A Direct Assay for Urinary Kallikrein

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The first protein-binding assay is described for the direct determination of kallikrein in rat urine. Isolation of urinary kallikrein and preparation of its specific antibody have been previously described. Outlined here are the methods for ^3H -labelling of kallikrein, for isolation of ^3H -labelled immunoreactive enzyme, and for separation of free and antibody-bound kallikrein with the aid of double antibody. Assay and equilibrium conditions were characterized and a protocol is presented for the measurement of kallikrein concentration. The direct assay is sensitive, accurate and it correlates well ($r = 0.81$) with a functional assay. The assay may hold promise to determine catalytically active and inactive enzyme in urine, tissues and biological fluids.

Urinary kallikrein is a renal enzyme (Nustad & Pierce, 1974) that catalyses the release of kinins from kininogen. Efforts have been made to develop specific (Oza *et al.*, 1974) and sensitive (Beaven *et al.*, 1971) assays for measurement of kallikrein activity in view of the postulated role of this enzymesystem in diuresis, natriuresis and in certain forms of hypertensive cardiovascular diseases (Mills & Ward, 1975; Margolius *et al.*, 1971). Since biological fluids and tissues contain many enzymes (Prado, 1970) capable of mimicking kallikrein-like activity, the functional assays (proteolytic or esterolytic) are rather limited in assessing true activities of kallikrein.

Rat urinary kallikrein has been isolated to apparent homogeneity and monospecific antibodies have been produced against this enzyme (Oza *et al.*, 1976). The present paper describes, for the first time, a protein-binding assay for the direct determination of rat urinary and, by homology (Webster, 1970), glandular kallikreins.

Materials and Methods

^3H -labelling of kallikrein

Rat urinary kallikrein was isolated by anion-exchange, affinity (Trasylol-Sepharose 4B) and Sephadex G-100 chromatography as described in a previous paper (Oza *et al.*, 1976).

Pure kallikrein was radiolabelled in 20–100 μg batches with 1 μCi of 1-fluoro-2,4-dinitro[3,5- ^3H]-benzene (specific radioactivity 24.1 Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) per 100 μg of enzyme. The reaction was performed in 0.5 M-sodium borate buffer, pH 9.0, at 25°C for 6 h. At the end of this time, 0.3 ml of 0.5 M-glycine amide

was added and left to react further for 1 h at 25°C. The reaction mixture was adjusted to a volume of 2.0 ml and bovine serum albumin was added to 0.1% (w/v) concentration of the carrier protein. The radiolabelled enzyme, [^3H]kallikrein, was dialysed for 18 h at 4°C against 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1% bovine serum albumin (assay buffer).

[^3H]Kallikrein was then passed through a column (1 cm $\times$ 50 cm) of Sephadex G-100 gel pre-equilibrated in the assay buffer. The void volume of this column was previously determined by the filtration of 1 mg of Blue Dextran 2000 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). Fractions (2 ml) were collected and a 5 μl sample of each fraction was mixed with 10 ml of solubilizer [18% BBS-3 solubilizer (Beckman Instruments, Fullerton, CA, U.S.A.) in scintillant (42 ml of Liquifluor Q.S. made up to 1 litre with toluene)] and counted for at least 10 min in a Packard Tri-Carb liquid-scintillation counter (wide-open window with efficiency of about 60%). All the fractions were also analysed for their ability to bind with the anti-kallikrein serum (by using the protocol described below). The immunoreactive fractions were pooled and stored frozen.

Assay

The nature of rabbit anti-(rat urinary kallikrein) has been described in a previous paper (Oza *et al.*, 1976). Briefly, the antibodies were found to be monospecific for urinary kallikrein and immunologically alike enzymes of certain tissues. As a routine, a double-antibody radioimmunoassay was performed. The double antibody was produced in goat against rabbit immunoglobulins (immunoglobulin G type). A single high-titre pool of anti-kallikrein and double antibody was used throughout the studies.

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Before use the anti-kallikrein, double antibody and normal rabbit sera were given acid-pH and heat treatment to denature complement and kallikrein inhibitors, which are normally present in the serum (Vogel & Werle, 1970). A sample of each serum was adjusted to pH 3.0 with 1 M-HCl, left to stand for 30 min at 25°C, re-adjusted to pH 7.4 with 1 M-NaOH and then heated at 56°C for 3 h. After centrifugation at 4000 rev./min for 30 min, the supernatants (called pretreated) were used in the assay at appropriate dilutions.

