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Studies on Acidic Arginine Esterase Excreted in Urine. II. Purification and Some Properties of Human Urinary Arginine Esterase[1)]

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Human urinary arginine esterase-2 (HUAE-2) was purified to homogeneity mainly by chromatographic methods. About 2950-fold purification was achieved, with yield of 30% of the initial *N*-α-tosyl-L-arginine methyl ester (Tos–Arg–Me) hydrolyzing activity. The specific activity of the finally purified HUAE-2 was 2.07 μmol/min/A_{280} for Tos–Arg–Me. The esterolytic and amidolytic actions of this enzyme showed broad specificities for arginine and lysine derivatives as substrates, and the substrate specificity of this enzyme was clearly different from that of partially purified human urinary arginine esterase-1 (HUAE-1). Aprotinin strongly suppressed the Val–Leu–Arg–*p*NA amidolytic activity of HUAE-2, while ovomucoid trypsin inhibitor and lima bean trypsin inhibitor were less effective. The isoelectric point (pI value) and optimum pH of this enzyme were determined to be pI 4.5 and pH 8.5, respectively.

Keywords——human urine; arginine ester hydrolyzing enzyme; enzyme purification; isoelectric point; substrate specificity

Recently, we reported[2)] that two forms of Tos–Arg–Me hydrolyzing activities which were absorbed on diethylaminoethyl (DEAE)-cellulose existed in the human urine, and we suggested that these enzymes, which have no vasodilator activity, are hitherto unidentified arginine esterases. The contents of these two new arginine esterases corresponded to about 10 to 20% of total Tos–Arg–Me esterolytic activity in human urine.[2)] In the previous paper,[3)] we reported the purification and characterization of dog urinary arginine esterases (DUAE). Nustad and Pierce separated new arginine ester hydrolyzing enzyme(s) in rat urine, so called "Esterase A,"[4)] and some work was done on the characterization of these enzymes.[5)] However, the function of these arginine esterases in urine remains unknown.

In the present paper, we report the purification and some properties of human urinary arginine esterases (HUAE).

Materials and Methods

Human Mixed Urine——This was collected from inpatients of either sex at the Sanraku Hospital excluding patients with renal diseases.

Materials——The following chemicals and proteins were obtained commercially: Sephadex G-100 and Sepharose 4B (Pharmacia Fine Chemical Co., Sweden). DEAE-cellulose (Nakarai Chemical Co., Kyoto, Japan). Lima bean trypsin inhibitor, ovomucoid trypsin inhibitor, aprotinin, *N*-α-tosyl-L-arginine methyl ester (Tos–Arg–Me), acetyl-glycyl-L-lysine methyl ester (Ac–Gly–Lys–Me), acetyl-L-lysine methyl ester (Ac–Lys–Me) and *N*-α-tosyl-L-lysine methyl ester (Tos–Lys–Me) (Sigma Chemical Co., U.S.A.). *N*-α-Benzoyl-L-arginine methyl ester (Bz–Arg–

Me), *N*-α-carbobenzoxy-L-lysine methyl ester (CBZ–Lys–Me), *N*-α-benzoyl-L-citrulline methyl ester (Bz–Cit–Me), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (Bz–Arg–*p*NA), *N*-α-carbobenzoxy-L-arginine-*p*-nitroanilide (CBZ–Arg–*p*NA) and D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (Val–Leu–Lys–*p*NA) (Serva Chemical Co., West Germany). D-Prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide (Pro–Phe–Arg–*p*NA) and D-valyl-L-leucyl-L-arginine-*p*-nitroanilide (Val–Leu–Arg–*p*NA) (Kabi Chemical Co., Sweden). Carrier ampholyte (pH range of 3.5 to 5.0) (LKB Produkter AB, Sweden).

All other chemicals used were of analytical reagent grade.

Enzyme Assay——Esterolytic activity was assayed by the method of Moriwaki *et al.*[6] by using a colorimetric method with chromotropic acid at pH 8.0, 30 °C. Amidolytic activity was measured by a modification of the method reported by Amundsen *et al.*[7] at pH 8.0, 30 °C. All esterolytic and amidolytic activities were expressed in terms of μmol of substrate hydrolyzed per min. Vasodilator activity was assayed by the method of Moriya *et al.* monitoring the increase in arterial blood flow in a dog.[8]

Protein Concentration——The protein concentration was estimated by measuring the absorbance at 280 nm in a 1 cm width cuvette.

Electrophoresis——Disc gel electrophoresis was performed with 7.5% gel and the gel was stained for protein with 0.2% Coomassie Brilliant Blue. Densitometry was done using a Jookoo densitometer "Densitron model PAN" (Jookoo Co., Japan).

