Existence of Prokallikrein in the Kidney
Its Biochemical Properties Compared to Three Active Glandular Kallikreins from the Kidney, Serum, and Urine of the Rat

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SUMMARY Prokallikrein in the kidney was partially purified with immunoaffinity and DEAE Sephadex A-50 column chromatographies, and its biochemical properties were studied in comparison to three active glandular kallikreins purified from kidney, serum, and urine of the rat. The properties of the enzyme obtained by trypsin activation of prokallikrein were identical with those of active glandular kallikreins from the kidney, serum, and urine of the rat. Apparent molecular weights of prokallikrein, trypsin-activated kallikrein, active renal kallikrein, and glandular kallikrein in rat serum were 38,000 and of active urinary kallikrein, 37,000. Prokallikrein fraction was activated only by trypsin, but not by acidification, pepsin, and rat urinary esterase A treatments. Renal kallikrein, purified in the presence of soybean trypsin inhibitor (SBTI), contained 85% prokallikrein, but the enzymic fraction, purified in the absence of SBTI, contained 23% prokallikrein. Prokallikrein contents of urinary kallikrein and glandular kallikrein in rat serum were 16% and 20% respectively. These results suggest that prokallikrein is produced in the kidney and activated easily by a trypsin-like enzyme. Since rat serum contains active glandular kallikrein, kallikrein in the kidney may be secreted not only into the urine, but also into the blood. (Hypertension 5: 205-210, 1983)

KEY WORDS  • prokallikrein • glandular kallikrein • immunoaffinity column chromatography • soybean trypsin inhibitor

RENAL kallikrein is an enzyme that liberates a kinin from kininogen.1 It has been suggested to be localized in the distal and convoluted tubular cells2 and to play an important role to control sodium and water balance in situ3 and also, to regulate renal blood flow.4

Corthorn et al.5 and Oza et al.6 reported that human urine contained inactive kallikrein that was activated by trypsin. There is no report whether inactive kallikrein is a proenzyme or an enzyme-inhibitor complex. We also do not know from where inactive kallikrein originates. Nishimura et al.7 reported that membrane-bound kallikrein in rat kidney was activated by detergent and by trypsin. Since membrane-bound kallikrein was quickly activated by solubilization from the membrane fraction, it was difficult to purify inactive kalli-

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Materials and Methods

Materials

Bradykinin (BK) was purchased from Protein Research Foundation (Osaka, Japan). H-D-Val-Leu-Arg-pNA (S-2266) was obtained from AB Kabi Diagnostica (Stockholm, Sweden). Aprotinin, SBTI, hog pancreatic kallikrein, trypsin, and pepsin were from Sigma Chemical Company (St. Louis, Missouri). CNBr-activated Sepharose 4B, DEAE Sephadex A-50, and Sephadex G-100 were from Pharmacia (Uppsala, Sweden), the DE-52 from Whatman (Maidstone, Kent, England), and the molecular weight marker kit from Boehringer Mannheim GmbH (Mannheim, West Germany).
Methods

Collection of Samples

All materials of kidney, serum, and urine were obtained from the same groups of 200 Wistar female rats. Twelve-hour rat urine (3.6 liters) was collected in the presence of toluene from 200 Wistar female rats, which were housed in the metabolic cages without food, but with water ad libitum. The pooled urine was dialyzed overnight against 5 mM sodium phosphate buffer, pH 7.4, and freeze-dried. After collection the 200 Wistar female rats (200-250 g) were killed by bleeding through the carotid artery. Kidneys and serum were collected and kept at -20°C.

Purification of Rat Urinary Kallikrein

Urinary kallikrein was purified with DE-52 cellulose, hydroxyapatite, DEAE Sephadex A-50 and Sephadex G-100 columns, according to the modified method of Nustad and Pierce. Antibodies were raised in three rabbits. A total of 330 µg protein from the purified urinary kallikrein was emulsified with Complete Freund's adjuvant and injected subcutaneously every 4 weeks. On Day 14 after the third injection, antiserum obtained by bleeding through the canulated carotid artery was heated for 30 minutes at 56°C. From the heated antiserum, further purified by precipitation with 40% ammonium sulfate showed one precipitation band with concentrated crude urine, but no discernible line with rat urinary kallikrein (protein, 1 mg/ml).

