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IDENTIFICATION OF T-KININOGEN IN RAT URINE

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Abstract—Studies were carried out in order to characterize the kininogen in rat urine. Rat urine contained a component which was cross-reactive with antibody to rat plasma T-kininogen. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of rat urine revealed a single antigenic band corresponding to the molecular weight of plasma T-kininogen. Induction of acute inflammation in rats by an injection of lipopolysaccharide caused an increase in the urinary excretion of immunoreactive T-kininogen in parallel with an elevation of plasma T-kininogen. Kininogen partially purified from rat urine by affinity chromatography using *S*-carboxymethylated papain-agarose liberated only T-kinin upon trypsinization, but not upon treatment with rat glandular kallikreins. From these results, we conclude that T-kininogen is the major kininogen present in rat urine.

The renal kallikrein–kinin system has been suggested to be involved in the regulation of salt and water excretion and in the pathophysiology of hypertension [1, 2], because kinins are potent vasoactive peptides with both natriuretic and diuretic properties [3, 4]. Considerable amounts of kinins, including bradykinin and lys-bradykinin, have been found in human urine [5, 6]. Urinary kinins are assumed to be generated intrarenally, since the proximal tubule is rich in kininases, which prevent filtered kinins from reaching the distal nephron [2]. While the occurrence of renal kallikrein is well documented [7], the origin of its substrate, kininogen, needed for the intrarenal generation of kinins has not been studied extensively. In humans, Proud *et al.* [8] found a kininogen corresponding to plasma low-molecular weight (LMW) kininogen in urine and cells of the distal nephron, and they concluded that the kidney is the source of urinary kininogen. However, no such information is yet available for the rat, despite the frequent use of this animal in studies of the renal kallikrein–kinin system.

Recently, Okamoto and Greenbaum [9, 10] found a new type of kininogen, T-kininogen, in rat plasma which is distinct from high-molecular-weight (HMW) and LMW kininogens. The kininogen was shown to release a vasoactive peptide, T-kinin (Ile-Ser-bradykinin), upon trypsinization, but to be resistant to the kinin-generating activity of glandular kallikrein [11]. Subsequently, Barlas *et al.* [12] demonstrated that elevated levels of plasma kininogen in rats with inflammatory lesions are due solely to the elevation of T-kininogen.

We now report on the characterization of rat urinary kininogen using anti-T-kininogen antibody. The data indicate that a major proportion of rat urinary kininogen consists of intact T-kininogen.

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MATERIALS AND METHODS

Materials. The following chemicals were obtained from commercial sources: bradykinin, [1-Tyr]-kallidin, T-kinin and pepstatin (Peptide Institute Inc., Osaka, Japan); hexadimethrine bromide (Aldrich, Milwaukee, WI); $Na^{125}I$ (New England Nuclear, Boston, MA); lipopolysaccharide (LPS) (*Staphylococcus typhosa* 0901; Difco, Detroit, MI); molecular weight standards for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, papain (type III) and trypsin (type XIII, TPCK-treated) (Sigma, St. Louis, MO); CM-23 (Whatman, Clifton, NJ); Affi-Gel 10 and Immuno-blotting Assay Kit (Bio-Rad, Richmond, CA); and Zysorbin (fixed and killed *Staphylococcus aureus*, Zymed Lab., San Francisco, CA). Rat plasma T-kininogen was purified as described previously [11]. Rat urinary kallikrein was purified by the method described by Geiger and Fritz [13]. Purified rat submandibular gland kallikrein was supplied by Dr. H. Kato (National Institute of Cardiovascular Disease, Osaka, Japan). Purified rat HMW-kininogen was supplied by Dr. S. Oh-ishi (Kitasato University, Tokyo, Japan).

Animals. Male Sprague–Dawley rats, weighing 180–220 g, were used.

Collection of urine. Twenty-four-hour samples of urine were each collected into a bottle containing 2.5 ml of inhibitor solution at 22° from rats housed separately in metabolic cages. The inhibitor solution consisted of benzamidine-HCl (1.8 mg/ml), hexadimethrine bromide (0.5 mg/ml), pepstatin (10 μg/ml), disodium EDTA (15 mg/ml) and 0.2% sodium azide in distilled water. These inhibitors were used for minimizing specific or non-specific degradation of kininogen by known or unknown proteinases in urine. Each urine sample was centrifuged at 9000 *g* for 20 min and stored at −25° until assay.

