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A NEW DIRECT RADIOIMMUNOASSAY OF RAT URINARY KININOGEN

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Abstract—A protein-binding radioimmunoassay (RIA) of rat low molecular weight (LMW) kininogen with the following characteristics has been developed: sensitivity, 2.5 ng/tube; inter-assay coefficient of variation, 12.4% (N = 28); and intra-assay coefficient of variation, 9.4% (N = 11). The new assay correlated ($r = 1$) with the determination of kinin equivalence of kininogen after trypsinization. The cross-reactivity of rabbit anti-rat LMW kininogen antibody was 2.5% with bovine LMW kininogen, 5.8% with rat plasma high molecular weight (HMW) kininogen, and none with kinin. Although the antibody appears to partially recognize des-kinin-kininogen, the low degree of cross-reactivity and the extremely low levels of kinin-free-kininogen allow accurate determination of total LMW kininogen in rat urines. The LMW kininogen formed 20% kinins with salivary kallikrein when compared with trypsin, suggesting that the preparation consists of both K- and T-kininogens (K = kallikrein susceptible; T = trypsin susceptible). The newly developed protein-binding RIA recognizes LMW kininogen of rat urine which consists of both K- and T-kininogens.

Many of the biological actions of the renal kallikrein-kinin system are due to generation of kinins in the kidney [1, 2]. A number of factors such as the amount of kallikrein in urine [2], urinary pH [3], as well as the electrolyte concentration [4] of the urine are important determinants of *in vivo* kininogenase activity. Recently, kinin generation was reported to be controlled also by the amount of urinary kininogen in human subjects [5].

Urinary kininogen has been measured hitherto by assaying kinins released by trypsin from urine samples. We reported previously the occurrence of kininogen in rat urine [6]. In the present work, low molecular weight (LMW) kininogen of rat plasma was isolated to apparent homogeneity and antibody was produced in rabbits. Using this polyclonal antibody, a direct, protein-binding radioimmunoassay (RIA) of LMW kininogen was developed and characterized. The antibody prepared against plasma LMW kininogen recognizes both the T- and K-kininogens. Therefore, the assay may be useful in the determination of total LMW kininogen (both T- and K-kininogens) of rat urine.

MATERIALS AND METHODS

Isolation of rat LMW kininogen. Citrated blood was obtained from the abdominal aorta of Sprague–Dawley rats. The blood was centrifuged, and clear plasma was collected and stored at −70°. Starting with 580 ml plasma, LMW kininogen was isolated as described by Bedi *et al.* [7] using: (1) DEAE-Sephadex A50 chromatography, (2) ammonium sulfate fractionation, (3) Sephacryl S-200 gel filtration, (4) SP Sephadex C-50 chromatography, and (5) repeat of Sephacryl S-200 gel filtration. Kininogen-containing fractions were identified either by their ability to bind with rabbit anti-rat LMW kininogen serum (courtesy of G. Bedi) or by the determination of bradykinin equivalence after digestion with excess trypsin. The molecular weight of the purified LMW kininogen was determined in 7.5% polyacrylamide gel and 0.1% sodium dodecyl sulfate (SDS) by electrophoresis as described by Weber and Osborn [8].

Preparation of kininogen antiserum. Four different disc-gel columns (7.5% polyacrylamide) were electrophoresed with 100 μg each of the LMW kininogen isolated by the above procedure. One disc-gel column was stained to locate the major band of LMW kininogen. The corresponding regions of the other three unstained disc-gel columns were eluted with 400 μl of sterile normal saline, and the eluates were pooled. Aliquots (50, 100 and 200 μl; approx. 12.5, 25 and 50 μg protein) were emulsified with 3 ml each of Freund's complete adjuant. The emulsion was intradermally injected on the shaven back surface of three New Zealand rabbits. The rabbits were boosted at weeks 10 and 15 following primary immunization with a similar emulsion of LMW kininogen. Two of the three rabbits gave peak titers at weeks 11 and 16 post primary immunization.

Radioimmunoassay of LMW kininogen. Pure LMW kininogen was ^{125}I-labeled in 50–100 μg batches by a procedure described previously [6] for radioiodination of rat urinary kallikrein. An RIA was established using approximately 4000 cpm ^{125}I-labeled LMW kininogen (2–4 μCi/μg) in 25 μl RIA buffer, 100 μl of 1:60,000 dilution of anti-kininogen serum, 10–100 μl of unlabeled standards and the balance of RIA buffer (265–365 μl) in a total volume of 0.5 ml. RIA buffer was 0.05 M Tris–HCl, 0.1% (w/v) neomycin and 0.1% (w/v) gelatin, pH 7.4. The tubes were incubated for 4 hr at 25°, and the bound tracer was separated from the free by the addition of a 50-μl suspension of particle-bound second antibody commercially obtained from Roche

Diagnostics, Nutley, NJ. Unspecific binding of under 3% was subtracted, and a standard curve was constructed with a net B_0 of about 50% by an automated data reduction program.

