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Structural studies of human urinary kallikrein (urokallikrein)

(molecular weight/amino acid composition/maintenance of native structure)

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ABSTRACT **Human urinary kallikrein (urokallikrein) has been purified by affinity chromatography with aprotinin coupled to CH-Sepharose and by gel filtration. The isolation procedure, which was performed under mild conditions, was completed in a 36-hr period and yielded an overall recovery of more than 75% and a purification of 1727-fold. Homogeneity of the urokallikrein was demonstrated by three criteria: the coincidence of the stained protein band and functional urokallikrein in duplicate gels after alkaline disc gel electrophoresis; the appearance of a single stained band of molecular weight 48,000 on sodium dodecyl sulfate/polyacrylamide gel electrophoresis of reduced and unreduced enzyme; and the finding of a single amino-terminal residue, namely alanine, after dansylation and acid hydrolysis of purified enzyme. The K_m of urokallikrein on N^{α}-*p*-tosyl-L-arginine methyl ester was 400 μM, and the V_{max} was 194 μmol/min per mg of protein, which is higher than that observed with any previous preparations. The molecular weight of 48,700 determined on gel filtration and the molecular weight of 48,000 observed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis are in good agreement with the molecular weight of 48,213 calculated from the amino acid composition. The finding of a molecular weight higher than those previously reported, namely 27,000–43,500, the increased functional activity on tosylarginine methyl ester, and the detection of a single amino-terminal residue are consistent with the isolation of a more native protein by the procedure described in this paper.**

Kallikreins or kininogenases (EC 3.4.21.8) are endopeptidases that generate kinin polypeptides from plasma α_2-globulin substrates, kininogens (1). Kallikreins have been identified in plasma (2) and urine (3) and in several organs, including kidney (4), submandibular glands (5), and pancreas (6). Recent investigations have implicated urinary kallikrein (urokallikrein) in the conversion of prorenin to renin (7), in the regulation of blood pressure in animal experimental models for hypertension (8, 9), and in human essential hypertension (10–12). Several investigators have isolated urinary kallikrein from animal (13–16) and human urine (16–22) either by lengthy conventional purification procedures or by affinity chromatography with aprotinin (Kunitz bovine basic trypsin inhibitor, Trasylol) directly coupled to Sepharose beads at alkaline pH (20, 22).

This study describes the isolation of human urinary kallikrein by a two-step procedure based upon affinity chromatography with aprotinin coupled to activated Sepharose bearing a six-carbon spacer arm. The coupling was performed at low pH so as to protect the lysine ϵ-amino group at the inhibitor binding site (21). The purification, which can be performed within a 36-hr period, yields a homogeneous single polypeptide chain protein as assessed by alkaline and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis and amino-terminal residue determination.

MATERIALS AND METHODS

Reagents. Reagents were obtained from the sources indicated: synthetic bradykinin triacetate (New England Nuclear); N^{α}-*p*-tosyl-L-arginine methyl ester (TosArgOMe), atropine sulfate, and Coomassie brilliant blue (Sigma); 4,5-dihydroxy-2,7-napthalenedisulfonic acid (chromotropic acid) and glacial acetic acid (Eastman); PM-10 and PM-30 Diaflo membranes (Amicon Corp., Lexington, MA); Sephadex G-100, activated CH-Sepharose 4B, and sizing standards (Pharmacia); aprotinin (Boehringer Mannheim Biochemicals, Indianapolis, IN); 99.9% pure methyl alcohol (Fisher); rheophoresis plates (Hyland Division, Travenol Laboratories Inc., Costa Mesa, CA); Ampholine carrier ampholytes (LKB, Inc., Hicksville, NY); 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) chloride (Sigma); dansyl-amino acids (Seikagaku Fine Biochemicals, Tokyo, Japan); polyamide sheets (Cheng Chin Trading Co., Ltd., Taiwan); $NaDodSO_4$, acrylamide, and *N*-*N*′-methylene-bisacrylamide (Bio-Rad); and ammonium persulfate (Schwarz/Mann). Monospecific rabbit anti-human urokallikrein IgG was prepared as described (21).

