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A Kinin-Generating Amidase Activated by Trypsin in Rat Urine

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An enzyme which hydrolyzes dog plasma kininogen and prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide (Pro–Phe–Arg–MCA) was separated from rat urinary kallikrein (RUK) by diethyl-aminoethyl-cellulose chromatography during the process of isolation of RUK. When some of the properties of this enzyme was examined, the enzyme was found to be a kinin-generating amidase with properties different from those of RUK, with respect to molecular weight and optimum pH for hydrolysis of Pro–Phe–Arg–MCA. Further, the kinin-generating amidase was activated with trypsin. The molecular weight of the kinin-generating amidase treated with or without trypsin was estimated to be 3.3×10^4 or 3.5×10^4, respectively, by gel filtration on a Sephadex G-100 column, these values being smaller than that of RUK (M.W. 3.8×10^4). These results indicate that rat urine contains a kinin-generating amidase of lower molecular weight than RUK, and that this kinin-generating amidase is activated with a reduction in molecular weight by trypsin treatment.

Keywords——rat urine; kallikrein; kinin-generating amidase; prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide; amidolytic activity; dog plasma kininogen; kinin-generating activity; trypsin treatment; molecular weight conversion

Kallikrein (EC 3.4.21.35) is a proteolytic enzyme that specifically splits its natural substrate, kininogen, to form a kinin. There are two kallikrein–kinin systems; one in plasma, involving plasma kallikrein, and the other in exocrine glands, involving the glandular kallikreins. Urinary kallikrein is also a glandular kallikrein, which is considered to be synthesized in the kidney and to be secreted into the tubular lumen at the distal segments of the nephron.[1–4] The enzyme activity is measured as an index of alterations in the activity of the renal kallikrein-kinin system. The excretion of kallikrein is known to be subnormal in patients with essential hypertension[5,6] and in animals with various forms of hypertension.[7–9]

Many assay methods using synthetic substrates have been developed for the measurement of urinary kallikrein activity. Esterolytic assays using sysnthetic esters of arginine are commonly used. However, since arginine esterases other than kallikrein are present in the urine of several species,[10–16] the method of esterolytic assay lacks specificity. On the other hand, amidolytic assays using synthetic amides of arginine have also been introduced for the measurement of activities of glandular kallikreins, including urinary kallikrein. We recently reported that an enzyme other than kallikrein could be detected in the urine of rats when prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide (Pro–Phe–Arg–MCA) was used as the substrate for urinary kallikrein.[16]

We now describe some properties of a rat urinary enzyme other than kallikrein (RUK) that hydrolyzes Pro–Phe–Arg–MCA and dog plasma kininogen, and we present evidence that this enzyme is activated by trypsin.

Materials and Methods

Chemicals——The following chemicals were obtained commercially: Pro–Phe–Arg–MCA, 7-amino-4-methylcoumarin (AMC) and bradykinin diacetate from the Protein Research Foundation (Osaka, Japan); diethylaminoethyl(DEAE)-cellulose (DE-52) from Whatman Ltd. Sephadex G-100 and blue dextran from Pharmacia Fine Chemicals AB (Uppsala, Sweden); trypsin (bovine pancreas Type III), soybean trypsin inhibitor (SBTI), ovomucoid (chicken egg white Type III), bovine serum albumin, ovalbumin, α-chymotrypsinogen A and cytochrome c from Sigma Chemical Co. (St. Louis, U.S.A.). A purified hog pancreatic kallikrein (49 KU/mg of protein), which was used as a standard preparation of kallikrein, was supplied by Fujimoto Pharmaceutical Co. (Osaka, Japan). Other chemicals used were of reagent grade.