The RIA was calibrated by determining the inter-assay and intra-assay coefficients of variation. Recovery of pure kininogen added to a rat urine pool also was determined. Parallelism of cross-reacting rat urinary antigen with the standard curve was evaluated. Cross-reactivities of bradykinin, bovine LMW kininogen, and rat high molecular weight (HMW) kininogen (courtesy of Dr. Alhenc-Gelas) against our anti-kininogen serum were determined.

The LMW kininogen content of twenty different rat urines was analyzed both by the direct RIA and by determining kinin equivalence by kinin RIA [6] after digestion with excess trypsin, in order to compare the two techniques.

In additional studies, pure LMW kininogen was digested separately with insoluble TPCK-trypsin (obtained commercially from the Pierce Chemical Co., Rockford, IL) with the insoluble rat salivary kallikrein. The salivary kallikrein was isolated from the rat salivary gland by extraction and DEAE-Sephadex A50 chromatography as described by Brandtzaeg *et al.* [9] and additionally by Trasylol-Sepharose affinity chromatography as previously described [10]. Rat salivary kallikrein was immobilized by a technique similar to the one employed for immobilization of Trasylol [10]. Trypsin and salivary kallikrein digests were passed through a molecular sieve gel column of Ultrogel ACA-44 (LKB, Bromma, Sweden), and emerging fractions were analyzed by kininogen RIA as well as by the kinin RIA. Since kinin antibody has approximately 1/1000th recognition for kininogen (see Fig. 4), the chromatographic separation should help to determine if kininogen antibody recognized kinin at any significant level. Furthermore, the experiment was intended to determine the rate of kinin formation by trypsin and by salivary kallikrein.

RESULTS

Figure 1 shows the electrophoretic mobility of 100 μg of rat LMW kininogen (middle column on right side of the figure corresponding to band No. 5) in SDS-gel electrophoresis and a calibration curve (left side of the figure). The current was 8 mA per gel column, and other conditions were exactly as described by Weber and Osborn [8]. The kininogen in band No. 5 had a mobility comparable to bovine serum albumin in band No. 4. A trace amount of contaminant (see middle gel column) was present, the molecular weight of which appeared to be about 135,000. It is conceivable that the trace contaminant was a dimer because the molecular weight of the major band (No. 5) was found to be 67,000 daltons.

The standard curve of LMW kininogen from 2.5 to 100 ng/tube, derived from seven experiments, is shown in Fig. 2. Also shown in the same figure are parallel curves obtained from increasing aliquots of four different rat urines. The parallelism indicates that the immunoreactivity of the antigen in these urines was monospecific. LMW kininogen excretion was found to be 9.7 ± 1.3 μg/ml (mean ± SEM) in eighteen different urines analyzed separately (direct RIA data of Fig. 3). The cross-reactivity of 25–200 ng bovine LMW kininogen (3.5%), 100–300 ng rat HMW kininogen (5.8%), and 25–200 ng bradykinin (none detectable) was not parallel to the standard curve as shown in this figure.

Inter-assay coefficient of variation was 12.4% (N = 28) and intra-assay coefficient of variation was 9.4% (N = 11). The recovery of exogenous kininogen was essentially quantitative, 110 ± 3.4% (mean ± SEM, N = 18), indicating that other substances present in the urine did not affect the deter-

