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ISOLATION AND CHARACTERIZATION OF RAT PLASMA GLANDULAR KALLIKREIN

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Abstract—A method has been developed to purify glandular kallikrein present in rat plasma by using Sepharose–Aprotinin affinity chromatography and elution of the enzyme with *p*-aminobenzamidine. The isolated enzyme liberated kinins from kininogen II of low molecular weight (sp. act. 14 ng kinins/min × mg) and *p*-nitroaniline (pNA) from the substrate S-2266 (sp. act. 1.23 nmoles pNA/min × mg); it was inhibited by aprotinin, benzamidine and rat urinary antikallikrein antibody but not by ovomucoid. In polyacrylamide gel electrophoresis, the enzymatic activities of the preparation were associated with two light protein bands of molecular weights equal to that of urinary kallikrein (35,000 daltons). Using this method, the recovery of [^{125}I]kallikrein added to the plasma was 82–88%. The concentration of the enzyme in normal rat plasma was equivalent to 6.1 ± 2.1 (S.D.) ng kallikrein/ml. The mean value found in nephrectomized rats was 20.0 ± 6.3 (S.D.) ng kallikrein/ml. This increment was highly significant ($P < 0.001$). Our results confirm the presence of glandular kallikrein in plasma which had been detected by other methods; they also demonstrate that the material purified from plasma is enzymatically active, suggesting that kallikrein may play a biological role in the control of blood circulation.

Glandular kallikreins are serin proteinases that can liberate bradykinin and kallidin from the plasma substrate called kininogen. The enzymes liberated by different exocrine glands, together with kidney and urinary kallikreins, present similar physicochemical and immunological characteristics for a given species [1–4]. Many workers have reported the existence in plasma of a material that reacts with the glandular antikallikrein antibodies [5–8]. However, radioimmunoassay (RIA) does not discriminate between the different forms (prokallikrein, kallikrein-inhibitor, active kallikrein) and their fragments, which are eventually found in blood. The presence of active glandular kallikrein has been detected in saliva [9] and lymph [10] but not in blood.

This work describes a purification method for an enzymatically active glandular kallikrein obtained from rat plasma (PGK).

MATERIALS AND METHODS

The following materials were obtained from commercial firms: CNBr-activated Sepharose 4-B (Pharmacia Fine Chemicals Inc.); acrylamide, bisacrylamide, *p*-aminobenzamidine, ovomucoid, bovine serum albumin (BSA), aprotinin, and Protein A (Sigma Chemical Co.); synthetic bradykinin (Sandoz AG); and S-2266 (Kabi AB). All other reagents, of analytical grade, were provided by Merck and by the Sigma Chemical Co.

Preparation of Sepharose–Aprotinin. The procedure used was similar to that described by Cuatrecasas *et al.* [11]: 15 g of CNBr-activated Sepharose 4-B was incubated for 15 min in 100 ml of 1 mM HCl, and then washed with 3 liters of the same acid solution in order to eliminate dextran and lactose from the gel; the binding of the ligand Aprotinin was carried out in bicarbonate buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0); 150 mg Aprotinin was dissolved in 50 ml bicarbonate buffer mixed with 37.6 g of the wet gel. The mixture was then gently stirred for 24 hr at 4°. The noncovalent bindings of the ligand were eliminated from the mixture by washing with the following buffer solutions in four 100-ml cycles: bicarbonate buffer; 0.2 M Tris–HCl, pH 7.5, 0.1 M sodium acetate, 1.0 M NaCl, pH 4.0. The mixture was then incubated with 300 ml of 1.0 M ethanolamine, pH 8.0, for 2 hr at room temperature, to block the remaining active groups of Sepharose 4-B. The gel was finally equilibrated in 0.1 M Tris–HCl buffer, pH 8.1. Sodium azide (0.1%) was added as a preservative, and the gel was stored at 4°.

