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RAPID SEPARATION AND MEASUREMENT OF RAT URINARY KALLIKREIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A CONTINUOUS FLOW ENZYME DETECTOR

YOSHIHIKO FUNAE*
Laboratory of Chemistry, Osaka City University Medical School, Asahi-machi, Abeno-ku, Osaka 545 (Japan)
HIROKO AKIYAMA
Osaka College of Pharmacy, 2-10-65 Kawai, Matsubara, Osaka 580 (Japan)
SUSUMU IMAOKA
Laboratory of Chemistry, Osaka City University Medical School, Asahi-machi, Abeno-ku, Osaka 545 (Japan)
and
MASANORI TAKAOKA and SHIRO MORIMOTO
Osaka College of Pharmacy, 2-10-65 Kawai, Matsubara, Osaka 580 (Japan)
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SUMMARY

Rat urinary kallikrein was separated by high-performance liquid chromatography (HPLC) using an ion-exchange or gel-permeation column. Kallikrein activity was monitored continuously with peptidase or esterase activity using a post-reactor system directly adapted to HPLC. A PTFE helically coiled tube served as the enzyme reactor vessel. Four and three peaks with peptidase and esterase activity, respectively, were detected on application of normal rat urine.

INTRODUCTION

Rat urinary kallikrein is heterogeneous and DEAE-cellulose chromatography is routinely used to separate the related kallikrein-like substances[1–3]. In chromatography, kallikrein activity is measured not continuously but rather by assessment of each fraction using synthetic substrates. Conventional ion-exchange chromatography requires a long separation time and its resolution is poor.

High-performance liquid chromatographic (HPLC) methods using gel-permeation or ion-exchange types of column have been developed to separate proteins[4–9]. The advantages of HPLC are high speed, resolution and reproducibility and automatic control over the chromatography and detection system. This method has been used to separate isoenzymes related to enzyme activity by a post-reactor system[10–14].

We now report a rapid and complete separation and a simple method of measurement of rat urinary kallikrein by continuously monitoring the peptidase and

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esterase activity using a post-reactor system. Peptidase and esterase activities were detected with prolylphenylalanylarginine-4-methylcoumaryl-7-amide (MCA) and N-α-tosyl-L-arginine methyl ester (TAME) as the enzyme substrate, respectively.

EXPERIMENTAL

Materials

MCA was obtained from the Protein Research Foundation (Minoh, Osaka, Japan) and TAME and aprotinin from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical-reagent grade and were used as received.

Urine samples and partially purified urinary kallikrein. Male Wistar rats weighing 210–240 g were kept in stainless-steel metabolic cages and were provided water but no solid food during urine collection. Urine was centrifuged at 3000 rpm for 30 min and dialysed at 4°C against deionized water for 24 h and lyophilized. The concentrations of protein, MCA peptidase and TAME esterase activities in the resulting urine sample were 33 mg/ml, 1314 peptidase unit (PU)/ml and 7.7 esterase unit (EU)/ml, respectively. Partially purified urinary kallikrein was prepared by DEAE-cellulose chromatography according to the method of Chao and Margolius[3]. MCA peptidase activity of the preparation was 1254 PU/mg protein. MCA peptidase and TAME esterase activities were measured as reported previously[15]. Protein was determined by the method of Lowry *et al.*[16] using bovine serum albumin as the standard.

Columns

Anion-exchange HPLC was performed on a 300 × 4 mm I.D. column of IEX-540 DEAE SIL (particle size 5 ± 1 μm) provided by Toyo Soda (Tokyo, Japan). Two columns of TSK-GEL G3000SW (Toyo Soda) which were directly connected were used on a gel-permeation HPLC system.

Apparatus

The HPLC instrument (Toyo Soda Model SP-8700) was equipped with a spectrophotometer (UV-8 model) fitted with an 8-μl flow cell. An RF-530 (Shimadzu, Tokyo, Japan) fitted with a 12-μl flow cell was used to detect fluorescence, a Model 4MD-3G four-channel (Ch-1, -2, -3 and -4) constamatic pump (Gasukuro Kogyo, Tokyo, Japan) was used for the post-reactor system and a Shimadzu C-R1A data processor served for calculation of peak areas.

