Rat Urinary Kallikrein: Purification and Properties†

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ABSTRACT: Rat urinary kallikrein was isolated with a purification factor of 87-fold and 19% yield. The steps involved included: concentration by ultrafiltration, acetone fractionation, gel filtration over Sephadex G-100, and affinity chromatography on a column of Sepharose-Arg-OMe. Finally, the protein was twice filtered over a Sephadex G-150 column. The material obtained at the end was homogeneous on polyacrylamide electrophoresis at pH 8.2 but was microheterogeneous on electrofocussing in a pH gradient of 3–6 with ampholine. The major component had pI = 4.18. The enzyme is a glycoprotein of molecular weight 33,100, as determined by filtration on Sephadex G-150. Biological characterization of the kallikrein included: liberation of a kinin from a precursor from dog plasma, tested on the isolated guinea pig ileum; direct action on the isolated rat uterus; and the lowering of rat arterial pressure by intravenous injection of the enzyme. Rat urinary kallikrein hydrolyzes Tos-L-Arg-OMe (K_m = 0.127 mM, k_{cat} = 4.06 sec^{-1}) with optimum pH at 8.55. Tos-L-Arg-OMe activates the enzyme at concentrations larger than 1 mM. The enzyme also hydrolyzes Bz-DL-Arg-NPNA and is competitively inhibited by substituted benzamidines, β-naphthamidine, and phenylguanidine. Its molarity was determined by titration with *p*-nitrophenyl *p'*-guanidinobenzoate (97.4% pure). The kallikrein is not inactivated by ethyl *p*-guanidinobenzoate or 1-chloro-3-tosylamido-7-amino-2-heptanone. Rat urinary kallikrein is inactivated by *p*-guanidinophenacyl bromide.

Kallikreins are proteases included in the group called kininogenases (EC 3.4.4.21). They are endogenous enzymes that rapidly and specifically liberate a kinin from plasma precursors called kininogens (Webster, 1970). The presence of a kallikrein in rat kidney and urine was described by Carvalho and Diniz (1963, 1966).

Work carried out with extensively dialyzed rat urine indicated that the enzyme had trypsin-like specificity, but was not inactivated by Tos-LysCH_2Cl[1] (Diniz *et al.*, 1965, 1966). The specificity of this enzyme was discussed by Mares-Guia and Diniz (1967b), and the action of several trypsin inhibitors and inactivators on the enzyme was studied by Mares-Guia and Diniz (1967a). Mares-Guia *et al.* (1970) presented a preliminary discussion on the nature of the active center topography of rat urinary kallikrein.

A description of the partial purification and reactions of this enzyme with active center reagents of trypsin was published by Mares-Guia *et al.* (1970). An interesting biological property of rat urinary kallikrein is the contraction of the isolated rat uterus that it causes by direct action, observed by Beraldo *et al.* (1966). Croxatto *et al.* (1971) and Nustad and Pierce (1974) have started investigating rat urinary kallikrein. The latter authors purified rat urinary kallikreins and their specific antibody.

In the present work we describe a purification procedure that is partially based on affinity chromatography and gel filtration, and a study of the kinetics of the enzyme on both synthetic and natural substrates, both in the presence and in the absence of inhibitors.

Experimental Procedure

Materials

Reagents. All chemicals used were reagent grade. Solvents were from E. Merck, Darmstadt; Sephadex gels were obtained from Pharmacia, Uppsala, Sweden. Bz-DL-Arg-NPNA and Tos-LysCH_2Cl were purchased from Cyclo Chemical Corp.; lysozyme and Tos-L-Arg-OMe were purchased from Sigma Chemical Co. Bradykinin was from Sandoz Co., Ltd; bovine serum albumin was from Calbiochem; and trypsinogen was from Worthington Biochemical Corp. Benzamidine was obtained from Aldrich Chemical Co. Phenylguanidine and *p*-aminobenzamidine were synthesized as described in Mares-Guia and Shaw (1965); β-naphthamidine and *m*- and *p*-toluamidines were prepared as described by Mares-Guia (1968).

The following compounds, *p*-chloro-, *p*-bromo-, *p*-hydroxy-, and *p*-nitrobenzamidines, as well as *p*-methylphenyl-, *p*-ethylphenyl-, and *p*-aminophenylguanidines, were synthesized according to literature methods, and had the correct melting point ranges and elemental analyses. Their detailed properties will appear in a coming paper.

β-Trypsin was prepared according to Schroeder and Shaw (1968). Sepharose-Arg-OMe was prepared in this laboratory by M. A. Almeida and D. L. Nelson according to the method of Cuatrecasas (1970). Partially purified dog plasma kininogen was supplied by Mr. J. Barroso. *p*-Guanidinophenacyl bromide, *p*-amidinophenacyl bromide, and Tos-LysCH_2Cl were previously supplied by Professor Elliott Shaw, from Brookhaven National Laboratories.