Purification of glandular kallikrein in plasma. Twelve male Sprague–Dawley rats (250–300 g) were bled through the carotid artery under ether anesthesia. The blood was collected in plastic tubes containing sodium citrate (3.5%, w/v) and Polybrene (5 μg/ml plasma) to inhibit the activation of plasma prokallikrein; it was centrifuged at 1500 *g* for 15 min; 25 ml of plasma was precipitated with ammonium sulfate to a final concentration of 30% saturation. The mixture was stirred gently for 60 min and centrifuged at 5000 *g* for 30 min. The supernatant fraction was brought to 70% saturation by adding 47 ml of the oversaturated solution of ammonium sulfate. The precipitate collected by centrifugation was resuspended in distilled water. The protein solution was

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dialyzed against distilled water at 4°, and changed four times in 24 hr.

Affinity chromatography. The sample described in the above paragraph was redialyzed against 0.1 M Tris–HCl buffer, pH 8.1, and applied to a column of Sepharose–Aprotinin (10 × 2 cm) equilibrated in a 0.1 M Tris–HCl buffer solution, pH 8.1. It was then washed with the same buffer added with 0.2 M NaCl until the absorbance of the effluent dropped to below 0.02 unit.

Kallikrein was eluted with 50 mM *p*-aminobenzamidine dissolved in 0.1 M Tris–HCl buffer, pH 8.1.

The column was kept at a constant flow of 10 ml/hr, and 3-ml fractions were collected. The PGK sample was exhaustively dialyzed against distilled water and lyophilized.

Amidase activity. The activity of the PGK samples was determined by incubating 50 μl of the enzyme solution with 100 μl of chromogenic substrate D-Val-Leu-Arg-pNA (S-2266) for 2 hr, according to the method of Amundsen *et al.* [12]. The activity was expressed in nmoles pNA liberated per min.

Kininogenase activity. Kininogenase activity was measured by using low molecular weight rat kininogen purified according to the method of Jacobsen and Kriz [13], and the liberated kinins were tested in rat isolated uterus [14]. Kininogen was not contaminated with kinins, and when incubated it did not release kinins. Under an excess of trypsin, it released 500 ng BK/mg protein. Synthetic bradykinin and/or purified rat urinary kallikrein was used as the standard [15]. The 50- to 100-μl samples were incubated for 10 min at 37° with 0.2 ml (1.5 mg) of low molecular weight kininogen in the presence of kininase inhibitors (1.0 mM phenantroline and 1 mM EDTA). Tyrode's solution was added to a final volume of 0.5 ml. The mixture was then put into a bath of 20 ml of Tyrode's solution containing a rat uterus.

Since 1 ng of standard kallikrein incubated at 37° with kininogen released 500 pg BK/min, the activity of the samples was expressed as ng kallikrein/ml plasma.

Protein determination. Protein concentration was determined according to the method of Lowry *et al.* [16] using BSA as the standard. Protein concentrations in the column effluents were monitored at 280 nm with an L.K.B. Absorbance Monitor model UVICORD 8301 A. An extinction coefficient of $E_{280} = 1.51$ was used for 1.0 mg/ml kallikrein [17].

Antibodies. Antikallikrein antibodies were obtained from New Zealand rabbits injected for 2 months with 1 mg pure urinary kallikrein [15]. The serum was heated at 56° for 0.5 hr and applied to a Sepharose Protein-A column, in order to obtain the fraction of immunoglobulins of the G type (IgG's) [18]. With double diffusion analysis and immunoelectrophoresis we showed that the IgG antikallikrein antibody was mono-specific [15]. Using the enzyme linking immunoassay [19], this serum exhibited a titer of 1:512. Non-specific antiserum corresponds to a normal rabbit serum treated in the same way described.

Electrophoresis. The method used for a vertical slab gel electrophoresis was that described by Laemmli and Favre [20] with slab gels 1 mm thick modified for the analysis of native proteins. Gels were prepared containing 11% acrylamide, 0.79% bisacrylamide in 0.375 M Tris–HCl buffer, pH 8.8. Polymerization of the gels was effected with 0.1% ammonium persulfate and 0.1% tetramethylethylenediamine. A voltage of 50 V was applied for 10–12 hr until the bromophenol blue dye emerged at the bottom of the separating gel. The gel was stained with Coomassie Brilliant Blue R-250 (1.25 g in 500 ml of 25% methanol and 10% acetic acid). It was destained with a mixture of methanol (25%) and acetic acid (10%). Two of the columns containing the sample of PGK and the standard kallikrein were left unstained and sectioned into 0.4-cm segments; they were homogenized in phosphate buffer and the supernatant fraction was used for measuring amidase and kininogenase activities.

