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RODENT KININ-FORMING ENZYME SYSTEMS—I

PURIFICATION AND CHARACTERIZATION OF PLASMA KININOGEN*†

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Abstract—Low molecular weight (LMW) kininogen was purified 70-fold with a 16% yield from fresh rat plasma by DEAE-Sephadex chromatography, ammonium sulfate precipitation, Sephadex G-200 gel filtration, SP-Sephadex chromatography, CM-cellulose chromatography, and Sephadex G-200 gel filtration. Ferguson plots of polyacrylamide gel electrophoretic patterns revealed four bands with relative molecular weights of 64,000, 123,500, 252,436 and 357,900 (ratio of 1:2:4:6). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis provided a single protein band with a molecular weight of 72,000, suggesting that the four kininogen bands had been caused by the aggregation of a single oligomeric protein. The purified LMW rat kininogen Fraction B (3.9 μg bradykinin/mg) was used to elicit an antiserum in the rabbit. Monospecificity of the antiserum was demonstrated by immunoelectrophoresis (Laurell rocket and Grabar methods) and, thus, the homogeneity of the kininogen was also. The purified kininogen (both Fractions A and B) formed kinin with human urinary kallikrein, rat urinary kallikrein and hog pancreatic kallikrein. Murphy–Sturm lymphosarcoma acid protease also formed kinin when incubated with the kininogen at pH 3.0. The isoelectric point for both fractions was at pH 4.3. Amino acid analyses showed the two kininogen fractions to be rich in acidic amino acids and to have a total carbohydrate content of 8.5% consisting of galactose (1.2 to 1.5%), mannose (1.9 to 2.1%), *N*-acetylglucosamine (4.3 to 5.1%), *N*-acetylgalactosamine (0.3%), and sialic acid (0.68%).

Two molecular species of kininogen, usually called low molecular weight (LMW) and high molecular weight (HMW) kininogens, have been reported to be present in rat plasma [1–3]. In a continuing effort to isolate and characterize a rat kininogen substrate suitable for the study of kinin-forming proteases from rodent transformed and malignant cells [4–7] and their formed vasopeptides [8], a kininogen with an apparent molecular weight of 110,000 was isolated and purified from rat plasma [9]. This kininogen yielded a single protein band on disc gel electrophoresis and formed vasopeptide kinins when incubated either with murine acid protease or crystalline trypsin. The present study reports the purification and characterization of yet another rat plasma kininogen with an apparent molecular weight of 72,000 and oligomeric properties.

MATERIALS AND METHODS

Acrylamide, *N,N'*-methylene bisacrylamide and *N,N,N',N'*-tetramethylene diamine were obtained from the Eastman Kodak Co., Rochester, NY. Agarose, ammonium persulfate, and Coomassie Brilliant Blue R-250 were purchased from Bio-Rad Laboratories, Richmond, CA. Tris base, trichloroacetic acid, sulfosalicylic acid and sodium dodecyl sulfate were products of the Sigma Chemical Co., St. Louis, MO. Polybrene was obtained from the Aldrich Chemical Co., Milwaukee, WI. Ampholines were procured from LKB Instruments, Inc., Rockville, MD. Sephadex G-200 (fine), DEAE-Sephadex A-50, and SP-Sephadex C-50 were products of Pharmacia Fine Chemicals, Piscataway, NJ. CM-cellulose was a product of Whatman Ltd., Springfield, Maidstone, U.K. Proteins used as molecular weight standards in gel electrophoresis were from the following sources: aldolase, catalase, chymotrypsinogen A, ovalbumin and ribonuclease from Pharmacia; transferrin from Sigma; soybean trypsin inhibitor from the Worthington Biochemical Corp., Freehold, NJ, and bovine serum from Calbiochem, La Jolla, CA. Rabbit anti-rat whole plasma was purchased from Miles Yeda, Ltd., Israel. Synthetic bradykinin was a product of U.S. Biochemicals, Cleveland, OH. All other reagents used were certified analytical grade chemicals.

Rat plasma kallikrein was prepared from citrated rat plasma. Kallikrein activity was monitored by either tosyl arginine methyl ester (TAME) hydrolytic activity [10] or by its ability to release kinin from heat-inactivated rat plasma [11]. Rat urinary kallikrein was obtained according to Margolius *et al.* [12]. Human plasma killikrein, human urinary kallikrein and hog pancreatic kallikrein were products of

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Bayer, A.G., West Germany. Trypsin was obtained from the Worthington Biochemical Corp. An acid protease was isolated from the Murphy–Sturm lymphosarcoma as described in the following paper [13].

Purification of rat plasma kininogen

Step 1: DEAE-Sephadex A-50 chromatography. All procedures were carried out at 4° unless otherwise specified. All fractions were concentrated by ultrafiltration through an Amicon PM-30 membrane and assayed for kininogen by the method of Diniz and Carvalho [14]. Citrated fresh rat plasma (380 ml), obtained from the abdominal aorta of Sprague–Dawley rats, was dialyzed at room temperature against two changes of 0.05 M Tris–Cl 0.001 M EDTA/0.005% Polybrene, pH 8.2, and loaded onto a DEAE-Sephadex A-50 (5 × 67 cm) column previously equilibrated in the same buffer. The column, washed initially with 2 liters of starting buffer, then was eluted with an 8-liter linear gradient of 0 to 0.5 M NaCl in the 0.05 M Tris–Cl buffer.

Step 2: Ammonium sulfate precipitation. Kininogen-containing fractions from Step 1 were pooled, and solid $(NH_4)_2SO_4$ was added in small increments to bring the fractions to 60% saturation. The precipitate was allowed to settle for 16 hr at 4° and was then separated by centrifugation in a Sorvall refrigerated centrifuge for 30 min at 12,000 *g*. The precipitate was dissolved in 100 ml of distilled water and dialyzed initially against cold water and then against 0.05 M sodium acetate, pH 6.5. After extensive dialysis a slight precipitate formed which was eliminated by centrifugation for 30 min at 12,000 *g*, and the supernatant fraction was concentrated.

