Purification and Properties of a Human Urinary Kallikrein (Kininogenase)†

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ABSTRACT: A highly purified kallikrein was isolated from human urine with 43% yield and a purification factor of 25. The enzyme is a glycoprotein with a molecular weight of 43,600. The protein is microheterogeneous on electrofocusing yielding p*I* values of 3.80, 3.95, and 4.06. It has a low catalytic activity on tosyl-L-arginine methyl ester (K_m = 1.14 mM, k_{cat} = 0.26 sec^{-1}), and this activity is inhibited by benzamidine. The kallikrein is not inactivated by Tos-LysCH_2Cl, nor can it be titrated by *p*-nitrophenyl *p'*-guanidinobenzoate. Its activity on kininogen is comparable to that of trypsin, but it is not inhibited by benzamidine. The kinin-releasing activity is inhibited by Trasylol and very slowly by iPr_2FP. The product of preincubation of kallikrein with kininogen relaxes the rat duodenum, as do the kinins, and the enzyme lowers the arterial blood pressure when injected intravenously into the rat. The differences in specificity observed by comparison with a preparation described earlier (Moriya, H., Pierce, J. V., and Webster, M. E. (1963), *Ann. N. Y. Acad. Sci. U. S. 104,* 172) have been attributed to environmental and/or genetic effects on the populations that supplied the urine. In the discussion of the results with small molecular synthetic substrates it is emphasized that the active center of different kallikreins may require amino acid sequences of different length for complexation, in order to display their full catalytic activity.

Kallikrein (EC 3.4.4.21) is defined as an endogenous enzyme which rapidly and specifically liberates a kinin from the kininogens in plasma (Webster, 1970). A historical account on the subject is found in the work by Werle (1970). Kallikreins have been studied in the urine, blood, plasma, and organs of several species (Pierce, 1970).

A kallikrein from human urine was purified by Moriya *et al.* (1963) that was able to hydrolyze the typical trypsin substrates Tos-L-Arg-OMe and Bz-L-Arg-OEt.[1] The ability to hydrolyze trypsin substrates is shared by kallikreins from different origins (Pierce, 1970; Prado, 1970). In a comparative study of urinary kallikreins from man, rat, and horse, Diniz *et al.* (1965) observed that their preparations from male human urine totally lacked, or had a minimum level of, esterase activity toward Bz-L-Arg-OEt, determined under identical conditions as Moriya *et al.* (1963). In addition, the kinin-releasing activity of these preparations could not be inhibited by benzamidine, a strong competitive inhibitor of trypsin (Mares-Guia and Shaw, 1965). These results have been repeatedly confirmed (Diniz *et al.*, 1966a,b). The findings of our group, apparently inexplicable to Pierce (1970), have now been fully confirmed with the isolation and characterization, chemical as well as kinetic, of a human urinary kallikrein that does not display any significant trypsin-like behavior toward the commonly used synthetic substrates of trypsin. A further interesting result is that no kinin-releasing activity was detected that could be associated with any significant level of Tos-L-Arg-OMe esterase activity in the urine samples we worked with. The implications of this finding are discussed in terms of possible genetic or environmental differences, or both, among the populations that supplied the urine to Pierce's group and to ours.

Experimental Procedure

Materials. Bz-L-Arg-OEt, Tos-L-Arg-OMe, Ac-L-Tyr-OEt, Z-L-Tyr-OPNP, iPr_2FP, iodoacetamide, twice crystallized bovine trypsin, and lysozyme were obtained from Sigma Chemical Co.; Bz-DL-Arg-NPNA was obtained from Cyclo Chemical Co.; and benzamidine-HCl was purchased from Aldrich Chemical Co. NPGB, Tos-LysCH_2Cl, and EPGB were supplied at an earlier opportunity by Professor Elliot Shaw, Brookhaven National Laboratories. Dioxane and Amido Schwartz-10B were obtained from Merck, Darmstadt, Germany, and Sephadex from Pharmacia, Uppsala, Sweden. All other chemicals were reagent grade, including those used in the organ-bath preparations.

Equipment. The titrations were carried out with a Radiometer TTT-1c titrator, equipped with an Auto-burette ABU-12, a SBR-2 recorder, and G-202 (glass) and K-605 (calomel) microelectrodes, at the temperature of 25 ± 1°. Spectrophotometric kinetic measurements were run in a double-beam Zeiss-Jena Specord spectrophotometer, using quartz cells of 1-cm light path. Electrofocusing was performed in an LKB analytical instrument, and amino acid

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[1] Abbreviations used are: Ac-L-Tyr-OEt, N^{α}-acetyl-L-tyrosine ethyl ester; BK, bradykinin; BKg, bradykininogen; Bz-DL-Arg-NPNA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; Bz-L-Arg-OEt, N^{α}-benzoyl-L-arginine ethyl ester; BzA, benzamidine; DEAE, diethylaminoethyl; iPr_2FP, diisopropyl fluorophosphate; DTNB, 5,5'-(dithiobis)nitrobenzoic acid; EDTA, ethylenediaminetetraacetate; EPGB, ethyl *p*-guanidinobenzoate; Gdn, guanidine; KPP, porcine pancreatic kallikrein; KUH, human urinary kallikrein; KSH, human salivary kallikrein; KUP, porcine urinary kallikrein; KUR, rat urinary kallikrein; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; PAS, periodic acid Schiff; Tos-LysCH_2Cl, L-(1-chloro-3-tosylamido-7-amino-2-heptanone); Tos-L-Arg-OMe, N^{α}-tosyl-L-arginine methyl ester; Z-L-Tyr-OPNP, N^{α}-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; *p*-HgBzOOH, *p*-hydroxymercuribenzoate.

analyses were carried out with a Beckman Model 120C analyzer. Absorbancies were measured with a Model 139 Hitachi–Perkin-Elmer spectrophotometer, equipped with photomultipliers. The Dale organ baths, with accessories, were made by Palmer, England. Arterial pressure was measured with a transducer linked to an EM-physiograph.