Inhibition study. Samples containing 10 ng of PGK were incubated for 10 min at 37° with Aprotinin (1 K.I.U.), benzamidine–HCl (25 mM), ovomucoid (40 μg), glandular antikallikrein antibodies (5 μl, 1:100 final dilution) or non-specific antibodies (5 μl, 1:100 final dilution).

The samples were reincubated for 100 min at 37° with 0.5 mg rat kininogen II in a 0.2-ml volume. The volume was completed to 0.5 ml with Tyrode's solution. Kininogenase activity of these samples, incubated in the presence of inhibitors, was measured by the bioassay already described.

Recovery studies. In a purification protocol, 600 pg of [^{125}I]kallikrein, labeled according to Hunter and Greenwood [21], was added into 25 ml plasma. Distribution of [^{125}I]kallikrein during the purification process was determined by drawing aliquots at the different steps of the process. They were measured in a Gamma LKB counter and expressed as the total counts per min.

[^{125}I]Kallikrein (600 pg) was added to four plasma samples of 3 ml each. The first sample was precipitated by means of ammonium sulfate, according to the procedure described, immediately after adding [^{125}I]kallikrein. The remaining samples were precipitated after 10, 120 and 240 min of incubation of the labeled kallikrein with plasma at 37°. All four samples were submitted to the purification procedure described, using 2-ml columns of Sepharose–Aprotinin. The counts that appeared in the eluate, which coincide with the activity of the PGK peak, were expressed as the percentage of the total counts added.

Kininogenase activity in control and nephrectomized rats. Six male Sprague–Dawley rats weighing 230–250 g were submitted to bilateral nephrectomy under ether anesthesia. Six other rats were sham operated and used as the controls. Twenty-four hours after the operation they were bled as described. The plasma obtained was purified by using a 2-ml Sepharose–Aprotinin column. Results are expressed as the mean ± standard deviation, and the differences were considered significant when the P value was < 0.05.

RESULTS

The results of the purification process are shown in Table 1. Fractionation of plasma with ammonium

Table 1. Purification of rat plasma glandular kallikrein*

Step	Volume (ml)	Total protein (mg)	Total units† (cpm)	Specific activity (cpm/mg)	Purification factor	Yield (%)
Plasma	25	1,260	21,300	17	1	100
30–70% Ammonium Sulfate fractionation	19	660	17,800	27	2	85
Aprotinin–Sepharose	6	3.57	15,200	4,250	250	71

* The given data represent the mean of the three preparations.
† These values were calculated by using [^{125}I]kallikrein as the tracer.

sulfate (30%) precipitated 506 mg protein. When [^{125}I]kallikrein was added to the plasma, only 10–12% of the added radioactivity was found in that fraction (2,250 cpm). Eighty-five percent of [^{125}I]-kallikrein (17,800 cpm) was precipitated with ammonium sulfate (70%) and a purification factor of 2 with 85% recovery was obtained. The specific activity for this stage was 27 cpm/mg. In the affinity chromatography step (Fig. 1), most of the protein (660 mg) (fractions 6–17) was eluted when the column was washed with the 0.1 M Tris–HCl, 0.2 M NaCl (pH 8.1) buffer. When [^{125}I]kallikrein was added during the purification process, it was observed that a small fraction of this protein (2000 cpm) was eluted with the proteins of the previous washing (fractions 8–10). No kininogenase or amidase activities were detected in the fraction. The remaining kallikrein retained in the column was effectively eluted by using 50 mM *p*-aminobenzamidine. When [^{125}I]kallikrein was added to the plasma, the recovery obtained by using the same elution method was 71% (15,200 cpm) (Table 1). The specific amidase activity found in the last stage of the purification procedure for 25 ml plasma was 1.23 nmoles pNA/min × mg, and the specific kininogenase activity was 14 ng kinin/min × mg.