Step 3: Sephadex G-200 gel filtration. The kininogen concentrate from Step 2 was applied to a Sephadex G-200 column (5 × 90 cm) in 0.05 M $C_2H_3NaO_2$, pH 6.5. Fractions containing kininogen were pooled, concentrated, and dialyzed against 0.05 M $C_2H_3NaO_2$/0.10 M NaCl, pH 4.5.

Step 4: SP-Sephadex C-50 chromatography. SP-Sephadex C-50 was preequilibrated with 0.05 M $C_2H_3NaO_2$/0.1 M NaCl, pH 4.5. After applying kininogen concentrate in this buffer from Step 3 to the column (2.6 × 40 cm) it was eluted with a 1.6-liter linear gradient of 0.1 M to 0.6 M NaCl in 0.05 M $C_2H_3NaO_2$, pH 4.5. Kininogen-containing fractions were pooled and concentrated.

Step 5: CM-cellulose chromatography. The concentrate from Step 4, after extensive dialysis against 0.05 M $C_2H_3NaO_2$, pH 5.0, was applied to a CM-cellulose column (1.5 × 30 cm) which then was washed with starting buffer and eluted with an 800-ml linear gradient of NaCl in 0.05 M acetate buffer, pH 5.0. The kininogen-containing fractions were pooled and concentrated to 10 ml.

Step 6: Sephadex G-200 gel filtration. The pooled kininogen fraction from Step 5 was applied to a Sephadex G-200 (fine) column, which was packed and eluted with 0.5% ammonium bicarbonate, pH 8.0. The collected fractions were assayed for kininogen content. The selected column fractions were subjected to polyacrylamide gel electrophoresis in the absence and presence of SDS and, also, to Laurell rocket immunoelectrophoresis [15]. Fractions A and B (tubes 105–125 and tubes 126–145, respectively) were pooled separately as shown in Table 1.

Polyacrylamide gel electrophoresis

The homogeneity of each preparation was determined by analytical disc gel electrophoresis carried out in 7% acrylamide gel prepared in 0.375 M Tris–0.06 M HCl buffer, pH 8.9, as described by Davis [16]. The electrophoresis was run in 0.05 M Tris–0.38 M glycine buffer, pH 8.3, at a constant current of 2 mA/tube for 2 to 2.5 hr at room temperature. Protein bands were stained with 0.1% Coomassie Brilliant Blue R-250 in 35% methanol, 12% trichloroacetic acid, 3.5% sulfosalicylic acid for 30 min at 60° [17] and were destained by diffusion in a 7% acetic acid, 5% methanol solution. The molecular weight of the rat plasma kininogen was estimated from the linear relation between the square root of the retardation coefficient (K_R) obtained from Ferguson plots [18] and the cube root of molecular weight [19]. Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Weber and Osborn [20]. The molecular weight of the kininogen monomer was estimated from the calibration curve obtained by plotting the logarithms of the molecular weights of the standard proteins versus their relative mobilities.

Preparation of kininogen antiserum

Antiserum against purified rat plasma kininogen Fraction B was raised in New Zealand male rabbits weighing 3.5 to 4.0 kg. Rabbits were immunized with 800 μg of the purified rat plasma kininogen in 1 ml saline emulsified with an equal volume of complete Freund's adjuvant injected subcutaneously and intramuscularly. This immunization step was repeated weekly for 4 weeks. After a 2-week rest period, a booster injection of 200 μg of the rat plasma kininogen was given by the intravenous route, and a week later blood was withdrawn from the marginal ear vein. The antiserum was obtained by incubating the blood at 37° for 1 hr, followed by incubation at 4° overnight. Potency of the serum was ascertained by Ouchterlony double-diffusion tests in 1% agar using serially diluted antiserum [21].

Immunoelectrophoresis procedures

Immunoelectrophoresis was carried out according to the method of Graber and Williams [22]. Gels were poured into Gel Bond polyester film and stored at 4° for 24 hr before use. Aliquots (10 μl) were placed into the wells, and electrophoresis was carried out for 6 hr at 10 V/cm in the LKB 2117 Multiphor. After electrophoresis, 50 μl of the antiserum was applied in the troughs running parallel to the direction of the run, and the plates were incubated at room temperature for 24 hr in a humidity chamber. The gels were washed extensively with 0.9% NaCl, dried, and stained with 0.1% Coomassie Brilliant Blue R-250 as described for polyacrylamide gel electrophoresis. Laurell rocket electrophoresis [15] in 1% agarose gels containing 2.5% antibody preparation was performed at 10 V/cm for 10 hr.

Isoelectric focusing

Isoelectric focusing was performed with an LKB apparatus, model 8101, according to the method of Vesterberg and Svensson [23]. Ampholine, pH range 3–10, was added at a concentration of 2% in a sucrose density gradient containing 5 mg of purified kininogen in a 110-ml column. Electrophoresis was carried out at 300 V for 3 days at 4°. After focusing, 2-ml fractions were collected and assayed for kininogen.

Amino acid and sugar analysis

Kininogen samples for amino acid analysis were prepared according to the method of Spackman *et al.* [24] and were analyzed on a Beckman Amino Acid Analyzer, model 121. Amino sugar analyses were carried out on a Beckman model 121 Amino Acid Analyzer after hydrolysis of the samples with 1 ml of 4 N HCl for 4 hr at 100°. Neutral sugars were determined as alditol acetates after hydrolysis with AG 50 (H^+) suspension in 0.02 N HCl (40%, v/v) for 40 hr [25] using arabitol as an internal standard. The acetylated sugars were analyzed by GLC on a column of 3% OV-225 at 190° isothermally using a Varian Gas Chromatography Series 3700. Sialic acid was determined according to the thiobarbituric acid procedure of Warren [26].