Methods. CHEMICAL AND KINETIC. Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard. The hydrolytic activity on Z-L-Tyr-OPNP was measured by following the release of *p*-nitrophenol at 410 nm as a function of time in the Specord instrument, essentially as described by Martin *et al.* (1959). The esterase activities toward Tos-L-Arg-OMe, Ac-L-Tyr-OEt, or Bz-L-Arg-OEt were measured in the Radiometer titrator, working as a pH-Stat, in a total volume of 2.0 ml, 0.10 M in NaCl and 2 mM in $CaCl_2$. Sodium hydroxide, 2.3 mM, was the usual titrant, delivered from a 2.5-ml syringe in the ABU-12 unit. The titrant was prepared daily from a stock 0.10 N solution, and was standardized at least twice daily against samples of potassium acid phthalate. During every assay a steady flow of humid nitrogen was passed over the sample solution. Exact substrate concentrations were obtained from total hydrolyses carried out with trypsin or chymotrypsin, according to the substrate used. Amidasic activity was determined with Bz-DL-Arg-NPNA, essentially as described by Erlanger *et al.* (1961) and Mares-Guia (1968). The incubation times were much longer (1 hr) in the kallikrein assays.

For the determination of kinin-releasing activity two methods were used. The first was essentially that of Diniz and Carvalho (1963) and the other one was that of Prado *et al.* (1962). The major change introduced was that, in either case, we used as substrate a partially purified kininogen, obtained from dog plasma by C. R. Diniz and J. Barroso (unpublished results).

BIOLOGICAL. The distal 10-cm piece of the ileum of a recently killed guinea pig was removed, washed with Tyrode, and maintained in Tyrode, pH 8.2, at 37° with aeration. A segment of about 3 cm was cut out; one of the ends was fixed to the bottom of a 4-ml glass cylinder in the Dale bath, and the other end was tied to an auxotonic lever. A smoked drum was used to register the effects. When rat duodenum was used, a 5-cm piece was cut from the proximal end of duodenum and treated and mounted as described above. In either case a solution of bradykinin, 1.5 μg/ml, was used as a standard.

For the measurements of lowering of arterial blood pressure in the rat, the kallikrein preparation was injected into the femoral vein of the heparinized and urethane anesthetized animal. The blood pressure was measured in the cannulated carotid artery with the help of a pressure transducer. A physiograph was used to record the pressure changes as a function of time.

TREATMENT OF KINETIC DATA. The constants K_m and V_m for Tos-L-Arg-OMe were determined according to Lineweaver and Burk (1934), using the variance of v as statistical weight (Wilkinson, 1961). The dissociation constant for benzamidine inhibition of Tos-L-Arg-OMe hydrolysis was determined according to Dixon (1953). K_m and V_m for Z-L-Tyr-OPNP hydrolysis and K_i for its inhibition by dioxane were obtained from replots of slopes and intercepts in Lineweaver–Burk and Dixon plots. In the latter case the method of solution was graphical. To avoid loss of accuracy, high quality graph paper and drawing equipment were used. In a few instances, computer evaluation and graphical calculation agreed within 5% or less, well within the range of values for the standard deviation in K_m.

Results

Purification. Human male urine was collected daily under toluene in a 5-l. erlenmeyer flask, after filtration through glass wool. When necessary, it was stored up to 4 days at 4° and was desalted by filtration through a large G-25 Sephadex column (void volume = 0.5 l.). Each 6-l. batch of desalted material was then lyophilized and used as starting material (step 1).

DEAE-SEPHADEX CHROMATOGRAPHY. A sample of *ca.* 5 g of the material just described was dissolved in about 60 ml of ammonium acetate buffer (0.1 M and at pH 7.0), centrifuged to remove any insoluble residue, and applied to a 4 × 44 cm column of DEAE-Sephadex, previously equilibrated with the same buffer, which was subsequently used for elution.

A NaCl gradient, in 0.1 M ammonium acetate buffer at pH 7.0, was started after the collection of 1 l. of eluate. The enzyme eluted from the column at 0.4 M NaCl, in the volume range 1900–2250 ml. The flow rate was 110 ml/hr; the fraction volume was 14 ml. The chromatography was run at 25°. After determining the kinin-releasing activity, as described under Methods, the active fractions were pooled, dialyzed against deionized water, and lyophilized (step 2).

SEPHADEX G-150 CHROMATOGRAPHY. A 50-mg sample of the material obtained in step 2, containing *ca.* 23 mg of protein, was dissolved in 4 ml of 0.01 M ammonium acetate buffer (pH 7.0, 0.10 M in NaCl). The solution was applied to a G-150 Sephadex column, 2.5 × 90 cm, previously equilibrated with the same buffer.