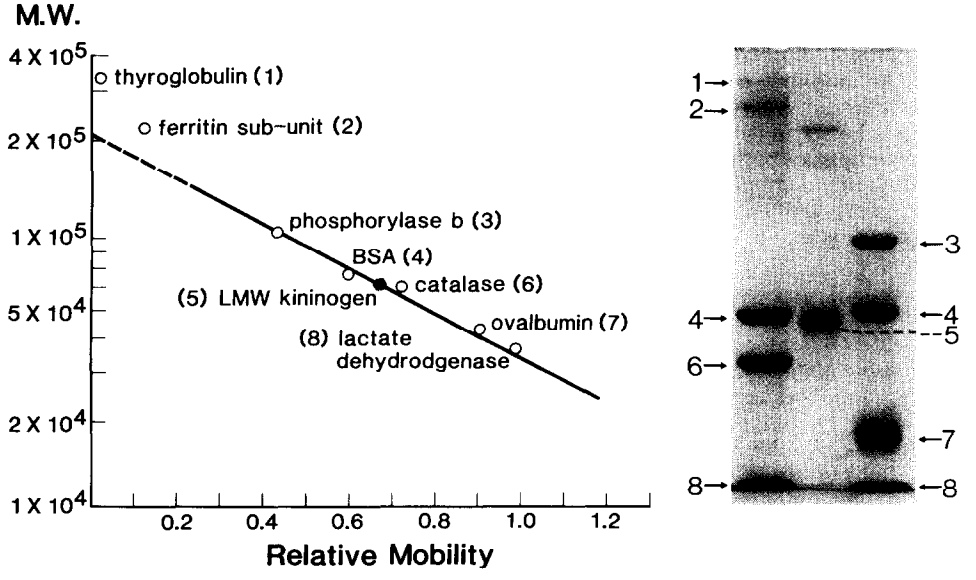

Fig. 1. Determination of molecular weight of rat plasma LMW kininogen by SDS-polyacrylamide disc gel electrophoresis. The mobility of 100 μg LMW kininogen (middle gel column on right) was determined in comparison with marker proteins electrophoresed in the left and right columns.

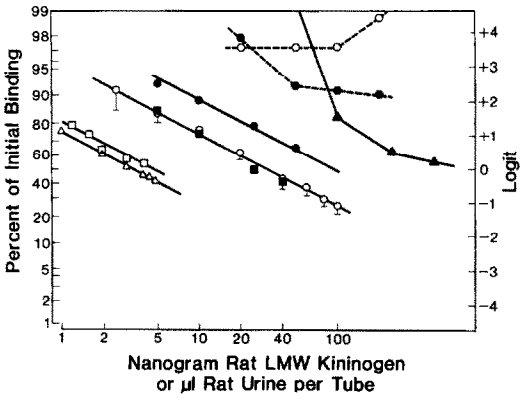

Fig. 2. Log-logit transformation of standard curves (N = 7) of rat urinary kininogen (○; mean ± SD); rat urine samples 1 (△—△), 2 (□—□), 3 (■—■) and 4 (●—●); cross-reactivity of 25–200 ng bradykinin (○—○), 25–200 ng bovine LMW kininogen (●—●) and 100–300 ng rat HMW kininogen (▲—▲).

mination of kininogen by this assay. The sensitivity of our assay is adequate to determine LMW kininogen in as little as 1 µl of rat urine.

The newly developed, direct assay of kininogen was compared with the kininogen determination by kinin equivalence assay in twenty random rat urines, as shown in Fig. 3. The data gave a straight line defined by $y = 11.92x + 10.86$ and the coefficient of correlation, r, was 1.00. Intercept on the Y-axis was not significantly different from the zero at $P = 0.05$.

Analysis of the digestion products released from LMW kininogen by trypsin or by rat salivary kallikrein is shown in Fig. 4. As shown in the upper half of the figure, untreated kininogen emerged at a peak effluent volume of 44 ml. Undigested kininogen after trypsin or kallikrein treatment emerged at a peak effluent volume of 42 ml. Although the reason for this very small shift is not quite apparent, it is conceivable that native kininogen shows some anomalous behavior on a molecular sieve column because of its carbohydrate moiety [11]. It is also evident

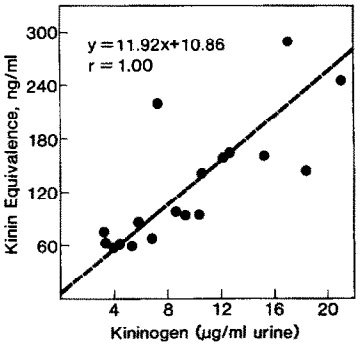

Fig. 3. Comparison of urinary kininogen determination by direct RIA (abscissa) and by assay of kinin equivalence after digestion with excess trypsin (ordinate). Twenty urines were analyzed from which the data of two urines, x = 65.2, y = 797 and x = 485, y = 5795, are not shown because of the scale limitation but included in the calculations for a straight line and for the r value.