Experimental Animals and Urine Collection——Male Wistar rats weighing 200—250 g were used. For at least 1 week before the study the rats were fed a standard laboratory chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan), and provided with tap water *ad libitum*. After being fasted for 16 h, the animals were given physiological saline equal to 2.5% of body weight *via* a stomach tube, and placed in individual stainless-steel metabolic cages. Eight-hour urine samples were collected in flasks containing toluene to prevent bacterial growth. All rats were deprived of food, but allowed free access to water during the time of urine collection. After the exclusion of toluene, the urine was centrifuged to remove the solid debris, and stored at −20 °C until required.

Assay of Enzyme Activity——Amidolytic activity in the urine and fractionated samples was assayed according to the method described previously.[17] After incubation of samples with Pro–Phe–Arg–MCA, the amount of AMC liberated from the substrate was measured fluorometrically with excitation at 380 nm and emission at 460 nm. One unit (AU) of the amidolytic activity was defined as the amount of the enzyme which hydrolyzed 1 nmol of Pro–Phe–Arg–MCA per min at 37 °C. Kinin-generating activity in the eluted sample was determined by the vasodilator assay of kinins generated after incubation with partially purified dog plasma kininogen. The plasma kininogen was diluted with 0.15 M NaCl containing 3 mM 1,10-phenanthroline and 10 mM disodium ethylenediaminetetraacetic acid, and adjusted to pH 8.5 with NaOH solution. One ml of the diluted plasma kininogen (3000 ng of bradykinin eq/ml) was incubated with 50 μl of enzyme solution in the presence of 10 nM SQ-14225 at 37 °C for 20 min. The enzymatic reaction was stopped by the addition of 1 ml of 20 mM malonate buffer (pH 5.0), and the mixture was heated in a boiling water bath. After centrifugation, the supernatant was adjusted to pH 7.0 with NaOH solution. One-half ml of diluted sample was injected into the femoral artery of anesthetized dogs, and the increase in arterial blood flow was measured. The quantity of kinins generated in the incubation mixture was determined from the calibration line obtained with 0.5 ml aliquots of standard bradykinin solutions (2—20 ng/ml). The kinin-generating activity was expressed as equivalents of bradykinin generated per min.

Trypsin Treatment——A mixture of 50 μl each of enzyme sample and trypsin (5.0—100 μg/ml) in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl was incubated at 37 °C for 15 min. The reaction was stopped by the addition of 50 μl of ovomucoid (500 μg/ml) in the same buffer. Then, the sample was assayed for amidolytic activity. The control of each sample was incubated with trypsin which had been mixed with ovomucoid before assaying the amidolytic activity.

Determination of Protein Concentration——The protein concentration in enzyme solution was determined by the method of Lowry *et al.*,[18] using bovine serum albumin as the standard.

Determination of Molecular Weight——A Sephadex G-100 column (1.6 × 90 cm) was used to estimate the molecular weights of enzymes. The column was eluted with 40 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl and 3 mM NaN_3 as an antimicrobial agent. The flow rate was 8.8 ml/h and 1 ml fractions were collected at 4 °C. The void volume of the column was estimated by using blue dextran. Bovine serum albumin (M.W. 6.7×10^4), ovalbumin (M.W. 4.5×10^4), α-chymotrypsinogen A (M.W. 2.5×10^4) and cytochrome c (M.W. 1.29×10^4) were used as molecular weight standards.

Results

A urine sample of 1330 ml (44 AU/mg protein) was first dialyzed against distilled water at 4 °C for 24 h, then against physiological saline at 4 °C for 24 h. The following procedures were then carried out for the isolation of RUK.

Step 1. Concentration

The dialyzed urine sample was concentrated to one-tenth of the original volume under low pressure in a collodion bag. This preparation showed a specific activity of 69 AU/mg protein.

Step 2. Acetone Fractionation

The concentrated urine sample was fractionated with two acetone cuts, one from 0 to

45% (v/v), the other from 45 to 65% (v/v). Since the latter precipitate contained 99% of the total urinary amidolytic activity, this precipitate (87 AU/mg protein) was dissolved in 50 mM ammonium acetate buffer (pH 6.7) containing 0.4 M NaCl.