Induction of acute inflammation. Acute inflammation was induced in rats by a single intraperitoneal injection of LPS (1 mg/kg). Twenty-four-hour sam-

ples of urine were collected before and after LPS injection. At different times after LPS injection, the tail-tip of each rat was cut with a razor and approximately 50 μl of blood was collected into an EDTA-treated capillary tube. The tubes was centrifuged at 1000 *g* for 20 min, and 20 μl of plasma was obtained.

Assay of kinin. Kinins were determined by radioimmunoassay (RIA) using ^{125}I-labeled [1-Tyr]-kallidin and rabbit anti-bradykinin serum as described previously [12]. Assuming the displacement of bound ^{125}I-labeled [1-Tyr]-kallidin by bradykinin to be 100%, the antiserum was found to recognize T-kinin (98%) and lys-bradykinin (88%) almost equally well.

Identification of kinin. Kinins were identified by CM-23 chromatography [9] with a slight modification as follows. Samples containing kinins were applied to a column (0.9 × 25 cm) of CM-23, previously equilibrated with 0.01 M ammonium acetate (pH 5.0). After washing the column with 40 ml of the equilibrium buffer, kinins were eluted with a linear gradient between 100 ml of the equilibrium buffer and 100 ml of 0.2 M ammonium acetate (pH 7.5). Fractions (2 ml) were collected at a flow rate of 30 ml/hr, and each fraction was subjected to kinin RIA.

Assay of kininogen. Kininogen was determined by assaying the amounts of kinin liberated by an excess amount of trypsin, as described previously [9, 10]. Liberated kinin was assayed by RIA.

Radioimmunoassay of T-kininogen. T-kininogen was assayed by RIA using ^{125}I-labeled T-kininogen and rabbit anti-T-kininogen serum. ^{125}I-labeled T-kininogen was prepared by the chloramine-T method [14] and separated from free ^{125}I by Sephadex G-50 chromatography. Antiserum for rat T-kininogen was prepared by injecting 0.5 mg of purified rat plasma T-kininogen with Freund's complete adjuvant into rabbits every 2 weeks for 2 months. The RIA was performed in 0.1 M Tris–HCl, pH 7.4, containing 0.2% gelatin and 0.002% sodium azide (RIA buffer). The incubation mixture contained 50 μl of ^{125}I-labeled T-kininogen (8000 cpm), 50 μl of either the T-kininogen standard (0.5 to 50 ng) or an unknown sample. Following the incubation at 4° for 18 hr, the sample was mixed with 20 μl of Zysorbin (1 mg/ml), kept for 30 min at room temperature and then centrifuged for 15 min at 2000 *g*. The supernatant fraction was removed, and the precipitate was washed once with 1 ml of RIA buffer. The pellet containing tracer bound to antibody was counted in a gamma counter (Aloka). A standard curve was obtained by plotting the percentage of initial binding against the T-kininogen standards. Duplicate estimations were made for each point on the curve, and the unknown samples were calculated by computer using logit transformation as described by Rodbard *et al.* [15]. The intra-assay coefficient of variation was estimated with five replicate urine samples and was 5.1%. The between-assay error was evaluated using the same urine samples as those employed in the five separate assays, and the coefficient of variation was 6.7%.

Immunoblot analysis of immunoreactive T-kininogen. Rat plasma and urine samples were subjected to SDS–polyacrylamide gel electrophoresis followed by immunoblot analysis. Plasma was collected from five rats before and 48 hr after LPS treatment, and each 50 μl aliquot of plasma from the rats was pooled. Twenty-four-hour samples of urine were also collected from the rats before and 24–48 hr after LPS treatment, and each 1 ml aliquot of urine was pooled. Pooled urine was then concentrated twenty times using Molecut II GC (Millipore, M_r < 10,000 cut-off). These samples of plasma and urine, which corresponded to 0.2 μl of plasma and 50 μl of original urine, were subjected to SDS–polyacrylamide gel electrophoresis in 7.5% gel according to the method of Laemmli [16]. Western blotting was carried out through electrophoretic transfer to a nitrocellulose filter [17]. The filter was first soaked in a blocking solution containing 3% gelatin, 20 mM Tris–HCl, pH 7.5, and 0.5 M NaCl for 1 hr. Then the blot was soaked in 200-fold-diluted anti-T-kininogen serum for 2 hr at room temperature, washed with the above buffer containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG for 1 hr at room temperature. The blot was then washed with the above buffer containing 0.05% Tween 20 and processed for color development according to the instruction manual issued by Bio-Rad Laboratories (Richmond, CA). The limit of detectability of the T-kininogen standard using these procedures was 10 ng.