A typical elution pattern showed two major peaks: that eluted from 140 to 170 ml contained the enzyme, whereas that eluted around 250–300 ml contained mainly pigment. Elution was carried out with 0.10 M ammonium acetate (pH 7.0, 0.10 M in NaCl). The flow rate was 10 ml/hr and the fractions measured 3.1 ml. The chromatography was carried out at 25°. The kinin-releasing fractions were pooled (step 3), dialyzed against water, and lyophilized.

SEPHADEX G-150 RECHROMATOGRAPHY. A 25-mg sample of material from step 3, containing *ca.* 23 mg of protein, was dissolved in 4 ml of buffer as in step 3 and chromatographed under identical conditions.

The result is shown in Figure 1. A protein peak of constant specific activity was obtained. The fractions were pooled as indicated and the material was dialyzed against water and then lyophilized (step 4). The lyophilized material is from now on called KUH.

A purification flow sheet is shown in Table I. The overall yield was 43.5%, and the purification factor was 25.

Physicochemical Characterization. POLYACRYLAMIDE ELECTROPHORESIS. Electrophoresis in polyacrylamide (7.5%) in Tris-glycine buffer, at pH 8.2, showed one single component after step 4. The runs were made at 4 mA/column, and the protein was stained with Amido Black in 7% acetic acid.

ELECTROFOCUSING. When KUH was submitted to electrofocusing in ampholine in a pH gradient from 3 to 5, three components active on Z-L-Tyr-OPNP were detected. Their p*I* values are 3.80, 3.95, and 4.06 as shown in Figure 2. No attempt has been carried out yet to separate them on a preparative scale.

CELLOGEL ELECTROPHORESIS. As all kallikreins known to this date are glycoproteins, we carried out cellogel

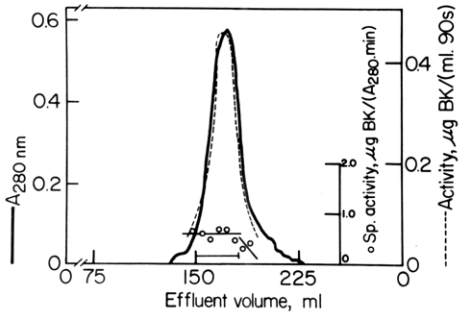

FIGURE 1: Sephadex G-150 rechromatography: 22.6 mg of protein was applied to a 2.5 × 90 cm column. Elution: ammonium acetate 0.01 M (pH 7.0), 0.10 M in NaCl. Flow rate was 12 ml/hr, and each fraction contained 3.1 ml; temperature, 25°.

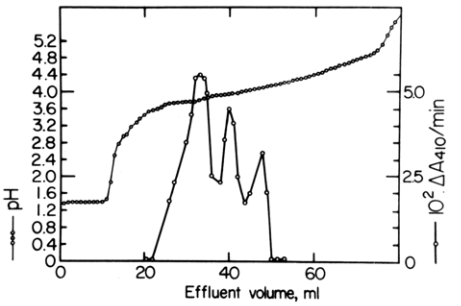

FIGURE 2: Electrofocusing of KUH (4 mg) in ampholine, pH range 3–5, 24 hr at 500–600 V and 4°. Fractions of 1 ml were collected after completion. The heavy line indicates activity measured with Z-L-Tyr-OPNP.

electrophoresis and stained two parallel strips with Amido Black-10B and a modified PAS technique (C. C. Oliveira, personal communication). The result is shown in Figure 3. The coincidence in the staining of the major peaks shows that KUH is also a glycoprotein. It is reasonable to suppose that the minor peaks represent the components detected by electrofocusing through activity measurements.

MOLECULAR WEIGHT DETERMINATION. The molecular weight of KUH was determined by filtration through a column of Sephadex G-150, run under identical conditions as described in Figure 1. Reference proteins were lysozyme, trypsinogen, and bovine serum albumin. The elution volume of KUH yielded a molecular weight of 43,600.

Amino Acid Analysis. One-milligram samples of KUH were hydrolyzed in sealed ampoules with constant boiling HCl, at 110 ± 2° for 20, 40, and 70 hr. After drying the hydrolysate in a desiccator with moist NaOH pellets the residue was dissolved in 0.5 ml of 0.2 N citrate buffer (pH 2.2). The analyses were carried out in the long column (resin AA-15) for neutral and acid amino acids and in the short column (resin PA-35) for basic amino acids. Methionine and cysteine were determined as methionine sulfone and cysteic acid, respectively, after performic acid oxidation according to Hirs (1967). Tryptophan was determined spectrophotometrically according to Spande and Witkop (1967).

The maximum number of residues obtained in the three hydrolysis times used was taken for the calculation of the amino acid composition of KUH. A summary of the results is shown in Table II. A minimum molecular weight of 5316.2 per hystidyl residue was obtained. Sulfhydryl groups were determined by reaction of KUH with DTNB and *p*-HgBzOOH. The results and conditions are shown in Table III. It can be seen that four SH groups/mol of protein were

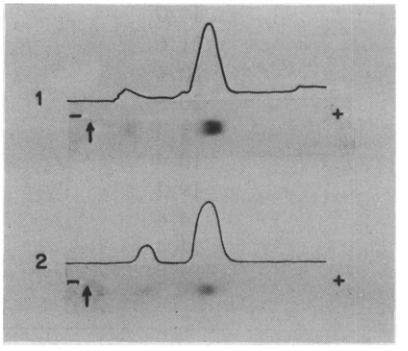

FIGURE 3: Cellogel electrophoresis of KUH in 0.1 M borate buffer (pH 9.5), for 50 min at 200 V and 1 mA/cm, 4°. One hundred micrograms of KUH was added to each strip. Strip 1 was stained with PAS and strip 2 with Amido Black 10B.

titrated, and that this number did not increase in the presence of a strong denaturing agent. The kinetics of the thiophenol liberation and mercaptide formation, in the absence of denaturants, indicate that all four SH groups are exposed, as full reaction is almost immediate. The carbohydrate composition of KUH is now under investigation.