Isoelectric Focusing——This was done with the Ampholine system[9] using a carrier ampholyte with a pH range of 3.5 to 5.0, and electrophoresis was performed for 40 h with a constant 500 V.

Results

Purification of HUAE-2

Mixed urine from humans of either sex (47 l) was dialyzed against tap water and then adsorbed on DEAE-cellulose by a batch method. After 2 h of adsorption, the DEAE-cellulose was packed in a column, and the column was washed with 0.02 M Tris–HCl buffer at pH 7.5. Elution was done with the above-mentioned buffer containing 0.15 M NaCl; the eluate had Tos–Arg–Me esterolytic activity, but not vasodilator activity. Accordingly, this preparation contained a HUAE. This preparation was dialyzed against the same buffer and then applied to a DEAE-cellulose column (1.5 × 70 cm), pre-equilibrated with 0.02 M Tris–HCl buffer at pH 7.5. The enzyme was eluted with an increasing gradient of NaCl concentration from 0 to 0.3 M in the same buffer. Two peaks of Tos–Arg–Me esterolytic activity were separated by this chromatography, and the fractions of the former peak (HUAE-2) were collected and dialyzed against 0.05 M Tris–HCl buffer pH 7.5, containing 0.05 M NaCl. These procedures were described previously[2] and the results obtained in these experiments are in good agreement with those of our recent report.[2] The dialyzed HUAE-2 preparation was applied to an aprotinin Sepharose column (2 × 20 cm) and the column was washed with 0.05 M Tris–HCl buffer at pH 7.5. The initial eluate with 0.05 M Tris–HCl buffer at pH 7.5 containing 0.5 M NaCl showed no esterolytic activity towards Tos–Arg–Me. HUAE-2 was eluted with 0.002 N HCl solution as shown in Fig. 1A. The active fractions were combined and adjusted to pH 7.5. The HUAE-2 preparation was applied to a Sephadex G-100 column (2 × 50 cm), pre-equilibrated with 0.05 M Tris–HCl buffer at pH 7.5. The elution profile is illustrated in Fig. 1B. The active fractions were pooled and the specific activity of this preparation was 2.07 μmol/min/A_{280} of Tos–Arg–Me esterolysis.

The overall results of the purification of HUAE-2 are summarized in Table I. The specific activity, measured in terms of Tos–Arg–Me esterolysis, of purified HUAE-2 was 2950 times as much as that of the eluate after DEAE-cellulose adsorption, and the activity recovery was about 30%. The purified HUAE-2 preparation was observed as a single peak on densitometry after disc gel electrophoresis (Fig. 2).

Properties of HUAE-2

Isoelectric Point——The result of measurement of the isoelectric point (pI value) of HUAE-2 by using the Ampholine system at pH 3.5 to 5.0 is shown in Fig. 3; HUAE-2 was

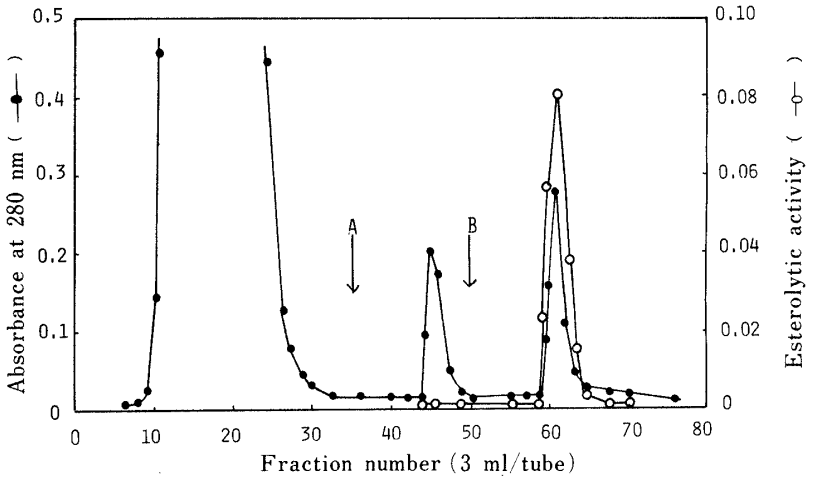

Fig. 1A. Elution Profile of HUAE-2 from Aprotinin Sepharose

A, eluted with 0.05 M Tris–HCl buffer at pH 7.5 containing 0.5 M NaCl; B, eluted with 0.002 N HCl solution. —●—, protein concentration; —○—, Tos–Arg–Me esterolytic activity. The other experimental details were given in the text.

