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Note

High-performance liquid chromatography with a continuous-flow enzyme detector for the demonstration of the conversion of rat urinary inactive kallikrein into its active form

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We previously reported[1] that an inactive kallikrein isolated from rat urine by DEAE-cellulose chromatography could be activated with trypsin. The molecular weight of this enzyme was estimated to be 44,000 by gel filtration on a Sephadex G-100 column, and the enzyme was converted into rat urinary kallikrein (RUK, mol.wt. 38,000) by trypsin treatment. In these experiments, the eluates were first collected from each column, followed by determining the kallikrein peptidase activity before and after trypsin treatment of each fraction. Therefore, in order to investigate the conversion of inactive kallikrein into RUK by trypsin treatment, much time was required for the separation and detection of enzymes. Recently, we reported[2] a new method for the separation and measurement of RUK by high-performance liquid chromatography (HPLC) with a continuous-flow enzyme detector. With this method, RUK could readily be separated, identified and measured.

We now describe a rapid method for the detection of the trypsin-activated form of inactive kallikrein by the HPLC technique using an ion-exchange or gel-permeation column, and confirm that an inactive kallikrein from rat urine can be converted into RUK by trypsin treatment.

EXPERIMENTAL

RUK and inactive kallikrein prepared from the urine of male Wistar rats according to the method described previously[1] were used. Enzyme solution (0.1 ml) was incubated for 15 min at 37°C with 0.1 ml of trypsin (50 μg/ml, bovine pancreas Type III; Sigma, St. Louis, MO, U.S.A.) in 0.1 *M* Tris–HCl buffer (pH 8.0) containing 0.15 *M* sodium chloride, and the reaction was stopped by the addition of 0.1 ml of ovomucoid (500 μg/ml, chicken egg white Type III, S igma) in the same buffer. The blank for each sample was incubated with trypsin which had been mixed with ovomucoid, before applying it to the column. Each 150 μl of sample containing about 40 μg of protein was subjected to chromatography.

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The HPLC analysis was conducted on a Toyo Soda Model SP-8700 instrument (Toyo Soda, Japan), equipped with a spectrophotometer (UV-8, Toyo Soda) fitted with a 8-μl flow cell and coupled with an automated post-column detector system, as reported previously[2]. Anion-exchange HPLC was carried out on a column of IEX-540 DEAE SIL (300 × 4.0 mm I.D.) provided by Toyo Soda. The flow-rate was 0.7 ml/min with a linear salt gradient of buffer A (20 m*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. Gel-permeation HPLC was performed on two coupled columns of TSK-GEL G3000SW (600 × 7.5 mm I.D., Toyo Soda). The flow-rate was 1.0 ml/min with 50 m*M* sodium phosphate buffer (pH 7.5) containing 0.1 *M* sodium chloride. All chromatograms were recorded at room temperature.

The assay solution for the post-column detection of kallikrein peptidase activity was 50 m*M* Tris–HCl (pH 8.2) containing 5 μ*M* prolylphenylalanylarginine-4-methylcoumaryl-7-amide (Pro-Phe-Arg-MCA; Protein Research Foundation, Japan). It was allowed to flow at 0.75 ml/min through a three-way cock made of PTFE, placed just after the UV detector, using a constamatic reagent pump. The enzymatic reaction was carried out in a 20-m helically coiled PTFE capillary tube (0.25 mm I.D.) at 40°C using a water-bath. The generated 7-amino-4-methylcoumarin was monitored at 460 nm using a fluorescence spectrometer (RF-530; Shimadzu, Japan) fitted with a 12-μl flow cell, with excitation at 380 nm. Peak areas were calculated using a data processor (C-R1A, Shimadzu).

