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Inhibitors of Acrosin and SH-Protease in Normal Human Urine (41209)

HIROYUKI SUMI*,[1] AND NAOTIKA TOKI†,[2]

*Biochemistry Section, Michael Reese Research Foundation, Chicago, Illinois 60616, and †Department of Dermatology, Hiroshima University School of Medicine, Kasumi 1-2-3, Hiroshima-734, Japan

Abstract. Trypsin inhibitors isolated from human urine and highly purified by affinity chromatography displayed molecular weights of 43,000 (H-UTI) and 22,000 (L-UTI) during sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These molecules not only inhibited trypsin [EC 3.4.21.4], chymotrypsin [EC 3.4.21.1], and plasmin [EC 3.4.21.7], but also showed strong inhibitory effects on the human sperm enzyme, acrosin [EC 3.4.21.10]. A novel protease inhibitor (anticin) which specifically reacted with SH-proteases, such as ficin [EC 3.4.22.3], papain [EC 3.4.22.2], and bromelain [EC 3.4.22.4], was also isolated from normal human urine.

Since the work reported by Bauer and Reich (1), many researchers have purified urinary trypsin inhibitors (UTI) of varying molecular weights, and most used acid treatment as the first step of concentration. Schulman (2) and Astrup *et al.* (3, 4) used trichloroacetic acid or hydrochloric acid, followed by alcohol and acetone precipitations, to isolate UTIs with molecular weights of 17,000 and 28,500, respectively. The inhibitors were active against trypsin [EC 3.4.21.4], chymotrypsin [EC 3.4.21.1], plasmin [EC 3.3.21.7], and plasma coagulation enzymes. Hochstrasser *et al.* (5) isolated two acid-stable UTIs with molecular weights of 22,000 and 44,000 from human urine after deproteinization by selective binding to a trypsin–cellulose resin. More recently Kessner *et al.* (6) used a chymotrypsin–agarose preparation for the purification, and obtained a UTI from normal human urine with a molecular weight of 28,500.

Acid treatment of urine may create modification of UTI molecules. Proksch *et al.* (7, 8) purified a UTI with high molecular weight (70,000) by DEAE-cellulose chromatography followed by gel filtration. They found that it is a heat-stable glycoprotein composed of 10% carbohydrate and possesses an immunological relationship with plasma inter-α-trypsin inhibitors. By using Arg–Sepharose column and gel filtration methods, we also confirmed the presence of one form of UTI (UTI-I; 67,000 MW) with high molecular weight by gel filtration in normal human urine, and found that it could be transformed into lower-molecular-weight forms of 45,000 (UTI-II) and 23,000 (UTI-III) following treatment with acid (9, 10). The weight modification was thought to be attributable to a pepsin-like enzyme present in urine which could be activated in acid conditions less than pH 3.5. Such enzymatic modifications were also confirmed by the treatment of purified UTI-I with papain and pronase (11, 12). The present report discusses the studies made on the inhibitory spectra of the two different types of highly purified UTIs and an SH-protease inhibitor that was first isolated from normal human urine.

Materials and Methods. *Substrates and enzymes.* N^{α}-tosyl-L-arginine methyl ester (TAMe), *N*-tosyl-L-tyrosine ethyl ester (ATEe), N^{α}-acetylglycyl-L-lysine methyl ester (AGLMe), *N*-acetyl-L-arginine methyl ester (AAMe), and N^{α}-benzoyl-L-arginine ethyl ester (BAEe) were purchased from the Foundation for Promotion of Protein Research, Osaka. Bovine trypsin (type II), α-chymotrypsin (type II), hog pancreas elastase [EC 3.4.21.11] (type II), hog stomach pepsin [EC 3.4.23.1], papain [EC

[1] Present address: Department of Physiology, Miyazaki Medical College, Miyazaki 889-16, Japan.

[2] To whom correspondence and requests for reprints should be sent at: Department of Dermatology, Hiroshima University School of Medicine, Kasumi 1-2-3, Hiroshima-734, Japan.