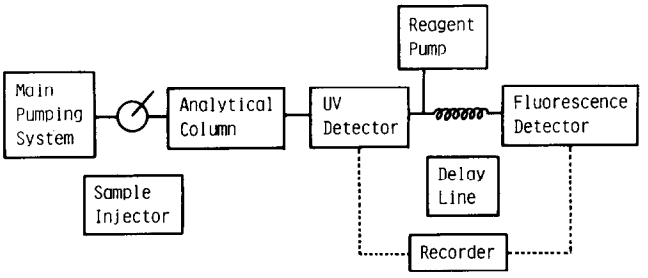

Fig. 1. Diagram of the HPLC system with continuous flow detector.

HPLC

Ion-exchange chromatography was carried out at a flow-rate of 0.7 ml/min with a linear salt gradient made by controlling buffer A (0.02 *M* Tris–acetate, pH 7.5) and buffer B (1.0 *M* sodium acetate added to buffer A, pH 7.5) using a solvent programmer, at 20–25°C. The mobile phase for gel-permeation chromatography was 0.05 *M* sodium phosphate buffer (pH 7.5) containing 0.1 *M* sodium chloride. The flow-rate was 0.5 ml/min and chromatography was carried out at 20–25°C. Bovine serum albumin (MW 68,000), ovalbumin (45,000), α-chymotrypsinogen (24,500) and cytochrome *c* (12,500) were used as standards for calculation of molecular weight.

Post-reactor system

The HPLC system with the continuous post-reactor is shown schematically in Fig. 1.

Peptidase activity. The principle of assay was according to Kato *et al.*[17]. The assay solution for the post-reactor detection of peptidase activities was 0.05 *M* Tris–HCl (pH 8.2) containing 5 μ*M* MCA. Aprotinin was added to the assay solution in the studies on inhibition. The assay solution was allowed to flow through a three-diagonal mixing joint made of PTFE, at a flow-rate of 0.75 ml/min just after the UV detector equipped for detection of the protein, using a constamatic reagent pump. Enzymatic reaction was carried out in a 20-m helically coiled PTFE capillary tube (0.25 mm I.D., 1.5 mm O.D.) at 40°C using a water-bath. The 7-amino-4-methylcoumarin generated was monitored fluorimetrically at 460 nm with excitation at 380 nm and the peak area was calculated using a data processor.

Esterase activity. The principle of the assay was according to Matsuda *et al.*[18]. The substrate solution of 0.1 *M* sodium phosphate buffer (pH 8.0) containing 10 m*M* TAME was combined with the eluate using pump Ch-1 at a flow-rate of 0.24 ml/min. The enzymatic reaction was carried out in a 20-m helically coiled PTFE capillary tube (0.25 mm I.D., 1.5 mm O.D.) at 40°C using a water-bath. Next, a mixed solution (1:2) of 0.1% potassium permanganate and 10% perchloric acid was added to the stream of reaction mixture using pump Ch-2 at a flow-rate of 0.5 ml/min, at 20–25°C to oxidize the generated methanol to formaldehyde. The length and inner diameter of this PTFE delay line tube were 20 m and 0.5 mm, respectively. To neutralize the reaction mixture, 0.1% hydroxylamine hydrochloride was added to the stream of reaction mixture at a flow-rate of 0.32 ml/min using pump Ch-3, at 20–25°C. The length and inner diameter of this PTFE tube were 20 m and 0.5 mm, respectively. Finally, to form a fluorescent compound, 2 *M* ammonium acetate containing 0.2% acetylacetone and 0.3% acetic acid was added to the stream using pump Ch-4 at a flow-rate 0.76 ml/min. The reaction was carried out at 80°C in a 10-m PTFE capillary tube (0.5 mm I.D., 1.5 mm O.D.) and the resulting fluorescence was measured at 510 nm with excitation at 410 nm.

