A Simple High-Yield Procedure for Isolation of Human Urinary Kallikreins

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Two human urinary kallikreins (fractions A-1 and A-2) were purified to apparently homogeneous forms. The two kallikreins were separated by affinity chromatography using Trasylol (aprotinin) covalently bound to Sepharose. The kallikreins were eluted with a pH gradient (pH 9.5–3.0). Fraction A-1 was eluted between pH 6.2 and 4.2 and fraction A-2 was eluted between pH 4.2 and 3.1. Final purification was obtained by chromatography on Sephacryl SS-200. Antibodies prepared against fraction A-2 were also reactive with fraction A-1; thus it may be possible to measure both by radioimmunoassay using the same antibody preparation.

Urinary kallikrein (EC 3.4.21.8) is an enzyme, derived from kidney (Nustad *et al.*, 1975), that acts on kininogen to release Lys-bradykinin (cf. Webster, 1970). A number of groups have reported that the rate of excretion of kallikrein varies in response to dietary NaCl and water, and to blood concentrations of mineralocorticoids (e.g. see Keiser *et al.*, 1976; Mills *et al.*, 1976). In addition, excretion rates are decreased in patients with essential hypertension and are markedly increased in patients with primary hyperaldosteronism (Margolius *et al.*, 1976). However, there is disagreement about which factors (e.g. naturesis versus diuresis; cf. Mills *et al.*, 1976; Keiser *et al.*, 1976) are primary determinants of kallikrein excretion, and about how the kallikrein–kinin system may function within the kidney.

Part of the controversy appears to arise from differences in the assays used to measure urinary kallikrein. Kallikrein, a trypsin-like enzyme, is commonly measured by its ability to hydrolyse *N*^α-acylarginine esters (e.g. *N*^α-tosylarginine methyl ester, see below). However, other urinary enzymes, e.g. urokinase (Imanari *et al.*, 1976), may also hydrolyse these substrates. Other workers (e.g. Croxatto & San Martin, 1970) have measured urinary kallikrein in terms of its ability to form kinins from known amounts of kininogen. However, it is not evident that the kinin-generation assays are free of interference from kallikrein inhibitors known to occur in kidney (Geiger & Mahn, 1976) and in urine (Beaven *et al.*, 1971).

The present work was begun to obtain pure urinary kallikrein and to prepare specific antibodies for the enzyme. These reagents will be used not only further to characterize the enzyme, but also to develop a specific radioimmunoassay. Such an assay should

eliminate or decrease the artifacts that interfere with current assays for urinary kallikrein.

Materials and Methods

N^α-Benzoyl-L-arginine ethyl ester, *N*^α-tosyl-L-arginine methyl ester and soya-bean trypsin inhibitor were from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose (DE-52) was a Whatman product (H. Reeve Angel and Co., Clifton, NJ, U.S.A.). CNBr-activated Sepharose 4B, Blue Dextran and Sephacryl SS-200 were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Trasylol (aprotinin) was a gift from AG Bayer, Leverkusen, Germany. Synthetic bradykinin was provided by A. Chung and G. H. Fisher of this laboratory.

Processing of human urine

Human urine was collected daily from healthy laboratory personnel (males) into an 18-litre carboy. Urine was filtered through glass-wool and was collected under a layer of toluene. Each 16–18-litre batch of urine was precipitated with (NH₄)₂SO₄ at 90% of saturation (at 4°C). After 18 h at 4°C the precipitate was collected by filtration using Whatman no. 1 filter paper. The proteins were dissolved in water and dialysed exhaustively against water. A fine precipitate was removed by centrifugation at 8000*g* for 30 min, and the supernatant was freeze-dried. A total of 212 litres of human urine was processed in this manner. In addition to these preparations, we also used a crude urinary protein fraction kindly supplied by Dr. Shinichi Morisue of the Green Cross Corp., Osaka, Japan.