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3.4.22.2], and bromelain [EC 3.4.22.4] were products of Sigma Chemical Company, St. Louis, Missouri. Ficin [EC 3.4.22.3] was acquired from Wako Pure Chemical Company, Osaka. Bovine thrombin [EC 3.4.21.5] for topical use was purchased from Mochida Pharmaceutical Company, Tokyo. Collagenase [EC 3.4.24.3] (*Clostridium histolyticum;* 1000 Mandl U/mg protein) was obtained from Seikagaku Kogyo Company, Tokyo. Human urinary kallikrein (40 KU/mg protein) and plasmin activated by urokinase (Midori PL-30) and urokinase (54,000 MW form, 60,000 IU/mg protein) were kindly supplied by The Green Cross Company, Osaka. Human plasma kallikrein activated by acetone was prepared as described by Tsutsumi (13); 1 mg of this preparation hydrolyzed 360 μmole of TAMe in 0.1 *M* Tris–HCl buffer, pH 8.5, in 30 min at 37°. The first components of the complement, $C\bar{1}r$ and $C\bar{1}s$, were purified from human plasma as described previously (14, 15); 1 mg of $C\bar{1}r$ hydrolyzed 81.0 μmole of AAMe and $C\bar{1}s$ hydrolyzed 1500 μmole of ATEe in 0.1 *M* phosphate buffer, pH 7.4, in 30 min at 37°. Human acrosin (1100 mU/mg protein) was purified by the method of Zaneveld *et al.* (16) except that Sephadex G-75 (fine, Pharmacia) was used for gel filtration in leiu of Sephadex G-50 gel. Lyophilized urine concentrate (273.8 U of UTI/mg protein) was prepared from normal human urine by the method of Sumi *et al.* (9, 17) using Arg–Sepharose.

Assay methods. UTI activity was determined by caseinolysis as described previously (9, 10). One unit of UTI activity was defined as that amount required to inhibit 1 μg of commercial trypsin (Sigma Chemical Co., type II); 42.3% was active by p-nitrophenyl-p-guanidinobenzoate titration (18). SH-protease inhibitor was assayed by a caseinolytic technique (15, 19), except that 0.1 *M* phosphate buffer containing 5 m*M* cysteine (pH 7.4) was used for the buffer; 0.1 ml of enzyme solution (30 μg of commercial ficin in 0.1 *M* phosphate buffer containing 5 m*M* cysteine, pH 7.4) and inhibitor solution was preincubated for 5 min at 37°, and then 0.25 ml of 8% casein solution (Hammarsten) was added (final volume; 1.0 ml). After 30 min at 37°, the reaction was stopped with 1.5 ml of 18% perchloric acid and the peptides released were determined by the reaction of Lowry *et al.* (20). One unit of SH-protease inhibitor was defined as that amount required to inhibit 1 μg of commercial ficin and was usually calculated from the amount causing 50% inhibition (30 μg of ficin without inhibitor produced A_{750} of approximately 0.75 under the assay conditions). The caseinolytic activities of other enzymes were also determined by Lowry's method (20) as reported previously (15, 19) using 2.0% casein substrate in 0.1 *M* phosphate buffer with (for SH-protease) or without 5 m*M* cysteine. Esterase activities on TAMe, ATEe, and AAMe were determined by Hestrin's method as modified by Roberts (21) (substrate concentration of 10 m*M* in 0.1 *M* phosphate buffer, pH 7.4). Acrosin activity was determined using BAEe as substrate by the method of Zaneveld *et al.* (16). One unit was defined as the amount of enzyme which caused a change in absorbance of 1.0/min at 253 nm under the assay conditions (0.05 *M* borate buffer containing 0.05 *M* $CaCl_2$, pH 8.0, and final volume 3.0 ml). Collagenase activity was determined by the method of Mandl (22) using collagen (Sigma Chemical Co.) as substrate. Elastase activity was determined by the method of Shotton (23) using congo red-elastin (Sigma Chemical Co.) as substrate. Pepsin activity was determined by the method of Umezawa (24) using casein as substrate. Kinin-forming activity of kallikrein was determined as described previously (13, 15, 19). To measure inhibitory activities, enzyme/inhibitor mixtures, except for those involving acrosin, were preincubated at 37° for 5 min after which residual enzyme activities were determined as described above. Acrosin inhibition was measured by preincubation at room temperature (22–23°) for 15 min. Protein concentration was determined by the method of Lowry *et al.* (20) using bovine serum albumin (Armour Pharmaceutical Co.) as a standard.