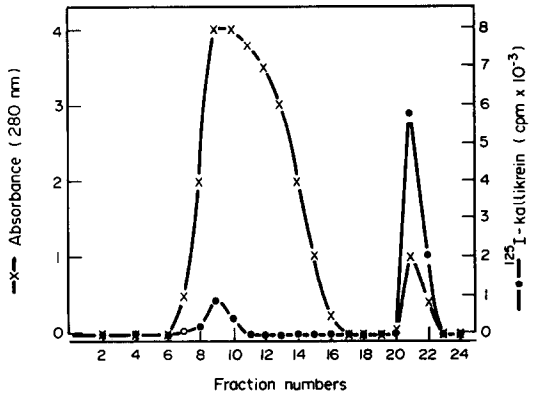

Fig. 1. Chromatography of 25 ml plasma with 600 pg [^{125}I]-kallikrein in a 10 × 2 cm Sepharose–Aprotinin column. The enzyme was eluted with 50 mM *p*-aminobenzamidine in 3-ml fractions. Key: (×——×) absorbance 280 nm; and (●——●) [^{125}I]kallikrein cpm.

Table 2. Effects of inhibitors over kininogenase activity of 10 ng of plasma glandular kallikrein

Inhibitor	Kininogenase activity (ng kallikrein)	Inhibition (%)
None	10	
Aprotinin (1 K.I.U.)	1	90
Benzamidine (2, 5 nm)	4	60
Ovomucoid (5 μg)	10	0
Antikallikrein antibodies (5 μl)	0	100
Non-specific antibodies (5 μl)	10	0

Figure 2 shows the results of the electrophoresis in 11% polyacrylamide gel. The purified PGK sample exhibited about twelve proteins bands, but amidase and kininogenase activities were observed in 5.0 to 6.2 cm slices of the gel associated with the two light protein bands. Both activities coincided with the kininogenase activity determined for the protein band which corresponds to 1 μg of purified rat urinary kallikrein used as the standard.

The percentages of recovery for [^{125}I]kallikrein incubated at 37° with the plasma samples during 0, 10, 120, and 240 min were 88, 87, 88 and 82% respectively. No significant differences were seen in these recovery values with respect to incubation time.

The effects of the different inhibitors upon the kininogenase activity of the purified PGK samples are shown in Table 2. Inhibition was observed with aprotinin (90%), benzamidine–HCl (60%) and rat urinary antikallikrein antibody (100%). No inhibition was produced when the samples were treated with ovomucoid or non-specific antibodies. Similar testing was routinely done in the various preparations of rat plasma, obtaining equal values.

Table 3 shows the individual and mean values ± S.D. of kininogenase activity in the purified PGK samples obtained from sham-operated rat plasma (6.1 ± ng kallikrein/ml) and from the plasma of rats nephrectomized 24 hr before (20.0 ± 6.3 ng