Laboratory Animals. Adult Wistar rats of both sexes, weighing 200–300 g, as well as rats of the Hooded strain

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[1] Abbreviations used are: Sepharose-Arg-OMe, Sepharose-L-arginine methyl ester; Tos-L-Arg-OMe, *p*-toluenesulfonyl-L-arginine methyl ester; Bz-DL-Arg-NPNA, benzoyl-DL-arginine *p*-nitroanilide; Bz-L-Arg-OEt, benzoyl-L-arginine ethyl ester; Tos-LysCH_2Cl, L-(1-chloro-3-tosylamido-7-amino-2-heptanone); BzA, benzamidine; KUR, rat urinary kallikrein; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; EPGB, ethyl *p*-guanidinobenzoate; APB, *p*-amidinophenacyl bromide; GPB, *p*-guanidinophenacyl bromide; BK, bradykinin; BKg, dog plasma kininogen, PAS, periodic acid Schiff.

weighing from 300 to 400 g, from the Johns Hopkins University, Baltimore, Md., and raised in our laboratory, were used. Guinea pigs of both sexes, weighing from 500 to 700 g, were from the School of Veterinary Medicine, of this University.

Equipment. Absorbance was measured with a Hitachi-Perkin-Elmer Model 139 spectrophotometer or a Beckman DU Model 2400. Isoelectric focusing was carried out with an LKB apparatus, Model 8100. Titrimetric enzyme assays were carried out with a Radiometer titrator (Model TTT-1c), equipped with an automatic syringe ABU-12, a SBR-2 recorder, G-202 glass electrode, and K-605 calomel electrode. Palmer organ baths with auxotonic levers were used for the biological assays with isolated organs.

Methods

Enzyme Activity. SYNTHETIC SUBSTRATES. Hydrolytic activity toward Bz-DL-Arg-NPNA was measured essentially according to Erlanger *et al.* (1961) and Mares-Guia (1968). The incubation times were, however, much longer (1 hr). When Tos-L-Arg-OMe was the substrate, a Radiometer titrator was used as a pH-Stat. The total volume was 2.0 ml, and the reaction mixture was 0.10 M in KCl, at the specified pH; temperature, 25°. The titrant was 2.3 mM sodium hydroxide, delivered from a 2.5-ml syringe in the ABU-12 unit. Humid nitrogen was continuously passed over the solution in the titrator. The substrate concentration was determined by total hydrolysis with β-trypsin.

KININ-RELEASING ACTIVITY. Routine determinations were carried out according to Prado *et al.* (1962). For the estimation of the apparent kinetic parameters with dog plasma kininogen the following method was used. To a volume of 0.20 ml containing from 0.25 to 1.0 mg kininogen in Tyrode solution at pH 8.2 was added 0.10 ml of a stock enzyme solution in deionized water, at a concentration of 0.45 mg/ml. After 1, 4, 7, and 10 min of incubation time at 37°, aliquots from 10 to 40 μl were removed and assayed for kinin content using the isolated guinea pig ileum. A standard bradykinin solution used as reference contained 1.5 μg/ml of the peptide in Tyrode (pH 8.2). Initial rates of kinin-releasing activity were obtained from the plot of the amount of kinin released *vs.* time.

Biological Assays. ISOLATED GUINEA PIG ILEUM. The distal 10–12-cm piece of the ileum of a recently killed guinea pig was removed, washed with Tyrode, and maintained in Tyrode at pH 8.2 and 37°, with aeration. A 3-cm segment was cut out and fixed in the cup of a Dale bath, with one end tied to an auxotonic lever. A smoked drum was used to register the effects. The guinea pigs were starved 15 hr before being killed.

ISOLATED RAT UTERUS. A virgin female rat was killed by a blow in the back of the head followed by section of the carotid arteries. One of the uterine horns was immediately mounted in the cup of an organ bath and covered with nutrient solution (Jalon solution, at pH 7.4), at 31°. One of the ends of the horn was tied to an isotonic lever, and the effects were registered on a smoked drum.

RAT ARTERIAL BLOOD PRESSURE was measured in the cannulated carotid artery with help of a pressure transducer. The rats were previously heparinized and anesthetized with urethane (140 mg/100 g body weight).

Treatment of Kinetic Data. The values of K_m and V_m for Tos-L-Arg-OMe hydrolysis were determined according to Lineweaver and Burk (1934), using the variance of v as statistical weight (Wilkinson, 1961). The calculations were carried out with the help of a Fortran program called KMVM.

The dissociation constants of benzamidine, β-naphthamidine, and phenylguanidine were determined with Bz-DL-Arg-NPNA, as described by Mares-Guia and Shaw (1965) and Mares-Guia (1968), and the calculation was carried out with the help of a program called INCOM. An IBM-1130 computer was used for these calculations.