Chromatographic Procedures. Six to eight liters of normal male urine were collected in an ice bath over a 4- to 6-hr period and concentrated 100-fold at 4°C by positive pressure ultrafiltration with PM-30 Diaflo membranes. The urine concentrate was dialyzed at 4°C for 12 hr against 0.1 M $NaHCO_3$, pH 8.5, made 0.5 M in NaCl. The dialyzed urine was centrifuged at 900 × *g* and the precipitate was washed with 5-ml portions of the dialysis solution until no detectable kinin-generating activity was eluted. The supernatant and pooled washes totaling 60–80 ml were directly incubated at 37°C for 60 min with 30 ml of settled aprotinin-Sepharose (10.4 mg of aprotinin per ml of Sepharose), which had been washed with the dialysis solution. Aprotinin was coupled to activated CH-Sepharose as described (21), with the exception that the pH of the 0.1 M potassium phosphate coupling buffer was lowered from 6.5 to 6.0 and the reaction time at 25°C was increased to 4 hr. The combined effect of these modifications increased coupling from about 50% to 75%. The mixture of aprotinin-Sepharose and concentrated, dialyzed urine was allowed to settle in a 2.5 × 24 cm column at 25°C for 60 min and the efficacy of urokallikrein adsorption was determined by screening a 200-μl portion of the breakthrough protein for kinin-generating activity. The column was transferred to 4°C and sequentially washed at a flow rate of 60 ml/hr with the same $NaHCO_3$/NaCl solution and with 0.05 M Tris·HCl, pH 6.5, containing 0.5 M NaCl until the absorbance of the 4-ml fractions at 280 nm was 0.002 at each step. Urokallikrein was eluted with 0.1 M sodium acetate/acetic acid buffer, pH 3.4, containing 1 M NaCl at a flow rate of 80 ml/hr, and 2-ml fractions were collected into tubes containing 2 ml

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Abbreviations: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; $NaDodSO_4$, sodium dodecyl sulfate; TosArgOMe, N^{α}-*p*-tosyl-L-arginine methyl ester.

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of 2 M Tris·HCl, pH 8.5. The fractions containing urokallikrein were pooled, concentrated to 6–9 ml, dialyzed against 0.05 M Tris·HCl buffer at pH 8.0 containing 0.2 M NaCl, and filtered on a 2.6 × 90 cm column of Sephadex G-100 previously equilibrated with the dialysis buffer. The column was run at a flow rate of 20 ml/hr and 5.6-ml fractions were collected. In both purification steps column fractions were screened for protein content at 280 nm, for kinin-generating activity with heat-inactivated plasma as the substrate (21), and for TosArgOMe-hydrolyzing activity (23, 24).

Analytical Techniques. Analytical polyacrylamide disc gel electrophoresis was performed in a Buchler apparatus as described by the manufacturer, using urokallikrein recovered from the Sephadex G-100 gel filtration step and quantitated by Folin analysis (25). One gel to which 17.5 μg of protein had been applied was sliced into 2-mm segments, and each of these was macerated and eluted with 400 μl of 0.2 M Tris·HCl, pH 8.0, containing 0.2 M NaCl. Five to 10 μl of gel eluates were screened for kinin-generating activity by using 200 μl of heat-inactivated plasma, and 50 μl was examined for urokallikrein antigen in rheophoresis plates. Another gel to which 35 μg of protein had been applied was stained with Coomassie brilliant blue.

$NaDodSO_4$/polyacrylamide gel electrophoresis was performed in 7.5% acrylamide gels (26). Samples containing 8 M urea were incubated for 60 min at 37°C in 0.1 M sodium phosphate buffer, pH 7.1, made 1% in $NaDodSO_4$ in either the presence or the absence of 2% (vol/vol) 2-mercaptoethanol and were then incubated in boiling water for 2 min. Subsequent alkylation with 0.2 mM iodoacetamide was carried out in the dark at 4°C for 30 min. The protein sizing standards were rabbit IgG, human serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A.

Analytical isoelectric focusing was performed at 4°C in 4 × 100 mm gels containing 4% acrylamide and 2% ampholyte with a pH range of 3.5 to 5.0 (27). At equilibrium the current was 3 mA and the applied potential was 400 V. After focusing, two gels were sliced into 5-mm segments, each of which was incubated for 2 hr in 200 μl of distilled water. After pH determination at 4°C, 200 μl of 0.05 M Tris·HCl (pH 8.0)/0.5 M NaCl was added to each slice and elution was continued for 12 hr at 4°C. The gel eluates were screened for kinin-generating activity and for urokallikrein antigen in rheophoresis plates with rabbit anti-urokallikrein IgG (21).