Preparation of Kallikrein Antibody

Antibodies were raised in three rabbits. A total of 330 µg protein from the purified urinary kallikrein was emulsified with Complete Freund's adjuvant and injected subcutaneously every 4 weeks. On Day 14 after the third injection, antiserum obtained by bleeding through the canulated carotid artery was heated for 30 minutes at 56°C. From the heated antiserum, further purified by precipitation with 40% ammonium sulfate and with a column of DE-52 cellulose equilibrated with 20 mM sodium phosphate buffer (pH 7.2), purified IgG fraction was obtained. The purified enzyme had a specific activity of 36.4 units/mg protein for S-2266 and 545.1 µg BK Eq/min/mg protein for kininogenase assay.

Preparation of Immunoaffinity Gel

CNBr-activated Sepharose 4B (30 g dry weight) was swollen and washed with 1 M HCl solution. IgG fraction (0.5 g in 10 ml), dialyzed for 3 hours against 0.1 M sodium phosphate buffer, pH 8.3, containing 0.5 M NaCl, was mixed with gel suspension and the suspension was stirred gently with a magnetic stirrer at 4°C overnight. Measurement of the supernatant at 280 nm showed that 99% of the protein was bound to the Sepharose 4B gel. The gel was incubated with 1 M monoethanolamine pH 8.0 to block any remaining active groups at room temperature for 3 hours and washed well with distilled water. Finally, the gel was washed with 10 mM sodium phosphate buffer, pH 7.4, containing 0.9% NaCl and 1% NaN3, and kept in a cold room at 4°C. When the purified urinary kallikrein was applied to an immunoaffinity column (1.0 x 2.0 cm), the recovery of the enzyme was 86% to 90% by the elution of 6 M urea. The binding capacity of the enzyme per ml of gel was 2.1 units for S-2266.

Assay of Kallikrein

Assay of kallikrein was performed by two methods. S-2266 Assay: Kallikrein assay was performed by the method of Amunsen et al., using S-2266 as substrate. The reaction mixture, containing 0.6 ml of 50 mM Tris-HCl buffer, pH 8.5, 0.1 ml of the enzyme, and 0.1 ml of 1 mM S-2266, was incubated at 37°C for 5 minutes. The reaction was stopped by the addition of 50% acetic acid (0.1 ml), and the absorbance was measured at the wavelength of 405 nm. One unit of the enzymatic activity was defined as that amount of the enzyme that hydrolyzed 1 µmole of S-2266 per minute at 37°C.

Kininogenase Assay: Kininogenase activity was determined using dog plasma as substrate, which was heated at 60°C for 30 minutes. The reaction mixture, containing 0.35 ml of 10 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl (PBS), 0.1 ml of dog plasma, and 0.05 ml of the enzyme, was incubated for 10 minutes at 37°C, and the reaction was stopped by boiling for 5 minutes. The product was bioassayed on an isolated rat uterus, using BK as standard. Kininogen content in dog plasma was determined to be 7.4 µg BK equivalents (Eq) per ml, using hog pancreatic kallikrein (protein, 1 mg/ml).

Protein concentration was measured by the method of Lowry et al., using bovine serum albumin as a standard.

Measurement of Prokallikrein

Prokallikrein was measured by the following method. The sample (0.3 ml) was incubated with 0.1 ml of trypsin (0.1 mg/ml in 10 mM Tris-HCl buffer, pH 7.4) for 10 minutes at 37°C, and the reaction was stopped by the addition of SBTI (0.1 ml of 5 mg/ml H2O). Then, the sample was assayed with S-2266 (total kallikrein). Maximum activation of prokallikrein was obtained at the concentration of trypsin from 10 to 500 µg/ml. Active kallikrein was also assayed with S-2266 in the presence of SBTI (1 mg/ml). Prokallikrein was calculated by total kallikrein minus active kallikrein. Percent prokallikrein was measured by calculating the total kallikrein minus active kallikrein total kallikrein x 100.