The incubation mixture for the assay consisted of approx. 4000 c.p.m. of [^3H]kallikrein in a volume of 0.1 ml, 0.1 ml of anti-kallikrein serum with a final dilution of 1:30000, unlabelled pure kallikrein (5–200 ng) (for the standard plot) or unknown samples and assay buffer to a total volume of 0.5 ml. Initial dilution of anti-kallikrein serum was made in the assay buffer, which, in addition, contained 1:400 dilution of pretreated normal rabbit serum for the enrichment of immunoglobulins. The reaction mixture was incubated for 2 h at 25°C and 0.1 ml of 1:5 dilution of double antibody was added to each tube. The tubes were, once again, incubated for 18 h at 4°C and centrifuged at 4000 rev./min for 30 min at 4°C. The supernatant (0.3 ml) was mixed with 10 ml of solubilizer and counted for free [^3H]kallikrein radioactivity. Pretreated normal rabbit serum was substituted for anti-kallikrein serum to determine total counts. The percentage of binding was determined by incubating [^3H]kallikrein and anti-kallikrein serum in the prescribed samples. Duplicate determinations were made and the unknown sample was interpolated from a plot of percentage initial binding versus the amount of added kallikrein by a computer program described by Rodbard *et al.* (1968).

The validity of the present component-binding assay was compared with a functional assay (Oza *et al.*, 1974) in which the urinary kallikrein activity was measured by the amount of kinins released. The kinins were determined by a radioimmunoassay adopted from that described by Talamo *et al.* (1969).

Results and Discussion

Sephadex G-100 gel filtration of [^3H]kallikrein is shown in Fig. 1. Before radiolabelling, the enzyme was homogeneous by criteria of disc gel electrophoresis, immunodouble diffusion and immunoelectrophoresis (see Oza *et al.*, 1976). However, two symmetrical peaks (see Fig. 1) were always obtained after ^3H -labelling. The immunoreactive [^3H]kallikrein emerged in a second uniform peak at about 2 times the void volume of the column (17 ml). Since the recovery of immunoreactive [^3H]kallikrein was in excess of 60% (in three different experiments), efforts were not made to identify the protein of the first peak that emerged

Fig. 1. Sephadex G-100 gel filtration of kallikrein after ^3H -labelling

The void volume (V_0) of the column (1 cm × 50 cm) was 17 ml. Fractions (2 ml) were collected and a 5 µl sample of each fraction was counted for radioactivity. Each fraction was also analysed for its ability to bind with anti-kallikrein. The fractions of the second peak (marked with a solid bar at the bottom), which emerged at twice the void volume of the column, contained immunoreactivity. These fractions were pooled and stored frozen until used in the assay.

with the void volume. It is conceivable that a small portion of the enzyme was denatured and aggregated during the ^3H -labelling and thus lost its immunoreactivity. Nonetheless, molecular sieving with Sephadex G-100 was useful to resolve immunoreactive [^3H]kallikrein from inactive enzyme. After combining the fractions of the second peak (Fig. 1), the specific radioactivity of the pool was found to be 25.6 Ci/mmol. Samples of this material were used directly in the assay after appropriate dilution.

The avidity of anti-kallikrein serum was proven by the fact that maximal binding, i.e. 45–55%, occurred within 2 h of first incubation. However, it was essential to carry out the second incubation with the double antibody for 18 h to obtain complete separation of free from antibody-bound [^3H]kallikrein. A standard plot of four different experiments is shown in Fig. 2. To determine inter-assay variation, a single urine sample was analysed for 7 consecutive days. The concentration of kallikrein was found to be $7.2 \pm 0.3 \mu\text{g/ml}$ (mean $\pm$ s.e.m.). In a separate assay, the intra-assay coefficient of variation, in six repetitive measurements, was found to be 7.64%. Furthermore, a correlation coefficient of 0.81 was obtained when 46 normal rat urine samples were compared by measuring kallikrein concentration and kinin-generating activity. Thus the present assay was found to be valid to determine kallikrein concentration in urine and other biological fluids. Since urinary kallikrein is similar to the kallikrein found in kidney,

Fig. 2. Standard curve of percentage of initial binding versus the amount of added unlabelled kallikrein (a) (b) Straight-line plot of the same data obtained by a logit plot (Rodbard *et al.*, 1968) to allow accurate interpolation of an unknown sample.

pancreas and salivary gland (Webster, 1970), the present assay should be useful to measure tissue enzyme concentration.

In functional assays, it is generally necessary to dialyse or partially purify urine samples to remove non-enzymic (salt, urea, inhibitors etc.) or enzymic (Keiser *et al.*, 1976) impurities. The assay described here is sensitive enough to measure 0.005 μg of kallikrein. Thus the concentration of kallikrein can be measured in as little as 1 μl of normal rat urine. In this small volume, the interference due to non-

enzymic impurities should be minimum. As expected, no differences were found when kallikrein concentration was compared in dialysed versus undialysed urine samples. The inherent specificity of immunoassay would indeed eliminate the interference due to other enzymic components of the urine.

The present assay eliminates the use of substrate and therefore the question of its purity (for kininogen) or stability (for esters) does not arise. A large number of samples can be analysed with consistency and the assay requires 2 days for its completion.

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