Purification

The urinary protein preparations were decolorized with charcoal; 22 g of crude kallikrein was dissolved

* Abbreviation used: Tos-Arg-OMe, *N*^α-tosyl-L-arginine methyl ester.

in 350ml of 0.1M-potassium phosphate buffer, pH6.7. The dark-brown solution was mixed intermittently with 2.75g of charcoal for 18h at 4°C. The mixture was centrifuged at 8000g for 30min. The supernatant was then chromatographed using DEAE-cellulose (column 5.2cm×40cm) equilibrated with 0.1M-potassium phosphate buffer, pH6.7. The column was washed with 2 litres of the starting buffer and then the enzyme was eluted with a linear NaCl gradient (0–1.0M). Kallikrein was eluted between 0.0 and 0.35M-NaCl. The Trasylol–Sephacryl affinity-chromatography procedure was described previously (Oza *et al.*, 1976). After developing a pH gradient (pH9.5–3.0), active fractions (A-1 and A-2) were obtained from the Trasylol–Sephacryl column. Both of these fractions were then chromatographed on a 2.8cm×110cm column of Sephacryl SS-200. The latter column was equilibrated and developed with 0.1M-Tris/HCl buffer, pH8.0, containing 0.5M-NaCl. The column had been calibrated previously with Blue Dextran 2000 (2000), aldolase (185000), ovalbumin (45000), chymotrypsinogen (25000) and ribonuclease (13700) (mol.wts. in parentheses).

Arginyl esterase activity

The assay of kallikrein in terms of the hydrolysis of Tos-Arg-OMe was performed as described by Nustad & Pierce (1974). Hydrolysis of *N*^α-benzoyl-L-arginine ethyl ester was performed as described previously (Oza *et al.*, 1976).

Kinin-generating activity

Human plasma, heated at 59°C for 3h, was used as the source of kininogen. Plasma thus treated was free of intrinsic kinin-generating activity, and, after the addition of EDTA, was free of kinin-inactivating activity. The heat-treated plasma (1.0ml) was mixed with 0.2ml of 0.15M-EDTA (disodium salt) and 0.8ml of 0.1M-sodium phosphate buffer, pH8.5. Enzyme, suitably diluted with the phosphate buffer, was added in a volume not exceeding 20μl, and the reaction mixture was incubated at 37°C for 15min. The reaction was stopped by heating in a boiling-water bath for 10min, and then 2.0ml of the phosphate buffer was added. A white precipitate was removed by centrifugation at 1500g for 30min. The kinin content of the supernatant was measured by bracket assay using an isolated guinea-pig ileum (Stewart *et al.*, 1970). The response of the ileum was standardized with synthetic bradykinin.

Protein determination

The protein concentration was determined by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

Immunization

Apparently pure human urinary kallikrein (360μg of fraction A-2) was emulsified with 3.0ml of complete Freund's adjuvant. A New Zealand rabbit (3kg body wt.) was injected intradermally at multiple sites (0.25ml per site). The rabbit was bled after 3 weeks and every week thereafter until the titre of antibody began to fall. The antiserum was heated at 56°C for 30min and was stored at –26°C. The development of antibody was monitored by Ouchterlony double immunodiffusion and also by its ability to inhibit the hydrolysis of Tos-Arg-OMe.

Results and Discussion

Urinary kallikrein behaved as a single substance during the first three steps of the purification. However, two active fractions (A-1 and A-2) were obtained by affinity chromatography with Trasylol–Sephacryl. Fraction A-1 was eluted between pH6.2 and 4.2, and fraction A-2 was eluted between pH4.2 and 3.1. It is not known at present whether fractions A-1 and A-2 differ in their affinities for Trasylol or whether fraction A-1 is more sensitive to reversible acid denaturation.

Fig. 1. Chromatography of fraction A-2 on Sephacryl SS-200

Fraction A-2 (4.0mg in 5.0ml of 0.1M-Tris/HCl buffer, pH8.0, containing 0.5M-NaCl) was applied to a column (2.8cm×110cm) of Sephacryl equilibrated with the same buffer. The flow rate of the column was 94ml/h. The material eluted between 284 and 340 ml was combined and ultrafiltered to 9ml (fraction A-2, step 5 as shown in the Table). ○, Arginyl esterase activity; —, A_{280} .