Results

Purification of Prokallikrein and Active Kallikrein from Rat Kidney

Rat kidney (300 g) was minced and washed with 10 mM sodium phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS) to exclude blood contamination. The tissue was homogenized with a mixer for 5 minutes in PBS (800 ml), containing SBTI (10 g) and stirred for 1 hour at 4°C with a magnetic stirrer after the addition of 0.5% Triton X-100. The homogenate was centrifuged at 10,000 g for 15 minutes and then the supernatant was ultracentrifuged at 100,000 g for 60 minutes.
Immunoaffinity Column Chromatography

The final supernatant (960 ml) was applied to the immunoaffinity column (2.6 x 20 cm), which was equilibrated with PBS and recirculated for 48 hours at 4°C with a pump speed of 40 ml/hr. The column was washed with PBS (2000 ml) and then with 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5% Triton X-100 and 1 M NaCl (1000 ml). The column was further washed with PBS (500 ml). The enzyme was finally eluted with 6 M urea in PBS (150 ml), shown in figure 1. Since 40% of the enzymic activity was destroyed when incubated with 6 M urea at room temperature for 3 hours, we added 3 ml of PBS to each fraction tube prior to collecting the eluate (each fraction volume = 3 ml). The enzymic fractions were separated into prokallikrein-enriched (fraction number = 18-28) and active kallikrein-enriched fractions (fraction number = 29-40). Prokallikrein was activated with trypsin (25 µg/ml) and measured. Also, there was no esterase activity in these fractions, which was inhibited by SBTI (5 mg/ml).

DEAE Sephadex A-50 Column Chromatography of Prokallikrein-Enriched Fractions

Prokallikrein-enriched fractions were pooled and concentrated to 20 ml with an Amicon PM 10 filter. The sample was dialyzed overnight against 10 mM sodium phosphate buffer, pH 6.0, and applied to the column (1.0 x 5.0 cm) of DEAE Sephadex A-50, which was equilibrated with the same buffer. The enzyme absorbed on the column was eluted with a linear gradient of 0-0.7 M NaCl (initial volume in gradient mixing chamber was 150 ml), shown in figure 2. Pro-
Fraction Number

Figure 3. DEAE Sephadex A-50 column chromatography of active kallikrein-enriched fractions. The arrow indicates the starting point of NaCl gradient (0-0.7 M). Applied sample: Active kallikrein-enriched fractions obtained by immunoaffinity column chromatography. Absorbance at 280 nm; prokallikrein; active kallikrein; conductivity.

Kallikrein fractions were separated from active kallikrein fractions, pooled, and concentrated to 3.0 ml with an Amicon PM 10 filter, which contained a trace amount of active kallikrein (less than 5%). Total activity of prokallikrein (3.0 ml), which was measured after trypsin treatment, was 1.7 units for S-2266 and the specific activity of the enzyme was 5.03 units/mg protein for S-2266 and 64.5 μg BK Eq/min/mg protein for kininogenase assay.

DEAE Sephadex A-50 Column Chromatography of Active Kallikrein-Enriched Fractions

Active kallikrein-enriched fractions were pooled and concentrated to 20 ml with an Amicon PM 10 filter. The sample was dialyzed overnight against 10 mM sodium phosphate buffer, pH 6.0, and applied to the column (1.0 x 5.0 cm) of DEAE Sephadex A-50, which was equilibrated with the same buffer. The enzyme was eluted with the same method described above. A single peak of active kallikrein and a trace amount of prokallikrein were obtained, shown in Figure 3. Active kallikrein fractions were pooled and concentrated to 3.0 ml with an Amicon PM 10 filter. Total activity of the enzyme was 2.4 units for S-2266 and the specific activity was 4.08 units/mg protein for S-2266 and 52.3 μg BK Eq/min/mg protein for kininogenase assay.

Purification of Glandular Kallikrein from Rat Serum

A total of 300 ml of the rat serum was applied to the immunoaffinity column (2.6 x 20 cm), which was equilibrated with PBS, and recirculated for 24 hours at 4°C with a pump speed of 40 ml/hr. The column was washed with PBS (500 ml) and with 0.1 M sodium phosphate buffer, pH 7.4, containing 0.5% Triton X-100 and 1 M NaCl (500 ml). Rat plasma kallikrein was eluted with 6 M urea in PBS (150 ml) with the same method, described above. The enzymic fractions were pooled and concentrated to 20 ml with an Amicon PM 10 filter. Rat serum contained a large amount of active glandular kallikrein (80%) and a small amount of prokallikrein (20%). The total enzymic activity obtained from 300 ml of rat serum was 1.12 units for S-2266 and the specific activity was 0.19 units/mg protein for S-2266 and 2.3 μg BK Eq/min/mg protein for kininogenase assay.