Kinetic Characterization. HYDROLYSIS OF ACYL-L-ARGININE DERIVATIVES. Under the conditions used for trypsin assay, no significant level of Tos-L-Arg-OMe hydrolysis could be detected with KUH. Only when the concentration of the titrant, sodium hydroxide, was lowered to 2.3 mM and the KUH concentration was raised to 0.125 mg/ml of incubation mixture could hydrolysis of the ester be measured, in 0.1 M NaCl–2 mM $CaCl_2$ at pH 8.0, 25°. The rate of hydrolysis was proportional to the protein concentration and varied as a function of substrate concentration according to the Michaelis–Menten model up to approximately 2 mM Tos-L-Arg-OMe. Beyond this value acti-

TABLE I: Purification of Human Urinary Kallikrein (Typical Results for a Sample of 16.4 l.).

Step	Procedure	Total Protein (mg)	Sp. Act. (μg of BK/(mg min))	Total Act. (μg of BK/min)	Yield (%)	Purif. Factor
1	Sephadex G-25 filtration	961	0.006	5.77	(100)	(1)
2	DEAE-Sephadex A-50 chromatography	63.7	0.058	3.70	64.10	9.7
3	Sephadex G-150 filtration	26.1	0.141	3.66	63.40	23.5
4	Second filtration through Sephadex G-150	16.7	0.150	2.51	43.50	25.0

TABLE II: Amino Acid Composition of Purified KUH and Comparison with Other Kallikreins.

Amino Acid	KUH		KPP[a]	KUP[a]	KSH[b]
	Amino Acid Residue	Nearest Integral No. (mol/mol)	(mol/mol)	(mol/mol)	(mol/mol)
Lys	15.2	15	13	16	11
His	8.0	8	10	10	4
Arg	17.6	18	4	8	5
Asp	40.0	40	33	31	14
Thr	24.8	25	18	21	8
Ser	36.0	36	17	20	13
Glu	50.4	50	28	29	17
Pro	48.0	48	20	27	17
Gly	44.0	44	27	24	13
Ala	21.6	22	16	16	13
Half-Cys	10.4[c]	10	10	10	12
Val	20.8	21	13	15	10
Met	2.4[c]	2	5	4	2
Ile	10.4	10	14	12	4–5
Leu	19.2	19	24	24	11
Tyr	14.4	14	9	8	4
Phe	14.4	14	13	11	5
Trp	2.2[d]	2	9	8	3
Mol wt		42,665.9	33,300	36,300	28,000[e]

[a] Frey *et al.* (1968). [b] Fujimoto *et al.* (1973). [c] Methionine and cysteine were determined as methionine sulfone and cysteic acid, respectively, according to Hirs (1967). [d] Tryptophan was spectrophotometrically determined by the method of Spande and Witkop (1967). [e] The molecular weight was determined by gel filtration on Sephadex G-150.

TABLE III: Sulfhydryl Groups in KUH; Reaction with DTNB and *p*-HgBzOOH.[a]

Conditions	Protein Concn (μM)	mol of SH/ mol of Protein
(1) 6 M Gdn-HCl in 0.05 M Tris-HCl (pH 8.0)–25 mM EDTA, containing 0.3 mM DTNB	4.2	4.3
(2) 6 M Gdn-HCl in 0.33 NaOAc (pH 4.6)–0.1 mM *p*-HgBzOOH	4.2	4.2
(3) Tris-HCl (0.5 M, pH 8.0)–25 mM EDTA–0.3 mM DTNB	5.2	3.9
(4) NaOAc (0.33 M, pH 4.6)–*p*-HgBzOOH	6.8	4.5

[a] An aqueous solution of KUH was added to the assay system already containing the SH reagents. Protein and reagent blanks were included.

TABLE IV: Kinetic Parameters for KUH-Catalyzed Hydrolysis of Synthetic Substrates and Inhibitors

	K_m (mM)	V_{max} (μmol/ (mg min))	k_{cat} (sec^{-1})	K_i (mM)
Tos-L-Arg-OMe[a]	1.14	0.37	0.26	
Bz-L-Arg-OEt[a]	1.34	0.34	0.25	
Z-L-Tyr-OPNP[b]	0.19	2.0	1.40	
Benzamidine[a]				6.42
Dioxane[b]				200

[a] NaCl, 0.1 M; $CaCl_2$, 2 mM; pH 8.0; 25°. [b] Tris, 0.05 M; methanol, 12% (v/v); pH 8.0; 25°.

vation by excess substrate was observed. A deviation from the simple model was clearly seen at the higher values of Tos-L-Arg-OMe concentration. From the data in the lower substrate concentration range, the values of K_m and V_m were calculated with the help of a computer program called KMVM, written as outlined elsewhere (Mares-Guia and Figueiredo, 1972). A K_m value of 1.14 mM and k_{cat} of 0.26 sec^{-1} were found (Table IV).