Step 3. Gel Filtration

The sample from the preceding step was applied to a Sephadex G-100 column (1.6 × 94 cm) equilibrated with 50 mM ammonium acetate buffer (pH 6.7) containing 0.4 M NaCl. Elution was carried out with the same buffer. The flow rate was 12 ml/h and 1 ml fractions were collected. A single peak of amidolytic activity was detected in the fractions eluted between ovalbumin (M.W. 4.5×10^4) and α-chymotrypsinogen A (M.W. 2.5×10^4), and 78% of the total urinary amidolytic activity was recovered in the active fractions. These fractions (115 AU/mg protein) were pooled, dialyzed against distilled water at 4 °C for 24 h, and lyophilized.

Step 4. DEAE-Cellulose Chromatography

The lyophilized sample was dissolved in 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and applied to a DEAE-cellulose column (1.0 × 15 cm) previously equilibrated with the same buffer. The column was washed with the same buffer until the absorbance of the eluate at 280 nm had disappeared, then eluted with 200 ml of a linear gradient of 0.1—0.5 M NaCl in the same buffer. The flow rate was 40 ml/h and 4 ml fractions were collected. As shown in Fig. 1, about 90% of the total amidolytic activity appeared in the eluates between fractions No. 125 and 134 (fraction II), reflecting the chromatographic behavior of a typical glandular kallikrein on a DEAE-cellulose column. This preparation of RUK showed a specific activity of 3805 AU/mg protein, this value being about 86 times that of the original urine sample. In this chromatography, the other peak of amidolytic activity (fraction I) was observed in the flow-through fraction. These results indicate that another enzyme hydrolyzing Pro–Phe–Arg–MCA is present in the rat urine besides RUK.

Next, we determined whether the other enzyme has kinin-generating activity, as well as RUK. When kinin-generating activity in each eluate fraction obtained by the DEAE-cellulose chromatography was measured with dog plasma kininogen as the substrate, the elution pattern of this activity was the same as that of amidolytic activity. About 10% of total kinin-generating activity was recovered in fraction I and the remaining activity was found in fraction II (RUK), as calculated from the recovered volume and the enzyme activity. The percent distributions were similar to those obtained by the assay of amidolytic activity. These data indicate that the kinin-generating activity per amidolytic unit in fraction I is similar to

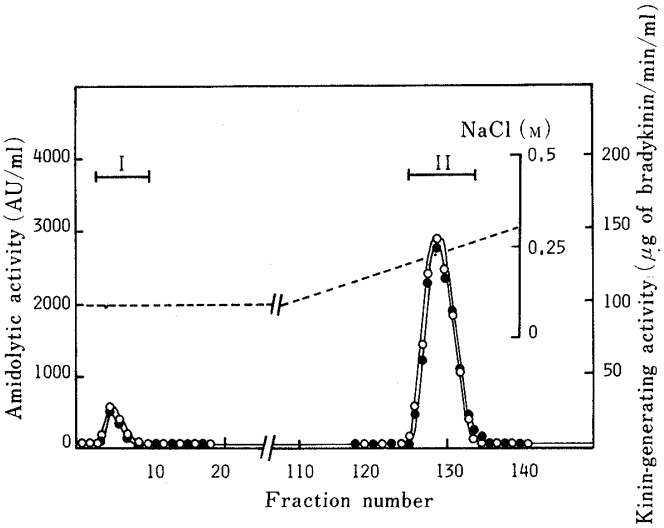

Fig. 1. Elution Patterns of Amidolytic and Kinin-Generating Activities on DEAE-Cellulose Chromatography

The sample obtained by gel filtration was chromatographed on a DEAE-cellulose column under the conditions described in the text. Eluates were assayed for amidolytic activity (○) and kinin-generating activity (●). (– – –), NaCl concentration.

that in the RUK fractions, although the kinin-generating activity in fraction I amounted to only about one-tenth of that in the RUK fractions. Neither RUK nor the other Pro–Phe–Arg–MCA-hydrolyzing enzyme, which we designate here as kinin-generating amidase, has yet been completely purified. However, for elucidation of the difference between the RUK and kinin-generating amidase, the preparations obtained by DEAE-cellulose chromatography were used in the following experiments.