Characterization of Prokallikrein and Active Kallikrein

The following four samples were used for the characterization of prokallikrein and active kallikrein: prokallikrein, which was purified with immunoaffinity and DEAE Sephadex A-50 columns from rat kidney; active renal kallikrein, which was purified with immunoaffinity and DEAE Sephadex A-50 columns from rat kidney; active glandular kallikrein in rat serum, which was purified with immunoaffinity column; and active urinary kallikrein, which was purified, according to the modified method of Nustad and Pierce.

Ratios (kininogenase assay/S-2266 assay) of trypsin-activated kallikrein, active renal kallikrein, active glandular kallikrein in rat serum, and urinary kallikrein were 12.8, 12.8, 12.0, and 14.9 respectively.

Effects of SBTI, Aprotinin and IgG Fraction (Kallikrein Antibody)

The enzymic activities (0.07-0.1 units/ml for S-2266) of trypsin-activated kallikrein, active renal kallikrein, active glandular kallikrein in rat serum, and urinary kallikrein were inhibited completely at the concentration of aprotinin (2 KIU/ml) and IgG fraction (12.1 mg/ml), but not at the concentration of SBTI (5.0 mg/ml), when the enzyme was assayed with S-2266.
**Molecular Weight Determination**

Molecular weight determination was carried out by gel filtration, calibrated with bovine serum albumin, hen egg albumin, horse radish peroxidase, and chymotrypsinogen A. A column of Sephadex G-100 was equilibrated with 10 mM sodium phosphate buffer, pH 7.4. Fractions (3.25 ml) were collected at a flow rate of 20 ml/hr. When each sample was applied to the column of Sephadex G-100, horse radish peroxidase was added and the molecular weight was calibrated exactly. Apparent molecular weights of prokallikrein, trypsin-activated kallikrein, active renal kallikrein, and glandular kallikrein in rat serum were 38,000 and that of active urinary kallikrein was 37,000, shown in figure 4.

**Km Values**

The Km values of trypsin-activated kallikrein and three active kallikreins for S-2266 were 0.1 mM.

**pH Optimum**

The pH optimums of the four kallikreins were 9.0 for S-2266.

**Activation of Kallikrein**

**Prokallikrein Contents of Renal Kallikrein, Glandular Kallikrein in Serum, and Urinary Kallikrein of the Rat**

Renal kallikreins were purified with immunoaffinity column in the presence of SBTI (25 mg/ml), and in the absence of SBTI from rat kidney (30 to 50 g). Fresh urines from five rats were dialyzed for 3 hours against PBS and used for this experiment (urinary kallikrein). Glandular kallikrein in rat serum was purified with immunoaffinity column. Each sample was activated with trypsin (25 μg/ml) and measured for S-2266. Renal kallikrein, purified in the presence of SBTI, contained 85% prokallikrein, but the enzymic fraction, purified in the absence of SBTI, contained 23% prokallikrein. Prokallikrein contents of urinary kallikrein and glandular kallikrein in rat serum were 16% and 20% respectively.

**Acidification**

Prokallikrein, which was purified with immunoaffinity and DEAE Sephadex A-50 columns from rat kidney, was dialyzed overnight against 10 mM glycine-HCl buffer at three different pHs (3.0, 3.3, and 4.0). The sample was neutralized with 1 M NaOH and measured for S-2266. The enzyme was not activated by acidification, but the enzyme after acidification was activated by trypsin (25 μg/ml).

**Pepsin**

When pepsin (2.5 μg, 25 μg, and 2.5 mg/ml) was incubated with prokallikrein in 10 mM glycine-HCl buffer, pH 3.3, at 37°C for 1 hour, there was no activation of prokallikrein.