Enzymatic release of kinin from kininogen

Comparative assays were carried out on the purified kininogen with rat plasma kallikrein, human plasma kallikrein, human urinary kallikrein, hog pancreatic kallikrein, rat urinary kallikrein, and acid protease isolated and purified from Murphy–Sturm lymphosarcoma (MSLS). The kininogen samples (1 mg/ml) were incubated at 37° for 10 min with 10 mU of the kallikreins in 0.1 M Tris buffer, pH 7.8, containing 30 mM EDTA or with 10 mU of the MSLS acid protease in 0.1 M citrate buffer, pH 3.0. One unit of kallikrein activity is defined as the amount of enzyme that hydrolyzes 1 μmole TAME/min, while a unit of acid protease represents the amount of enzyme that liberates 1 μmole tyrosine equivalent/min from hemoglobin substrate. The reaction was stopped by immersing the incubation mixture into boiling water for 10 min. The samples were cooled and assayed for the kinin released. Total releasable kinin activity was determined by incubating the kininogen with 50 μg (≃10 units) of trypsin. For further comparison, the enzymes were studied under identical conditions for their kinin-releasing activities on rat plasma that had been heated at 61° for 60 min. An amount of 10 mg of heated rat plasma was used per incubation.

RESULTS

Purification of rat plasma kininogen

The purification of rat plasma kininogen is summarized in Table 1. The kininogen that eluted from the DEAE-Sephadex column at 0.3 M NaCl resulted in a 2.0-fold purification of the kininogen. Prekallikrein was eliminated during the first part of the salt gradient. An increase in the total kininogen during this step presumably was due to the removal of some inhibitor(s) of kinin-forming activity. The $(NH_4)_2SO_4$-precipitated proteins were resolved into three peaks by Sephadex G-200 gel filtration. Most of the kininogen was present in the last peak, tubes 63–80. In the SP-Sephadex C-50 gradient elution chromatography of the G-200 kininogen peak, the kininogen eluted between 0.25 and 0.3 M NaCl in 0.05 M $C_2H_3NaO_2$, pH 4.5. While CM-cellulose chromatography was effective in removing approximately 45% of the protein from the kininogen pool of the SP-Sephadex C-50 step, the specific activity increased only slightly because of a loss of 29% of kininogen activity (Table 1).

Polyacrylamide disc gel electrophoresis of the pooled fractions from the above CM-cellulose column resulted in the appearance of multiple components when stained for protein bands (Fig. 1). Each of the protein bands that eluted from slices of a simultaneously-run gel contained kininogen activity (Fig. 1). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed

Table 1. Summary table of purification of rat plasma kininogen

Step	Treatment	Total protein (mg)	Kininogen (μg Bk eq.)	Specific activity*	Purification factor	Yield (%)
	Plasma	12,920	798	62	1.0	100
1.	DEAE-Sephadex A-50 chromatography	7,796	1,396	178	2.9	178
2.	60% $(NH_4)_2SO_4$ precipitate	1,540	644	418	6.8	81
3.	Sephadex G-200 gel filtration	372	625	1,680	27.2	78
4.	SP-Sephadex C-50 chromatography	203	548	2,698	43.6	69
5.	CM-cellulose chromatography	112	319	2,851	46.2	40
6.	Sephadex G-200 gel filtration					
	Fraction A (105–125)	25	114	4,587	74.2	14
	Fraction B (126–145)	39	151	3,987	63.1	19

* Expressed as ng Bk equivalent/mg protein.

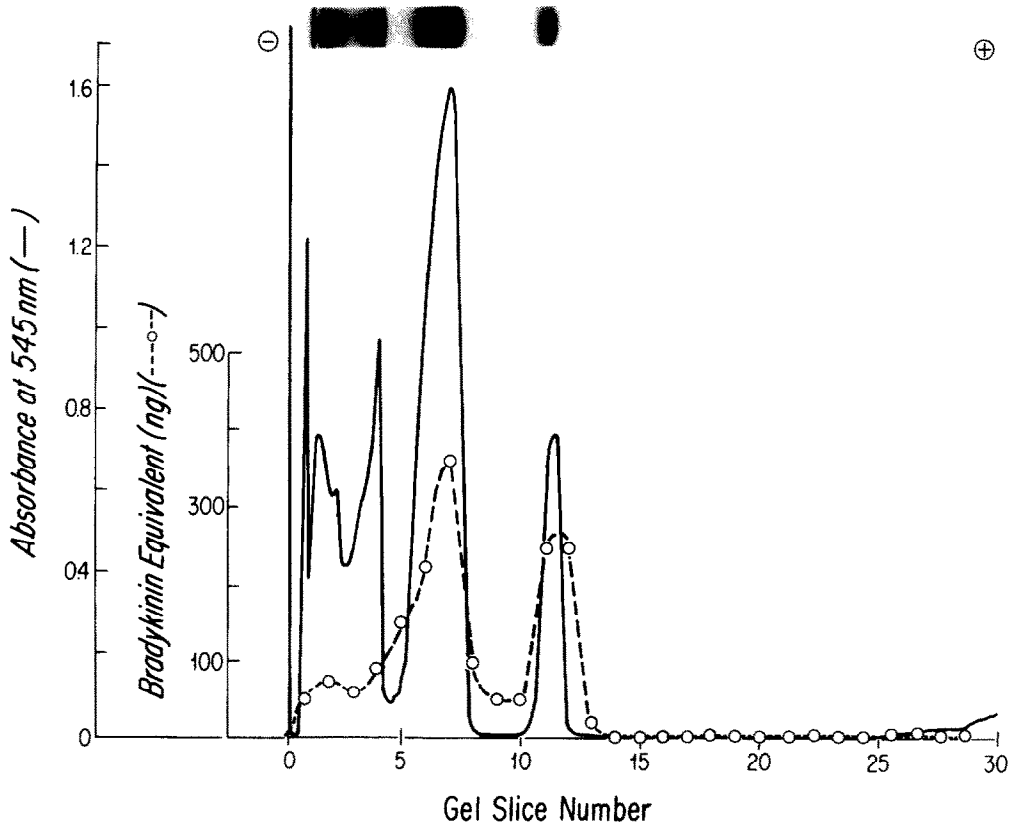

Fig. 1. Polyacrylamide gel electrophoretic pattern and densitometric scan of rat plasma kininogen from the CM-cellulose column. Protein bands were stained with 0.1% Coomassie Brilliant Blue R-250. An identical gel was sliced and each band-containing slice was eluted and assayed for kininogen.

one major band and a few minor bands (data not shown).