The K_i values for substituted benzamidines and phenylguanidines were evaluated by reference to the K_i for benzamidine, through measurement of enzyme activity at two different inhibitor concentrations. Plots were made of v_0/v *vs.* inhibitor concentration according to eq 1, where v_0 is the reaction rate in the absence of inhibitor and v is the rate in the presence of inhibitor (Laidler, 1958).

$$\frac{v_0}{v} = 1 + \frac{[I]}{K_i(1 + [S]/K_m)} \quad (1)$$

The dissociation constant, K_i, for any inhibitor is related to the K_i for benzamidine (BzA) through the following equation

$$K_i(\text{inhib.}) = K_i(\text{BzA})[\text{slope(BzA)}/\text{slope(inhib.)}] \quad (2)$$

where slope(BzA) is the slope of eq 1 for benzamidine and slope(inhib.) is the slope of eq 1 for any other inhibitor. The apparent values of V_m and K_m for kininogen hydrolysis were graphically evaluated from a Lineweaver–Burk plot.

Results

(1) Purification. Urine was collected overnight from rats in a metabolic cage placed over a funnel provided with a nylon screen to separate feces. During day time the rats were given food and water at will; at night they were given only water. The urine samples were collected every morning, filtered, and stored under toluene in the refrigerator in dark bottles.

CONCENTRATION. A sample of 16.7 l. of urine was filtered, dialyzed against running water at 4° for 72 hr and then against 0.9% NaCl for an additional 24 hr, and fed into a concentrating device formed by a vacuum conical flask containing a long dialysis tubing of 1-cm diameter. Applying low vacuum to the outside of the membrane, pervaporation took place, allowing the volume to be reduced to 500–600 ml.

ACETONE PRECIPITATION. Two acetone cuts were made, one from 0 to 45% (v/v), the other from 45 to 65% (v/v). Table I shows that the 45–65% cut contained most of the activity.

GEL FILTRATION OVER SEPHADEX G-100. Figure 1 shows the filtration of a sample of 500 mg of material from the preceding step, containing 282.5 mg of protein. The sample was dissolved in 10 ml of 0.05 M ammonium acetate, 0.40 M in NaCl, at pH 6.7. Elution was carried out with the same buffer. The active fractions were identified with Bz-DL-Arg-NPNA, and the active peak was in a trough in the protein profile.

After pooling the active fractions, they were dialyzed against deionized water at 4° and lyophilized. A purification of 4.3-fold was obtained up to this point (Table I).

AFFINITY CHROMATOGRAPHY. The material from the preceding step was then chromatographed in a 1.7 × 6.0 cm column of Sepharose-Arg-OMe. Seven-milligram samples, containing 5 mg of protein, were applied and eluted with

TABLE I: Purification of Rat Urinary Kallikrein.

Step	Treatment	Protein (g)	Sp. Act. (μmol/(mg min)) Amidasic	Esterasic	Kinino-genic	Total Amidasic Act. (μmol/min)	Purif. Factor[a]	Yield (%)[a]
1	Pervaporation	10.7	0.004			42.8	1	100
2	Acetone cut, 45–65%	5.30	0.007	0.69	0.11	37.1	1.75	86.7
3	Sephadex G-100	1.40	0.017	1.78	0.26	23.8	4.25	55.6
4	Sepharose-Arg-OMe	0.18	0.113	4.56	0.27	20.3	28.3	47.4
5	Twice Sephadex G-150	0.023	0.348	7.42	31.7	8.04	87.0	18.8

[a] Based on amidasic activity.

100 ml of 0.05 M Tris (pH 8.1) followed by 160 ml of a linear ionic strength gradient from 0 to 0.4 M NaCl in 0.05 M Tris (pH 8.1). Finally, 10 ml of 1 M arginine (pH 8.1) was passed through the column. The major active component was eluted with the ionic strength gradient and was further studied. A 6.6-fold purification factor was obtained, in relation to the previous step (Table I). The preparation of the column material, as well as the technical details, will appear in a separate paper dealing with affinity chromatography of trypsin-like enzymes. The material obtained was dialyzed against deionized water at 4° and lyophilized.

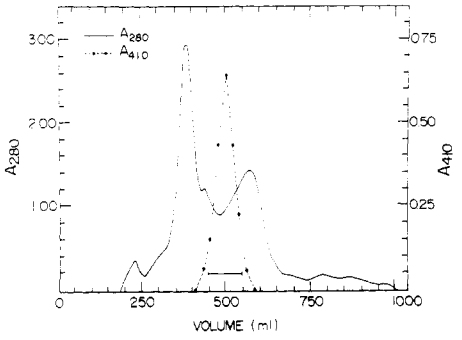

FIGURE 1: Gel filtration over Sephadex G-100: 282.5 mg of protein from the 45–65% acetone cut added to two columns in series, 2.3 × 103 cm and 2.5 × 90 cm, respectively. Elution: 0.05 M acetate buffer, 0.40 M in NaCl, containing merthiolate at 1:50,000 (%), at pH 6.7; flow rate was 20 ml/hr, with 4.5-ml fractions being collected at 4°. Activity was measured with Bz-DL-Arg-NPNA.