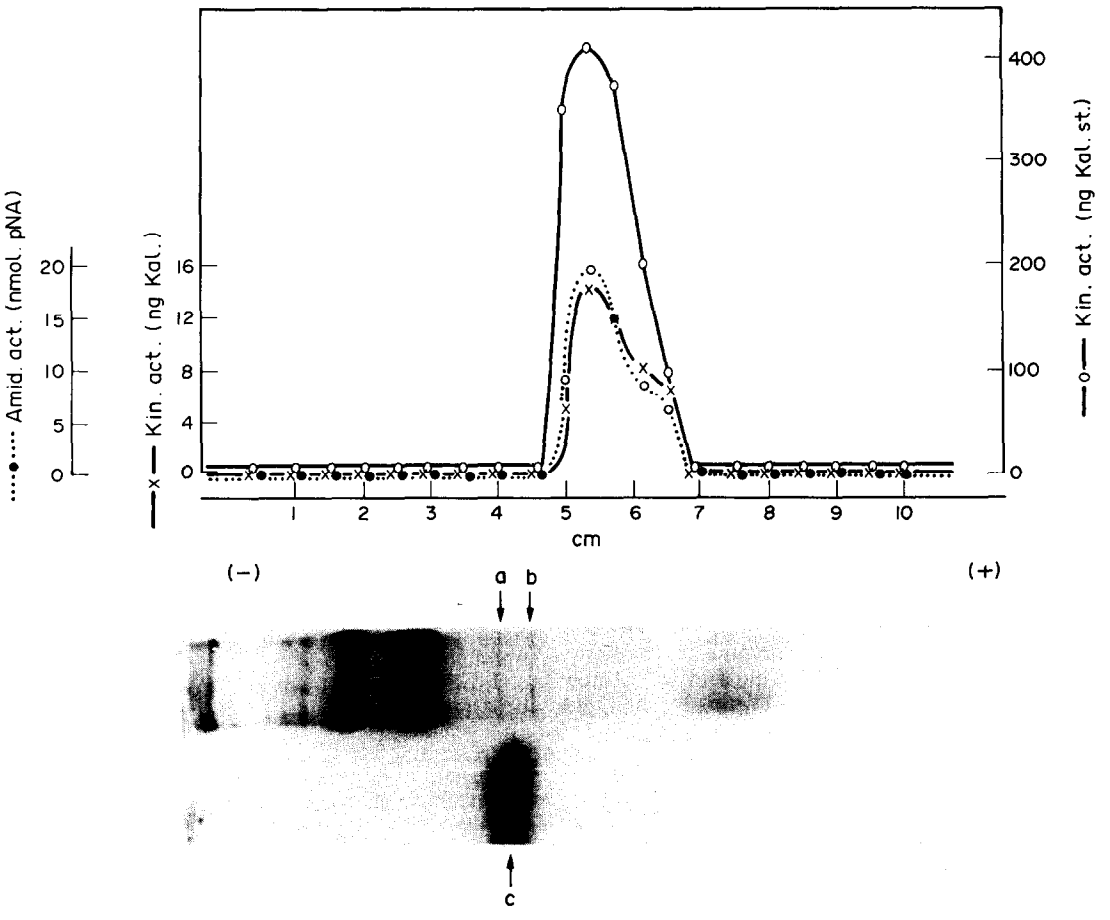

Fig. 2. Electrophoresis of plasma glandular kallikrein and standard urinary kallikrein in 11% polyacrylamide gels. The kininogenase (×——×) and amidase (●——●) activities of the sample coincide with the activities found for standard kallikrein (○——○). Both activities correspond to the material associated with the two protein bands (a) and (b) located opposite to band (c), which is equivalent to 1 μg of pure standard urinary kallikrein.

kallikrein/ml). These results show that bilateral nephrectomy produced a highly significant increase in the PGK concentration ($P < 0.001$).

DISCUSSION

The presence of glandular kallikrein in blood has been demonstrated by RIA [5–8]. However, owing to the low concentrations of immunoreactive material found [6], it has not been possible to measure the enzymatic activity over specific synthetic or natural substrates. By immunoaffinity chromatography Geiger *et al.* [22] have isolated an enzymatically active glandular kallikrein from human plasma. The procedure described in this work allows one to purify by affinity chromatography and to characterize an active glandular kallikrein from rat plasma. It is feasible to postulate that this material corresponds to a kallikrein freely circulating in blood. However, we cannot rule out that it corresponds to a glandular kallikrein totally or partially linked to an unknown low molecular weight inhibitor, which during the purification procedure is set free. The active material eluted by *p*-aminobenzamidine can be identified as a kallikrein of the glandular type because it shows enzymatic activity against the natural substrate, the low molecular weight kininogen obtained from rat plasma [13]; it hydrolyzes the S-2266 synthetic substrate which is also suitable for glandular kallikrein [12]; and these enzymatic activities are completely inhibited by urinary kallikrein antibodies.