To eliminate contaminating proteins and to separate the various bands showing kininogen activity, the pooled CM-cellulose fraction was subjected to Sephadex G-200 gel filtration in a 2.6 × 180 cm column (Fig. 2C). The kininogen-containing fractions were found in the molecular weight range of 68,000 (bovine serum albumin) to 440,000 (ferritin). The specific activity remained essentially constant in fraction numbers 105–145 but showed a continuous decrease in fractions 146–180. When selected fractions were subjected to polyacrylamide disc gel electrophoresis (PAGE) at pH 8.9, a progression of molecular weight forms from high to low was observed (Fig. 2A).

On SDS-polyacrylamide gel electrophoresis, fractions 115–158 showed one single band in the molecular weight range of 72,000, while fractions 165–180 showed additional low molecular weight protein bands (Fig. 2B). Immunoelectrophoresis in anti-kininogen antiserum containing agarose (Laurell rocket immunoelectrophoresis) of these fractions showed a pattern consistent with the bioassay data for kininogen (Fig. 2D). The kininogen-containing fractions were divided into two pools. Fraction A and Fraction B (Table 1). Fractions A and B exhibited single bands on SDS-PAGE and were used for further characterization.

Homogeneity of rat plasma kininogen

Homogeneity of the purified kininogen was confirmed by the presence of a single precipitin line formed between kininogen and anti-kininogen antiserum and rabbit anti-rat whole plasma antiserum (Fig. 3). Further evidence of homogeneity was obtained from the SDS-PAGE study (see Figs. 2 and 6) and from isoelectric focusing in which plasma kininogen yielded a single symmetrical peak with an isoelectric point at pH 4.3.

Molecular weight determination

Since the purified kininogen tended to aggregate even in the presence of high salt concentration, it was not possible to estimate the molecular weight of the kininogen by gel filtration on Sephadex G-200. Thus, the molecular weight of the kininogen was determined by use of a Ferguson plot [18]. Four protein bands were obtained at each gel concentration. The plots of gel concentration versus log R_f for each band yielded a straight line which, on extrapolation, merged at a common point at a 3% gel concentration (Fig. 4); this is the predicted behaviour of proteins bearing an identical charge at a given pH but varying in their molecular sizes [19], thus confirming that different bands arise from the aggregation of a single oligomeric protein. An estimate of the molecular size of each band was made using