Miscellaneous techniques. Immunoelectrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–

PAGE) and double diffusion in agar gels were performed as described previously (25). Molecular weights were determined using SDS–PAGE from a simultaneous run of a number of reference proteins which included bovine serum albumin (67,000 MW), egg albumin (45,000 MW), chymotrypsinogen A (25,000 MW), and cytochrome C (13,500 MW). Trypsin–Sepharose resin was prepared as described previously; trypsin was coupled to Sepharose 4B (Pharmacia) with cyanogen bromide as described by Cuatrecasas (26) and mentioned in a previous report (150 mg of trypsin and 20 ml of Sepharose suspension were used) (10). The material obtained was washed thoroughly with 0.01 *N* HCl containing 0.2 *M* NaCl followed by phosphate–NaCl buffer (0.1 *M* phosphate containing 0.2 *M* NaCl, pH 7.4), and stored at 4° until used.

Results and Discussion. *Purification of UTI.* Twenty five milligrams of urine concentrate was dissolved in 10 ml of distilled water and dialyzed against phosphate–NaCl buffer and then applied to a trypsin–Sepharose column (2.0 × 7.5 cm). As shown in Fig. 1, after washing with the same buffer, UTI was eluted with 0.01 *N* HCl containing 0.2 *M* NaCl. The active fractions (Tubes 23–27; Fig. 1B) were collected and neutralized to pH 7.4 with 0.1 *N* NaOH. The same procedures were repeated three times and UTI fractions were mixed. After concentrating the fractions to about 2.0 ml by ultrafiltration (PM 10 membrane; Amicon Corp.) they were subjected to Sephadex G-100 gel filtration (fine, Pharmacia; 1.0 × 152 cm). Elution was performed with 0.15 *M* ammonium bicarbonate at a rate of 5.0 ml/hr, and 2.0-ml fractions were collected. Two peaks of UTI activity (H-UTI; Tubes 19–26, L-UTI; Tubes 33–39), which corresponded to molecular weights of about 67,000 and 23,000, were detected. These fractions were concentrated to about 2.0 ml by ultrafiltration followed by rechromatography on Sephadex G-100 gel filtration under the same conditions. By this procedure, highly purified UTIs with specific activities of about 1090 U/mg protein (H-UTI) and 1230 U/mg protein (L-UTI) were obtained.

The molecular weights calculated by SDS–PAGE (Fig. 2) were about 43,000 ± 2000 for H-UTI and 22,000 ± 2000 for L-UTI, respectively. The latter weight corresponded closely with that determined by gel filtration, but the former was much less. This phenomenon may be attributable to

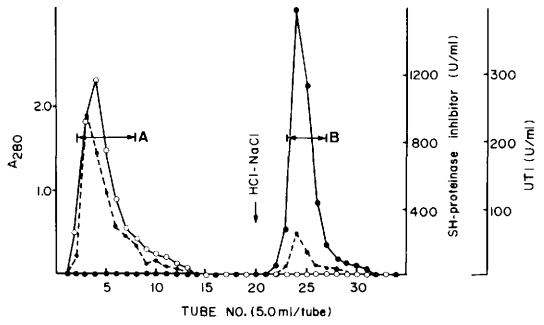

FIG. 1. Elution profile of urinary protein concentrate obtained by trypsin–Sepharose. Column; 2.0 ×7.5 cm, sample; 25 mg in 10 ml, fractions; 5.0 ml, flow rate; 17 ml/hr. Starting buffer was 0.2 *M* NaCl in 0.1 *M* phosphate buffer (pH 7.4). Elution was started at the vertical arrow with 0.2 *M* NaCl in 0.01 *N* HCl. The fractions pooled were indicated by horizontal arrows. ●—●, protein (A_{280}), ●——●, UTI (U/ml), ○——○, SH-protease inhibitor (U/ml).