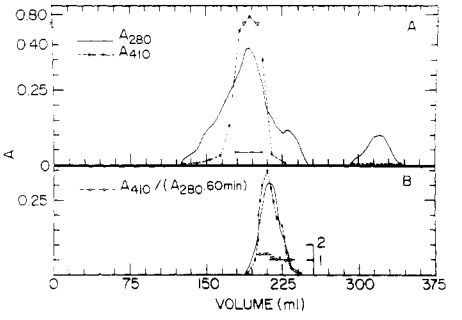

FIGURE 2: Gel filtration over Sephadex G-150: (A) 39 mg of protein applied to a 2.2 × 88 cm column, equilibrated with 0.10 M ammonium acetate buffer, pH 7.0, 0.10 M in NaCl. *Elution* took place with same buffer, at a flow rate of 12 ml/hr, and fractions of 3.2 ml were collected, at room temperature; activity was determined with Bz-DL-Arg-NPNA; (B) 8 mg of protein obtained in A was applied to the same column and eluted under identical conditions.

GEL FILTRATION OVER SEPHADEX G-150. Material from the previous step containing 39 mg of protein was dissolved in 3.5 ml of 0.01 M ammonium acetate, 0.1 M in NaCl, at pH 7.0, and filtered through a 2.2 × 88 cm column of Sephadex G-150. Elution was carried out with the same buffer. The results are shown in Figure 2A. The active fractions were pooled, dialyzed against deionized water at 4°, and lyophilized. Eight milligrams of protein were dissolved in 2.0 ml of the same buffer and applied to the same column. Elution took place under the same conditions, and the pattern of rechromatography is shown in Figure 2B. The protein peak was fairly homogeneous, but it contained two close levels of specific activity, measured with Bz-DL-Arg-NPNA, and registered in Figure 2B. The fractions were pooled as indicated, dialyzed against deionized water, and lyophilized. The analytical results are shown in Table I. It is seen from Table I that an 87-fold purification was obtained with 18.8% yield, measured from the concentrate.

(*2*) *Physicochemical Characterization.* POLYACRYLAMIDE ELECTROPHORESIS. The fraction obtained by Sephadex G-150 rechromatography showed a single component after electrophoresis in 7.5% polyacrylamide for 80 min in Tris-glycine at pH 8.2. This component will now be referred to as KUR, rat urinary kallikrein.

ELECTROFOCUSING. KUR was submitted to electrofocusing in an ampholine pH gradient from 3 to 6. After 22 hr at 4°, the pattern shown in Figure 3 was observed. It is clearly seen that two major components with p*I* = 3.97 and 4.18 were obtained, whereas three minor components had p*I* = 4.50, 4.87, and 5.30.

CELLOGEL ELECTROPHORESIS. The glycoprotein nature of KUR was made evident through cellogel electrophoresis, followed by staining by the Schiff's periodic acid

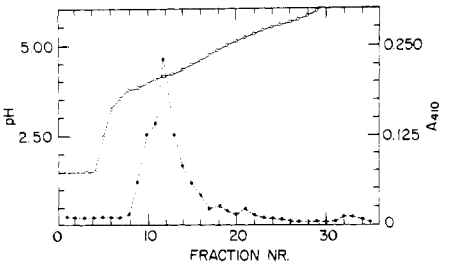

FIGURE 3: Electrofocusing of KUR in ampholine. The pH gradient was 3–6; 3.5 mg of KUR was dissolved in the dense solution and applied to an LKB 8100 column (110 ml). Conditions: 1.7 W, 22 hr, at 4°. Fractions of 3 ml were collected: (– O) pH; (- - ●) activity, measured with Bz-DL-Arg-NPNA.

(PAS) technique. The results in Figure 4 show two parallel strips stained for carbohydrate and protein, respectively. Thus, the microheterogeneity brought about by electrofocusing is again seen on cellogel electrophoresis.

MOLECULAR WEIGHT DETERMINATION. Filtration of KUR over a Sephadex G-150 column calibrated with lysozyme, trypsinogen, and bovine serum albumim permitted the determination of its molecular weight, after plotting V_e/V_0 *vs.* log (mol wt). A straight line was obtained, from which a molecular weight of 33,100 was calculated. The void volume was determined with blue dextran (Pharmacia).