RESULTS

Peptidase activity

The elution profiles of urine protein and MCA peptidase activity are shown in Fig. 2. Proteins were clearly separated into more than ten peaks, and this profile was similar to that seen with plasma in previous work[9]. The highest peak in the centre of

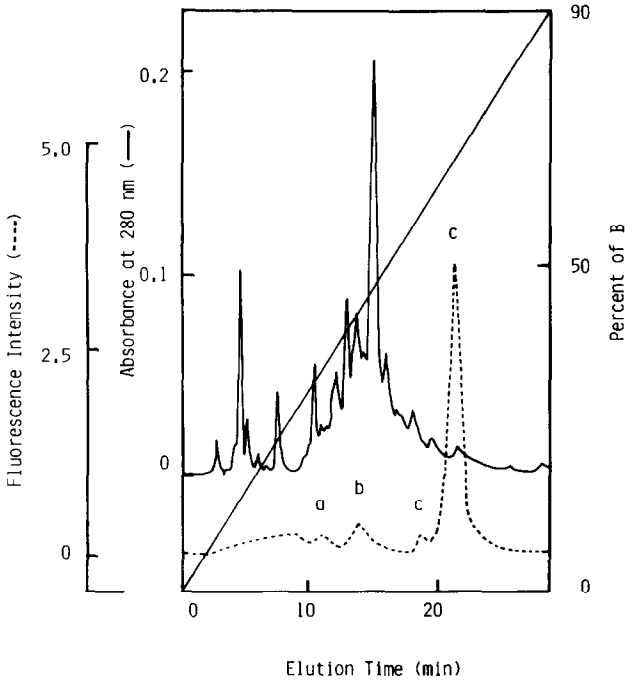

Fig. 2. Elution profile of rat urinary MCA peptidases. The urine sample (10 μl) was directly chromatographed on a DEAE-type of column with a linear gradient (0–1 *M* sodium acetate) at a flow-rate of 0.7 ml/min. The MCA solution (5 μM) was added at 0.75 ml/min. The delay line (0.25 mm I.D.) was 20 m long and the post-reactor temperature was 40°C. Percent of B indicates the percentage of buffer B.

the chromatogram of protein was assumed to be albumin. Three small peaks (peaks a, b and c') and a large peak (c) with peptidase activity were apparent. Peak c was identified as urinary kallikrein, based on the following evidence. The elution profile of MCA peptidases of partially purified urinary kallikrein is shown in Fig. 3. Only one peak was detected. The retention time of this peak (22.6 min) was the same as that of peak c (Fig. 2). The molecular weight of MCA peptidases was measured by gel-permeation HPLC and the elution profile is shown in Fig. 4. One large peak and two small MCA peptidase peaks were detected and the molecular weights were calculated to be 38,000, 35,000 and 34,000, respectively. The major peak (molecular weight 38,000) was considered to correspond to peak c, and two small peaks seemed to relate to peak a or b, because of the intensity of fluorescence.

The fluorescence sensitivity (peak area) of the peaks of peptidase activity was influenced by the sample volume, concentration of substrate (MCA), reaction time, length of the PTFE tube and incubation temperature. The effect of the sample volume on the area of peak c is shown in Fig. 5. The relationship between the peak area computed by a data processor and the volume of sample was linear in the range 2.5–10 μl, under the conditions of the post-reactor described in Fig. 5. These results indicate that this method should facilitate quantitative studies of kallikrein. A 2.5-μl volume of sample contained 82 μg of protein and the peptidase and esterase activities were 3.3 PU and 0.02 EU, respectively. When 5 μl of dialysed normal rat urine

