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Excretion Patterns of Urinary Enzymes Having Amidolytic and Esterolytic Activities in the Urine of Male and Female Rats

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Excretion patterns of urinary enzymes having amidolytic and esterolytic activities were examined in the urine of male and female rats. Kallikrein and non-kallikrein [esterase A1 (EA1) and esterase A2 (EA2)] fractions were separated by diethylaminoethyl-cellulose chromatography and amidolytic and esterolytic activities were assayed by using prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide and *N*-α-tosyl-L-arginine methyl ester. In male rat urine, about 25% of total esterolytic activity was due to kallikrein, and the remainder was attributable to EA1 and EA2. In female rat urine, about 45% of total esterolytic activity was due to kallikrein and the remainder was mostly attributable to EA2. EA1 in female rats accounted for less than one-tenth of total esterolytic activity. On the other hand, about 87 and 13% of total amidolytic activity were due to kallikrein and EA2, respectively, in both male and female rats. However, EA1 had no amidolytic activity. These findings indicate that most of the urinary amidolytic activity in both sexes is associated with kallikrein, in contrast to urinary esterolytic activity, and that EA1 is not relevant to the results of urinary amidolytic assay.

Keywords——rat urine; kallikrein; esterase A1; esterase A2; *N*-α-tosyl-L-arginine methyl ester; esterolytic activity; prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide; amidolytic activity; diethylaminoethyl-cellulose chromatography

Urinary kallikrein is generally considered to be synthesized in the kidney and to be secreted into the tubular lumen.[1-3] It is known that urinary kallikrein resembles renal and other glandular kallikreins in biochemical characteristics.[3-7] Many investigators have measured the enzyme activity as an index of changes in the renal kallikrein-kinin system, under various conditions.

The level of urinary kallikrein has been determined by assaying the vasodilative, kinin-generating or synthetic substrate-hydrolyzing activity of this enzyme. Of these assays, esterolytic assay using *N*-α-tosyl-L-arginine methyl ester (TAME), a synthetic N-substituted arginine ester, has been widely used in mammals including humans, because this assay method is simple and the substrate is commonly available. However, since it has been shown that the urine from the human, dog and rat contains one or more non-kallikrein arginine esterases,[8-11] the esterolytic assay is unlikely to be a valid method for the measurement of urinary kallikrein. Therefore, the separation of urinary kallikrein from non-kallikrein arginine esterases is necessary for determining the esterolytic activity of urinary kallikrein.[8,12]

Recently, Morita *et al.*[13] synthesized various fluorogenic compounds of the peptidyl-4-methylcoumaryl-7-amide type, and found that prolyl-phenylalanyl-arginine-4-methylcoumaryl-7-amide (Pro-Phe-Arg-MCA) was a suitable substrate for pancreatic and urinary kallikreins. Thereafter, several investigators employed this substrate for the measurement of kallikrein in rat stomach, human urine and rabbit kidneys.[14-18] A previous study in our laboratory indicated that the results of the assay of amidolytic activity correlated well

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with those of vasodilative and kinin-generating activities in the male rat urine.[19] However, it is unclear whether kallikrein is the only urinary enzyme which hydrolyzes Pro–Phe–Arg–MCA. The present study was designed, therefore, to determine the amidolytic activity as well as esterolytic activity of kallikrein and non-kallikrein(s) in fractions obtained by diethylaminoethyl (DEAE)-cellulose chromatography of the urine from male and female rats.

Materials and Methods

Chemicals——The following chemicals were commercially obtained: Pro–Phe–Arg–MCA and 7-amino-4-methylcoumarin (AMC) from the Protein Research Foundation (Minoh, Japan); TAME from Sigma Chemicals Co. (Saint Louis, U.S.A.); DEAE-cellulose (DE-52) from Whatman, Ltd. (Kent, U.K.). Other chemicals used were of reagent grade.

Urine Collection——Male (318 ± 7.8 g) and female (236 ± 4.4 g) Wistar rats, 9—10 weeks old, were used. For 1 week before the study, the rats were fed a standard laboratory chow, Oriental MF (Oriental Yeast, Tokyo, Japan) and provided with tap water *ad libitum*. The animals were placed in individual stainless-steel metabolic cages, and 24 h urine samples were collected in flasks containing toluene to prevent bacterial growth. 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 solid debris, dialyzed against distilled water at 4 °C for 24 h, and lyophilized.