TITRATION WITH NPGB. KUR was titrated by NPGB, an active center titrant of trypsin described by Chase and Shaw (1967). After the initial burst of *p*-nitrophenol, a slow hydrolysis was also observed. Calculation of enzyme molarity indicated that KUR was 97.4% pure in active centers. For this calculation the molecular weight was taken as 33,100 (see above) and protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

(*3*) *Kinetic Characterization.* The *amidasic activity* of KUR toward Bz-DL-Arg-NPNA was inhibited by benzamidine, β-naphthamidine, and phenylguanidine and the results are shown in Table II. The inhibition was competitive in nature, as determined according to Mares-Guia and Shaw (1965). Several substituted benzamidines were also found to be competitive inhibitors of KUR. The K_i values, calculated according to eq 2, are given in Table II.

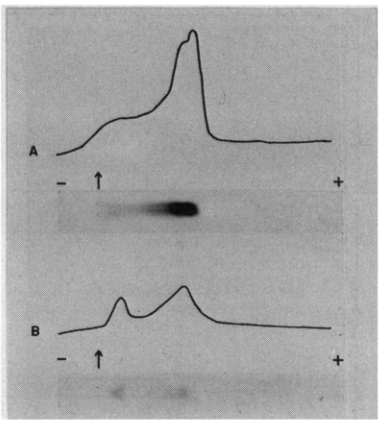

FIGURE 4: Cellogel electrophoresis of KUR. Fifty micrograms of KUR was added to each of two 5.7 × 14 cm strips and developed for 40 min, at 1 mA/cm and 200 V, in 0.1 M borate buffer (pH 9.5): (A) stained with Amido Black 10B, 0.5%; (B) stained with Schiff's periodic acid.

TABLE II: Competitive Inhibitors of Rat Urinary Kallikrein (Tris 0.10 M, pH 8.1, 37°).

Inhibitor	10^4K_i (M)	10^4SD (M)
β-Naphthamidine·HCl	0.46[a]	0.16
Benzamidine·HCl	2.40[a]	0.90
Phenylguanidine·$0.5H_2SO_4$	10.0[a]	2.00
p-Aminobenzamidine·HCl	0.36[b]	
m-Methylbenzamidine·HCl	0.79	
p-Hydroxybenzamidine·HCl	1.01	
p-Methylbenzamidine·HCl	1.02	
p-Chlorobenzamidine·HCl	3.01	
p-Bromobenzamidine·HCl	3.32	
p-Nitrobenzamidine·HCl	4.22	
p-Aminophenylguanidine·$0.5H_2SO_4$	2.51	

[a] Determined according to Mares-Guia and Shaw (1965). [b] All the K_i values in this group were calculated as described under the section on Treatment of Kinetic Data (Methods). Incubation mixtures (2.0 ml) contained 10 μg of KUR and were 0.40 mM in Bz-DL-Arg-NPNA and 0.10 M in Tris, at pH 8.1. Incubation lasted 60 min at 37°.

During these experiments the interesting observation was made that substituted guanidines are activators of KUR in the same concentration range where amidines are inhibitors. *p*-Tolylguanidine shows a slight inhibitory action that gives way to activation at higher concentrations.

The *esterasic activity* of KUR toward Tos-L-Arg-OMe was very little dependent on the ionic strength in the range $\mu = 0.1$–0.8 but, as expected, the rate of reaction depended on the enzyme concentration.

The enzyme was activated by the substrate at concentrations greater than 1 mM (Figure 5). The optimum pH for hydrolysis was 8.5, as shown in the inset of Figure 5. The kinetic parameters for Tos-L-Arg-OMe hydrolysis at concentrations smaller than 1 mM are shown in Table III.

Several *active center reagents* of trypsin were tested upon rat urinary kallikrein. Thus, Tos-LysCH_2Cl, EPGB, and APB did not inactivate KUR at final concentrations in the range 2–3 mM, in 0.05 M Veronal buffer (pH 8.1). EPGB, however, displayed a slight activation, also observed with other guanidines, whereas Tos-LysCH_2Cl showed a slight reversible inhibition. Another alkylating agent of trypsin described by Schroeder and Shaw (1971), *p*-guanidinophenacyl bromide (GPB), inactivated KUR under the same conditions already described, yielding a second-order rate constant of 3.0 M^{-1} min^{-1}.

TABLE III: Kinetic Parameters of Rat Urinary Kallikrein and β-Trypsin.