Enzymatic Properties of Kinin-Generating Amidase and Kallikrein Isolated from Rat Urine

Table I summarizes the enzymatic properties of kinin-generating amidase, in comparison with those of RUK. The K_m values for Pro–Phe–Arg–MCA hydrolysis by the kinin-generating amidase and RUK were 0.14 and 0.12 mM, respectively. The optimum pH of kinin-generating amidase was 8.0 for Pro–Phe–Arg–MCA. On the other hand, the optimum pH of the RUK was 11.0 under the same conditions. In addition, the amidolytic activity of kinin-generating amidase was inhibited by aprotinin in a dose-dependent manner, as was that of RUK, but it was more strongly inhibited by SBTI compared with that of RUK. However, the activities of these enzymes were not inhibited by ovomucoid.

Amidolytic and Vasodilator Activities of Kinin-Generating Amidase Following Trypsin Treatment

To further investigate the properties of kinin-generating amidase, this enzyme was treated with various concentrations of trypsin. When the enzyme was incubated in the presence of 20 μg/ml trypsin, the amidolytic activity was enhanced, the value being about 1.6 times that of the enzyme solution incubated without trypsin (Fig. 2). Increasing the concentration of trypsin resulted in a dose-related increase in the amidolytic activity of kinin-generating amidase during incubation at 37 °C for 15 min. At a concentration of 50 μg/ml, the amidolytic activity reached the maximum value, which was about 4 times that of the enzyme solution incubated without trypsin. However, RUK was not activated by trypsin at any concentation up to 100 μg/ml.

We also examined the vasodilator actions of RUK and the kinin-generating amidase treated with or without trypsin, because the vasodilator activity seems to be an index of the

TABLE I. Enzymatic Properties of Kinin-Generating Amidase and Kallikrein Isolated from Rat Urine

		Rat urinary kallikrein	Kinin-generating amidase
K_m value	(mM)	0.12	0.14
Optimum pH		11.0	8.0
		Inhibition (%)	
Aprotinin	500 KIU/ml	95.0	95.0
	50	84.0	76.0
	5.0	72.0	50.0
SBTI	500 μg/ml	78.0	97.0
	50	19.0	81.0
	5.0	4.6	45.0
Ovomucoid	500 μg/ml	0	0
	50	0	0
	5.0	0	0

K_m values for Pro–Phe–Arg–MCA hydrolysis were determined from Lineweaver–Burk plots. The pH optimum for Pro–Phe–Arg–MCA was measured in 0.04 M Britton–Robinson's wide-range buffer (pH range of 6.0 to 12.0) consisting of H_3PO_4, CH_3COOH, H_3BO_3, and NaOH. Inhibition studies were performed as follows; the RUK and kinin-generating amidase were diluted with 0.1 M Tris-HCl buffer (pH 8.0) so that the final concentration became 50 AU per ml. One-tenth ml of the diluted enzyme solution was incubated with 0.3 ml of the indicated amount of a trypsin inhibitor dissolved in the same buffer at 37 °C for 1 h. Then, the amidolytic activity of incubation samples was assayed by using Pro–Phe–Arg–MCA as the substrate.