In order to exclude the possibility that the activation observed at high Tos-L-Arg-OMe concentration could be due to an ionic strength effect instead of a specific one, we carried out control experiments where the ionic strength was increased by the addition of NaCl to the incubation mixture. The results in Figure 4 demonstrate that ionic strength *per se* does not cause enzyme activation.

The KUH-catalyzed hydrolysis of Tos-L-Arg-OMe is competitively inhibited by benzamidine, a trypsin inhibitor (Mares-Guia and Shaw, 1965). A K_i value of 6.42 mM was obtained from the intersection of the two lines in the Dixon plot (Figure 5). The slopes of the lines were obtained by a least-squares treatment of the data.

Under similar experimental conditions KUH hydrolyzed Bz-L-Arg-OEt. The same treatment of data yielded a K_m of 1.34 mM and k_{cat} equal to 0.25 sec^{-1}. On the other hand,

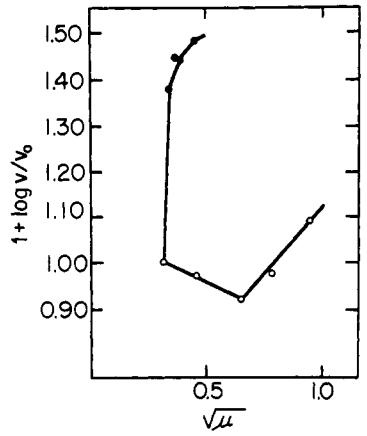

FIGURE 4: Ionic strength effect on KUH-catalyzed Tos-L-Arg-OMe hydrolysis. Conditions: reaction volume was 2.0 ml, KUH concentration was 0.088 mg/ml, at pH 8.0 and 25°; (○) substrate concentration was 1 mM and the ionic strength was varied by increasing NaCl molarity; (●) NaCl concentration constant at 0.1 M and ionic strength was varied by increasing substrate concentration beyond 1 mM.

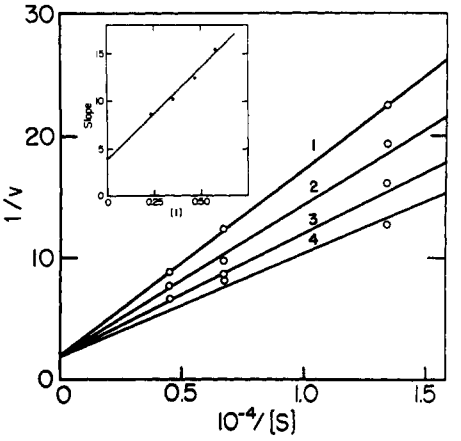

FIGURE 6: Dioxane inhibition of KUH-catalyzed Z-L-Tyr-OPNP hydrolysis. Initial rates expressed as ΔA_{410}/min, measured in Tris (0.05 M), 12% in methanol (v/v) at pH 8.0 and 25°; total volume 2 ml; enzyme concentration, 0.016 mg/ml; dioxane concentrations, line 1, 0.588 M; line 2, 0.470 M; line 3, 0.352 M; line 4, 0.235 M; inset, slope replot. Each point represents duplicate determinations.

when KUH was incubated up to 24 hr with Bz-DL-Arg-NPNA under similar conditions, no hydrolysis of the substrate was detected.

Attempts at titrating the enzyme active center with NPGB, a specific titrant of trypsin, did not succeed. The enzyme was not titrated nor did it hydrolyze the reagent under the conditions described by Chase and Shaw (1967).

HYDROLYSIS OF ACYL-L-TYROSINE ESTERS. Given the low values of k_{cat} observed with acylarginine esters, we decided to test KUH action on acyltyrosine esters. At pH 8.0, 25°, and a KUH concentration of 0.13 mg/ml no enzyme-catalyzed hydrolysis of Ac-L-Tyr-OEt was detected in the pH-Stat. However, when carbobenzoxy-L-tyrosine *p*-nitrophenyl ester was used under similar conditions a clearcut hydrolysis was detected spectrophotometrically. The assays with Z-L-Tyr-OPNP were done essentially as described by Martin *et al.* (1959), using dioxane as the solvent for the substrate stock solution. In Figure 6 is shown the Lineweaver–Burk plot of KUH-catalyzed hydrolysis of Z-L-Tyr-OPNP in the presence of different dioxane concentrations. A Dixon plot confirmed the competitive nature of the dioxane inhibition of the reaction. From the replots—that of Figure 6 is shown as an inset—the values of K_m, 0.19 mM, k_{cat}, 1.40 sec^{-1}, and K_i for dioxane, 0.2 M, were calculated (Table IV). Benzamidine did not inhibit this reaction even at the 2 mM level. Thus, Z-L-Tyr-OPNP became a very convenient substrate for routine assays of KUH.