DEAE-Cellulose Chromatography of Rat Urine——The lyophilized urine sample was dissolved in 0.01 M sodium phosphate buffer (pH 7.0) and applied to a DEAE-cellulose column (1.0 × 15 cm) previously equilibrated with the same buffer. The column was washed with 200 ml of the same buffer, then eluted with 200 ml of a linear gradient of 0—0.5 M NaCl in the same buffer. The flow rate was 40 ml/h and 4 ml fractions were collected.

Assays of Enzyme Activity——Enzyme activity in the urine and fractionated samples was assayed according to the method described previously.[19] Amidolytic activity was measured with Pro–Phe–Arg–MCA as the substrate. After incubation of samples with the substrate, the amount of AMC liberated from Pro–Phe–Arg–MCA was fluorometrically measured 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. Esterolytic activity was measured spectrophotometrically using TAME as the substrate. The amount of methanol liberated from the substrate was estimated from the calibration line obtained with standard methanol solutions (0—10 μmol/ml). One unit (EU) of the esterolytic activity was defined as the amount of the enzyme which hydrolyzed 1 μmol of TAME per min at 30 °C.

Statistical Analysis——Student's *t*-test was used to determine whether differences between male and female rats were significant. Differences were not considered significant if $p > 0.05$.

Results

Urine Volume, and Amidolytic and Esterolytic Activities in the Urine

As shown in Table I, no significant difference could be detected in urine volume per 100 g of body weight for 24 h between male and female rats. Esterolytic activity in the urine was significantly different ($p < 0.01$) between males and females, the value for males being 1.5 times that for females. On the other hand, amidolytic activity in the urine was significantly higher ($p < 0.001$) in females than in males.

TABLE I. Urine Volume, and Amidolytic and Esterolytic Activities in the Urine of Male and Female Rats

	Urine volume (ml/100 g/24 h)	Esterolytic activity (EU/100 g/24 h)	Amidolytic activity (AU/100 g/24 h)
Male	7.54 ± 0.9	2.90 ± 0.3	474 ± 46
Female	6.94 ± 1.2	1.92 ± 0.2[a]	744 ± 39[b]

All values are means ± S.E. of 10 rats.
a) Significantly different from the male value ($p < 0.01$).
b) Significantly different from the male value ($p < 0.001$).

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Correlation between Amidolytic and Esterolytic Activities in the Urine of Male and Female Rats

Figure 1 shows the correlation between amidolytic and esterolytic activities in urine samples from male and female rats. A significant positive correlation ($r=0.92, p<0.001$) was observed in female urine samples. The linear regression line passed through the vertical axis at -0.05 EU/ml, which was not significantly different from zero ($p>0.1$). Similarly, a positive correlation ($r=0.74, p<0.01$) was obtained with male urine samples, but the r value was lower than that of female urine samples. The y-intercept (0.24 EU/ml) of the linear regression line was significantly different from zero ($p<0.001$). These data suggest the existence in male rat urine of a non-kallikrein arginine esterase that has no amidolytic activity.

DEAE-Cellulose Chromatography of Rat Urine

Lyophilized urine samples from male and female rats were each chromatographed on a DEAE-cellulose column. As shown in Fig. 2(A), when the male rat urine was applied to the column, the chromatogram revealed three peaks of esterolytic activity. One of the three peaks appeared in the flow-through fraction (peak I), and the remaining two peaks were obtained at 0.05 M NaCl (peak II) and 0.25 M NaCl (peak III). On the other hand, most of the amidolytic activity was observed in peak III fractions, which showed chromatographic behavior of typical glandular kallikrein on the DEAE column. The remainder of this activity appeared in peak II fractions, and none was found in the fractions corresponding to peak I. From the elution profiles of these enzymes detected by the esterolytic assay, we found that peaks I, II and III qualitatively correspond to esterase A1 (EA1), esterase A2 (EA2) and kallikrein, respectively, obtained in male Sprague-Dawley rats by McPartland *et al.*[11] Similarly, a DEAE-cellulose chromatogram of female rat urine is shown in Fig. 2(B). Elution patterns of amidolytic and esterolytic activities in peak II (EA2) and III (kallikrein) fractions were similar to those obtained by the chromatography of male rat urine. However, peak I (EA1) fractions contained a very small amount of esterolytic activity as compared with the corresponding fractions of male rat urine.