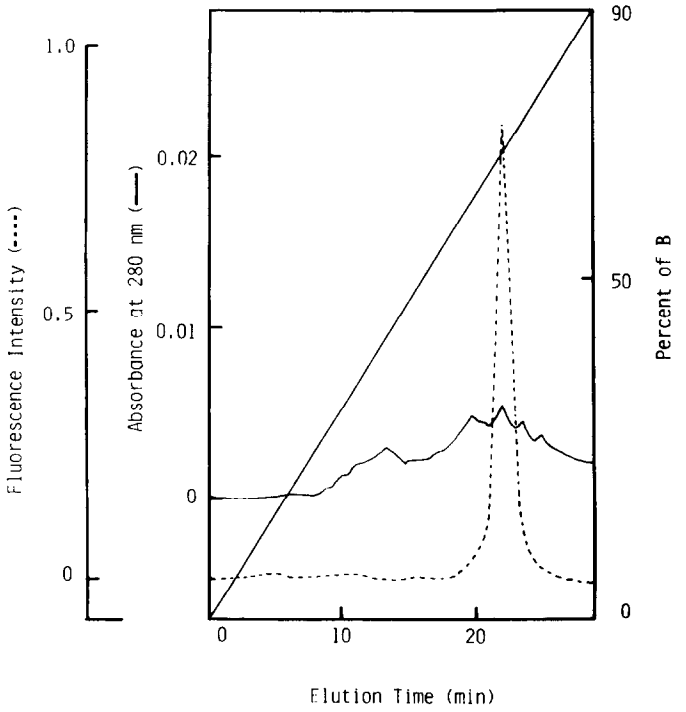

Fig. 3. Elution profile of partially purified urinary kallikrein. Conditions as in Fig. 2. Sample volume, 50 μl.

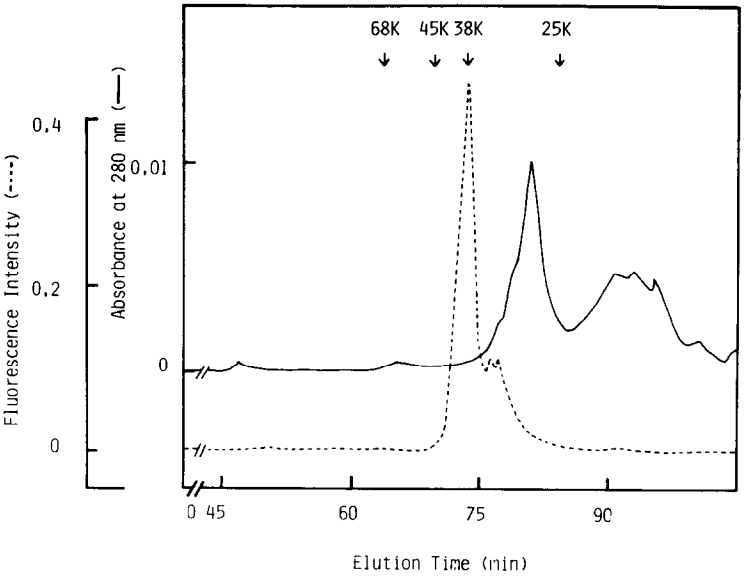

Fig. 4. Elution profile of urinary MCA peptidases on gel-permeation HPLC. A urine sample (10 μl) was chromatographed on a TSK G-3000 SW (60 cm × 2) column with 0.05 *M* phosphate buffer (pH 7.5) containing 0.1 *M* sodium chloride at a flow-rate 0.5 ml/min. Conditions of post-reactor system as in Fig. 2. K = kilodalton.

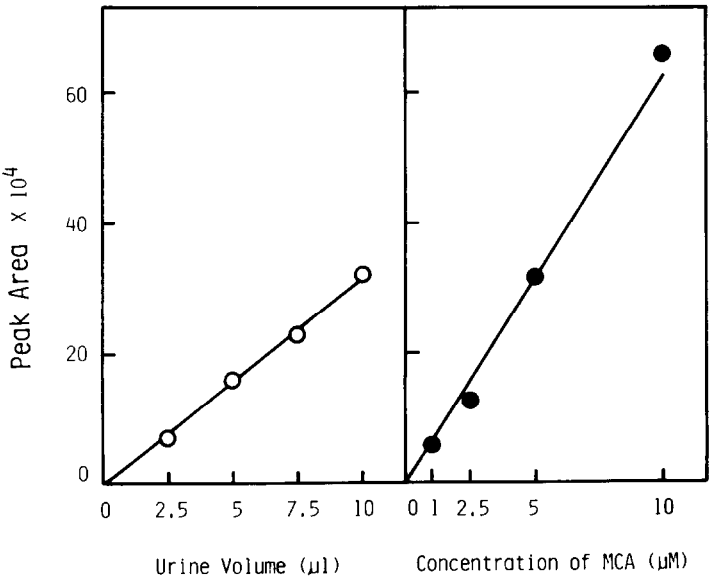

Fig. 5. Correlation of sample volume and the concentration of MCA with area of peak c. Sample volumes were 2.5, 5.0, 7.5 and 10.0 μl. Concentrations of MCA were 1.0, 2.5, 5.0 and 10.0 μM. Other conditions of HPLC and post-reactor as in Fig. 2.

were injected on to the column under the same conditions as for Fig. 2, MCA peptidase activity was clearly detected.