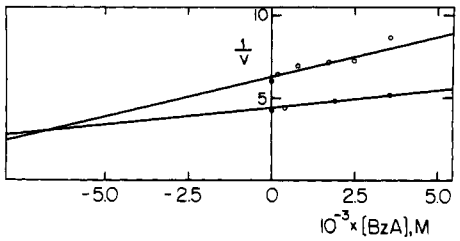

FIGURE 5: Benzamidine inhibition of KUH-catalyzed Tos-L-Arg-OMe hydrolysis: upper line, 1.25 mM substrate; lower line, 2.46 mM substrate; enzyme concentration, 0.104 mg/ml. Experiments conducted in NaCl 0.1 M, 2 mM in $CaCl_2$, at pH 8.0 and 25°. Reaction volume was 2.0 ml. Each point represents duplicate determinations. For more details see Methods.

REACTION WITH IRREVERSIBLE INHIBITORS. The irreversible trypsin inhibitors Tos-LysCH_2Cl and EPGB were unable to inactivate KUH at pH 8.0 and 25° when preincubated with enzyme up to 80 min, at concentrations that rapidly inactivate trypsin. Both the kinin-releasing activity and esterase activity on Tos-L-Arg-OMe were used to follow the reaction. Preincubation of KUH with 25 mM iodoacetamide in 0.1 M Veronal buffer (pH 8.1) failed to inactivate the enzyme, as measured by its esterase activity on Z-L-Tyr-OPNP. However, when KUH was preincubated with 3 mM iPr_2FP in 0.1 M Tris, pH 8.1 at 25°, a slow inactivation ensued. After 8 hr of incubation the activity had dropped to 83% of the control containing isopropyl alcohol, the solvent used for iPr_2FP. At the end of 24 hr of reaction the iPr_2FP concentration was raised to 10 mM. The Z-L-Tyr-OPNP and kinin-releasing activities dropped to 47 and 45% of the controls, respectively, after 4 additional hours of incubation.

KININ-RELEASING ACTIVITY. One of the difficulties in comparing the kinin-releasing activities of different kallikreins lays in the absence of a pure kininogen available to

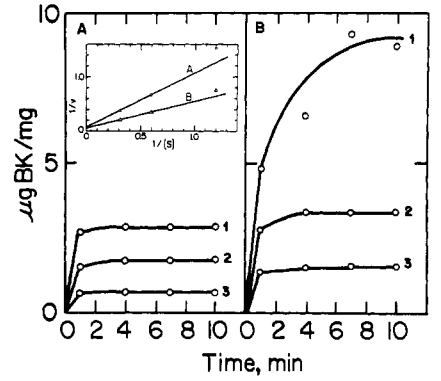

FIGURE 7: Effect of kininogen concentration on the initial rate of kinin liberation by KUH and β-trypsin; preincubations in Tyrode (pH 8.2), at 37°; KUH concentration was 0.58 mg/ml; β-trypsin was 0.39 mg/ml. The kininogen concentrations were: line 1, 3.20 mg/ml; line 2, 1.65 mg/ml; line 3, 0.83 mg/ml. Total volume was 0.30 ml. At the indicated times aliquots of 10–40 μl were withdrawn and added to the organ bath for the estimation of liberated kinin; inset, Lineweaver–Burk plot. Each point represents duplicate determinations: (A) KUH; (B) β-trypsin.

all workers in the field. In addition to that, it is a frequent occurrence that published specific activities are not measured under conditions where the substrate is saturating, which renders these data less reliable for comparisons.

In order to circumvent this difficulty we used three different kininogen concentrations and measured the initial rates of kinin release from the plots in Figure 7. Trypsin was also used for comparison. One sees that KUH practically liberates all the kinin within 1 min of contact with kininogen. The partially purified kininogen we used contained 0.9 μg of Bk/mg of protein. A Lineweaver–Burk plot for both enzymes is shown in the inset of Figure 7. As the kininogen we used is only partially purified, K_m(app) is expressed as milligrams/milliliter, and V_m as micrograms of bradykinin/milligram of protein minute). The results are contained in Table V. The reference specific activity of KUH is, therefore, 12.5 μg of BK/(mg min). The kinin-releasing activity of KUH is not at all inhibited by 9 mM benzamidine, at pH 8.2, 37°. On the other hand, Trasylol, a well-known kallikrein inhibitor, effectively blocked the kinin-releasing activity of KUH under the following conditions: 1100 U.I.C. Trasylol was incubated for 10 min at 37° with 1.1 mg of KUH in 0.30 ml of Tyrode (pH 8.2). Aliquots were then withdrawn and assayed in the organ bath.

Biological Characterization. In addition to the contraction of guinea pig ileum, the kinins are able to cause relaxation of rat duodenum and to lower blood pressure. We have demonstrated that KUH acts on kininogen liberating a substance that fulfills these criteria for biological characterization as a kinin (bradykinin or analogs). Thus, when 0.40 mg of KUH was preincubated with 0.80 mg of kininogen in 0.60 ml of Tyrode at pH 8.2 for 30 min at 37° and aliquots of the reaction mixture were added to a perfusion of rat duodenum, a relaxation was observed. On the other hand, endevenous injection of 0.14 mg of KUH in a urethane anesthetized rat caused an appreciable lowering of arterial blood pressure.