Table 1. *Purification of human urinary kallikreins*

Step	Treatment	Fraction	Total protein (mg)	Specific activity* ($\mu\text{mol}/\text{min}$ per mg)	Specific activity† (μg of bradykinin/min per mg)	Percentage recovery* of activity	Purification
1	Crude enzyme		14840	0.12	1.9	100	1
2	Charcoal		9828	0.18	—	99	1.5
3	DEAE-cellulose		3872	0.26	—	57	2.2
4	Affinity	A-1	4.8	71	—		592
		A-2	4.0	86	—	39‡	717
5A	Sephacryl	A-1-1	0.204	61	941		—
	SS-200 of fraction A-1	A-1-2	0.390	76	1231		—
		A-1-3	1.8	119	750		992
5B	Sephacryl	A-2	2.88	100	1094	31§	833
	SS-200 of fraction A-2						

* Based on μmol of Tos-Arg-OMe hydrolysed/min per mg of protein at pH 8.0, 30°C.

† Based on μg equiv. of bradykinin formed/min per mg at pH 8.5, 37°C.

‡ Recovery of fractions A-1 and A-2.

§ Recovery of fractions A-1-1, A-1-2, A-1-3 and A-2.

The elution profile of fraction A-2 on Sephacryl SS-200 is shown in Fig. 1. A symmetrical peak of the enzyme emerged at $V_e/V_0 = 1.54$. The elution volume of kallikrein (319 ml) was not distinguished from that of ovalbumin (319 ml, mol.wt. 45000). Fraction A-1 was eluted in the same volume, but its elution profile suggested the presence of a faster-moving contaminant (apparent $V_e/V_0 = 1.46$). Therefore the peak of kallikrein activity was divided into three fractions: A-1-1 (average $V_e/V_0 = 1.40$), A-1-2 (average $V_e/V_0 = 1.46$) and A-1-3 (average $V_e/V_0 = 1.58$).

A summary of the purification is presented in Table 1. As is evident from the Table, the affinity-chromatography step alone resulted in a 326-fold purification. To our knowledge, the specific activities (arginyl esterase and kinin-generating activities) are the highest of any preparations reported so far (cf. Nustad & Pierce, 1974; Hial *et al.*, 1974).

Fractions A-1-2, A-1-3 and A-2 appeared to be pure. Each reacted with antibody (raised against fraction A-2) to give a single precipitin line on Ouchterlony immunodiffusion. Conversely, the antibody reacted with crude urinary proteins (step 1, Table 1) to give a single precipitin line. In addition, the antiserum, but not normal rabbit serum, inhibited the hydrolysis of Tos-Arg-OMe by fraction A-1.

Both fractions A-1 and A-2 were inhibited by Trasylol, but not by soya-bean trypsin inhibitor. Trasylol, at a concentration of $2.3 \mu\text{g}/\text{ml}$, completely inhibited the hydrolysis of Tos-Arg-OMe. Soya-bean trypsin inhibitor at $90 \mu\text{g}/\text{ml}$ had no effect.

Thus, in a relatively simple procedure, two human urinary kallikreins can be purified to apparently homogeneous forms. Results of studies underway at

present indicate that our purification procedure can be made simpler still. Concentration of urinary proteins by ultrafiltration eliminates the need for precipitation of proteins with $(\text{NH}_4)_2\text{SO}_4$ and for decolorizing with charcoal.

In contrast with the human urinary protein(s) purified by Hial *et al.* (1974), the kallikreins obtained by our method possess strong arginyl esterase activities (fraction A-1-2, $76 \mu\text{mol}/\text{min}$ per mg of protein; fraction A-2, $100 \mu\text{mol}/\text{min}$ per mg of protein). Hial *et al.* (1974) purified a kinin-generating enzyme that appeared to hydrolyse benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester rapidly (V_{max} , $2.0 \mu\text{mol}/\text{min}$ per mg of protein), but that hydrolysed Tos-Arg-OMe slowly (V_{max} , $0.37 \mu\text{mol}/\text{min}$ per mg of protein). Although we did not obtain a perfect correlation of kinin-generating activity with arginyl esterase activity, the differences among fractions A-1-1, A-1-2, A-1-3 and A-2 are well within the experimental error for measurement of kinin generation, an assay that has an experimental error of $\pm 15\%$.

Our method of purifying human urinary kallikreins relies heavily on the use of Trasylol bound to Sepharose. Indeed this one step results in a 300-fold purification. However, the step selects for enzymes having an affinity for Trasylol. Thus whether human urine contains kinin-generating enzymes that have no affinity for Trasylol remains to be determined.

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