Preparation of S-carboxymethylated papain-agarose. *S*-carboxymethylated papain-agarose was prepared according to the method described by Barrett [18] with slight modification. Papain (100 mg) was carboxymethylated with iodoacetate and dialyzed against 0.1 M 3-[*N*-morpholino]propanesulfonic acid–NaOH buffer, pH 7.5 (MOPS buffer). The solution (15 ml) was then mixed with 5 ml of Affi-Gel 10 suspended in MOPS buffer at 4° for 4 hr. The gel was further mixed with 0.1 M glycine for 1 hr and washed thoroughly with 0.1 M glycine–HCl (pH 2.5), 50 mM trisodium phosphate (pH 12.0), and 0.1 M sodium phosphate (pH 7.4). Approximately 80 mg of papain was coupled with 5 ml of gel.

Partial purification of urinary kininogen. Rat urine (1 liter) was collected from ten normal rats as described above and concentrated to 250 ml by ultrafiltration (UM-10, Amicon) at 4°. The precipitates were removed by centrifugation at 9000 *g* for 20 min, and the supernatant fraction was applied to a column (1.5 × 6 cm) of *S*-carboxymethylated papain-agarose, previously equilibrated with 20 mM sodium phosphate, pH 7.4, in 0.15 M NaCl, at a flow rate of 30 ml/hr. The column was then washed with 150 ml of 20 mM sodium phosphate, pH 7.4, in 0.5 M NaCl. Adsorbed protein was eluted with 2 M sodium thiocyanate in 0.1 M Tris–HCl, pH 8.0. Kininogen, which was determined by assaying the amount of kinin liberated by trypsin, was found in the eluate, and the fractions were pooled and dialyzed against distilled water at 4°. The dialysate was lyophilized and then dissolved in 3 ml of 20 mM Tris–HCl, pH 8.0.

Assay of protein. Protein was determined by the method of Bradford [19] using bovine gamma-globulin as the standard.

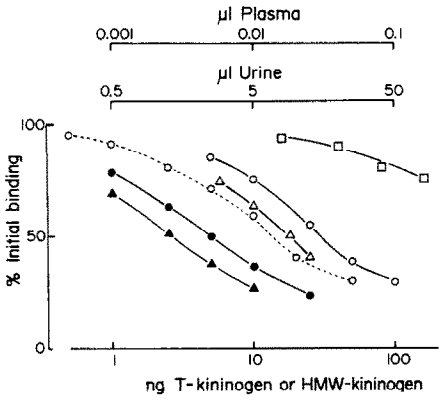

Fig. 1. Displacements of ^{125}I-labeled T-kininogen bound to antibody with rat plasma and urine. Several amounts of rat plasma and urine shown in the figure were subjected to T-kininogen radioimmunoassay. Key: (O---O) purified plasma T-kininogen; (□—□) purified rat HMW-kininogen; (O—O) normal rat plasma; (●—●) rat plasma collected 48 hr after LPS treatment; (△—△) normal rat urine; and (▲—▲) rat urine collected 24–48 hr after LPS treatment. Each point represents the mean of duplicate assays.

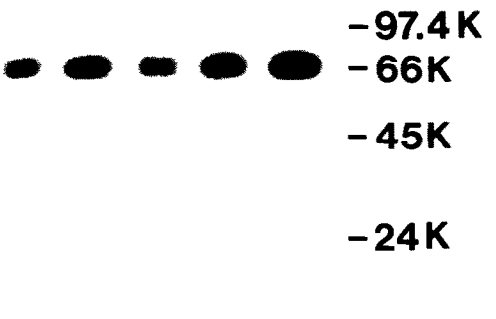

Fig. 2. Immunoblot analysis of immunoreactive T-kininogen in rat plasma and urine. Rat plasma and urine were subjected to SDS–polyacrylamide gel electrophoresis in 7.5% gel, followed by Western blotting. Antigen to T-kininogen antibody was detected immunochemically. Lane 1, normal rat plasma (0.2 μl); Lane 2, rat plasma collected 48 hr after LPS treatment (0.2 μl); Lane 3, normal rat urine (50 μl); Lane 4, rat urine collected 24–48 hr after LPS treatment (50 μl); Lane 5, purified rat plasma T-kininogen (0.2 μg). The marker proteins were phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and trypsinogen (24 kDa) respectively.