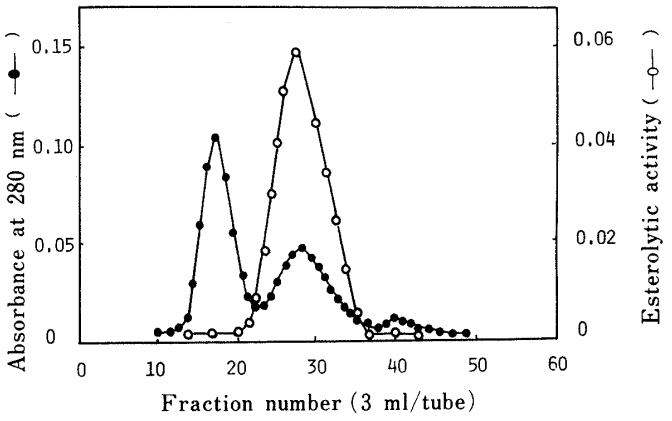

Fig. 1B. Gel Filtration of HUAE-2 on Sephadex G-100

—●—, protein concentration; —○—, Tos–Arg–Me esterolytic activity. See the text for other experimental details.

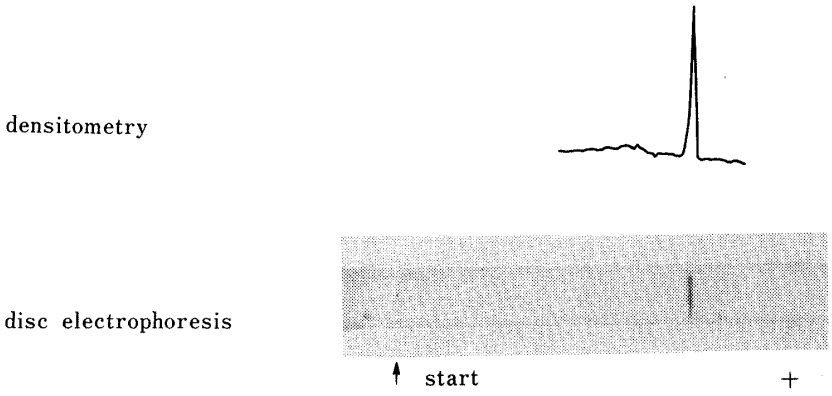

Fig. 2. Disc Gel Electrophoresis of Purified HUAE-2

found as a single Val–Leu–Arg–*p*NA amidolytic active peak having an isoelectric point of 4.5.

pH Dependency of Esterolytic Activity of HUAE-2——This was monitored by its esterolytic activity toward Tos–Arg–Me, and was determined with 0.08 M modified Britton–

TABLE I. Summary of Purification of HUAE-2 from 47 l of Human Urine

Procedure	Protein	Tos–Arg–Me esterolytic activity		
	Recovery (%)	Recovery (%)	Specific activity (μmol/min/A_{280})	p.f.[a]
DEAE-cellulose adsorption and elution	100	100	0.0007	1
DEAE-cellulose chromatography	31.7	67.6	0.0015	2.2
Aprotinin Sepharose affinity adsorption	0.021	33.5	1.19	1590
Sephadex G-100 gel filtration	0.010	30.3	2.07	2950

a) p.f.: purification factor.

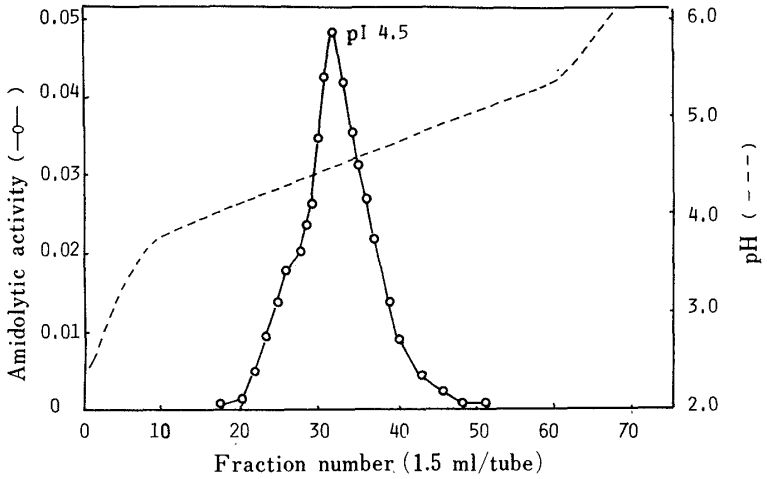

Fig. 3. Isoelectric Focusing of Purified HUAE-2

—○—, Val–Leu–Arg–*p*NA amidolytic activity; ----, pH.