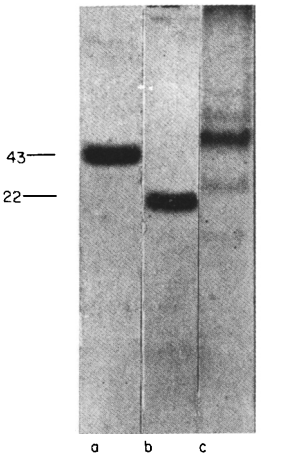

FIG. 2. SDS–polyacrylamide gel electrophoresis of purified UTIs and SH-protease inhibitor. Sample diluent consisted of 6.0 *M* urea, 1.0% SDS, and 0.1 *M* phosphate buffer, pH 7.4. The numbers on the left indicate the molecular weight in thousands. (a) H-UTI, (b) L-UTI, and (c) SH-protease inhibitor, respectively.

the difference in carbohydrate content of each molecule of the inhibitor. As reported previously (9, 10), the authors demonstrated the presence of a native UTI with a molecular weight of 67,000 (UTI-I) in normal human urine, and two modified forms with low molecular weights of 45,000 (UTI-II) and 23,000 (UTI-III) following treatment of urine with acid. The molecular weight of H-UTI by gel filtration was identical to that of the native UTI, whereas the molecular weight of L-UTI was close to that of UTI-III or that reported by Hochstrasser *et al.* (22,000 MW) (5)). Recently, Kessner *et al.* (6) used a chymotrypsin–agarose instead of a trypsin–agarose preparation for purification of UTI. They applied the urine sample directly on the column and obtained one low-molecular-weight form of UTI (28,500 MW). Considering the easy transformation of UTI molecules to lower-molecular-weight forms by enzyme treatment (11, 12), and the fact that when the adsorbed H-UTI was left for a long time in the column, the molecular weight of the inhibitor gradually changed to low-molecular-weight forms (unpublished), the forms may be enzymatically degraded products of UTI-I. Such modifications may be caused by insolubilized enzymes or more probably by contaminated urinary enzymes. In the present experiments, the yield of H-UTI and L-UTI were 47,000 and 24,000 U, respectively. Both preparations were homogeneous on SDS–PAGE and immunoelectrophoresis. With rabbit anti-human inter-α-trypsin inhibitor antiserum (Behringwerke), it was confirmed that H-UTI migrated into the prealbumin fraction, and L-UTI into the α,β-globulin fraction. These results are identical to those with the partially purified materials reported previously (9, 10).

Purification of SH-protease inhibitor. As shown in Fig. 1, when the urine concentrate was applied to a trypsin–Sepharose column, strong inhibitory activity against SH-protease was detected in the nonadsorbed fraction. The fractions were pooled as indicated in Fig. 1A (Tubes 2–8). The inhibitor fractions obtained from three separate columns were concentrated to about 2.0 ml by ultrafiltration followed by Sephadex G-100 gel filtration under the same conditions as those described above. A single broad peak of inhibitory activity (Tube 18–26) was detected, but this fraction concentrated by ultrafiltration was inhomogeneous on SDS–PAGE (Fig. 2c). The molecular weight of SH-protease inhibitor roughly estimated by gel filtration on Sephadex G-100 was 70,000. The yield was 32,300 U and the specific activity was 926 U/mg protein. In double immunodiffusion, this inhibitor did not react with the rabbit antiserum against α1-antitrypsin, α2-macroglobulin, antithrombin III, or inter-α-trypsin inhibitor (Behringwerke).

Properties of Purified UTI. The inhibitory effects of purified UTIs on 16 different enzymes were examined, and the concentrations required for 50% inhibition are shown in Table 1. Both H-UTI and L-UTI strongly inhibited trypsin and had a lesser effect on chymotrypsin and plasmin. In order to inhibit equivalent amounts of these enzymes, it is necessary to use about four times the amount of UTIs for chymotrypsin than that for trypsin and about 30–40 times the amount for plasmin. These results confirmed the results obtained in previous reports (2, 10). However, Egelblad and Astrup (4) have reported that UTI (which they had called "mingin") inhibits the urinary plasminogen activator, urokinase, and blood coagulation enzyme. We have also examined with crude preparations and reported the inhibitory effects of UTIs on elastase and kallikrein (10). Current experiments with a highly purified system, however, failed to show any effects of either H-UTI or L-UTI on urokinase, thrombin, elastase, kallikrein, pepsin, ficin, bromelain, and the plasma complement components, $C1\bar{r}$ and $C1\bar{s}$. In contrast, we first observed a strong inhibition of UTIs on the sperm trypsin-like enzyme, acrosin. By Dixson's plot (27), the inhibition was shown to be competitive. Assuming molecular weights of 43,000 for H-UTI and 22,000 for L-UTI, the dissociation constant K_i calculated was 1.2×10^{-8} M for H-UTI and 2.0×10^{-8} M for L-UTI, respectively. These values were about five times lower than those of leupeptin ($K_i = 8.6 \times 10^{-8}$ M)

