Evidence of Two Forms of Basic Arginine Esterases in Human Male Urine

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Two forms of basic arginine ester hydrolyzing enzymes were found in human male urine, and their partial purification was performed using lima bean trypsin inhibitor (LBTI) affinity adsorption and Cellulofine GCL-2000 gel chromatography. The forms tentatively called basic human urinary arginine esterase-1 (BHUAE-1) and -2 (BHUAE-2), had respective estimated molecular weights of about 4.5×10^4 and 1.8×10^4 daltons, with pH optima observed at 9.5 and 10.0. These two new enzymes in the human male urine were different from human renal urokinases.

Keywords human male urine; esterolytic enzyme; amidolytic enzyme; *N*-α-tosyl-L-arginine methyl ester; urokinase

Introduction

Human urine contains the proteolytic enzymes kallikrein (EC 3.4.21.35) and urokinase (EC 3.4.21.31), and the properties of these enzymes were clarified recently.[1,2] We reported previously[3] the finding of two forms of acidic arginine ester hydrolyzing enzymes, called human urinary arginine esterase-1 (HUAE-1) and -2 (HUAE-2) excreted in the human urine, and HUAE-2 was purified and characterized.[4]

McPartland *et al.*[5] reported the basic arginine ester hydrolyzing enzyme, called Esterase A-1, is only excreted in the male urine. The purification and characterization of this enzyme was described by Nakamura *et al.*,[6] who did not find this enzyme activity in the rat male urine collected from the bladder or ureter.[6] We reported on a similar enzyme in the human male urine,[7] and the content level of basic human urinary arginine esterase (BHUAE) was calculated to be about only 3 per cent or less of the total *N*-α-tosyl-L-arginine methyl ester (Tos–Arg–Me) hydrolyzing activity in the human male urine.[7]

The present paper describes some properties of two forms of BHUAE, comparing them with urokinase.

Materials and Methods

Human Mixed Male Urine This was freshly collected from inpatients at Sanraku Hospital excluding those with renal diseases, and dialyzed against tap water overnight. Precipitates appearing during the dialysis were removed by centrifugation and the supernatant was used in these studies.

Materials Formyl Cellulofine and Cellulofine GCL-2000 were obtained from Seikagaku Kogyo Co., Tokyo, Japan. Urokinase (human kidney), *N*-α-tosyl-L-arginine methyl ester (Tos–Arg–Me), acetyl-glycyl-L-lysine methyl ester (Ac–Gly–Lys–Me), cytochrome c, soybean trypsin inhibitor (SBTI), lima bean trypsin inhibitor (LBTI), egg white albumin and bovine serum albumin were purchased from Sigma Chemical Co., U.S.A. D-Valyl-L-leucyl-L-arginine-*p*-nitroanilide (Val–Leu–Arg–*p*NA), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (Bz–Arg–*p*NA) and human fibrinogen were obtained from Kabi Chemical Co., Sweden. D-Valyl-L-leucyl-L-lysine-*p*-nitroanilide (Val–Leu–Lys–*p*NA), benzoyl-L-phenylalanyl-L-leucyl-L-arginine-*p*-nitroanilide (Bz–Phe–Val–Arg–*p*NA), succinyl-L-alanyl-L-alanyl-L-prolyl-L-leucine-*p*-nitroanilide (Suc–$(Ala)_2$–Pro–Leu–*p*NA), *N*-α-benzoyl-DL-lysine-*p*-nitroanilide (Bz–Lys–*p*NA) and benzoyl-L-citruline methyl esters (Bz–Cit–Me) were products of Serva Chemicals, Germany. Carboxymethyl (CM)-cellulose was obtained from Nakarai Chemical Co., Kyoto, Japan. Human thrombin was from Mochida Pharmaceutical Co., Japan.

All other chemicals and materials used were of analytical reagent grades.

Enzyme Assay Esterolytic and amidolytic activities were assayed using basic amino acid derivatives as substrates by the methods of Moriwaki *et al.*[8] and Amundsen *et al.*[9] with substrate concentration slightly modified of 10 and 0.5 mM, respectively, in 0.05 M Tris–HCl buffer containing 0.15 M NaCl at pH 8.0 for 30 °C. All esterolytic and amidolytic activities are shown in terms of pmol or nmol of substrate hydrolyzed per min (pmol/min or nmol/min), under the above conditions.