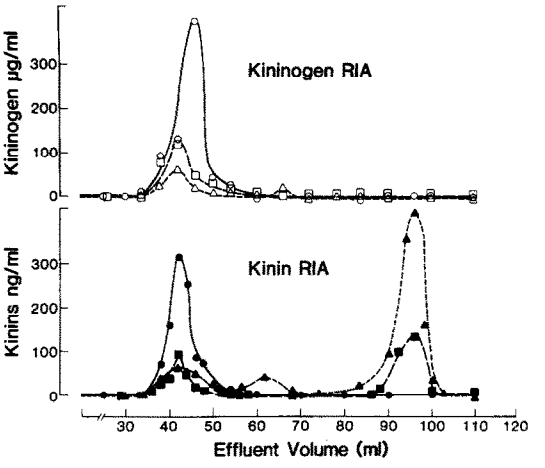

Fig. 4. Analysis of the digestion products released from LMW kininogen by trypsin or by rat salivary kallikrein. Aliquots of rat plasma LMW kininogen (1.44 mg) were subjected to three separate treatments. The first was the control; the second was treated with 0.2 mg particle-bound TPCK-trypsin; and the third was treated with 0.26 mg particle-bound rat salivary kallikrein. All three reactions were incubated at 37° for 24 hr, centrifuged to remove particle-bound enzymes, and individually gel-filtered by gravity on a 1.5 × 40 cm column (0.1 M sodium phosphate buffer, pH 8.5) of ACA-44. Two-ml fractions were collected, and each fraction was analyzed by a kininogen RIA (upper half of the figure) and by a kinin RIA (lower half of the figure). Key: (○) control kininogen, (△) trypsin-digested kininogen, and (□) salivary kallikrein-digested kininogen were all analyzed by the direct RIA of kininogen; (●) control kininogen, (▲) trypsin-digested kininogen and (●) kallikrein-digested kininogen were all analyzed by the kinin RIA (see text).

from these data that: (1) Both glandular kallikrein and trypsin liberated kinins from our LMW kininogen preparation. Trypsin was about five times as active as kallikrein (compare right hand peaks in bottom half of figure). (2) Our antiserum against kininogen showed recognition only in the elution area where intact kininogen was migrating, suggesting that the antibody had essentially no recognition for kinins. (Note the absence of peaks beyond 55 ml effluent volume in the upper part of the figure.) Therefore, the kininogen RIA is able to determine intact kininogen accurately even in the presence of kinins which are normally present in rat urines.

DISCUSSION

Using the standard trypsin-digestion assay, we previously reported that kininogen is present in rat urine [6]. However, the origin of LMW kininogen in rat urine is not known. Human renal kininogen is reportedly [12] localized in distal tubular cells and in cortical and medullary collecting ducts; nonetheless, no detectable mRNA for kininogen was found in the bovine kidney [13]. Thus, it is conceivable that the kininogen in urine is derived mainly from the glomerular filtration. Furthermore, rat plasma is also a rich source of LMW kininogen (400–500 µg/ml) and,

therefore, rat plasma was used as a starting material for our preparation.

A recent study [14] showed that LMW kininogens consist of two microheterogeneous counterparts, T-kininogen and K-kininogen. T- and K-kininogen mRNAs show a remarkable 90% homology not only in their nucleotide sequences but also in their amino acid sequences. Furthermore, the T- and K-kininogens appear to have similar molecular sizes [7, 15]. Thus, the separation of T- and K-kininogens should be very difficult. Even if such a resolution was possible, the topologies of antigenic epitopes of T- and K-kininogens were unlikely to be significantly different. Therefore, polyclonal antibody prepared against T- and K-kininogens or an admixture should have recognition for T- as well as K-kininogen. In view of these expectations, no special effort was even attempted to further resolve LMW kininogen into its counterparts.

The nature of kininogen in rat urine is not known. Rat plasma reportedly [16] contains 90% kininogen in LMW form (60% T- and 30% K-). If the source of urinary kininogen is indeed from the plasma [13], then it is possible that rat urine contains T- as well as K-kininogen in spite of the fact that T-kinin was not detectable in the rat urine [17]. To test this possibility we took seven different rat urines (fresh 24-hr collection), in an experiment not reported here, and determined pre-formed kinins as previously reported [6]. Thereafter, in one set of aliquots the endogenous kininogen was digested for 24 hr with a thousand-fold excess of pure rat urinary kallikrein. In another set of aliquots, the endogenous kininogen was digested with an excess of TPCK-trypsin. The resulting kinins from both the instances were analyzed by a kinin RIA. The data [(kallikrein-digested kinins − endogenous kinins)/(trypsin-digested kinins − endogenous kinins)] × 100) suggest that the normal rat urine contains approximately 45% K-kininogen and 55% T-kininogen (data to be published elsewhere). These urinary levels of K- and T-kininogen appear comparable to those reported in the rat plasma [16], suggesting the notion that the kininogen in rat urine is probably derived from the glomerular filtration.