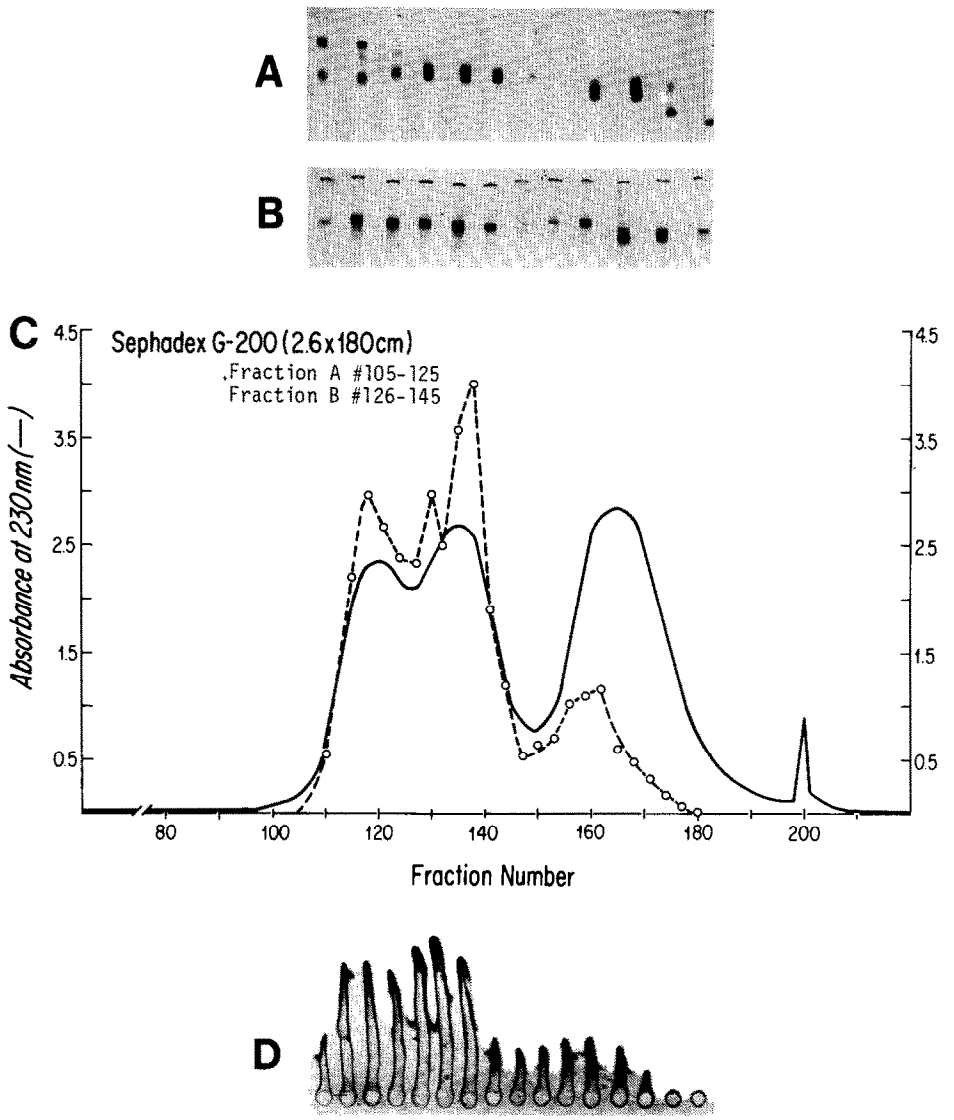

Fig. 2. Gel filtration on a Sephadex G-200 column (2.6 × 180 cm) of rat kininogen from step 5. The kininogen concentrate was applied to the column in 0.5% ammonium bicarbonate, pH 8.0. Fractions of 3.5 ml were collected at a flow rate of 15 ml/hr. Kininogen-containing fractions were subjected to polyacrylamide gel electrophoresis (A), SDS-PAGE (B), and Laurell rocket immunoelectrophoresis in 1% agarose gels (D).

the linear relationship between the square root of the retardation coefficient (K_R, the slope of log R_f versus gel concentration) and the cube root of the molecular weight [19], shown in Fig. 5. The following molecular weights were estimated for each band: 64,000 (Band 1), 123,500 (Band 2), 252,436 (Band 3), and 357,900 (Band 4). Values obtained show an approximate ratio of 1:2:4:6. The molecular weight of the monomer, estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate on 7.5% gels, was 72,000 (Fig. 6).

Amino acid and carbohydrate composition

Table 2 describes the composition of purified rat plasma kininogen. Fractions A and B. Within experimental error, the amino acid compositions of the two fractions were identical. The total carbohydrate content of the kininogen was 8.5% consisting of galactose (1.2 to 1.5%), mannose (1.9 to 2.1%), *N*-acetylglucosamine (4.3 to 5.1%), *N*-acetylgalactosamine (0.3%), and sialic acid (0.68%). Based on a molecular weight estimate of 72,000, rat plasma kininogen appears to have 6 galactose, 8–9 mannose, 14–16 *N*-acetylglucosamine, 1 *N*-acetylgalactosamine and 1.6 sialic acid residues.

Enzymatic release of kinin from kininogen

The susceptibility of the purified rat plasma kininogen to various enzymes is summarized in Table 3. As noted, neither rat nor human plasma kallikrein

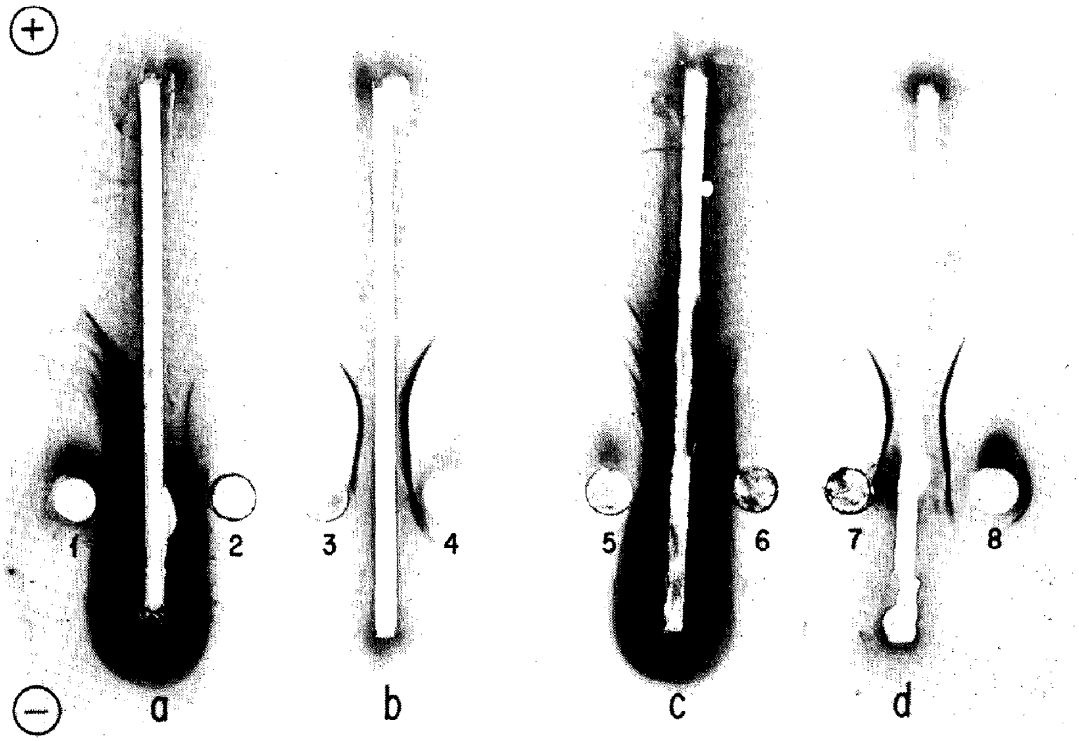

Fig. 3. Immunoelectrophoretic demonstration of homogeneity of purified rat plasma kininogen. Troughs a and c contained rabbit anti-rat whole plasma antiserum and troughs b and d contained rabbit anti-rat plasma kininogen antiserum. Wells 2 and 3 contained rat plasma kininogen (Fraction A) and wells 6 and 7 contained rat plasma kininogen (Fraction B).