The fibrinolytic and plasminogen activator activities were assayed qualitatively to make a comparison with standard plasmin and urokinase, respectively, by a slightly modified method of Mullerz and Lasson.[10]

Protein Concentration Protein concentration was estimated to determine the absorbance at 280 nm in a 1 cm width cuvette.

Results

Separation and Partial Purification of BHUAE We used 35 l of mixed human male urine in this experiment.

The dialyzed mixed urine was diluted with deionized water (1.5 mS/cm or less), and then adsorbed on a CM-cellulose by a batch method. After 2 h of adsorbtion, the CM-cellulose was packed in a column and washed with 0.01 M phosphate buffer at pH 7.0 (buffer A). Solution which contained Tos–Arg–Me esterolytic activity was eluted with buffer A containing 0.5 M NaCl. This solution (with pH adjusted to 8.0) was applied to a lima bean trypsin inhibitor (LBTI) cellulofine column, pre-equilibrated with 0.02 M Tris–HCl buffer at pH 8.0 (buffer B), and the column was washed with the buffer. The elution was made with HCl solution at pH 2.0 (a profile of this procedure is shown in Fig. 1). The fractions containing Tos–Arg–Me esterolytic activity were collected. The enzyme preparation obtained from this procedure was tentatively called BHUAE. Thereafter pH was adjusted to 8.0, the BHUAE preparation was applied to a Cellulofine GCL-2000 column, pre-equilibrated with buffer B containing 0.15 M NaCl. The elution profile is shown

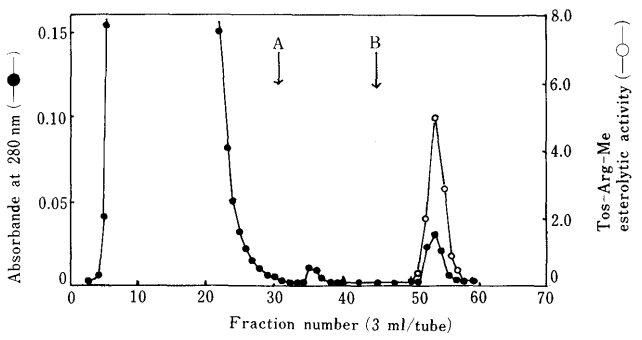

Fig. 1. Lima Bean Trypsin Inhibitor Affinity Adsorption and Elution of BHUAE

A: Elution with 0.02 M Tris–HCl buffer at pH 8.0. B: Elution with HCl solution at pH 2.0. —●—, absorbance at 280 nm; —○—, Tos–Arg–Me esterolytic activity (nmol/min/ml).

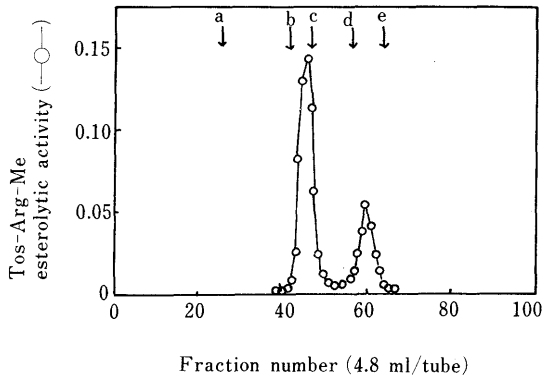

Fig. 2. Cellulofine GCL-2000 Gel Filtration of BHUAE

a, b, c, d, and e indicate the eluted fractions of the blue Dextran, Bovine serum albumin, egg white albumin, soy bean trypsin inhibitor and cytochrome c, respectively —○—, Tos-Arg-Me esterolytic activity (nmol/min/ml).

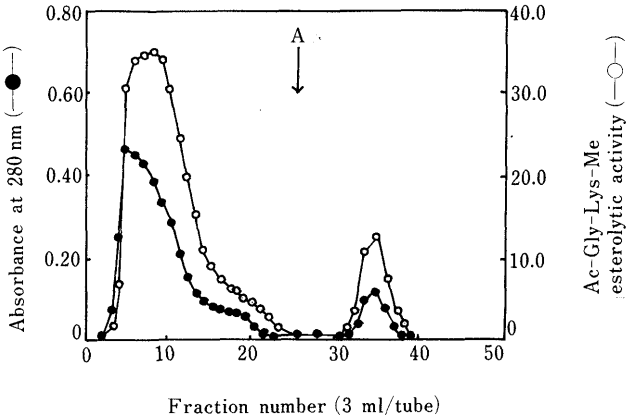

Fig. 3. Lima Bean Trypsin Inhibitor Affinity Adsorption and Elution of Human Kidney Urokinase