	Tos-L-Arg-OMe				Dog Plasma Kininogen			
Enzyme	V_m (μmol/(min mg))	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})	V_m (μg of BK/(min mg))	K_m (mg of BKg/ml)	k_{cat} (sec^{-1})	k_{cat}/K_m (ml/(mg of BKg sec))
β-Trypsin		15.0[a]	270	1.80×10^7	20.0[c]	10.2	0.0075	0.74×10^{-3}
KUR	7.36	130.0[b]	4.0	3.08×10^4	100[c]	27.2	0.0520	1.91×10^{-3}

[a] pH 8.0, 37°; unpublished results of N. M. Magalhães and M. Mares-Guia. [b] pH 8.0, 25°. [c] pH 8.2, 37°; apparent parameters calculated from lines in Figure 6.

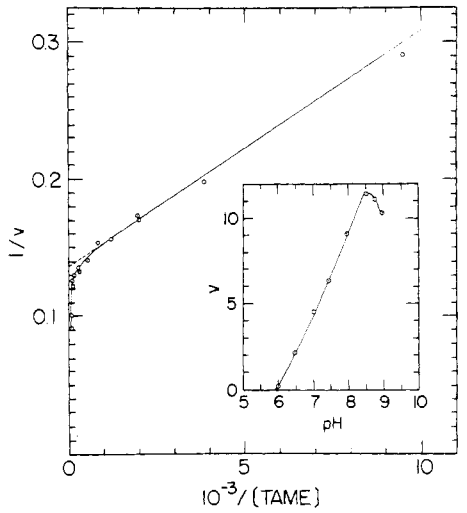

FIGURE 5: Lineweaver-Burk plot for KUR-catalyzed Tos-L-Arg-OMe hydrolysis. Conditions: pH 8.0, 25°; 10 μg of KUR/2.0 ml of incubation mixture; solvent was 0.10 M KCl containing the substrate. Ordinate, reciprocal of rate, in (min mg)/μmol; abscissa, reciprocal of molar concentration of substrate. Inset: pH dependence of rate, measured as above, with 0.108 M substrate.

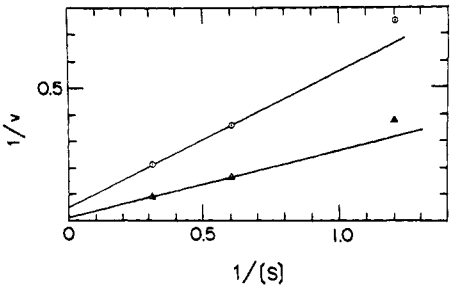

FIGURE 6: Lineweaver-Burk plot for the kinin-releasing activities of KUR and β-trypsin. Conditions: 45.2 μg of KUR or 38.7 μg of β-trypsin were preincubated with 0.25, 0.49, and 0.98 mg of dog plasma kininogen, in 0.30 ml at pH 8.2, 37°. Activity measurement: 10–40 μl of incubation mixture was added to the 4-ml cup containing the isolated guinea pig ileum. The heights of contraction were registered on a smoked drum, and compared with standard bradykinin contractions. Ordinates, reciprocal of rate, in (min mg)/μg of bradykinin released; abscissa, reciprocal of kininogen concentration, in ml/mg; (○) β-trypsin; (△) KUR.

The *kinin-releasing activity* of KUR was assayed with a partially purified dog plasma kininogen. When the kininogen was preincubated with KUR or with trypsin, a kinin was released, according to the assays carried out with the help of the isolated guinea pig ileum. After a rapid kinin liberation within the first minute of incubation, there followed a plateau in the time curves of both KUR and β-trypsin. The initial rates of kinin liberation originated a Lineweaver-Burk plot (Figure 6) from which apparent kinetic parameters were calculated and included in Table III. A justification for the use of these parameters will be given under the Discussion Section.

(*4*) *Biological Characterization.* In order to further characterize KUR as a kallikrein, three other techniques were used. When KUR was added to the isolated guinea pig preparation to which dog plasma kininogen had already been added, a contraction was observed. This contraction would not be elicited by either KUR or kininogen alone.

Intravenous injection of 31 μg of KUR into a urethane anesthetized rat caused an arterial blood pressure drop of 30 mm, indicating the liberation of kinin in the circulating blood of the rat. Finally, KUR caused a direct contraction of the isolated rat uterus. By reference to bradykinin, it was possible to determine that 0.075 μg of KUR has the same activity on the isolated rat uterus as 0.001 μg of bradykinin.

Discussion

Purification. A review of purification procedures for kallikreins was recently published by Pierce (1970). Acetone fractionation has been previously used by Moriya *et al.* (1963), in the purification of human urinary kallikrein, and by Prado *et al.* (1962, 1963) in the purification of horse urinary kallikrein. Croxatto *et al.* (1971) used it in work with rat urinary kallikrein, as well as Moriya *et al.* (1965, 1969) who used it for the purification of hog pancreatic kallikrein. Gel filtration through Sephadex G-100 has also been used by different workers in the purification of kallikreins from several origins.