Table 2. Amino acid and carbohydrate compositions of rat plasma kininogen (Fractions A and B)

	Fraction A		Fraction B	
	g/100 g (% composition)	moles percent	g/100 g (% composition)	moles percent
Amino acid				
Lysine	7.7	7.4	7.9	7.7
Histidine	3.3	3.0	3.4	3.1
Arginine	3.8	3.0	3.8	3.0
Aspartic acid	9.7	10.3	10.0	10.5
Threonine	6.2	7.3	6.3	7.5
Serine	4.5	6.1	4.4	5.9
Glutamic acid	13.7	13.2	13.9	13.3
Proline	5.2	6.8	5.0	6.1
Glycine	3.4	6.3	3.6	6.7
Alanine	4.2	6.7	4.3	6.8
Half cystine	2.3	2.7	2.6	3.0
Valine	4.7	5.7	4.5	5.4
Methionine	1.25	1.2	0.6	0.6
Isoleucine	3.4	3.7	3.4	3.7
Leucine	7.4	8.0	7.2	7.7
Tyrosine	5.5	4.3	5.6	4.4
Phenylalanine	5.1	4.4	5.3	4.5
Sugars				
Glucosamine	4.3		5.1	
Galactosamine	0.3		0.3	
Mannose	2.1		1.9	
Galactose	1.5		1.2	
Sialic acid	0.68		N.D.*	

* Not done.

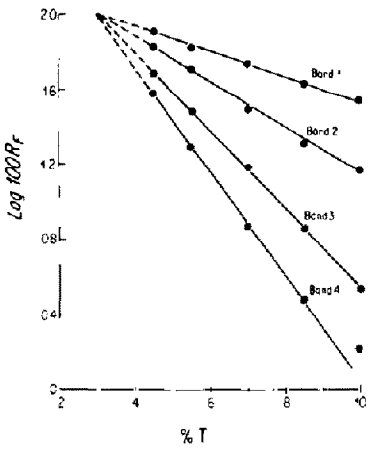

Fig. 4. Ferguson plot of rat plasma kininogen. The plots of gel concentration versus log R_f for four protein bands. (Shown are the averages of at least two determinations.)

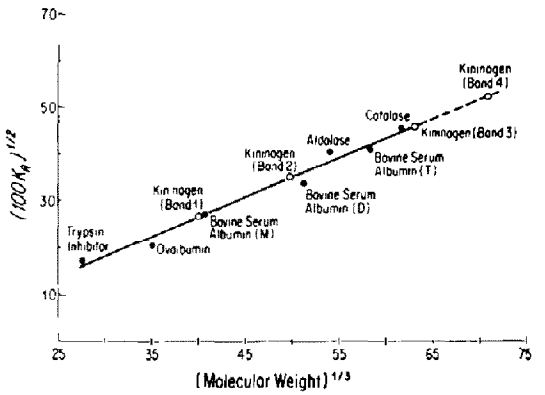

Fig. 5. Polyacrylamide gel electrophoresis molecular weight determination of rat plasma kininogen from Ferguson plot data obtained from Fig. 4. The K_R for kininogen and standard proteins was estimated from the slope of the log R_f versus gel concentration (shown for kininogen in Fig. 4).

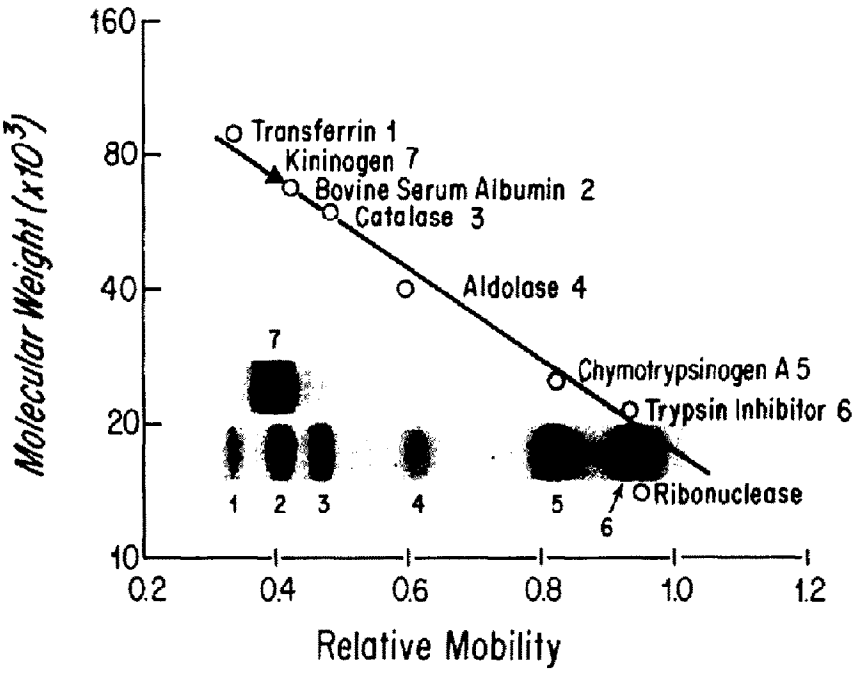

Fig. 6. Molecular weight determination of kininogen by SDS-polyacrylamide gel electrophoresis.

Table 3. Release of kinin from purified rat plasma kininogen and heated rat plasma by various proteases

Enzyme	Kinin released (ng) Kininogen	Heated rat plasma
Trypsin	2520	2400
Rat plasma kallikrein	0	41.0
Human plasma kallikrein	0	50.0
Human urinary kallikrein	74.0	23.0
Hog pancreatic kallikrein	60.0	49.0
MSLS acid protease	200.0	ND*
Rat urinary kallikrein	720.0	ND

* Not done.

released kinin from the purified kininogen substrate. However, both hog pancreatic kallikrein and human urinary kallikrein released small but equivalent amounts (60–74 ng) of kinin. Rat urinary kallikrein released about 30% of the total kinin content from the kininogen preparation compared to that released by trypsin, whereas the acid protease released approximately 10% of the amount released by trypsin. Incubation of the heated rat plasma as the kininogen substrate under identical conditions resulted in the release of 20–50 ng bradykinin equivalents with all of the kallkrein preparations. While the MSLS acid protease released 3- to 4-fold more kinin from the purified rat plasma kininogen, these comparative values are difficult to interpret since the unit of activity of this enzyme is different.