The effect of the concentration of substrate (MCA) on the area of peak c is shown in Fig. 5. This area increased with increase in the concentration of MCA. The enzyme reaction time could be controlled by the length of the PTFE tube in the post-reactor system. When 10- and 20-m lengths of PTFE tube were used, the areas of peak c were $15.6 \cdot 10^4$ and $31.4 \cdot 10^4$ $\mu V \cdot$ sec, respectively. The peak area also increased with increasing incubation time. When HPLC was carried out under the conditions described in Fig. 2, the incubation time of the enzyme reaction was 40 sec and here the temperature affected the peak area. The peak area at 40°C ($31.4 \cdot 10^4$ $\mu V \cdot$ sec) was larger than that at 25°C ($14.6 \cdot 10^4$ $\mu V \cdot$ sec).

Esterase activity

The elution profiles of urine protein and TAME esterase activity are shown in Fig. 6. The elution profile of protein was slightly different from that of the assay of peptidase activity because the flow-rate of the column and the volume of sample differed with the assay system. Three peaks (I, II and III) with esterase activity were detected. As the sensitivity of the esterase activity detection system was lower than that of peptidase activity detection system, ten times the volume of sample was injected. The flow-rate of the ion-exchange chromatography was reduced to 0.5 ml/min as the back-pressure was high with four reactor pumps, the total flow-rate of which was 1.82 ml/min. The post-reactor system for detection of the esterase activity was more complicated than that for the peptidase activity, as four different reagent pumps were required. In the first step, substrate solution (TAME) flowed to generate meth-

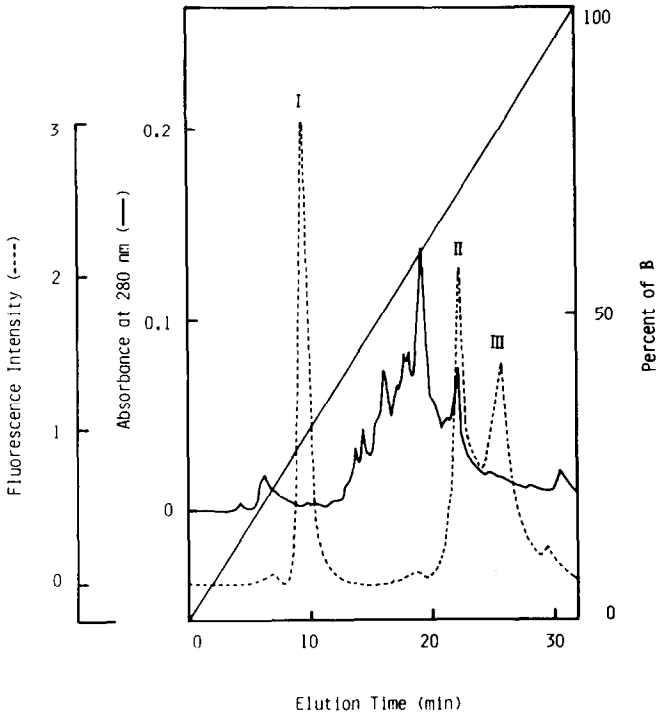

Fig. 6. Elution profile of TAME esterase. A urine sample (10 μl) was chromatographed on a DEAE-type of column at a flow-rate of 0.5 ml/min. Conditions of the post-reactor system as described in the text.

anol using pump Ch-1 at a flow-rate of 0.24 ml/min. The conditions (delay line and incubation temperature) of the enzyme reaction were the same as for detection of the peptidase activity. In the second step, acetic acid was used instead of perchloric acid for pump protection but the sensitivity was poor. In this step, potassium permanganate was reduced to manganese dioxide, which dissolved only slightly in the mobile phase. The resulting microparticles sometimes plugged the 0.25 mm I.D. PTFE tube. PTFE tubing of 0.5 mm I.D. was therefore used. At an incubation temperature of 60°C, good results were not always obtained. This step is critical in this post-reactor system. The final step producing the fluorescent compound by reaction with acetylacetone was fairly easy to control. A high concentration of acetylacetone (0.6%) and a longer delay line (20 m) did not affect the peak area.