RESULTS

RIA for T-kininogen. Using chloramine T, approximately 20 μCi/μg of radiolabeled iodine was incorporated into T-kininogen. A high-titer antiserum for T-kininogen was employed in the direct RIA for T-kininogen. At a dilution of 1 : 9000, the antiserum bound approximately 40% of the tracer antigen. Figure 1 shows a typical standard curve, together with the displacements of tracer antigen by plasma and urine obtained from one rat before and after the induction of acute inflammation with LPS. The displacement curves of plasma and urine from a normal rat were shifted to the left by the induction of acute inflammation. The fact that these curves were parallel to that of standard T-kininogen suggests that the antibody recognized T-kininogen in plasma and urine. The minor cross-reactivity of rat HMW kininogen was non-parallel.

Immunoblot analysis of immunoreactive T-kininogen in rat urine. To characterize the immunoreactive T-kininogen in rat urine, pooled urine collected from five rats before and after LPS treatment was subjected to SDS–polyacrylamide gel electrophoresis, and the immunoreactive T-kininogen was immunochemically localized following Western blotting. Pooled plasma from normal and LPS-treated rats was also subjected to analysis. As shown in Fig. 2, urine collected from either normal or LPS-treated rats gave a virtually single immunoreactive band, which corresponded to the immunoreactive band of either purified T-kininogen (M_r = 69,000) or plasma.

Plasma level and urinary excretion of immunoreactive T-kininogen in rats following the injection of LPS. Following the induction of acute inflammation in rats by LPS injection, the levels of immunoreactive T-kininogen in plasma and urine were determined by T-kininogen RIA (Fig. 3). The concentration of immunoreactive T-kininogen in plasma was increased about 8-fold at 48 hr after the treatment. In parallel with the increases in plasma levels, the amounts of immunoreactive T-kininogen excreted in

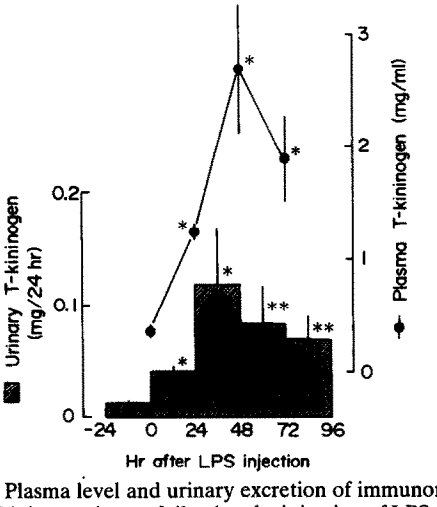

Fig. 3. Plasma level and urinary excretion of immunoreactive T-kininogen in rats following the injection of LPS. LPS (1 mg/kg) was injected i.p. into five rats at 0 hr. Plasma and 24 hr urine were collected before and after LPS treatment. The plasma levels of immunoreactive T-kininogen and the amounts of immunoreactive T-kininogen excreted in 24 hr urine were determined by T-kininogen radioimmunoassay. Vertical bars represent standard errors. A single or double asterisk indicates significantly different from the levels before LPS treatment (* $P < 0.001$, and ** $P < 0.05$).

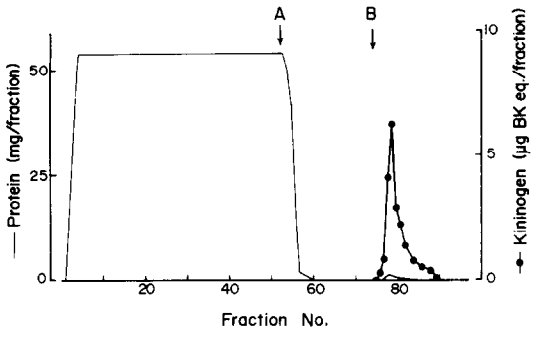

Fig. 4. Affinity chromatography of rat urinary kininogen on *S*-carboxymethylated papain-agarose. Normal rat urine (1 liter) was concentrated to 250 ml and applied to a column (1.5 × 6 cm) of *S*-carboxymethylated papain-agarose, previously equilibrated with 20 mM sodium phosphate, pH 7.4, in 0.15 M NaCl. The column was washed with 20 mM sodium phosphate, pH 7.4, in 0.5 m NaCl (A), followed by elution with 2 M sodium thiocyanate in 0.1 M Tris–HCl, pH 8.0 (B). The flow rate was 30 ml/hr and 5 ml fractions were collected. Kininogen in each fraction was determined by assaying the amounts of kinin liberated by trypsin.