The application of affinity chromatography as a step in the purification scheme was the consequence of previous work of this laboratory which indicates that rat urinary kallikrein is related to trypsin in terms of action on synthetic substrates and inhibition by benzamidine (Diniz *et al.*, 1965). Other researchers have applied affinity chromatography in kallikrein purification: Fritz *et al.* (1972) for the purification of hog and human serum kallikreins; Fritz and Förg-Brey (1972) for the purification of several hog kallikreins; and Takahashi *et al.* (1972) for the purification of bovine plasma prekallikrein.

A single, fairly symmetrical component was obtained after rechromatography on Sephadex G-150 (Figure 2B). Takahashi *et al.* (1972) used Sephadex G-150 chromatography in the purification of bovine plasma prekallikrein. Hial (1973) and Hial *et al.* (1974) also used it as a final step and obtained a homogeneous preparation of human urinary kallikrein.

Referring back to Table I, it should be noted that the kinin-releasing activity had a large improvement only after the Sephadex G-150 chromatographies. This finding was confirmed in all repetitions of the process. It is interpreted as being due to the removal, at the later steps, of a ligand of KUR that severely affected binding of protein substrates but apparently did not prevent action on the small synthetic substrates used.

Criteria of Purity. The rat urinary kallikrein described in this work was considered pure by the following criteria: (i) it gave a single peak upon rechromatography on Sephadex G-150 (Figure 2B); (ii) it produced a single band on polyacrylamide electrophoresis at pH 8.2; (iii) the titration of the enzyme active center with NPGB gave 97.4% purity, related to a molecular weight of 33,100 found by gel filtration on Sephadex G-150. This estimate of purity carries an uncertainty related to the estimation of the molecular weight in Sephadex. The enzyme is, however, microheterogeneous, as demonstrated by electrofocusing in the pH gradient 3–6 (Figure 3) and by cellogel electrophoresis at pH 9.5 (Figure 4). The major component has a p*I* of 4.18, a lesser component has a p*I* of 3.97, whereas minor components have p*I* values of 4.50, 4.87, and 5.30. The microheterogeneity of kallikreins is most probably due to their glycoprotein nature, as discussed by Fritz *et al.* (1967).

Physicochemical Properties. The value of the molecular weight found for KUR by gel filtration (33,100), the isoelectric points observed by electrofocusing, as well as the glycoprotein nature of KUR are all within the set of proper-

ties that have been observed for kallikreins by other research workers (see Frey *et al.* 1968, for a review). The molecular weight given here is subject to some uncertainty, as glycoproteins may be more hydrated in solution than typical globular proteins (Andrews, 1965). Very recently Nustad and Pierce (1974) reported p*I* values in the range 3.50–3.80 for rat urinary kallikrein.

Kinetics. (i) AMIDASIC ACTIVITY. The hydrolysis of Bz-DL-Arg-NPNA by rat urinary kallikrein as well as its inhibition by benzamidine, β-naphthamidine, and phenylguanidine were first shown by Mares-Guia and Diniz (1967a), with a partially purified preparation.

The K_i values in Table II are very similar to those obtained with the impure preparation. From Table II it is clear that *p*-aminobenzamidine and β-naphthamidine are the best inhibitors of the group, a finding also observed for trypsin (Mares-Guia, 1968). Another similarity between KUR and trypsin is that amidines are better inhibitors than guanidines for both enzymes (Table II; Mares-Guia and Shaw, 1965). Although there are quantitative differences in the K_i values found for each inhibitor with either enzyme, it is clear that the pattern of binding is similar. Thus, the active center of KUR contains a binding site for the side chain of Arg or Lys, probably formed by an anionic site and a hydrophobic site (Mares-Guia and Shaw, 1965, 1967; Mares-Guia *et al.*, 1970). From Table II one can also conclude that the substituted benzamidines carrying an electron-releasing group are better inhibitors than those carrying electron-withdrawing groups. A similar conclusion was reached with trypsin in this laboratory, and its detailed interpretation will be published soon.

An interesting result that contrasts with that found for trypsin is the *activation* caused by *p*-tolylguanidine and *p*-ethylphenylguanidine. This phenomenon is now undergoing investigation.

(ii) ESTERASIC ACTIVITY. Using a crude rat urinary kallikrein preparation, Diniz *et al.* (1965, 1966) showed that it hydrolyzed Bz-L-Arg-OEt. The activity was inhibited by benzamidine, but was resistant to inactivation by Tos-LysCH_2Cl.