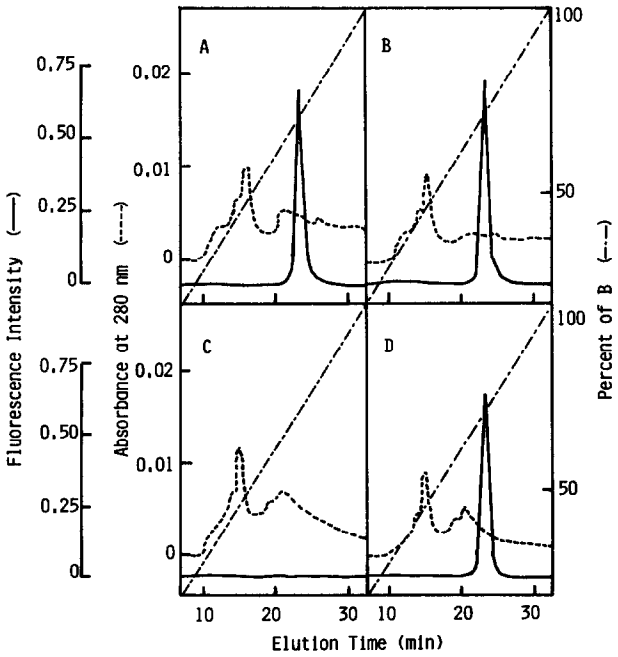

Fig. 1. Elution profiles of rat urinary kallikrein (A, B) and inactiive kallikrein (C, D) obtained by ion-exchange HPLC, with (B, D) or without (A, C) trypsin treatment. Each sample (150 μl) was chromatographed on a IEX-540 DEAE SIL column, under the HPLC and post-reactor conditions described in the text. "Percent of B" indicates the percentage of buffer B.

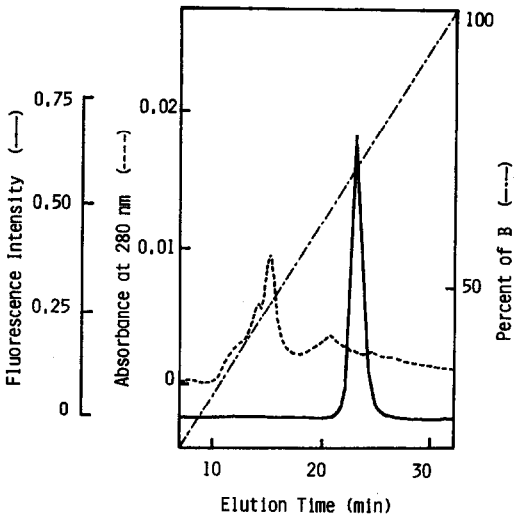

Fig. 2. Elution profile of a mixture of rat urinary kallikrein and the trypsin-activated form of inactive kallikrein obtained by ion-exchange HPLC.

RESULTS AND DISCUSSION

Fig. 1 presents elution profiles of RUK and inactive kallikrein with or without trypsin treatment, obtained by ion-exchange HPLC. The peptidase activity of RUK showed a single peak with a retention time of 22.6 min (Fig. 1A). When RUK was first treated with trypsin and then applied to the column, the retention time and peak area of peptidase activity (Fig. 1B) were exactly the same as before (Fig. 1A). On the other hand, the chromatogram of inactive kallikrein revealed no peak of peptidase activity (Fig. 1C). However, trypsin treatment of this enzyme resulted in a complete activation, and the peptidase activity showed a single peak with a retention time of 22.6 min (Fig. 1D), the same as for the RUK with or without trypsin treatment. In addition, when a mixture of RUK and the trypsin-activated form of inactive kallikrein was applied to the column, only one peak was detected. The retention time of this peak was also 22.6 min (Fig. 2).

To evaluate the effect of aprotinin on the activity of the trypsin-activated form of inactive kallikrein, the concentration of aprotinin added to the assay solution was increased progressively from 10 to 1000 kallikrein inhibitor units (KIU) per ml (Fig. 3). Compared with the blank containing no aprotinin (Fig. 3A), increasing concentrations of aprotinin resulted in a dose-related decrease in the peptidase activity of the trypsin-activated form of inactive kallikrein. At a concentration of 1000 KIU/ml, aprotinin mostly inhibited the activity. These results were similar to those obtained with RUK[2]. From the observation that the separation and subsequent detection of the trypsin-activated form of inactive kallikrein was made in less than 30 min in a single analysis, it is clear that ion-exchange HPLC with a continuous-flow enzyme detector can enable a rapid analysis of the conversion of inactive kallikrein into RUK compared with conventional ion-exchange chromatography.