24 hr urine were also increased about 10-fold at 24–48 hr after LPS treatment.

Partial purification of kininogen from rat urine. To characterize urinary kininogen further, kininogen was partially purified from 1 liter of normal rat urine. Concentrated urine was applied to a column of *S*-carboxylated papain-agarose, which has an affinity for cysteine proteinase inhibitors [18, 20, 21], including kininogen [22, 23]. As shown in Fig. 4, kininogen, which was detected by assaying the amounts of kinin liberated upon trypsin treatment, was adsorbed onto the column and eluted with 2 M sodium thiocyanate. Table 1 summarizes the purification step. The amount of kininogen recovered by affinity chromatography was 11.0 μg bradykinin equivalents, and the specific activity was increased 144-fold from that in the original urine, with a recovery of 49%.

Kinin liberation from partially purified urinary kininogen by trypsin and glandular kallikrein. To ascertain whether the partially purified kininogen from pooled urine contained kininogens other than T-kininogen, the susceptibility of the preparation to rat glandular kallikrein was examined by determining the amount of kinin liberated (Table 2). An excess amount (1 mg/ml) of trypsin, which is known to bring about complete liberation of kinin from rat plasma [9, 10], was used to determine the total amount of kinin available from the kininogen preparation. As shown in Table 2, only a small amount of kinin, corresponding to less than 5% of the total amount, was detected in the reaction mixture of the partially purified preparation of urinary kininogen and either rat urinary kallikrein (10 μg/ml) or rat submandibular gland kallikrein (10 μg/ml). Small amounts of kinin were also detected in a reaction mixture containing a purified preparation of rat plasma T-kininogen and kallikreins with a substrate to enzyme ratio of 30 to 1.

Identification of kinin liberated by trypsin from partially purified urinary kininogen. Kinin liberated by trypsin from partially purified urinary kininogen was identified by CM-23 chromatography (Fig. 5). Standard kinins were eluted in the order of T-kinin followed by bradykinin, as shown in Fig. 5A. Kinin

Table 1. Purification of kininogen from rat urine

	Protein (mg)	Kininogen (μg BK equiv.)	Kininogen (μg BK equiv./mg)	Yield (%)
Urine (1 liter)	5793	22.5	0.0039	100
Affinity chromatography on *S*-carboxymethylated papain-agarose (55 ml)	19.5	11.0	0.563	49

Table 2. Kinin liberation from partially purified rat urinary kininogen and purified rat plasma T-kininogen by trypsin and kallikreins

Kininogenase	Kinin liberation (μg BK equiv./mg protein)	
	Urinary kininogen	Plasma T-kininogen
Trypsin (1 mg/ml)	0.56	15.28
(10 μg/ml)	0.48	12.11
Rat urinary kallikrein (10 μg/ml)	0.02	0.57
Rat submandibular gland kallikrein (10 μg/ml)	0.03	0.71

Protein (30 μg) of partially purified rat urinary kininogen or purified rat plasma T-kininogen was incubated in 100 μl of 0.1 M Tris–HCl, pH 8.0, with kininogenases at the concentrations shown in parentheses at 37° for 1 hr. Kinin liberation is expressed as the amount of kinin (μg bradykinin equivalents) liberated from 1 mg protein of kininogen preparation. Values represent means of three experiments.

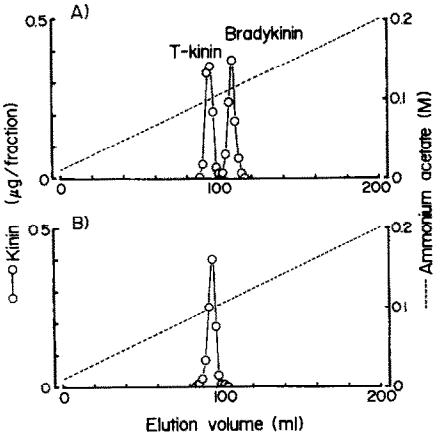

Fig. 5. CM-23 chromatography of kinin liberated by trypsin from partially purified rat urinary kininogen. Partially purified kininogen (2.4 mg) from rat urine was heated in boiling water for 5 min and then incubated with trypsin (200 μg) in 200 μl of 20 mM Tris–HCl, pH 8.0, at 37° for 1 hr. The sample was diluted in 5 ml of 0.01 M ammonium acetate, pH 5.0, and then applied to a column (0.9 × 25 cm) of CM-23. Kinin was eluted using the procedure described in Materials and Methods. (A) Elution profiles of standard T-kinin and bradykinin (each 1 μg). (B) Elution profile of kinin (1.34 μg bradykinin equivalent) liberated from urinary kininogen.