**Rat Urinary Esterase A**

Rat urinary esterase A (1-10 units/ml for S-2266), which was purified with DE-52 cellulose column from rat urine, was incubated with prokallikrein for 20 minutes at 37°C in PBS and the reaction was stopped with SBTI (25 mg/ml). There was no activation of prokallikrein.

**Discussion**

We succeeded in partial purification of prokallikrein from rat kidney, using immunoaffinity column chromatography in the presence of SBTI. We compared its biochemical properties to those of active glandular kallikreins purified from kidney, serum, and urine of the rat.

Membrane-bound kallikrein was quickly activated by detergent, trypsin, and phospholipase A2.7 Renal kallikrein was solubilized by 0.5% Triton X-100 in the presence of SBTI (25 mg/ml) and purified with an immunoaffinity column. Of the total kallikrein in the kidney, 85% was obtained as prokallikrein. On the other hand, the prokallikrein fraction obtained in the absence of SBTI was only 23%. Since tissue was minced and washed well with PBS, contamination of serum and urine was negligible. These results mean that prokallikrein is produced in the kidney and converted to active kallikrein in the absence of SBTI. Corthorn et al.14 and Nolly et al.15 have reported the same data. Prokallikrein content in serum and urine was 16% to 20%. We have no data, however, as to whether serum prokallikrein is produced in the kidney or not.

The molecular weight of prokallikrein was estimated to be 38,000. This is very similar to that of pancreatic prokallikrein, reported by Matsas et al.16 Molecular weights of trypsin-activated kallikrein and active kallikreins in serum and kidney were 38,000. Change of molecular weight from prokallikrein to active kallikrein was suggested to be less than 1000. Matsas et al.16 reported that a Kazar-type trypsin inhibitor was bound to pancreatic prokallikrein and separated using immunoaffinity column at the final step. We have no
data as to whether a Kazar-type trypsin inhibitor exists in the kidney. We used an immunoaffinity column in the first step and succeeded in the purification of prokallikrein.

The following two results suggest that prokallikrein is not an enzyme-inhibitor complex. First, acid may be expected to dissociate a low molecular weight inhibitor like aprotinin, but the prokallikrein fraction was not activated by dialysis against glycine-HCl buffer, pH 3.0. Second, the molecular weight of prokallikrein was almost the same as that of active renal kallikrein.

On the other hand, Geiger and Mann 17 reported that kidney inhibitor in renal tubules had a molecular weight of 4700.

Enzymic properties of trypsin-activated kallikrein in the kidney were identical with those of active glandular kallikreins in kidney, serum and urine, except for molecular weight. The molecular weight of urinary kallikrein was 37,000. The difference in molecular weight of 1000 by gel filtration is usually negligible, but we used the same column and measured the distance of the enzyme from the marker enzyme (horse radish peroxidase) every time.

There was no activation of prokallikrein by acidification, pepsin, and rat urinary esterase A. Maximum activation of prokallikrein was obtained at the concentration of trypsin from 10 μg to 500 μg/ml.13 More than 500 μg of trypsin degraded kallikrein, but 25 μg of trypsin did not change enzymic properties such as ratio of enzymic activity (kininogenase assay/S-2266 assay), Km value, pH optimum, and molecular weight. Since SBTI inhibited activation of prokallikrein in the homogenate, trypsin-like enzyme may play an important role in activation of the enzyme.

Lawton et al. 18 separated three peaks of immunoreactive glandular kallikrein in rat plasma by gel filtration and purified only the third peak and showed it was an inactive form. They concluded glandular kallikrein circulated in rat plasma in an inactive form, but they did not describe anything about the first and second peaks. We could purify an active glandular kallikrein from 300 ml of rat serum by an immunoaffinity column, which had a larger molecular weight than that of urinary kallikrein. This is very similar to the report of Geiger et al., 19 who purified an enzymatically active glandular kallikrein from human plasma by an immunoaffinity column.

Our results suggest that prokallikrein is produced in the kidney, kept in the tubular membrane fraction, and is activated easily by a trypsin-like enzyme. Rat serum contained active glandular kallikrein. Rabito et al. 20 reported that most of glandular kallikrein in plasma originated from the salivary gland. On the other hand, Roblero et al. 21 reported that kidneys released kallikrein into the circulation. The origin and physiological function of active glandular kallikrein in plasma is still unknown.

References
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