A: Elution with HCl solution at pH 2.0. —●—, absorbance at 280 nm; —○—, Ac-Gly-Lys-Me esterolytic activity (nmol/min/ml).

in Fig. 2; two peaks of Tos-Arg-Me esterolytic activity are observed in this chromatography, the first tentatively called BHUAE-1 and the latter BHUAE-2. Their respective approximate molecular weights were estimated to be about 4.5×10^4 and 1.8×10^4 daltons (Da), by means of the above mentioned Cellulofine GCL-2000 gel filtration following the method of Andrews.[11]

Lima Bean Trypsin Inhibitor Affinity Adsorption and Elution, and Cellulofine GCL-2000 Gel Filtration of Urokinase A commercially available urokinase preparation from human kidney including high and low molecular weight forms was dissolved in buffer B, and then was applied to a LBTI cellulofine column, pre-equilibrated with the buffer. The column was washed with buffer B, and the elution was done with HCl solution at pH 2.0. Two forms of Ac-Gly-Lys-Me esterolytic enzyme were found, both in the non-adsorbed fraction (preparation A) and eluted fractions with HCl (preparation B) as shown in Fig. 3. Each preparation (A and B) was separately applied to a Cellulofine GCL-2000 gel column, pre-equilibrated with buffer B containing 0.15 M NaCl. Figure 4 shows the result of chromatograms of preparation A (above) and preparation B (below). Only one peak of the Ac-Gly-Lys-Me esterolytic activity was found in the gel chromatography of each preparations A and B. The approximate molecular

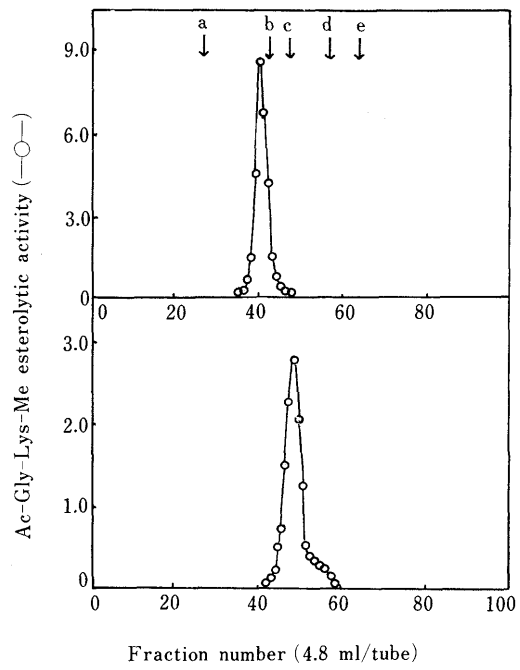

Fig. 4. Cellulofine GCL-2000 Gel Filtration of Preparations A (Above) and B (Below) of Human Kidney Urokinase Separated from Lima Bean Trypsin Inhibitor Affinity Adsorption and Elution

a, b, c, d and e indicate the eluted fraction of the blue dextran, bovine serum albumin, egg white albumin, soybean trypsin inhibitor and cytochrome c, respectively. —○—, Ac-Gly-Lys-Me esterolytic activity (nmol/min/ml).

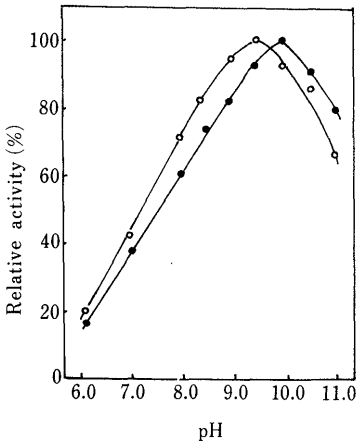

Fig. 5. pH Optimum of BHUAE-1 and -2

pH optimum of BHUAE-1 (—○—) and -2 (—●—) were measured using the substrate with Val-Leu-Arg-*p*NA at various pH's in modified Britton-Robinson's wide range buffer (0.08 M). The activity was expressed as a percentage of that at optimum pH.