Figure 3 illustrates the distribution patterns of amidolytic and esterolytic activities in EA1, EA2 and kallikrein fractions obtained by DEAE chromatography. The percent distribution was calculated from the recovered volumes and the enzyme activities. In male rat urine, $46\pm8\%$ of total esterolytic activity was recovered in the EA1 fractions, which had no amidolytic activity. The remainder was found in the EA2 ($29\pm5\%$) and kallikrein ($25\pm3\%$) fractions. On the other hand, $87\pm1\%$ of total amidolytic activity was observed in the kallikrein fractions, and the remainder was found in the EA2 fractions ($13\pm1\%$). In female

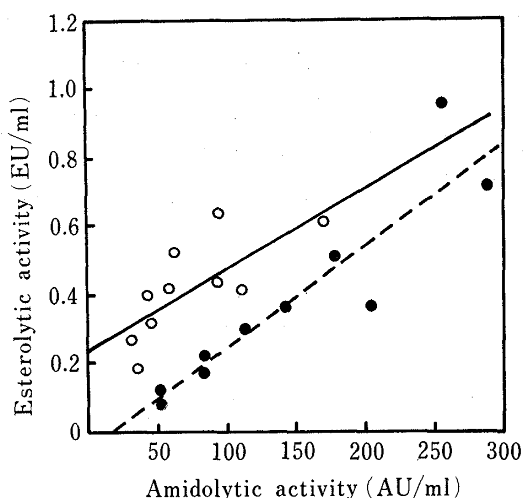

Fig. 1. Correlation between Amidolytic and Esterolytic Activities in the Urine of Male and Female Rats

Regression lines were obtained by the method of least mean squares. The equation for male rat urine is $y=0.0024x+0.24$, $r=0.74$, $p<0.01$. The equation for female rat urine is $y=0.0030x-0.05$, $r=0.92$, $p<0.001$.

—○—, male; ---●---, female.

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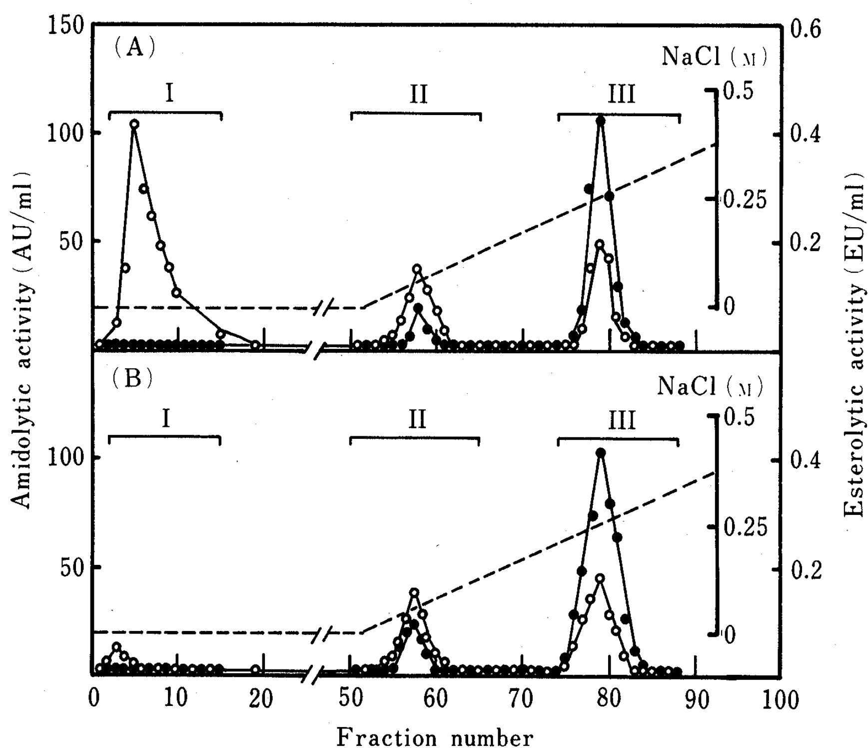

Fig. 2. DEAE-Cellulose Chromatographies of Urine Samples from Male and Female Rats

Urine samples (24 h) from male (A) and female (B) rats were applied to the column. Eluates were assayed for amidolytic (●) or esterolytic activity (○). (----), NaCl concentration.

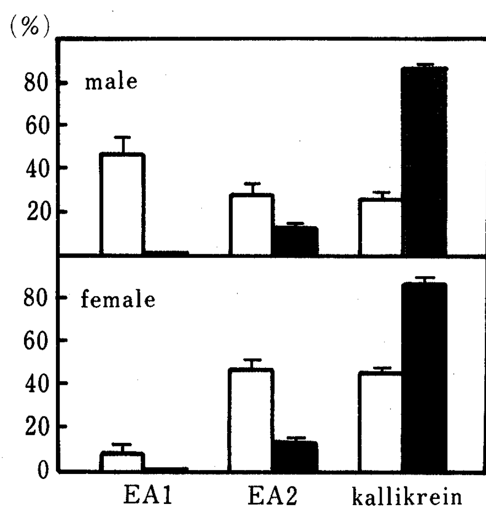

Fig. 3. Distribution Patterns of Amidolytic and Esterolytic Activities in Esterase A1, Esterase A2 and Kallikrein Fractions Following DEAE-Cellulose Chromatography of Rat Urine