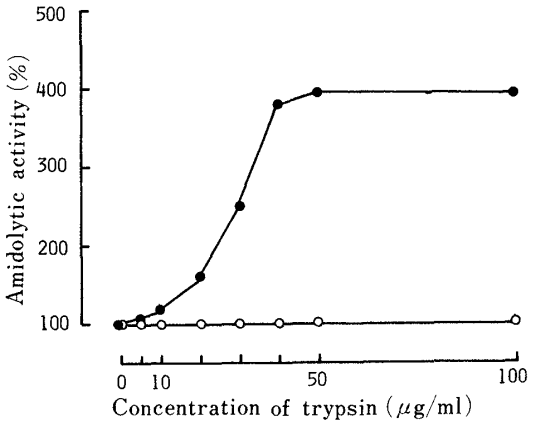

Fig. 2. Effect of Trypsin Concentration on the Amidolytic Activities of Rat Urinary Kallikrein and Kinin-Generating Amidase

RUK and kinin-generating amidase were diluted with Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl so that the enzyme solution contained 50 AU per ml. The diluted enzyme sample was treated with various concentrations of trypsin (0—100 μg/ml). The enzyme activity is expressed as a percentage of the amidolytic activity at 0 μg/ml trypsin concentration. Each point represents the mean of triplicate analyses. (○), RUK; (●), kinin-generating amidase.

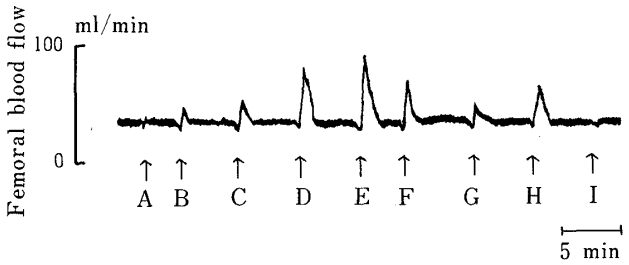

Fig. 3. Responses of Femoral Blood Flow to Intraarterial Injection of Rat Urinary Kallikrein and Kinin-Generating Amidase in Anesthetized Dogs

Each sample (0.2 ml) was injected into the femoral artery of the dog. A, saline; B, C, D and E, 0.015, 0.03, 0.06 and 0.12 KU of the standard kallikrein; F, RUK (1 AU); G, kinin-generating amidase treated without trypsin (0.25 AU); H, kinin-generating amidase treated with trypsin (1 AU); I, a mixture of trypsin and ovomucoid.

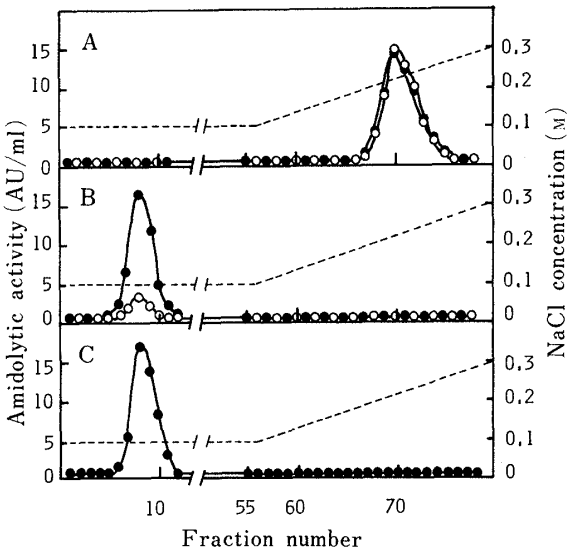

Fig. 4. Elution Profiles of Kallikrein and Kinin-Generating Amidase by DEAE-Cellulose Chromatography

The elution profile of RUK is shown in (A), and those of kinin-generating amidase treated with or without trypsin are shown in (C) or (B). Each sample (1.5 ml) was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, and applied to a DEAE-cellulose column (1.0 × 5.0 cm) previously equilibrated with the same buffer. The column was washed with 50 ml of the same buffer, and subsequent elution was carried out with 50 ml of a linear gradient from 0.1 M up to 0.5 M NaCl in the same buffer. The flow rate was 40 ml/h and 1 ml fractions were collected. In (A) and (B), eluates were treated with (●) and without (○) trypsin, and then assayed for amidolytic activity. In (C), kinin-generating amidase treated with trypsin was applied to the column and the eluates were assayed for amidolytic activity.

physiological action of kallikrein. As shown in Fig. 3, the injection of RUK resulted in an increase of arterial blood flow. An increase of the arterial blood flow was also observed after the administration of kinin-generating amidase. Trypsin treatment of the kinin-generating amidase produced a further marked vasodilator action, while the mixture of trypsin and ovomucoid used for the treatment of this enzyme caused no change in the arterial blood flow.