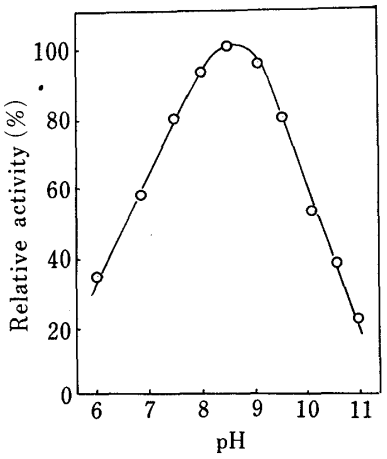

Fig. 4. pH Dependency on the Esterolytic Activity of HUAE-2

pH Dependency was measured for Tos–Arg–Me esterolysis. The activity was expressed as a percentage of that at the optimum pH.

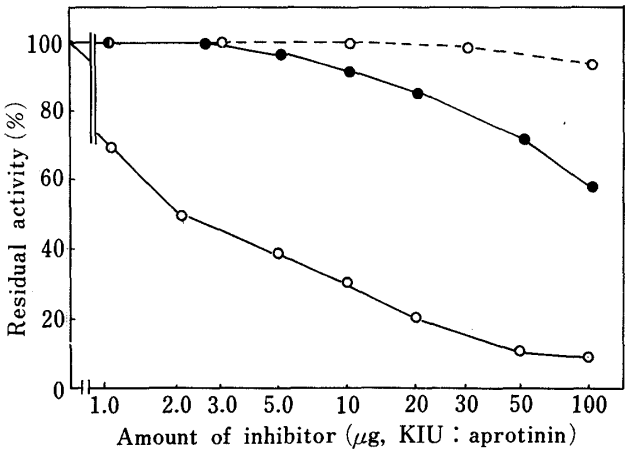

Fig. 5. Behavior of Proteinase Inhibitors towards HUAE-2

A mixture of the designated amounts of inhibitor (μg or kellikrein inhibitor unit (KIU)) and HUAE-2 (2.2×10^{-3} A_{280}) was preincubated at 30 °C, pH 8.0 for 10 min, and the remaining activity was assayed with Val–Leu–Arg–*p*NA as the substrate. The activity was expressed as a percentage of the control. ---○---, ovomucoid trypsin inhibitor; —●—, lima bean trypsin inhibitor; —○—, aprotinin.

TABLE II. Esterolytic and Amidolytic Activities of HUAE-2 and Partial Purified HUAE-1 towards Synthetic Arginine, Lysine and Citrulline Derivative Substrates

Substrate	HUAE-2		HUAE-1	DUAE-2[a]	Human urinary kallikrein[b]
	Activity	Ratio	Ratio	Ratio	Ratio
Tos–Arg–Me	2.07	1	1	1	1
Bz–Arg–Me	2.42	1.17	0.68	0.13	0.61
Tos–Lys–Me	2.05	0.97	0.39	1.46	0.27
Ac–Lys–Me	2.09	1.01	0.19	0.74	—[c]
Ac–Gly–Lys–Me	2.18	1.05	0.41	0.99	0.17
CBZ–Lys–Me	2.98	1.44	0.11	—[c]	—[c]
Bz–Cit–Me	3.50	1.69	0.29	—[c]	—[c]
Pro–Phe–Arg–*p*NA	0.401	0.193	0.0034	—[c]	0.005
Val–Leu–Arg–*p*NA	0.375	0.181	—[c]	0.48	0.041
Val–Leu–Lys–*p*NA	0.149	0.072	0.0087	—[c]	—[c]
Bz–Arg–*p*NA	0.097	0.047	—[c]	0.029	—[c]
CBZ–Arg–*p*NA	0.043	0.021	0.0006	—[c]	—[c]

The specific activity of HUAE-1 was 0.218 μmol/min/A_{280} of Tos–Arg–Me esterolysis. The concentrations of methyl ester and *p*-nitroanilide substrates were 2×10^{-2} and 10^{-3} M, respectively. The activities were expressed in terms of μmol of substrate hydrolyzed/min/A_{280}, and the ratio of the activity was given relative to standard Tos–Arg–Me. *a*) Data from ref. 3. *b*) Data from ref. 10. *c*) Not measured.

Robinson's wide range buffer from pH 6.0 to 11.0. The maximum relative Tos–Arg–Me esterolytic activity of HUAE-2 was found at pH 8.5, as shown in Fig. 4.