Discussion

Purification. The use of DEAE-Sephadex A-50 as an initial step in the purification of human urinary kallikrein (Moriya *et al.*, 1963) was suggested by Pierce (1970). Sephadex G-150 was successfully used by Takahashi *et al.* (1972) for the purification of a prekallikrein from bovine plasma. In the present work, the purification scheme had three phases: DEAE-Sephadex A-50 chromatography, filtration over Sephadex G-150, followed by a second filtration through the same column. The yield was 43.5%, with a 25-fold increase in specific activity (kinin release) from step 1. When contrasted with data by Moriya *et al.* (1963), who purified a human urinary kallikrein 5700 times with a 6% yield, it becomes clear that the major difference in the purification factor is the result of the choice of starting material and substrate for assay: these authors took optical density at 280 nm as a measure of protein, and determined activity by Tos-L-Arg-OMe hydrolysis. As urine contains pigments and other compounds that strongly absorb at 280 nm, we did not use this method for protein evaluation, at least in the earlier steps of purification. Furthermore, our material did not practically hydrolyze Tos-L-Arg-OMe. Therefore, an attempt at further comparing the two purification procedures seems impractical.

Criteria of Purity. The enzyme was considered pure by the following criteria: it yields a single peak on rechromatography in a Sephadex G-150 column, with constant specific activity measured as μg of BK/(A_{280} min) (Figure 1); it shows a single band upon electrophoresis in polyacrylamide at 7.5%. The amino acid analysis gave data that needed minimum rounding-off when the minimum molecular weight was multiplied by 8 to give molecular weight close to that found by Sephadex G-150 chromatography (Table II).

By electrofocusing a microheterogeneity was observed, that is, three peaks appeared that had p*I* values of 3.80, 3.95, and 4.06. Fiedler and Werle (1968) observed similar results with their hog pancreatic kallikrein. By treatment with neuraminidase the microheterogeneity of their preparation disappeared and a single protein peak was evident on electrofocusing. It is quite possible that our findings be also due to variations in the carbohydrate moiety of the glycoprotein enzyme.

Chemical and Physicochemical Characteristics. Using sedimentation equilibrium Moriya *et al.* (1963) obtained a molecular weight of 40,500 for their preparation of human urinary kallikrein. The value of 43,600 found for the enzyme described in the present paper was found by gel filtration on Sephadex G-150. This value may be higher than real molecular weight, as discussed by Silva *et al.* (1974). The data in Table II show that the amino acid composition of KUH compares well with that of other kallikreins obtained from hog and man.

Of the ten half-cystine residues found by amino acid analysis (Table II), four are free sulfhydryl groups, easily accessible to sulfhydryl reagents, as was shown by the data in Table III. Preliminary experiments have shown that

TABLE V: Comparison between Kinetic Parameters of Several Kininogenases and of KUH.

Enzyme	Tos-L-Arg-OMe			Bz-L-Arg-OEt			BKg[e]	
	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})	K_m (mg/ml)	V_m (μg of BK/(mg min))
β-Trypsin	0.015[a]	270	1.8×10^7	0.011[a]	64.8	5.9×10^6	10.2	20.0
KUR	0.130[b]	4.04	3.1×10^4					
KPP	0.06[c]	3.8	6.3×10^4	0.13[c]	129	1.0×10^6		
KUH	1.14[d]	0.26	2.2×10^2	1.34[d]	0.25	1.9×10^2	12.0	12.5

[a] NaCl, 0.1 M; $CaCl_2$, 5 mM; pH 8.0; 25° (N. Magalhães and M. Mares-Guia, personal communication). [b] KCl, 0.1 M; pH 8.0; 25° (Silva *et al.*, 1974). [c] NaCl, 0.1 M; thioglycolate, 0.1 mM; pH 8.0; 25° (Fielder and Werle, 1968). [d] NaCl, 0.1 M; $CaCl_2$, 2 mM; pH 8.0; 25° (this work). [e] Tyrode, pH 8.2; 37° (this work).

treatment of KUH with 25 mM iodoacetamide for 4 hr at pH 8.0 did not affect the hydrolytic activity on Z-L-Tyr-OPNP. On the other hand, the reaction with iPr_2FP, although a slow one, may be taken as an evidence for serine involvement in the catalytic mechanism of KUH.

Kinetic Characterization. SYNTHETIC SUBSTRATES. The low level of esterase activity of KUH toward synthetic substrates of trypsin is shown by the data in Table V, where a comparison is made to similar activity of other kallikreins and trypsin. Using k_{cat}/K_m as a measure of catalytic activity it is seen that trypsin is *ca.* 530 times more efficient than KUR, but is about 10^5 times more efficient than KUH when Tos-L-Arg-OMe is used as a substrate. With Bz-L-Arg-OEt, trypsin is 3×10^4 times more efficient than KUH. The difference between our enzyme and Pierce's is made fully evident by the following result: whereas Pierce (1970) reported a specific activity on Tos-L-Arg-OMe of 27 μmol/(mg min), KUH has a V_m of 0.37 μmol/(mg min), determined under practically identical conditions.

The activation observed at high Tos-L-Arg-OMe concentrations has been found for other kallikreins and, in the case of KUH, is not due to an ionic strength effect (Figure 4). Although benzamidine did inhibit the KUH-catalyzed hydrolysis of Tos-L-Arg-OMe (Figure 5), the enzyme was not titrated by NPGB, nor was it inactivated by Tos-LysCH_2Cl or EPGB. In addition to that, it was found that KUH does not hydrolyze Bz-DL-Arg-NPNA, a trypsin substrate, even after 24 hr of incubation at pH 8.0, in contrast to the observation that rat urinary kallikrein hydrolyzes this substance (Mares-Guia and Diniz, 1967).