liberated from urinary kininogen was found only in the fractions corresponding to T-kinin (Fig. 5B). No immunoreactive kinin was detected in the fractions corresponding to bradykinin.

DISCUSSION

In the present study, we demonstrated for the first time the presence of T-kininogen in rat urine on the basis of the following evidence. First, rat urine contained a component that was cross-reactive with antibody for T-kininogen. The molecular weight of the immunoreactive T-kininogen in urine could not be distinguished from that of plasma T-kininogen, as indicated by immunoblot analysis. Second, the urinary excretion of the immunoreactive T-kininogen was increased in rats following the induction of acute inflammation, which caused elevation of the plasma T-kininogen level. Third, a kininogen preparation obtained from rat urine was not susceptible to rat glandular kallikrein, but released T-kinin upon trypsinization.

Weinberg *et al.* [24] have already demonstrated the presence of kininogen in rat urine by assaying kinin in trypsin-treated urine. However, they were unable to explain the relatively high concentration of kininogen in rat urine despite the presence of active urinary kallikrein. This question now seems to be answered in the light of our present finding that rat urine contains T-kininogen, which yields T-kinin biologically and immunologically indistinguishable from bradykinin upon treatment with trypsin and which is resistant to urinary kallikrein.

Three types of kininogens, i.e. HMW kininogen, LMW kininogen and T-kininogen, have been found in rat plasma; T-kininogens accounts for more than 70% of the kininogen in this plasma that is able to release kinin upon trypsinization [10, 12]. The induction of acute inflammation in rats is known to produce an increase in the plasma T-kininogen level, but not in the levels of other kininogens [12]. A similar result was obtained in the present study using a direct immunoassay method for T-kininogen (Fig. 3). In parallel with this elevation in the plasma T-kininogen level, the urinary excretion of T-kininogen was also found to increase (Fig. 3), suggesting that the urinary T-kininogen originates from the circulation.

To characterize urinary kininogen further, 24 hr rat urine was employed for the purification of kininogen by affinity chromatography using *S*-carboxymethylated papain-agarose (Fig. 4), which has been employed previously for the purification of cysteine proteinase inhibitors [18, 20, 21] and recently for purification of human kininogen [22, 23]. Elution of kininogen from the affinity column was carried out using 2 M sodium thiocyanate, instead of employing an acidic [20] or alkaline pH [18, 21–23], in order to minimize any denaturation. By this procedure, the kininogen was effectively extracted from urine with a good recovery (Table 1). The kininogen preparation obtained did not liberate a definite amount of kinin upon treatment with a sufficient amount of rat glandular kallikrein (Table 2). Although only a small amount of kinin, corresponding to less than 5% of the total amount made available by trypsin, was detected by incubation with kallikrein, a purified preparation of T-kininogen also liberated small amounts of kinin upon treatment with glandular kallikrein (Table 2). In addition to this evidence, the only kinin liberated by trypsin from this preparation was identified to be T-kinin (Fig. 5). These results support the contention that T-kininogen is a major kininogen in rat urine, as is the case for plasma [25].

Recently, Mindroiu *et al.* [26] demonstrated the presence of bradykinin and lys-bradykinin, but not T-kinin, in urine collected from ureteral catheters in rats. As kinins in urine are liberated from kininogen by renal kallikrein in the distal nephron [2] and at least lys-bradykinin is not derived from T-kininogen, the evidence seems to indicate the possibility that kininogen other than T-kininogen is also excreted into rat urine, as has been demonstrated for the excretion of LMW kininogen in human urine [8]. However, in the present study, no evidence indicating the presence of HMW kininogen or LMW kininogen in rat urine was obtained. The reason is obscure, but we speculate that HMW and LMW kininogens, even if they are excreted into urine, may be degraded by the active kallikrein during their period of retention in the bladder [2]. Therefore, in order to confirm whether kininogens other than T-kininogen are excreted into rat urine, further studies will be required.

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