In the present work we demonstrate the hydrolysis of Tos-L-Arg-OMe by KUR (Figure 5). The Michaelis–Menten mechanism is obeyed up to 1 mM substrate. At higher Tos-L-Arg-OMe concentrations, substrate activation of the enzyme ensues, as shown by the downward deviation of the line in the Lineweaver–Burk plot in Figure 5. The inset of Figure 5 shows an apparent optimum pH of 8.5 for the hydrolysis of the substrate by KUR. The curve was not corrected for the substrate dissociation in the alkaline range. A comparison of the parameters for Tos-L-Arg-OMe hydrolysis by KUR and β-trypsin is presented in Table III. A pure human urinary kallikrein prepared by Hial *et al.* (1974) had a very low level of hydrolytic activity toward Tos-L-Arg-OMe, as indicated by a k_{cat}/K_m ratio of 220 in contrast to the values shown in Table III.

Fiedler and Werle (1968) reported the Tos-L-Arg-OMe activation of hog pancreatic kallikrein. This phenomenon, which was observed with trypsin by Trowbridge *et al.* (1963) and Bechet and Yon (1964), and in human urinary kallikrein by Hial *et al.* (1974), may be a general pattern in proteases of the trypsin type of activity. Kinetically, it is related to binding at a *secondary binding site* in the active center of the enzymes, and in the case of KUR, its relation to activation by substituted phenylguanidines is now being investigated.

(iii) Several active center reagents of trypsin were tested on KUR. Tos-LysCH_2Cl did not inactivate KUR as it does trypsin (Shaw *et al.*, 1965), but caused a slight reversible inhibition, probably competitive. Another reagent, EPGB (Mares-Guia and Shaw, 1967), also failed to inactivate KUR and, indeed, caused a slight activation of the enzyme, a fact observed with substituted phenylguanidines. From two trypsin alkylating agents described by Schroeder and Shaw (1971) one, APB, was inert toward KUR, whereas the other, GPB, inactivated KUR (see Results). At the present time we do not know the site of alkylation of KUR by GPB.

The lack of reaction of Tos-LysCH_2Cl with human urinary kallikrein, equine urinary kallikrein, and rat urinary kallikrein was observed earlier by Diniz *et al.* (1965, 1966). As emphasized by these authors, it cannot be taken as an indication of lack of a histidyl residue in the enzyme catalytic site. The pH dependence of V_m for Bz-L-Arg-OEt hydrolysis, where a group dissociating with a pK' of 6.75 was observed, may indeed bbbe a strong indication for histidine involvement in KUR catalysis. In that case, the lack of reaction with reagents such as Tos-LysCH_2Cl may be the consequence of a stereochemical arrangement in the E · S complex that does not favor alkylation (Mares-Guia and Diniz, 1967a).

The set of kinetic results obtained up to the present with KUR, and also with other kallikreins by several authors, seems to support a basic scheme for the active center of these enzymes along the lines proposed by Mares-Guia *et al.* (1970). A *secondary binding* site is required for the understanding of substrate and guanidine activation, as shown in this paper. One should not assume, however, that trypsin-like kallikreins and trypsin have closely similar or identical active centers. As discussed by Hial *et al.* (1974), the specificity site of kallikreins may require the binding of more than *one* amino acid residue in the natural substrate sequence, causing the remarkable protein specificity of these enzymes.

(iv) The kinin-releasing activity of KUR was quantitatively determined with a partially purified dog plasma kininogen. Although a partially purified substrate was used, we felt that calculation of apparent provisional kinetic parameters, K_m and V_m, would be very useful in comparing the activities of different kininogenases (Figure 6, Table III). This is so because frequently in the literature these enzymes have been compared on the basis of activity determinations at a single substrate concentration, most of the time not saturating. Thus, apparent K_m and V_m values offer a better basis for reporting the activity of kallikreins, until a pure protein substrate is available to everybody working in this field.

A comparison between the kinin-releasing activities of KUR and β-trypsin (Table III) shows that KUR has a k_{cat} value *ca.* 7 times greater than β-trypsin, but their k_{cat}/K_m ratios differ by a factor of 2.6.

The results in Table III further emphasize the important point that continued study with synthetic substrates of the type used up to the present may lead to an oversimplified model of the active centers of kallikreins. Indeed, Hial *et al.* (1974) have purified from human urine a kallikrein that has extremely low activity toward Tos-L-Arg-OMe, but has a kinin-releasing activity comparable to that of β-trypsin or KUR.

Biological Properties. The characterization of a kallikrein requires biological assay due to the nature of the reac-

tion product between enzyme and substrate, a peptide that is able to lower blood pressure or to elicit contraction of the isolated guinea pig ileum, among other activities. In the case of KUR, a unique test is the direct action of the enzyme upon the rat uterus, causing contraction. The lowering of blood pressure caused by injection of KUR into the rat makes evident the interaction of this enzyme with a plasma substrate of its own species, causing a marked drop in arterial blood pressure.

Final Comment. The next steps in this work include, besides those mentioned in the text, the identification of the peptide liberated when KUR is incubated with kininogen, a necessary step in the definition of the specificity of the enzyme.

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