Amino Acid Composition. Forty micrograms of purified urokallikrein in 1.0 ml of 0.05 M Tris·HCl buffer, pH 8.0, containing 0.2 M NaCl and 1.0 ml of the buffer were separately dialyzed against distilled water, lyophilized, and subjected to either amino-terminal (28) or amino acid composition analysis (29). For the former procedure the sample and the buffer control were dissolved in 25 μl of 0.1 M $NaHCO_3$ and incubated with 25 μl of a 25 mg/ml solution of dansyl chloride in acetone for 30 min at 37°C. The reaction mixtures were dried and hydrolyzed under reduced pressure in 50 μl of 6 M HCl at 110°C for 24 hr. The hydrolysates were dried, dissolved in pyridine/water (1:1, vol/vol), spotted on three polyamide sheets, and developed by standard two-dimensional chromatography with a four-solvent system (30). Dansyl amino acid standards were spotted on the reverse side of each sheet.

For the determination of amino acid composition 40 or 46 μg of lyophilized urokallikrein and the buffer control were separately hydrolyzed in 6 M HCl containing 1% phenol at 110°C for 24 hr. The samples were dried, dissolved in pyridine/water (1:1) and subjected to amino acid analysis in a Durrum D 500 analyzer.

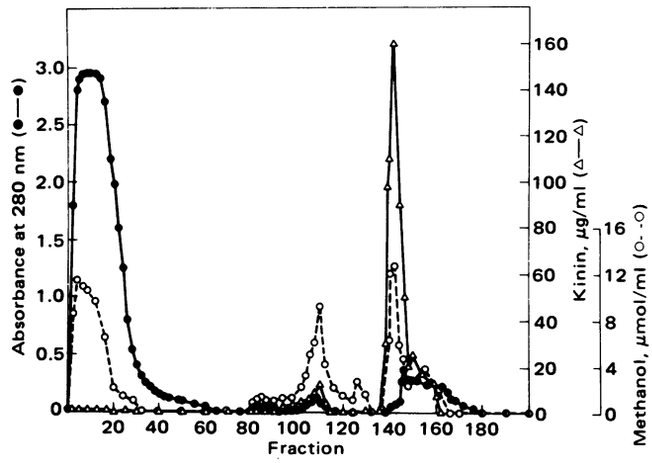

FIG. 1. Aprotinin-Sepharose affinity chromatography of pooled concentrated urine. Column fractions were assessed for protein (●), esterolytic activity on TosArgOMe (○), and kinin-generating activity (△).

RESULTS

Preparation of Urokallikrein. More than 99% of kinin-generating activity in the 100-fold concentrated, centrifuged urine was adsorbed to the aprotinin-Sepharose affinity column. Washing the column with 0.1 M $NaHCO_3$ (pH 8.5)/0.5 M NaCl removed approximately 80% of the applied protein (Fig. 1), 50% of the TosArgOMe-hydrolyzing activity, and less than 1% of the kinin-generating activity. Elution with the pH 6.5 buffer removed approximately 5% of the applied protein, 30% of the TosArgOMe-hydrolyzing activity, and less than 5% of the kinin-generating activity.

More than 95% of the kinin-generating activity was eluted in fractions 140–160 after application of the pH 3.4 buffer. These fractions were pooled, concentrated, and filtered through the Sephadex G-100 column (Fig. 2). Approximately half of the protein applied and a small amount of TosArgOMe-hydrolyzing activity appeared in the exclusion volume. The included kinin-generating and TosArgOMe-hydrolyzing activities were concordant and appeared in association with a small protein

