.44	0.75	0.78	96
10	0.89	1.10	1.23	89
10	2.23	2.75	2.57	107

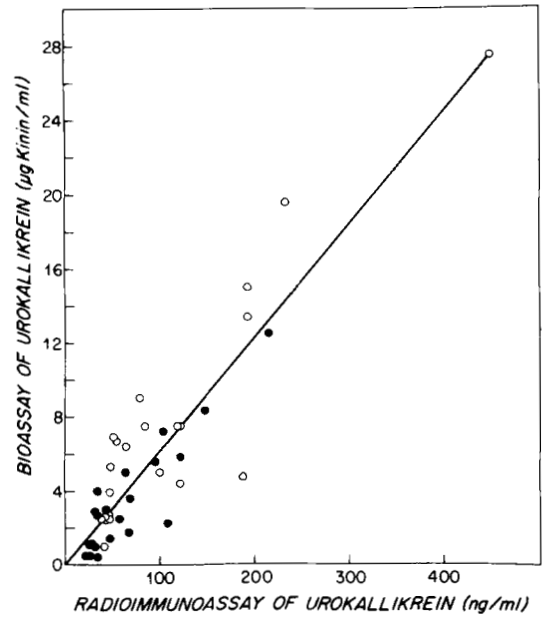

Figure 2. Comparison of the urokallikrein content of 21 random urine samples determined by radioimmunoassay and by bioassay, both with (○) and without (●) trypsin treatment. The calculated linear regression line is shown, for which the correlation coefficient r = 0.91 ($p < 0.001$).

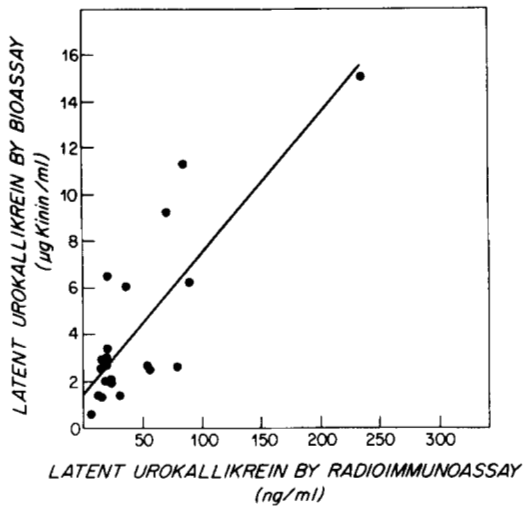

Figure 3. Comparison of latent urokallikrein levels in 21 random urine samples determined by radioimmunoassay and by bioassay. The calculated linear regression line is shown, for which the correlation coefficient $r = 0.82$ ($p < 0.001$).

urokallikrein without a detectable lag period and in a dose-related fashion (8). The average inhibition constant for the monovalent Fab fragments, 22 nM, was the same as that observed for the specific IgG when kinin-generating activity was examined. When the inhibition of the esterolytic activity of the enzyme by the Fab fragments was analyzed by Dixon plots of the data, there was competitive inhibition at the lower concentrations of Fab with an average inhibitory constant of 250 nM and mixed inhibition at higher concentrations of the fragments. These data suggest that the high affinity antibodies were directed toward the active site of the enzyme and that by selection of appropriate dilutions of the antibody, it would be possible to develop an active site-specific radioimmunoassay. In order to favor the involvement of the antibody with high affinity for the active site in the competition between urokallikrein in biologic fluids and radiolabeled urokallikrein, the radioligand was separated from free counts by gel filtration and from denatured radiolabeled urokallikrein by affinity chromatography for the active site. The radioligand that retained intact active sites represented only about 5% of the total iodinated protein. The concentration of specific IgG selected for the assay bound approximately 80% of the radioligand and the radioligand was displaced in a dose-related fashion by 0.2 to 2.5 ng of unlabeled urokallikrein (Fig. 1). The mean percent variation from the mean of each pair of 50 duplicate values in 10 standard curves was 3.4%. Increasing volumes of crude urine gave normalized values of urokallikrein per milliliter of urine that fell within a mean standard deviation of 7.6% of the mean for each urine. The recovery of purified urokallikrein added to normal urine averaged 98%. Thus, the radioimmunoassay for urokallikrein was sensitive, reproducible, and specific.

When 21 random urine samples were assessed for endogenous urokallikrein by both active site radioimmunoassay and kinin generation, there was a linear relationship between the results of the two assays with a correlation coefficient $r = 0.89$. The fact that the radioimmunoassay recognized the active site was then confirmed by trypsin activation of each urine and repeat analysis that showed that the linear regression line for results of the bioassay and the active site radioimmunoassay exhibited a correlation coefficient $r = 0.91$. A combined correlation plot of the results of the bioassay and the active site radioimmunoassay for the 21 urine samples before and after trypsin activation gave a linear regression line with a correlation coefficient $r = 0.91$ (Fig. 2). The finding that trypsin activation increased the urokallikrein concentration of urine to a similar extent in both the radioimmunoassay and the bioassay (Fig. 3) means that latent urokallikrein was not detected until its active site was uncovered.

The use of insolubilized trypsin to uncover latent urokallikrein made it possible to remove the trypsin before bioassay of urokallikrein by sedimentation rather than by blocking the trypsin by the addition of a competitive inhibitor. The latent form of the urokallikrein prepared by DE-52 chromatography and G-100 gel filtration, as defined by trypsin-induced esterolytic activity, has a m.w. of 50,000 (7), which would be considerably larger than urokallikrein purified by conventional techniques (14–17). However, urokallikrein purified by affinity chromatography from fresh urine under mild conditions over a limited time period has a m.w. of 48,000 by gel filtration, 48,000 by SDS-PAGE, and 48,214 as calculated from amino acid composition (9). Although the nature of the trypsin-induced change in latent urokallikrein is unknown, the existence of the latent form is clearly demonstrated by the correlation of the net increase in trypsin-inducible activity in crude urine measured by bioassay and active site radioimmunoassay (Fig. 3).

The multiplicity of alkaline arginine esterases in urine (5, 6) makes it unlikely that cleavage of synthetic substrates, irrespective of the sensitivity of the detection system, will offer an assay that is specific for urokallikrein. The assay of urokallikrein by kinin generation offers specificity but has an important limitation imposed by the presence of kinin-inactivating enzymes (kininases) in urine (18). This problem, although not evident in the current studies of normal urine, might appear in circumstances where the urinary urokallikrein concentration was reduced without alteration of the kininase activity. A radial immunodiffusion assay of urokallikrein employing the same antiserum as used for the active site radioimmunoassay was sensitive in the range of 1 to 10 μg/ml (6). Since it required concentration of the urine before testing and a relatively high concentration of the antiserum, it is likely that the precipitin lines observed in the radial immunodiffusion assay depended on all the specificities for urokallikrein present in the antiserum. Other recently described radioimmunoassays appear to detect urokallikrein without distinguishing its active from its latent form and the reports of these assays have not considered the relative effects of these two physicochemical states on the competition with the radioligand for the antibody (19, 20). The active-site radioimmunoassay described herein not only is specific and reproducible, but its sensitivity exceeds that of any other immunoassay described for human urokallikrein (6, 19, 20). Its combination with trypsin activation of urine allows assessment of latent as well as endogenous urokallikrein content.

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