TABLE 1. INHIBITORY SPECTRA OF URINARY PROTEINASE INHIBITORS

Enzyme	Substrate	Inhibitor conc. for 50% inhibition (μg)		
		H-UTI[a]	L-UTI[a]	SH-proteinase inhibitor (anticin)
Trypsin (2.0 μg)	Casein	1.83	1.63	—[b]
	TAMe	1.77	1.70	—
Chymotrypsin	Casein	7.51	6.47	—
(2.0 μg)	ATEe	6.63	6.00	ND[c]
Plasmin (0.25 CU)	Casein	72.4	54.0	—
Ficin (30 μg)	Casein	—	—	16.2
Papain (30 μg)	Casein	—	—	45.7
Bromelain (30 μg)	Casein	—	—	50.1
Acrosin (13.5 mU)	BAEe	11.9	9.77	—

[a] TAMe hydrolysis of urinary kallikrein (0.53 KU), plasma kallikrein (6.3 μmole TAMe/37°, 30 min), and thrombin (50 NIU), AGLMe hydrolysis of urokinase (40 IU), ATEe hydrolysis of $C\bar{1}s$ (10.3 μmole ATEe/37°, 30 min), AAMe hydrolysis of $C\bar{1}r$ (7.5 μmole AAMe/37°, 30 min), proteolysis of pepsin (4.0 μg), elastase (2.4 U), collagenase (100 Mandl U) and kinin-forming activity of kallikrein (0.43–0.55 μmole TAMe/37°, 30 min) were not affected by 50–1000 μg of both UTI preparations.

[b] No inhibition.

[c] Not determined.

and antipain ($K_i = 7.1 \times 10^{-8}$ *M*) (28). Acrosin is essential for sperm passage through the zona pellucida of the ovum, and thus is a key enzyme in the fertilization process. In the presence of inhibitors of this acrosomal protease, fertilization is obstructed both *in vivo* and *in vitro* (29–31). Recently, Fritz *et al.* (32, 33) have isolated a trypsin–acrosin inhibitor (HUST-II) from human seminal plasma. Although HUST-II is also acid stable and possesses strong inhibitory activity toward human acrosin ($K_i = 9 \times 10^{-10}$ *M*; *N*-benzoyl-DL-arginine-*p*-nitroanilide as substrate), the molecular weight (4,000–6,500) is much lower than UTI and has no effect on chymotrypsin and plasmin. The molecular structure and biological significance of each UTI remain to be elucidated.

Properties of SH-protease inhibitor. Recently we treated UTIs with papain, ficin, and the bacterial protease, pronase, and succeeded in obtaining active fragments with a molecular weight of less than 10,000 (11, 12). In the course of the experiments, we frequently encountered the problem of obtaining fragmentation of UTIs by papain or ficin when using crude UTIs as starting materials. We were free of such difficulties when experimenting with purified materials, and it was found that the cause was the presence of SH-protease inhibitor in crude materials. This material was named "anticin" because of its strong effect on ficin. Anticin could be distinguished from UTIs either by its elution profile on trypsin–Sepharose, by gel filtration, or by immunoassay. As shown in Table 1 (right column), anticin had no effect on several serine proteases, but strongly inhibited SH-proteases, such as ficin, papain, and bromelain. The activity was so high that its content in normal human urine could be determined after dialysis against saline; the mean value as determined based on measurements of 10 normal persons (24-hr pooled urine, five males and five females) aged between 25 and 47 was 18.16 ± 6.82 U/ml. In 1946 Beloff (34), and more recently Hayashi (35) and Tokaji (36) indicated that the prolonged phase of inflammation depends on the presence of SH-proteases which are influenced by blood and tissue inhibitors. Anticin may be related to these inhibitors. Studies on the physicochemical properties of the purified material and its biological significance are presently in progress.

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