We isolated rat plasma LMW kininogen to apparent homogeneity as indicative by the SDS-gel electrophoresis. This technique assumes [8] that the individual charge pattern of each protein is totally changed by the binding of SDS anions, rendering all molecules negatively charged. Subsequent resolution is therefore by the molecular size and not by charge. Thus, the LMW kininogen band in Fig. 1 may consist of K-kininogen, T-kininogen or a mixture of the two, assuming that the molecular weights of T- and K-kininogens are within 10% of each other (SDS-gel electrophoresis recognizes differences in excess of 10%). LMW kininogen is a glycoprotein consisting approximately of 20% weight due to carbohydrate moieties [11] and, therefore, the molecular weight of 67 kD which we found for our preparation is apparent, not absolute. Others [7, 11] have found a comparable molecular size of 72 kD for their rat plasma LMW kininogen preparations.

The rabbit anti-rat plasma LMW kininogen serum showed approximately 5.8% cross-reactivity with HMW kininogen. HMW and LMW kininogen have similar heavy chains of 50–60 kD followed by the kinin moieties. The LMW kininogen differs from the HMW in the fact that the light chain in LMW is rudimentary consisting only of 5–6 kD, whereas the light chain of HMW consists of 45–58 kD [15]. Low cross-reactivity, 5.8%, of our antibody suggests that, in spite of similarities in the heavy chains, the antigenic determinants on the two chains must be sufficiently different to invoke a somewhat selective response.

As shown in Fig. 2, the antibody did not recognize kinin or bovine LMW kininogen in any significant manner. Although there was a perfect correlation between the direct assay and the kinin equivalence assay (Fig. 3), the recognition of des-kinin-kininogen by the antibody cannot be ruled out because a perfect relationship can occur if the concentration of kinin-free-kininogen is exceedingly small (in proportion to the observed level of urinary kinins). Furthermore, there was a measurable amount of kininogen-like material even after extensive digestion with trypsin and kallikrein (see Fig. 4, 35–50 ml effluent volume in upper panel). This observation supports the contention that the kininogen antibody reported here recognizes des-kinin-kininogen at least partially. However, the degree of cross-reactivity was not apparent from the present data.

The ^{125}I-labeled LMW-kininogen-antibody conjugate was displaced by four representative rat urines in parallel to the standard curve (see Fig. 2). Although the parallelism suggests the presence of a single antigen in the rat urine, it does not exclude the presence of another antigen having subtle or no differences in the antigenic epitopes such as those which might be present in the T- and K-kininogens.

The newly developed direct RIA of LMW kininogen was compared with the kinin equivalence assay in twenty random rat urines. The kininogen concentration determined by the direct RIA was not significantly different from that determined by the kinin-equivalence assay. The direct RIA of kininogen also correlated extremely well with the kinin-equivalence assay. In a recent report, Jacob *et al.* [18] described a direct radioimmunoassay for the measurement of T-kininogen in rat plasma. Although the assay is very similar to the one described here, its major emphasis is in the determination of kininogen in plasma, not in the urine as reported here.

Analysis of the fractions from molecular sieve chromatography of trypsin-digested and kallikrein-digested LMW kininogen revealed (upper half of Fig. 4) that the kininogen antibody did not recognize kinins that were emerging in effluent volume 85–100 ml as recognized by the kinin antibody in the lower half of the figure. Almost five times more kinins were formed by trypsin than by kallikrein (compare effluent volume 85–100 ml peak of trypsin digest with kallikrein digest in the lower half of the figure). These data are evidence that the LMW kininogen, isolated here, is a mixture of T- and K-kininogens.

In summary, we have isolated LMW kininogen from rat plasma, the apparent molecular weight of which is 67 kD. Although it migrated as a single

protein in SDS-gel electrophoresis, in all probability it is microheterogeneous because only 20% kinins were released from the substrate by kallikrein compared with trypsin (T-kinin has 100% cross-reactivity in our kinin RIA). This conclusion is further supported from the previous reports showing 90% homologies in the amino acid sequences of T- and K-kininogens [14] and a very close similarity in the molecular weights of T- and K-kininogens [15]. The antibody produced against the LMW kininogen had no significant recognition for kinins, bovine LMW kininogen, or rat HMW kininogen. On the other hand, the antibody appeared to have partial recognition for des-kinin-kininogen and full recognition for precursor proteins (T- and K-kininogens) of kinins present in the rat urine. Therefore, the assay appears useful in situations wherein the urinary excretion of T- and K-kininogens (total LMW kininogen) is of interest.

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