DISCUSSION

LMW rat plasma kininogen was purified and characterized in an effort to identify the various components of an acid protease kinin-forming system in the transplantable solid Murphy–Sturm lymphosarcoma (MSLS). Both an alkaline [4] and acid protease [5] kinin-forming system were found present in the MSLS, and the substrate requirements for these proteases have been under investigation. Earlier studies with rat plasma by Fasciolo and Halvorsen [27] led to the conclusion that rat plasma may lack a substrate that will react with glandular kallikrein. However, Jacobsen [1] found two kininogen species (LMW and HMW) in rat plasma, one (LMW) that reacted with glandular kallikrein and a second (HMW) that also formed kinin with plasma kallikrein. More recently, Uchida and Katori [3] reported equal levels of HMW and LMW kininogens present in rat plasma. Prior to our studies [8, 28], no previous investigations on the purification and characterization of rat plasma kininogen have been reported. Our laboratory previously reported the 100-fold purification of a rat plasma kininogen with an apparent molecular weight of 110,000 [8]. These purification data compare favorably with those reported for LMW human kininogen [29] and bovine kininogen [30] and exceed those reported by Habal *et al.* [31] for the purification of human kininogen and, more recently, by Nakayasu and Nagasawa [32]. Hamberg *et al.* [33] achieved an average 260-fold purification of LMW human kininogen, whereas Pierce and Guimaraes [34] reported a 500-fold purification of various forms of human plasma kininogen.

Since the purified rat plasma kininogen tended to aggregate, even in the presence of high salt concentration, the molecular weights of the aggregate forms were determined by Ferguson plot [18], whereas the molecular weight of the monomer was estimated to be 72,000 by SDS-polyacrylamide gel electrophoresis. The molecular weight values estimated for each band showed an approximate ratio of 1:2:4:6, suggesting self association of the monomer into dimer, tetramer, and hexamer forms. Despite the fact that both of the above techniques yield data on molecular weights that are subject to some uncertainty due to the differences in structure between glycoprotein kininogen and the protein molecular weight markers, there is consistency with regard to relative size distribution of the monomeric and aggregate forms. A similar state of aggregation was found in human HMW [35] as well as in LMW kininogen (H. Fritz, personal communication). Schiffman *et al.* [36, 37] have suggested that human HMW kininogen may circulate in dimeric form. Habal *et al.* [38] also have reported the dimerization of human HMW kininogen. The 72,000 molecular weight of the rat plasma kininogen was in the range reported for LMW human [39, 40] and rabbit [41] kininogen. Molecular weights ranging from 48,000 to 56,000 also have been reported for LMW human [33, 42], bovine [43], and rabbit [44] kininogens.

The purified protein was designated as LMW kininogen on the basis that it formed kinin with glandular kallikrein (hog pancreatic, human and rat urinary) but not with the plasma kallikrein (human and rat). The HMW kininogen previously isolated from rat plasma [8] formed kinin following incubation both with glandular and plasma kallikrein. The fact that the purified kininogen did not correct the clotting defect of human plasma that was deficient in HMW kininogen (R. W. Colman, personal communication) whilst rodent plasma [45] and HMW kininogens do [37, 46–51] further supports the suggestion that the kininogen is a LMW form.

Rat urinary kallikrein [12] released approximately 30% of the total kinin content available from the kininogen compared to that released by trypsin. The difference in the kinin amount released may be explained by the different kinin forms released by each enzyme. Trypsin releases bradykinin whereas rat urinary kallikrein releases kallidin which is known to have only 1/3–1/2 the rat uterine muscle stimulating activity of bradykinin. Thus, an equimolar release of both peptides would show two to three times more kinin activity in the tryptic digest of kininogen in conformity with the activity ratios obtained in our study. Hog pancreatic kallikrein also released very low concentrations of kinin from our purified rat plasma kininogen, an observation consistent with that of Bennett and West [52].

The rat plasma kininogen (both Fractions A and B) herein reported was relatively rich in acidic amino acids, consistent with the acidic pI value for this protein. The amino acid composition was very similar to that of LMW bovine kininogen [53]. However, the total carbohydrate content of rat kininogen was 8.5%, whereas bovine kininogen had a carbohydrate content of 18%. The carbohydrate moieties of each of kininogens A and B were essentially the same.

REFERENCES

1. S. Jacobsen, *Br. J. Pharmac. Chemother.* **28**, 64 (1966).
2. M. Katori, S. Mineshita and T. Shigei, in *Bradykinin and Related Peptides* (Eds. F. Sicuteri, M. Rocha e Silva and N. Back), p. 273. Plenum Press, New York (1970).
3. Y. Uchida and M. Katori, *Thromb. Res.* **15**, 127 (1979).
4. N. Back and R. Steger, in *Bradykinin and Related Peptides* (Eds. F. Sicuteri, M. Rocha e Silva and N. Back), p. 225. Plenum Press, New York (1970).
5. N. Back and R. Steger, in *Vasopeptides: Chemistry, Pharmacology and Pathophysiology* (Eds. N. Back and F. Sicuteri), p. 417. Plenum Press, New York (1972).