Fig. 4 presents elution profiles of RUK and inactive kallikrein obtained by

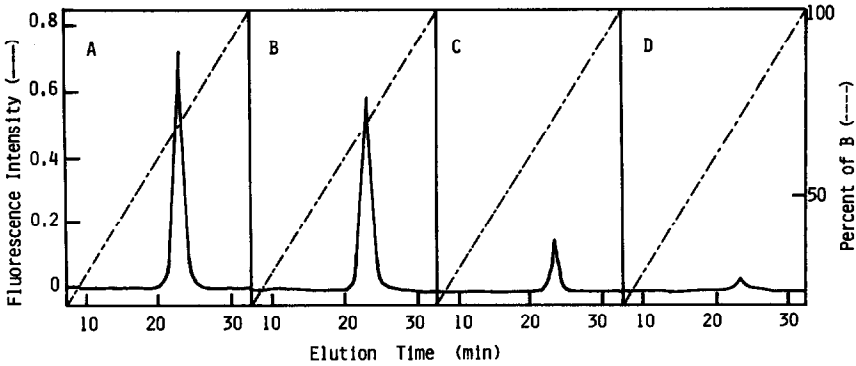

Fig. 3. Inhibitory effect of aprotinin on the trypsin-activated form of inactive kallikrein. Aprotinin was added to the assay solution containing 5 μM Pro-Phe-Arg-MCA at a concentration of 0 (A), 10 (B), 100 (C) or 1000 KIU/ml (D). Other HPLC and post-reactor conditions as in Fig. 1.

gel-permeation HPLC. The peptidase activity of RUK with or without trypsin treatment showed a single peak with a retention time of 36.5 min and with a molecular weight of 38,000 (Fig. 4A and B). On the other hand, the chromatogram of inactive kallikrein showed no peptidase activity (Fig. 4C). However, when inactive kallikrein was treated with trypsin and then applied to the column, a peak of peptidase activity

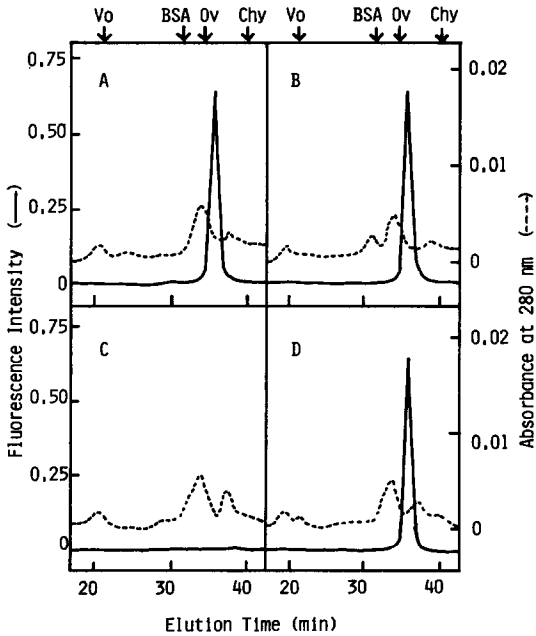

Fig. 4. Elution profiles of rat urinary kallikrein (A, B) and inactive kallikrein (C, D) by gel-permeation HPLC, with (B, D) or without trypsin treatment (A, C). Each sample (150 μl) was chromatographed on a TSK G3000SW column, under the HPLC and post-reactor conditions described in the text. Elutions of molecular weight (M_r) standards are indicated by arrows: void volume (V_0); bovine serum albumin (BSA, M_r 68,000); ovalbumin (Ov, M_r 45,000); α-chymotrypsinogen A (Chy, M_r 25,000).

appeared at a retention time of 36.5 min corresponding to a molecular weight of 38,000, in addition to RUK (Fig. 4D). These molecular weight results were similar to those estimated using a Sephadex G-100 column[1]. The use of gel-permeation HPLC with a continuous-flow enzyme detector made it possible to determine the change in molecular weight on conversion of inactive kallikrein into RUK within 40 min, whereas conventional gel filtration usually requires overnight elution followed by measurements of enzyme activity in each eluate.

In recent years, an HPLC method has been developed to separate isoenzymes related to enzyme activity using a post-reactor system[3–6]. In the present study, we found that HPLC with a continuous-flow enzyme detector is applicable to the demonstration of the conversion of inactive kallikrein into its active form. The advantages of this method compared with conventional column chromatography are high speed and automatic control of the chromatography and detection system. Accordingly, HPLC may be instrumental in the successful investigation of the conversion of other physiologically inactive enzymes into their active forms.

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