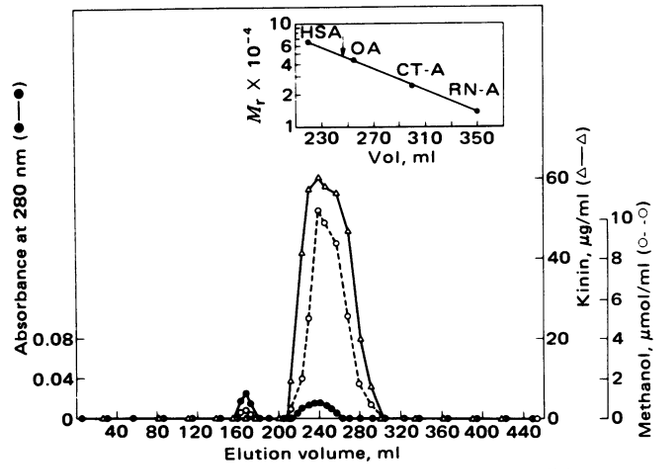

FIG. 2. Sephadex G-100 gel filtration of the urokallikrein recovered from aprotinin-Sepharose affinity chromatography. Fractions were assessed for absorbance at 280 nm (●), TosArgOMe-hydrolyzing activity (○), and kinin-generating activity (△). (*Inset*) Elution volume plotted against the molecular weight of the sizing standards: human serum albumin (HSA), ovalbumin (OA), chymotrypsinogen A (CT-A), and ribonuclease A (RN-A). Urokallikrein filtered at the position indicated by the arrow.

Table 1. Purification of human urinary kallikrein*

Preparative step	Total protein, mg	Total kinin-generating activity, μg/min	Specific activity, μg/min per mg	Yield, %	Purification factor
Crude urine concentrate	432.50	731.2	1.7	100.0	1
Aprotinin-Sepharose affinity chromatography	5.85	648.0	110.8	88.6	66
Sephadex G-100 gel filtration	0.19	563.4	2919.0	77.1	1727

* The data represent the mean of three separate preparations.

peak. Urokallikrein function filtered in the molecular weight range of 47,000–50,000 with a mean of 48,700 in three separate experiments. The two-step purification procedure yielded an enzyme capable of generating a mean of 2919 μg of kinin/min per mg of enzyme when incubated with excess heat-inactivated plasma, an average purification factor of 1727-fold, and an overall recovery of 77% (Table 1).

Physicochemical Characterization. Alkaline disc gel electrophoresis of 35 μg of protein recovered from Sephadex G-100 gel filtration revealed a single protein band in the stained gel (Fig. 3). Ninety-one percent of the kinin-generating activity was recovered in the same region of a duplicate sliced gel. When gel eluates were screened in rheophoresis immunodiffusion plates, precipitates were observed with eluates from slices 17–20. Isoelectric focusing in polyacrylamide gels using Ampholines with a pH range of 3.5–5.0 and 15 μg of enzyme showed kinin-generating activity and urokallikrein antigen in the range of pH 3.99–4.30, with 70% of the activity recovered between pH 4.10 and 4.25.

$NaDodSO_4$/polyacrylamide gel electrophoresis of 34 μg of purified, reduced, and alkylated urokallikrein gave a single protein band with a molecular weight of 48,000 (Fig. 4), under conditions in which rabbit IgG was cleaved to its heavy and light chain components. An equivalent amount of enzyme that was alkylated without prior reduction had the same apparent size as the reduced and alkylated protein.

Dansyl-amino acid standards were used to identify the amino-terminal residue, which in three separate experiments was identified as alanine. The mean recovery of amino acid residues in two hydrolysis experiments with 40 μg is shown in Table 2. The amount of each amino acid is presented as a net obtained by subtraction of the amount of each amino acid found in the dialyzed buffer from the total recovered in the protein hydrolysate. The former did not exceed 5% of total residues recovered and the two analyses for each residue varied by no more than 4%. No analysis for tryptophan was performed, and independent cysteine analyses were not done. Because deamination of glutamine and asparagine residues during hydrolysis yielded the free acids, values are given for the sum of each amine/acid pair (Table 2). The minimum molecular weight calculated from these data is 6887. When multiplied by 7 this yields a molecular weight for urokallikrein of 48,213, a value that agrees well with the molecular weights obtained by $NaDodSO_4$/polyacrylamide gel electrophoresis and Sephadex gel filtration.