6. P. P. LeBlanc and N. Back, *J. natn. Cancer Inst.* **54**, 1107 (1975).
7. H. C. Li, W. F. McLimans and N. Back, *Biochem. Pharmac.* **26**, 1187 (1977).
8. H. C. Li and N. Back, *Prep. Biochem.* **9**, 229 (1979).
9. H. C. Li and N. Back, *Prep. Biochem.* **10**, 561 (1980).
10. A. M. Siegelman, A. S. Carlson and T. Robertson, *Archs. Biochem. Biophys.* **97**, 159 (1962).
11. Y. Fujimoto, C. Moriwaki and H. Moriya, *J. Biochem., Tokyo* **74**, 247 (1973).
12. H. S. Margolius, R. Geller, W. deJong, J. J. Pisano and A. Sjoerdsma, *Circulation Res.* **30**, 358 (1972).
13. G. S. Bedi, J. Balwierczak and N. Back, *Biochem. Pharmac.* **32**, 2071 (1983).
14. C. R. Diniz and S. F. Carvalho, *Ann. N.Y. Acad. Sci.* **104**, 77 (1963).
15. C. B. Laurell, *Analyt. Biochem.* **15**, 45 (1966).
16. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
17. O. Vesterberg, L. Hansen and A. Sjosten, *Biochim. biophys. Acta* **491**, 160 (1977).
18. K. A. Ferguson, *Metabolism* **13**, 985 (1964).
19. D. Rodbard and A. Chrambach, in *Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel* (Eds. R. C. Allen and H. R. Maurer), p. 28. Walter de Gruyter, New York (1974).
20. K. Weber and M. Osborn, *J. biol. Chem.* **44**, 4406 (1969).
21. Q. Ouchterlony, *Ark. Kemi* **1**, 43 (1949).
22. P. Graber and C. A. Williams, Jr., *Biochim. Biophys. Acta* **10**, 193 (1953).
23. O. Vesterberg and H. Svensson, *Acta chem. scand.* **20**, 820 (1966).
24. D. H. Spackman, W. H. Stein and S. Moore, *Analyt. Chem.* **30**, 1190 (1958).
25. W. F. Lehnhardt and R. J. Winzler, *J. Chromat.* **34**, 471 (1968).
26. L. Warren, *J. biol. Chem.* **234**, 1971 (1959).
27. J. C. Fasciolo and K. Halvorsen, *Am. J. Physiol.* **207**, 901 (1964).
28. G. S. Bedi, J. Balwierczak and N. Back, *Fedn Proc.* **40**, 686 (1981).
29. S. Jacobsen and M. Kriz, *Br. J. Pharmac. Chemother.* **29**, 25 (1967).
30. M. Yano, K. Kato, S. Nagasawa and T. Suzuki, *J. Biochem., Tokyo* **62**, 386 (1967).
31. F. M. Habal, H. Z. Movat and C. E. Burrows, *Biochem. Pharmac.* **23**, 2291 (1974).
32. T. Nakayasu and S. Nagasawa, *J. Biochem., Tokyo* **85**, 249 (1979).
33. U. Hamberg, P. Elg, E. Nissinen and P. Stelwagen, *Int. J. Pept. Protein Res.* **7**, 261 (1975).
34. J. V. Pierce and J. A. Guimaraes, in *Chemistry and Biology of the Kallikrein-kinin System in Health and Disease* (Eds. J. J. Pisano and K. F. Austen), p. 113. U.S. Government Printing Office, Washington, DC (1977).
35. B. Dittman, A. Steger, R. Wimmer and H. Fritz, *Hoppe-Seyler's Z. physiol. Chem.* **362**, 919 (1981).
36. S. Schiffman, C. Mannhalter and K. D. Tyner, *J. biol. Chem.* **255**, 6433 (1980).
37. S. Schiffman and P. Lee, *J. clin. Invest.* **56**, 1082 (1975).
38. F. M. Habal, B. J. Underdown and H. Z. Movat, *Biochem. Pharmac.* **24**, 1241 (1975).
39. J. Spragg and K. F. Austen, *Biochem. Pharmac.* **23**, 781 (1974).
40. S. Nagasawa and T. Nakayasu, in *Chemistry and Biology of the Kallikrein-kinin System in Health and Disease* (Eds. J. J. Pisano and K. F. Austen), p. 139. U.S. Government Printing Office, Washington, DC (1977).
41. K. D. Wuepper and C. G. Cochrane, in *Biochemistry of the Acute Allergic Reactions* (Eds. K. F. Austen and E. L. Becker), p. 299. Blackwell, Oxford (1971).
42. F. M. Habal and H. Z. Movat, *Semin. Thromb. Hemostas.* **3**, 27 (1976).
43. T. Suzuki and H. Kato, in *Chemistry and Biology of the Kallikrein-kinin System in Health and Disease* (Eds. J. J. Pisano and K. F. Austen), p. 107. U.S. Government Printing Office, Washington, DC (1977).
44. T. P. Egorova, E. B. Rossinskaya and T. S. Paskhina, *Biokhimiya* **41**, 1052 (1975).
45. H. Saito, G. Goldsmith and R. Waldman, *Blood* **48**, 941 (1976).
46. H. L. Heier, J. V. Pierce, R. W. Colman and A. P. Kaplan, *J. clin. Invest.* **60**, 18 (1977).
47. R. W. Colman, A. Bagdassarian, R. C. Talmo, C. F. Scott, M. Seavey, J. A. Guimarease, J. V. Pierce and A. P. Kaplan, *J. clin. Invest.* **56**, 1650 (1975).
48. K. D. Wuepper, D. R. Miller and M. J. LaCombe, *J. clin. Invest.* **56**, 1663 (1975).
49. V. H. Donaldson, H. I. Glueck, M. A. Miller, H. Z. Movat and F. Habal, *J. Lab. clin. Med.* **87**, 327 (1976).
50. H. Saito, *J. clin. Invest.* **60**, 584 (1977).
51. M. Silverberg, J. E. Nicoll and A. P. Kaplan, *Thromb. Res.* **20**, 173 (1980).
52. A. J. Bennett and G. B. West, *Br. J. Pharmac.* **69**, 625 (1980).
53. S. Nagasawa, Y. Mizushima, T. Sato, S. Iwanaga and T. Suzuki, *J. Biochem., Tokyo* **60**, 643 (1966).