It seems that in comparing the activities of kallikreins and trypsin one finds a clear-cut difference: no kallikrein was found to date that is inactivated by Tos-LysCH_2Cl. On the other hand, there seems to be a gradation on the hydrolytic ability toward esters that are typical trypsin substrates, the kallikrein reported here having the lowest level found to date.

The low level of activity on arginine esters led to experiments with tyrosine esters. Although Ac-L-Tyr-OEt was not hydrolyzed, Z-L-Tyr-OPNP was, and the kinetic parameters were given in Table IV.

This observation provided a useful and rapid routine test for purified KUH. The reaction was not inhibited by benzamidine, but was by dioxane, the solvent for the substrate. Interestingly, the K_i value found was rather low, 0.20 M, when contrasted with values found for the inhibition of chymotrypsin by dioxane, K_i = 0.32 M (Clement and Bender, 1963), or trypsin, K_i = 2.0 M (Mares-Guia and Figueiredo, 1972).

PROTEIN SUBSTRATE. Using a partially purified dog plasma kininogen we determined apparent kinetic parameters for the kinin-releasing activity of KUH (Figure 7, Table V). It is clear that KUH and trypsin have very similar kinetic parameters when acting on dog kininogen. However, it is shown in Figure 7 that β-trypsin liberates more kinin from the kininogen used than KUH, although the initial rates of kinin release are similar. The data were confirmed with a purer kininogen. An explanation for these findings is now being pursued using purified human kininogen. The use of apparent values of K_m and V_m as parameters for comparison of kinin-releasing enzymes constitutes a step forward in comparing kallikreins in the literature, where single values of specific activities have been usually reported, and frequently the substrate concentration has not been given.

The fact that a partially purified substrate is being used is not a very serious drawback, because the data can be corrected whenever pure kininogen becomes widely available to researches in the field.

Final Comment

The differences in the kinetic parameters observed with small substrates may be explained with a model of kallikrein active center that includes more extensive interactions between the enzyme and the substrate. One such model would require, for example, that optimum catalytic activity of a given kallikrein would occur only when a longer substrate sequence such as Met-Lys-Arg-Pro, for instance, were bound in the Michaelis complex. Kallikreins that had different activities upon synthetic substrates but that had similar activities on kininogen would differ by the details of substrate binding to subsites in the active center.

This proposition finds support in recently published work by Kurachi *et al.* (1973), who showed an improvement in peptide chloroketones as chymotrypsin inactivators as they contained a larger number of amino acid residues. Similar results were obtained for elastase by Thompson and Blout (1973).

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Lipoprotein Lipase: Properties of the Enzyme Isolated from Post-Heparin Plasma†

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ABSTRACT: Lipoprotein lipase purified from rat post-heparin plasma was characterized in terms of its amino acid, carbohydrate, and lipid composition. Molecular weight determinations by several procedures indicate a monomeric molecular weight of about 37,000; apparent dimer and tetramer forms were also identified. The purified lipase is a glycoprotein, but it does not contain heparin. It did not bind heparin in solution or covalently attached to agarose beads. The purified lipase retained approximately 1 mol of phospholipid/mol of protein.

Heparin releases from the endothelial membrane of the blood capillaries of the extrahepatic tissues a lipase (post-heparin lipoprotein lipase) active on the triglycerides of the natural lipoprotein substrates (chylomicrons, very low density lipoproteins). This enzyme has been purified in a number of laboratories (Fielding, 1969, 1970; Nilsson-Ehle *et al.*, 1971; Yasuoka and Fujii, 1971). The protein moiety of the lipoprotein substrates stimulates the hydrolysis of synthetic neutral lipid substrates by the purified enzyme (Havel *et al.*, 1973). This lipase has a high specificity for reaction with emulsified substrates (Fielding, 1973). It is released from its membrane site by high molecular weight polyanions, in particular polysaccharide sulfates such as heparin (Korn, 1957). In an attempt to define further the molecular basis of these properties we have undertaken a chemical and physical characterization of this enzyme, which is the subject of the present report.

Experimental Section

Materials

Unlabeled heparin was purchased from Invenex Company, San Francisco. [*N-sulfonate*-^{35}S]Heparin (initial radioactivity 13.5 mCi/g) (mol wt 14,500 by sedimentation equilibrium for $\bar{v}$ = 0.47 cm^3/g) (Barlow *et al.*, 1961) was from Amersham-Searle, Chicago, and was used without dilution with unlabeled heparin. No difference was found in the amount or properties of lipase released by unlabeled or radioactive heparin species. Linolenic acid (Sigma Chemical Co., St. Louis, Mo.), 99% pure by thin-layer chromatography, was used as the potassium salt after conversion with aqueous KOH. Sodium deoxycholate was from Mann Research, New York, N. Y., and Intralipid 20% triglyceride emulsion with lecithin was the gift of Vitrum AB, Stockholm, Sweden. Animal plasma donors were male Sprague-Dawley rats, fed ad libitum; 10–12 ml of plasma was obtained from each animal of 400–500 g body weight by aortic cannulation.

Methods

Lipase Preparation. Lipoprotein lipase was purified from blood plasma of rats obtained 3–5 min after the injection of heparin (1 mg/kg body weight). Lipase activity of plasma and at various stages of purification was assayed with triglyceride–lecithin emulsion in the presence of recalcified

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