To purify this active material, Sepharose–Apro-

Table 3. Measurement of plasma kallikrein in nephrectomized rats

Rat no.	Kallikrein (ng/ml) Sham	Nephrectomy
1	6.7	8.3
2	10.0	18.5
3	5.7	24.0
4	4.4	20.0
5	4.8	25.0
6	4.8	24.0
$\bar{x}$	6.1	20.0*
S.D.	2.1	6.3

* $P < 0.001$.

tinin chromatography and *p*-aminobenzamidine were used in order to elute it specifically. In this way, a 250-fold purification was obtained with a yield of 71%. These values were calculated by using [^{125}I]-kallikrein as the tracer. The kininogenase activity of the purified preparation and the amidase activity against the synthetic substrate S-2266 are associated with two light protein bands whose electrophoretic migration was equal to that of the urinary kallikrein used as the standard.

It was confirmed that the two complex forms of kallikrein–inhibitor described by Hojima *et al.* [23], which occur in rat plasma, are immunologically different from renal kallikrein [7] and from PGK [15]. When the plasma fractions, free of PGK but with both kallikrein–inhibitor forms, were passed through the Sepharose–Aprotinin column, no free active kallikrein was detectable. The presence of activated plasma kallikrein in the eluted sample must be ruled out, since our active material migrated together with the standard urinary kallikrein of approximately 35,000 dalton mol. wt and its enzymatic activity was not inhibited by ovomucoid, a specific inhibitor for plasma kallikrein, but it was inhibited by specific urinary antikallikrein antibodies, by aprotinin, and by benzamidine. The presence in plasma of this material, which behaves as a glandular kallikrein, can be ascribed to a direct delivery from different exocrine glands such as the pancreas [24], the salivary glands [25] and the kidney [26–28].

Our results differ from those reported by Lawton *et al.* [7] who employed a Bio-Gel-A column and found an immunoreactive material similar to the glandular kallikrein, with no kininogenase or amidase activity. We believe that these differences may be due to the fact that our active material (35,000 dalton mol. wt) is different from peak 3 (29,500 dalton mol. wt) described by Lawton. This Lawton's peak 3 might be a product of degradation of active kallikrein, produced by proteolysis during the characterization process, resulting in a peptide with immunological activity but not enzymatic activity.

Our recovery studies by incubating samples of [^{125}I]kallikrein of renal origin with intact plasma indicate that the binding of active kallikrein to plasma protein *in vitro* occurs very slowly. Other investigators [27] have demonstrated that porcine pancreatic kallikrein is also inactivated very slowly by human serum; they found only 66% inactivation after a 14-hr incubation. This suggests that the rapid inactivation of glandular kallikrein *in vivo* could be ascribed to the metabolic action occurring in some organs such as the kidney, rather than to the binding with plasma inhibitors.

The higher values of glandular kallikrein that we found in plasma of nephrectomized rats than in normal rats are in agreement with the results obtained by Rabito *et al.* [6] and Lawton *et al.* [7] using RIA. The increase of glandular kallikrein activity in plasma of nephrectomized rats supports the concept that the kidney may play a role in the clearance or metabolism of glandular kallikrein. However, the nephrectomy experiments do not preclude the possibility that *in vivo* the kidney might deliver glandular kallikrein into the circulation. It has been shown that kallikrein is associated with the plasma membrane and basolateral infolding of the tubular cells [28–29], that it is released into the renal lymph at a rate correlated with its release into the urine during saline infusion [30], and that it is found in the venous effluent of the isolated perfused kidney [26]. The differences we obtained in the absolute values of kallikrein activity in control and nephrectomized rats, as compared to those reported by the authors mentioned, might be due to the fact that the RIA could be showing all the kallikreins forms: active kallikrein, prokallikrein, kallikrein inhibitor, or inactive fragments such as the one described by Lawton which share antigenic sites recognized by the antikallikrein antibodies.

The presence of glandular kallikrein in plasma demonstrated by RIA [5–8], by immuno-affinity chromatography [22], and through enzymatic activity in the present paper suggests that this enzyme could play a physiological role in the control of blood circulation, because of its capacity to generate kinins, which are peptides of an important vasodilator action.

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