weights of two enzymes having Ac-Gly-Lys-Me esterolytic activity in preparations A and B from human kidney urokinase were estimated to be about 5×10^4 and 3×10^4 Da, respectively, calculated by the method of Andrews[11] using the above gel chromatography (Fig. 4); these molecular weights corresponded well with the high and low molecular weights of urokinases from human urine.[12]

Optimum pH of BHUAE The optimum pH was monitored by its amidolytic activity towards Val-Leu-Arg-*p*NA in modified Britton-Robinson's wide range buffer (0.08 M) from pH 6.0 through 11.0. As can be seen in Fig. 6, the apparent optimum pH of BHUAE-1 and -2 was found at 9.5 and 10.0, respectively.

TABLE I. Substrate Specificity of HUAE-1, -2, Urokinase Preparations A and B

Substrate	Enzyme			
	Urokinase		BHUAE	
	Preparation A	Preparation B	BHUAE-1	BHUAE-2
Val–Leu–Arg–*p*NA	1.0	1.0	1.0	1.0
Val–Leu–Lys–*p*NA	0.58	0.49	0.13	0.04
Bz–Phe–Val–Arg–*p*NA	0.20	0.35	0.48	0.62
Bz–DL-Arg–*p*NA	0.15	3.9	0.17	1.36
Bz–DL-Lys–*p*NA	0.13	3.1	0.07	0.75
Suc–$(Ala)_2$–Leu–Pro–*p*NA	0.20	0.20	0.42	0.36

The ratio of activity was given relative to standard Val–Leu–Arg–*p*NA. Val–Leu–Arg–*p*NA activity of BHUAE-1, -2 and urokinase preparations A and B was 11.2, 5.3, 90.0 and 13.6 pmol/min/ml, respectively.

Substrate Specificity The ability of amidolysis of the present enzymes and two preparations of urokinases obtained in this investigation primarily toward basic amino acid derivatives as substrate were measured, and the results are summarized in Table I. The profiles of the substrate specificity spectrum of BHUAE-1 and -2 did not agree with the respective ones of preparations A and B from urokinase. However, the best substrates for the present two enzymes were the same as those of urokinase preparations A and B, respectively. The best substrate of BHUAE-1 and urokinase preparation A was found to be Val–Leu–Arg–*p*NA, and Bz–DL-Arg–*p*NA was the best one for BHUAE-2 and urokinase preparation B among those tested. Val–Leu–Lys–*p*NA was also a good substrate for urokinase preparation A, but was not good for BHUAE-1. The ratio of activities against substrates Val–Leu–Arg–*p*NA *versus* Val–Leu–Lys–*p*NA of the four enzymes differed.

Plasmin and Plasminogen Activator Activities of BHUAE Non-plasminogen activator activity was observed by BHUAE-1 and -2, but these enzymes showed plasmin-like activity.

Discussion

In the present investigation, we found that two forms of basic arginine ester (amido) hydrolyzing enzymes, which were separated by gel filtration on a Cellulofine GCL-2000 column (Fig. 2), were excreted in the human male urine; these eluted preparations were tentatively called BHUAE-1 and BHUAE-2. The results of our experiments showed that the newly found basic arginine esterases in the male urine of the human (present study) are clearly different from the esterase A-1 in rat male urine,[5,6] because in rat esterase A-1 is one form.

Using lima bean trypsin inhibitor affinity adsorption and elution of urokinase (Fig. 3), the urokinase from human kidney was separated into a non-adsorbed preparation (preparation A) and HCl eluted fractions (preparation B). The molecular weights of preparations A and B, estimated by Cellulofine GCL-2000 gel filtration, were in good agreement with the high and low molecular weights of urokinases from human urine,[12] respectively. However, the two forms of new basic arginine esterases found in human male urine were adsorbed well to the lima bean trypsin inhibitor affinity column (Fig. 1), and their molecular weights (Fig. 2) differed from the human urinary urokinases.[12] The optimum pH of the present enzymes also differed and were higher than the human urokinases having optimum pH of 7.8 when Ac–Gly–Lys–Me was used as the substrate.[13] Different substrate specificity spectrum among these four enzymes were observed in this investigation (Table I). Furthermore, the two new enzymes have no plasminogen activator activity, but did show weak plasmin-like activity. All these results might indicate that the newly recognized basic arginine esterases BHUAE-1 and -2 are different from human urokinases.

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