Inhibition studies with aprotinin

To elucidate which peaks were for kallikrein, we investigated the peaks that decreased with aprotinin treatment. In the assay for peptidase activity, peak c (Fig. 2) decreased as a function of concentration whereas the other peaks remained unchanged. The percentage inhibition of peak c by aprotinin is shown in Table I. When the concentration of aprotinin was high (over 1000 kallikrein inhibitor units (KIU) per ml under these experimental conditions), peak c was completely inhibited. In the assay system for esterase activity, only peak III was inhibited by aprotinin.

TABLE I

INHIBITION OF MCA PEPTIDASE WITH APROTININ

Percentage inhibition of peak c shown in Fig. 2 by four different concentrations of aprotinin is shown. Peak area was computed by data processor. Conditions of HPLC and post-reactor system as in Fig. 2. Aprotinin was added to MCA solution.

Concentration of aprotinin (KIU/ml)	*Peak area ($\times 10^3$) ($\mu V \cdot sec$)*	*Inhibition (%)*
0	314.0	0
10	225.6	28.2
100	54.7	82.6
1000	6.4	97.9

DISCUSSION

The most commonly used technique for the separation of enzymes is ion-exchange chromatography. However, the slow flow-rates make it unsuitable for routine analytical work. Manual methods are extremely tedious and, because only a given eluate fraction is subsequently measured, one cannot be sure of the resolution of one fraction from another. An ion-exchange type of HPLC column for the separation of proteins and a continuous flow detector of enzyme activity have been developed[10–14]. Our approach differs in that a PTFE tube is used as the enzyme reactor vessel. Schroeder *et al.*[11] used a stainless-steel tube as an enzyme reactor, but PTFE is probably better as there is no problem with corrosion. Schlabach *et al.*[12] and Huber *et al.*[13] found that a packed-column reactor was more efficient than a capillary tube. However, in our post-reactor system, band spreading did not occur to a measurable extent because the width of the peaks of enzyme activity did not spread as seen with the proteins. PTFE tubing as an enzyme reactor facilitates easy control of the incubation time by appropriate selection of the tube length and the incubation temperature for the reaction.

The detection of peptidase activity was superior to that of esterase activity with regard to sensitivity of the enzyme activity, probably because with the system used to determine the esterase activity it was necessary to drive the four reactor pumps concomitantly. Thus, it is difficult to regulate each step for optimal conditions (flow-rate, reaction temperature and concentration of carrier solution). Another reason is that oxidation of the generated methanol with potassium permanganate does not completely terminate in formaldehyde but proceeds partially to formic acid. As potassium permanganate oxidizes not only methanol but also tris(hydroxymethyl)aminomethane, it is difficult to control this step with optimal conditions.

Synthetic substrates (MCA and TAME) were used to detect kallikrein activity; however, these substrates are not specific for kallikrein, but rather are substrates showing peptidase or esterase activity. We identified the kallikrein peak in the following way. Peaks c and III were identified as kallikrein peaks and both were considered to be the same enzyme. Although the retention times of peaks c and III differed because the flow-rates in HPLC were different, the eluted postions of peaks c and III

seemed to be the same in comparison with the protein elution profile. Kallikrein is inhibited by aprotinin[19]. Only the two peaks c and III were inhibited by aprotinin, other peaks being little affected. The molecular weight (38,000) of the peak c enzyme was compatible with that of urinary kallikrein reported by other workers[1,3], indicating that peak c was urinary kallikrein. The retention time (Fig. 3) of partially purified urinary kallikrein which had biological kallikrein activity was the same as that of peak c, and therefore peak c is undoubtedly urinary kallikrein.

The activity of kallikrein is usually measured using a synthetic substrate nonspecific for kallikrein because it is tedious to measure the biological and kinin-forming activities. With our new technique, urinary kallikrein can be readily separated, identified and measured.

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