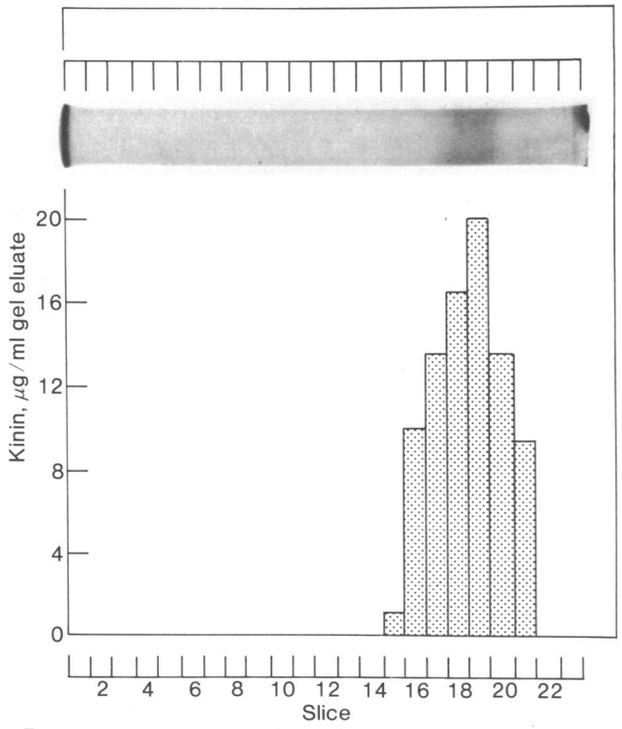

FIG. 3. Alkaline disc gel electrophoresis of purified urokallikrein. The kinin-generating activity of eluates from gel slices was measured with heat-inactivated plasma used as the substrate source. (*Inset*) Second gel prepared with 35 μg of protein and stained with Coomassie brilliant blue. The anode was at the right.

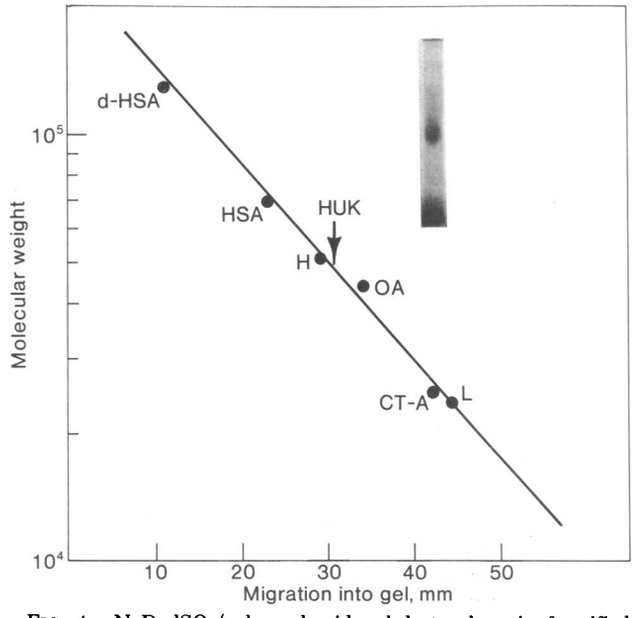

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified, reduced, and alkylated urokallikrein (HUK). Electrophoresis was from top to bottom and was carried out until the tracking dye reached the bottom of the gel. The standards include human serum albumin dimer (d-HSA), human serum albumin (HSA), rabbit IgG heavy (H) and light (L) chains, ovalbumin (OA), and chymotrypsinogen A (CT-A).

Table 2. Amino acid composition of human urokallikrein

Amino acid	Amount, nmol
Aspartic acid/asparagine	52.02 (1.97)
Alanine	25.95 (1.00)
Arginine	9.75 (0.38)
Cysteine	1.42 (0.16)
Glutamic acid/glutamine	56.80 (2.18)
Glycine	40.50 (1.47)
Histidine	18.93 (0.73)
Isoleucine	14.88 (0.57)
Leucine	43.70 (1.68)
Lysine	14.86 (0.57)
Methionine	6.42 (0.25)
Phenylalanine	11.62 (0.45)
Proline	25.60 (0.99)
Serine	28.65 (1.03)
Threonine	25.15 (0.97)
Tyrosine	5.60 (0.22)
Valine	35.50 (1.37)

Values in parentheses are the relative amounts obtained by normalizing to the amount of alanine found, arbitrarily set at 1.00.