Chromatographic Behavior of Kinin-Generating Amidase Following Trypsin Treatment

As the enzyme activities of kinin-generating amidase increased markedly after trypsin treatment, we examined whether this enzyme could be converted to RUK by the same treatment. Figure 4A shows an elution profile of RUK obtained in ion-exchange chromatography on a DEAE-cellulose column (1.0 × 5.0 cm). The amidolytic activity of RUK showed a single peak at 0.23 M NaCl, but the activity was not augmented after trypsin treatment of these

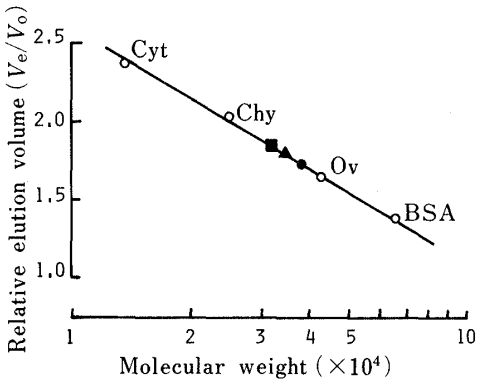

Fig. 5. Determination of Molecular Weights of Rat Urinary Kallikrein and Kinin-Generating Amidase By Gel Filtration on a Sephadex G-100 Column

Bovine serum albumin (BSA), ovalbumin (Ov), α-chymotrypsinogen (Chy), and cytochrome *c* (Cyt) were used as molecular weight standards. Experimental conditions are described in the text. (●), RUK; (■), kinin-generating amidase treated with trypsin; (▲), kinin-generating amidase treated without trypsin.

peak fractions. On the other hand, the chromatogram of kinin-generating amidase showed a single peak of amidolytic activity in the flow-through fraction, and trypsin treatment of these fractions resulted in an extensive activation (Fig. 4B). When the kinin-generating amidase was first activated with trypsin, then applied to the column, the activity was also observed in the flow-through fraction (Fig. 4C).

In addition, the RUK and kinin-generating amidase treated with or without trypsin were subjected to gel filtration on a Sephadex G-100 column. Figure 5 shows the calibration curve obtained with standard proteins. By comparison with the relative mobilities of standard proteins, the molecular weight of kinin-generating amidase treated with or without trypsin was estimated to be 3.3×10^4 or 3.5×10^4, respectively, each value being smaller than that of RUK (M.W. 3.8×10^4). These results indicate that the kinin-generating amidase, separated from RUK by DEAE-cellulose chromatography, was activated by trypsin without conversion to RUK.

Discussion

The present study showed that an enzyme hydrolyzing both Pro–Phe–Arg–MCA and dog plasma kininogen can be separated from RUK by DEAE-cellulose chromatography during the process of isolation of the RUK. This enzyme was found to have properties distinctly different from those of RUK, although complete purification was not achieved.

The optimum pH of RUK was 11.0 for Pro–Phe–Arg–MCA hydrolysis. Since the kallikreins in human urine, hog pancreas and rat stomach have been reported to show highly alkaline pH optima of 11.0 for this substrate,[19,20] it seems that such an alkaline optimum pH is a common property of glandular kallikreins including RUK. On the other hand, the optimum pH of kinin-generating amidase was 8.0, which is different from that of RUK. This optimum pH value for Pro–Phe–Arg–MCA hydrolysis is the same as that of rat plasma kallikrein previously found by Uchida *et al.*[20] However, the molecular weight of kinin-generating amidase was about 3.5×10^4 (Fig. 5), which is lower than that of plasma kallikrein reported by several investigators.[20-24] Therefore, it seems that the kinin-generating amidase in the rat urine may also be different from plasma kallikrein.