Abscissa: esterase A1 (EA1), esterase A2 (EA2) and kallikrein fractions. Ordinate: percent of the total enzyme activity obtained from EA1, EA2 and kallikrein fractions. ▩, amidolytic activity; □, esterolytic activity.

rat urine, $8 \pm 4\%$ of total esterolytic activity was detected in the EA1 fractions. The remainder of this enzyme activity was found in the EA2 ($47 \pm 4\%$) and kallikrein ($45 \pm 2\%$) fractions. On the other hand, the percent distribution of amidolytic activity in these fractions was the same as that of males.

Discussion

The present DEAE-cellulose chromatography studies showed that kallikrein and two

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types of non-kallikreins (EA1 and EA2) are present in the rat urine, as reported by McPartland *et al.*[11] They demonstrated that kallikrein, EA1 and EA2 having esterolytic activity were found in male rat urine, whereas EA1 could not be detected in female rat urine. However, in the present study, a small amount of esterolytic activity was consistently observed in the EA1 fractions of female rats. This discrepancy might be due to the differences of experimental conditions: 1) the strain of rats used in this study was different from that used in their work, and 2) differences of assay procedures may have caused variations in the results for EA1. In their experiments, the residual TAME after incubation with the urine sample was measured as hydroxamate–ferric complex, whereas the methanol liberated from TAME was assayed in our experiments. Concerning this, Moriwaki *et al.*[20] have demonstrated that the latter assay method is 10 times more sensitive than the above hydroxamate method. Thus, it was assumed that both EA1 and EA2 were excreted into the urine in male and female rats.

The measurements of esterolytic activity in the three enzyme fractions after DEAE-cellulose chromatography revealed that the percent distribution of the activity was different in male and female rats. About half of the total activity was due to EA1 in males, while the activity of this enzyme was less than one-tenth of the total activity in females. On the other hand, approximately 25 and 45% of total activity were associated with EA2 in males and females, respectively. Similar percentage values were noted in the kallikrein fractions from male and female rats. These results indicate that at least half of the total esterolytic activity is associated with non-kallikrein esterases (EA1 and EA2). This implies a lack of specificity of esterolytic assay for the estimation of kallikrein enzyme in whole urine samples.

On the other hand, the measurements of amidolytic activity using Pro–Phe–Arg–MCA demonstrated that most of the total activity was recovered in the kallikrein fractions and the remainder was found in the EA2 fractions in both male and female rats. The EA1 fractions had no amidolytic activity in both sexes, indicating that this enzyme is not relevant to the results of urinary amidolytic assay. Recently, McPartland *et al.*[11] reported that the kinin-generating activity of kallikrein fractions was 12 times greater than that of EA2 fractions, although the EA1 fractions had no kinin-generating activity. In our previous study,[19] a good correlation ($r=0.99$) was obtained between the amidolytic and kinin-generating activities in male rat urine. In addition, we have preliminary data indicating that the kinin-generating activity per amidolytic unit in the kallikrein fractions is similar to that in the EA2 fractions (unpublished data). These results suggest that the assay of amidolytic activity correlates well with that of kinin-generating activity in the EA2 and kallikrein fractions.

On the basis of the above findings, we calculated the excretion rate of kallikrein from the recovered activity in the kallikrein fractions. The average kallikrein excretion rate of males was 412 AU or 0.73 EU per 100 g of body weight for 24 h, while that of females was 647 AU or 0.86 EU per 100 g of body weight for 24 h. Accordingly, it seems that urinary excretion of kallikrein in females is greater than in males. This sex difference in kallikrein excretion of Wistar strain rats agrees qualitatively with that obtained with Dahl salt-sensitive and salt-resistant rats.[12] The higher value of esterolytic activity of the whole urine in male rats relative to female rats (Table I) might be attributed to the higher urinary excretion of EA1. The contribution of EA1 to total esterolytic activity was also observed in the linear regression analysis for the male urine samples (Fig. 1); substantial esterolytic activity could be observed even when the amidolytic activity was zero.

The present study indicates that most of the urinary amidolytic activity is associated with kallikrein, in contrast to urinary esterolytic activity, in both sexes of Wistar rats and that EA1 is not relevant to the results of urinary amidolytic assay.

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