Kinetic Parameters. Dose–response and kinetic experiments were carried out with various amounts of TosArgOMe and enzyme. When 0.5, 0.2, and 0.1 μg of urokallikrein were incubated with doses of TosArgOMe from 4 to 200 mM, Lineweaver–Burk plots gave three straight lines that intersected at a single point on the abscissa, yielding a K_m of 400 μM. The V_{max} was 194 μmol/min per mg of protein.

DISCUSSION

Human urinary kallikrein has been purified to homogeneity by a two-step procedure with aprotinin coupled to CH-Sepharose as a ligand for affinity chromatography followed by gel filtration on Sephadex G-100. To avoid denaturing conditions, the urine was collected in an ice bath during a 6- to 8-hr period, concentrated at 4°C and, with the exception of a 1-hr adsorption to the affinity column at 37°C, chromatographed at 4°C. The entire procedure, which was performed in 36 hr, permitted a 74–83% recovery of the kinin-generating activity as compared to the starting urine concentrate (Table 1). The aprotinin-Sepharose affinity column (Fig. 1) separated the urokallikrein from 95% of the other urinary proteins, including nonurokallikrein TosArgOMe esterase activity, and yielded a 57- to 75-fold increase in specific activity. Subsequent concentration and dialysis removed low molecular weight contaminants, while gel filtration separated additional proteins and nonkallikrein TosArgOMe esterase activity from urokallikrein (Fig. 2). The overall purification of 1727-fold is about 3 times that obtained by conventional chromatographic techniques (24) and almost twice that observed when affinity chromatography and gel filtration followed an initial isolation of urokallikrein by anion-exchange chromatography on DE-52 DEAE-cellulose (21). The anion exchange column was eliminated because it did not separate nonurokallikrein TosArgOMe esterases from urokallikrein, doubled the time required to isolate urokallikrein, and consistently resulted in a 40% loss of activity. As assessed by gel filtration, the urokallikrein has an apparent molecular weight of 48,700 (Fig. 2) as compared to 39,500 (21) when the anion exchange chromatographic step was included in the isolation sequence.

Geiger *et al.* (20), who used a combination of three gel filtration steps and aprotinin affinity chromatography without a spacer arm, achieved a 212-fold purification of human urokallikrein. They observed a molecular weight of 64,000 by gel filtration in distilled water. Oza and Ryan (22), who utilized salt precipitation, decolorization with charcoal, anion exchange chromatography, aprotinin-Sepharose affinity chromatography, and gel filtration, obtained approximately a 1000-fold purification and observed a molecular weight of 45,000 on Sephacryl S-200 gel filtration in 0.1 M Tris·HCl (pH 8.0)/0.5 M NaCl. Lower molecular weights of 27,000–29,000 (19) and 43,600 (17) by gel filtration, of 37,000 by $NaDodSO_4$/polyacrylamide gel electrophoresis (18), and of 35,400 (18) and 42,665 (17) by calculation from amino acid composition data have been previously reported for human urokallikrein purified by classical chromatographic procedures.

Homogeneity of human urokallikrein isolated by affinity chromatography and gel filtration was established by three criteria. The urokallikrein appeared as a single stained band on alkaline disc gel electrophoresis coincident with the region in which urokallikrein was identified functionally and antigenically in a parallel gel (Fig. 3). $NaDodSO_4$/polyacrylamide gel electrophoresis of 35 μg of material revealed a single protein band of molecular weight 48,000, which was not altered by reduction before $NaDodSO_4$ gel electrophoresis (Fig. 4). Finally, after dansylation and hydrolysis, the purified urokallikrein yielded a single amino-terminal residue, namely alanine, in three separate determinations conducted with two different enzyme preparations.

The K_m value for urokallikrein on TosArgOMe of 400 μM is comparable to that previously obtained with enzyme purified by procedures that included an affinity chromatography step (20, 21), namely, 8700 μM and 670 μM; the K_m for enzyme obtained by conventional purification techniques has been higher, ranging from 4350 μM (24) to 1140 μM (17). The V_{max} of 194 μmol/min per mg of protein is higher than that observed in procedures employing conventional (17, 24) or affinity chromatography (20, 21).