Pro–Phe–Arg–MCA is considered to be a suitable substrate for glandular kallikreins including pancreatic and urinary kallikreins, among various fluorogenic compounds of the peptidyl-MCA type.[25] A previous study in our laboratory indicated that the results of assay of amidolytic activity correlated well with kinin-generating activity ($r=0.99$) in rat urine.[17] In the present work, the measurement of kinin-generating activity in fractions I and II obtained by DEAE-cellulose chromatography (Fig. 1) revealed that the distribution pattern of the activity was the same as that of amidolytic activity, confirming that the results of assay of amidolytic activity correlated well with kinin-generating activity in the two enzyme fractions.

These findings indicate that urinary amidolytic activity can be used as an index of changes in the urinary kinin-generating activity. However, when the enzymatic activity of RUK is assayed by using Pro–Phe–Arg–MCA or dog plasma kininogen as the substrate, the presence of the kinin-generating amidase should be taken into consideration.

It has been demonstrated that a kinin-generating enzyme other than kallikrein is present in rat urine.[10,14] This enzyme was first designated as "esterase A" by Nustad and Pierce,[10] and then as "esterase A2" by McPartland *et al.*[14] In a previous study, we showed that about 90% of the total urinary amidolytic activity is due to RUK, and the remaining activity is associated with "esterase A2."[16] The properties of the kinin-generating amidase, which we isolated in this work, are similar to those of "esterase A," or "esterase A2" with respect to the ability to hydrolyze dog plasma kininogen or Pro–Phe–Arg–MCA. Thus, it is possible that the kinin-generating amidase might be identical with "esterase A," or "esterase A2." On the other hand, in recent studies, the molecular weights of the purified "esterase A" of Chao,[15] and "esterase A2" of McPartland *et al.*[26] were estimated to be 3.3×10^4 and 4.1×10^4 on Sephacryl S-200, respectively. These values are somewhat different from that of the kinin-generating amidase (M.W. 3.5×10^4) determined on Sephadex G-100. The difference in molecular weight may be due to the experimental conditions or the extent of purification of the sample used. To determine whether or not the kinin-generating amidase is identical with "esterase A" or "esterase A2," additional experiments are under way.

The most interesting result of the present study was the activation of the kinin-generating amidase by trypsin. The amidolytic and vasodilator activities of this enzyme were both activated, but those of RUK were not. Recently, we have demonstrated that an inactive kallikrein, the molecular weight of which is slightly larger than that of RUK, is present in rat urine and is converted to RUK by trypsin treatment.[27] In the present work, we also examined whether the kinin-generating amidase could be converted to RUK by trypsin treatment. However, the activity of kinin-generating amidase following trypsin treatment was not observed in the eluate corresponding to RUK on the DEAE-cellulose column. Furthermore, the molecular weights of kinin-generating amidase (M.W. 3.5×10^4) and its activated form (M.W. 3.3×10^4) were less than that of RUK (M.W. 3.8×10^4). These results indicate that the kinin-generating amidase is activated by trypsin without conversion to RUK, and that possible contamination of inactive kallikrein in the kinin-generating amidase can be ruled out.

This is the first report showing that amidolytic and vasodilator activities of a urinary kinin-generating amidase, probably "esterase A," or "esterase A2" as designated by other investigators, are enhanced by trypsin treatment. It seems that the activation of this enzyme is accompanied by the release of a polypeptide fragment with a molecular weight of about 2000. However, we cannot yet rule out the possibility that the kinin-generating amidase is a complex with a protein binding near the active site of this enzyme, so that the trypsin-activation of the enzyme may be due to the cleavage of this binding protein. Further studies on the precise mechanism of activation of the kinin-generating amidase by trypsin are in progress.

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