Behavior of Proteinase Inhibitors——The effects of several proteinase inhibitors, aprotinin, lima bean trypsin inhibitor and ovomucoid trypsin inhibitor, on the Val–Leu–Arg–*p*NA amidolytic activity of HUAE-2 were examined after preincubation at 30 °C for 10 min, pH 8.0, and the results are shown in Fig. 5. Aprotinin strongly suppressed the amidolytic activity of HUAE-2. The other two trypsin inhibitors were less inhibitory towards HUAE-2.

Substrate Specificity——Esterolytic and amidolytic actions of purified HUAE-2 and partially purified HUAE-1 towards mainly synthetic arginine and lysine derivatives as substrates were investigated, and the results are summarized in Table II. The substrate specificities of DUAE-2 and human urinary kallikrein are also shown for reference.[3,10] Among the substrates examined, esterolytic substrates, especially Bz–Cit–Me and CBZ–Lys–Me, were easily hydrolyzed by HUAE-2, and the most effective substrate for HUAE-1 was Tos–Arg–Me. The hydrolytic activities of HUAE-1 and -2 towards amidolytic substrates were lower than those towards esterolytic activities.

Discussion

In a previous report,[2] we showed that two new non-kallikrein arginine ester hydrolyzing enzymes called HUAE-1 and HUAE-2 were present in human urine, and the levels of these enzymes (total HUAE), determined by measuring Tos–Arg–Me esterolysis at pH 8.0, 30 °C, were found to be 0.34 to 0.86 nmol/min/ml of urine ($n=12$), accounting for about 10 to 20% of the total urinary activities. The ratio of HUAE-1 and -2 in human urine was not clear because of the presence of individual differences. It was found that the estimated approximate molecular weights of partially purified HUAE-1 ($M_r = 4.8 \times 10^4$ daltons) and -2 ($M_r = 3.0 \times 10^4$ daltons) were distinguishable.[2] Two forms of non-kallikrein arginine ester hydrolyzing enzymes in the urine, which were adsorbed on an anion exchanger, were also found in dog[3] and rat.[4,5]

The specific activity of finally purified HUAE-2 as a homogeneous preparation (Fig. 2) was 2.07 μmol/min/A_{280} of Tos–Arg–Me esterolysis; this value was 6 times lower than that of DUAE-2, which is a similar type of enzyme in dog urine,[3] and 7 times lower than that of human urinary kallikrein.[10,11] The substrate specificity of purified HUAE-2 towards synthetic arginine, lysine and citrulline derivatives as substrates was clearly different from those of partially purified HUAE-1, DUAE-2[3] and human urinary kallikrein,[10] and also from those of bovine trypsin, bovine plasmin and hog sperma acrosin.[10] The behavior of proteinase inhibitors with the present enzyme was in contrast to that with DUAE-2. The amidolytic activity with Val–Leu–Arg–*p*NA of the present enzyme was strongly suppressed by aprotinin, while this inhibitor had little effect on DUAE-2.[4] These result might suggest that the catalytic action of HUAE-2 is different from those of DUAE-2, though the isoelectric points of both enzymes were pI 4.5 (Fig. 6).

McPartland *et al.* showed[5a] that the level of rat urinary arginine esterase A-1[4] was dependent upon the sex of the animal, and they also reported that the activity in male urine was stimulated by administration of testosterone.[5a] We found that the content of HUAE was increased significantly in partients with primary aldosteronism,[12] as was also found with human urinary kallikrein.[13,14] These results suggest that the non-kallikrein arginine esterases in the urine are related to the function of the kidney.

References and Notes

1) Abbreviations: HUAE-2, human urinary arginine esterase-2; Tos–Arg–Me, *N*-α-tosyl-L-arginine methyl ester; Bz–Arg–Me, *N*-α-benzoyl-L-arginine methyl ester; Tos–Lys–Me, *N*-α-tosyl-L-lysine methyl ester; Ac–Lys–Me, acetyl-L-lysine methyl ester; Ac–Gly–Lys–Me, acetyl-glycyl-L-lysine methyl ester; CBZ–Lys–Me, *N*-α-carbobenzoxy-L-lysine methyl ester; Bz–Cit–Me, *N*-α-benzoyl-L-citrulline methyl ester; Pro–Phe–Arg–*p*NA, D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide; Val–Leu–Arg–*p*NA, D-valyl-L-leucyl-L-arginine-*p*-nitroanilide; Val–Leu–Lys–*p*NA, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; Bz–Arg–*p*NA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide; CBZ–Arg–*p*NA, *N*-α-carbobenzoxy-L-arginine-*p*-nitroanilide.
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