The amino acid analysis of the 48,000 molecular weight urokallikrein (Table 2) differs substantially from the analyses reported by others, which, in turn, differed markedly from each other. Urokallikreins purified by Hial *et al.* and Porcelli *et al.* with molecular weights of 43,600 (17) and 37,000 (18), respectively, were not established to be homogeneous by amino-terminal determination or $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction and alkylation. The fact that rapid isolation with a minimum of manipulation yields an enzyme with a higher molecular weight (Figs. 2, 4), a lower K_m, and a higher V_{max} than previously observed with other preparations of urokallikrein is attributed to the more native state of the protein isolated in this fashion.

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1. Webster, M. E. & Pierce, J. V. (1963) *Ann. N. Y. Acad. Sci.* **104**, 91–107.
2. Kraut, H., Frey, E. K. & Bauer, E. (1928) *Z. Physiol. Chem.* **175**, 97–114.
3. Frey, E. K. & Kraut, H. (1926) *Z. Physiol. Chem.* **157**, 32–61.
4. Beraldo, W. T., Feldberg, W. & Hilton, S. M. (1956) *J. Physiol. (London)* **133**, 558–570.
5. Werle, E. & von Roden, P. (1936) *Biochem. Z.* **286**, 213–219.
6. Kraut, H., Frey, E. K. & Werle, E. (1930) *Z. Physiol. Chem.* **189**, 97–106.
7. Sealey, J., Atlas, S. A., Laragh, J., Oza, N. & Ryan, J. W. (1978) *Nature (London)* **275**, 144–145.
8. Keiser, H. R., Andrews, M. J., Guyton, R. A., Margolius, H. S. & Pisano, J. J. (1976) *Proc. Soc. Exp. Biol. Med.* **151**, 53–56.

9. Carretero, O. A., Vasudev, M. A., Ocholik, T., Scicli, A. & Koch, J. (1978) *Circ. Res.* **42,** 727–731.
10. Margolius, H. S., Geller, R., Pisano, J. J. & Sjoerdsma, A. (1971) *Lancet* **ii,** 1063–1065.
11. Adetuyibi, A. & Mills, I. H. (1972) *Lancet* **ii,** 203–207.
12. McGiff, J. C. & Nasjletti, A. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35,** 172–174.
13. Nustad, K. & Pierce, J. V. (1974) *Biochemistry* **13,** 2312–2319.
14. Silva, E., Diniz, C. R. & Mares-Guia, M. (1974) *Biochemistry* **13,** 4304–4310.
15. Oza, N. B., Amin, V. M., McGregor, R. K., Scicli, A. F. & Carretero, O. A. (1976) *Biochem. Pharmacol.* **25,** 1607–1612.
16. Moriya, H., Pierce, J. V. & Webster, M. E. (1963) *Ann. N. Y. Acad. Sci.* **104,** 172–185.
17. Hial, V., Diniz, C. R. & Mares-Guia, M. (1974) *Biochemistry* **13,** 4311–4318.
18. Porcelli, G., Marini-Bettolo, G. B., Croxatto, H. R. & Di Iorio, M. (1974) *Ital. J. Biochem.* **23,** 43–55.
19. Matsuda, Y., Miyazaki, K., Moriya, H., Fujimoto, Y., Hojima, Y. & Moriwaki, C. (1976) *J. Biochem.* **80,** 671–679.
20. Geiger, R., Mann, K. & Bettels, T. (1977) *J. Clin. Chem. Clin. Biochem.* **15,** 479–483.
21. ole-MoiYoi, O., Spragg, J. & Austen, K. F. (1978) *J. Immunol.* **121,** 66–71.
22. Oza, N. B. & Ryan, J. W. (1978) *Biochem. J.* **171,** 285–288.
23. Siegelman, A. M., Carlson, A. S. & Robertson, T. (1962) *Arch. Biochem. Biophys.* **97,** 159–163.
24. ole-MoiYoi, O., Austen, K. F. & Spragg, J. (1977) *Biochem. Pharmacol.* **26,** 1893–1900.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
26. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
27. Righetti, P. G. & Drysdale, J. W. (1971) *Biochim. Biophys. Acta* **236,** 17–28.
28. Gray, W. R. (1972) *Methods Enzymol.* **25,** 121–138.
29. Spackmann, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30,** 1190–1206.
30. Narita, K., Hisayuki, M. & Nakajima, T. (1975) in *Protein Sequence Determination,* ed. Needleman, S. B. (Springer, New York), p. 30.