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PURIFICATION OF HUMAN PLASMA KALLIKREIN

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Abstract—Attempts to purify human plasma kallikrein activated by contact with a glass surface are described. Fresh human plasma was shaken with glass beads and the adsorbed protein was eluted from the glass surface with glycinate–NaCl buffer, pH 10. The kallikrein obtained was further purified by exclusion chromatography on DEAE-cellulose followed by chromatography on P-cellulose or SP-Sephadex. The most active preparation obtained had a specific activity of 460 (μg-equivalents bradykinin/mg protein) and an esterase activity of 27·4 (μM/mg protein per min), using TAME as substrate.

Various methods of activation of plasma kallikrein have been described using casein [1], acetone [2], acid [3, 4], negatively charged surfaces such as glass, kaolin, and Celite [5], as well as trypsin, papain and dilution [6]. More recently Wendel *et al.* [7] studied the conditions of activation of plasma kallikrein by quartz surface. The initial specific activity obtained by this method of activation is very low compared with the activity of the eluates obtained after glass activation of plasma kallikrein by the method published by Henriques and Allan [8]. The high purification obtained in this first step of activation facilitates the preparation of highly purified kallikrein.

In this paper we describe the further purification of human plasma kallikrein after the initial step of activation by adsorption onto a glass surface.

MATERIAL AND METHODS

Bradykinin. Synthetic bradykinin was kindly supplied by Sandoz Products Ltd., London. Loss of bradykinin activity in the diluted solutions was prevented by the addition of oxalic acid in the final concentration of 1 mM, a fresh solution being prepared daily.

Human plasma. Blood was collected in the Provincial Bank, Havana. The equipment used for the collection of blood and separation of plasma was silicone-treated, and care was taken in order to prevent loss of kallikrein by adsorption. 96 ml of a solution containing 0·8% citric acid, 2·2% sodium citrate and 2·2% dextrose in bidistilled water was added to each 440 ml of fresh blood.

Plasma kallikrein. Plasma kallikrein was prepared from plasma by batch adsorption onto glass beads, as previously described [8, 9]; 500 mg of beads per ml plasma were used in each adsorption with a period of 30 sec shaking. Two adsorptions were made with each sample of plasma and each batch of beads was eluted twice with glycinate buffer after being thoroughly washed with 0·9% NaCl; Ten ml buffer was used for each 25 g beads. Large samples of plasma were divided in portions of 50 ml for the adsorption and subsequent washing and elution as the use of larger samples leads to preparations with lower specific activity. The corresponding eluates were then mixed and dialysed against 5 mM sodium phosphate, pH 7·4.

Buffer. Glycine–NaCl buffer, 0·1 M, pH 10, was prepared according to Sörensen.

Glass beads. 75–105 μm glass beads, obtained from Sigma, were activated with 4 M HCl.

Chromatography. CM-cellulose, CM-52, DEAE-cellulose, DE-52 and P-cellulose, P-11 were obtained from Whatman. All the columns were 1·5 × 5 cm.

Heat-treated plasma. Human plasma was heated at 60° for 1 hr [8]. Before use this crude substrate was always checked to make sure it contained enough substrate in the volume used for the measurement of kallikrein activity.

Kininogenase determination. The activity of the kallikrein preparations was tested on isolated guinea-pig ileum, using Tyrode solution containing diphenhydramine and atropine, by incubation with heat-treated human plasma; its activity is expressed in μg-equivalents of bradykinin released by 1 mg of protein. Further details were given in a previous paper [8] where it can be seen also that the kinin nature of the substance released from heated plasma by glass-activated plasma kallikrein was proved by parallel assays on cat jejunum, rat colon, rat duodenum and guinea-pig ileum, using the method of superfusion in cascade described by Vane [10].

Esterase activity. Esterase activity was determined by the method of Brown [11] on *p*-toluenesulfonyl-L-arginine methyl ester (TAME). The activities are expressed in μmoles of ester hydrolysed per mg of protein/min.

Protein. Protein was determined according to the method of Lowry *et al.* [12].

RESULTS

The chromatographic behaviour of the glass-activated kallikrein eluates was studied using several ion-exchangers. In order to exclude dialysis of the purified enzyme solutions at the final phases of purification, 5 mM phosphate buffer, pH 7·4, was used in most of the columns, as in this solution the samples can be incubated with the substrate directly in the ileum bath.

Table 1. Purification of glass-activated human plasma kallikrein

Ion-exchanger	Prot. (mg)	Kininogenase specific activity					Purification factor (over previous stage)	
		Initial	Peak	(yield %)	Pool act. fs.	(yield %)	Peak	Pool act. fs.
CM-cellulose	21·6	22	50	(4)	30	(40)	2·2	1·4
DEAE-cellulose	5·0	14	40	(70)	28	(87)	2·8	2
P-cellulose	4·3	22	221	(27)	196	(52)	10	9

The ion-exchangers were equilibrated with 5 mM sodium phosphate buffer, pH 7·4. All the columns were 1·5 × 5 cm. Kininogenase activity was measured on isolated guinea-pig ileum and is expressed in μg Br/mg protein, liberated from human plasma substrate (heated plasma). Exclusion chromatography was carried out on CM- and DEAE-cellulose. Kallikrein was eluted with NaCl from P-cellulose.

The results of representative experiments are shown in Table 1. At pH 7·4 the kallikrein was not adsorbed either on DEAE- or CM-cellulose but it was purified approximately twice on CM-cellulose and slightly more on DEAE-cellulose, by exclusion chromatography, the yield being higher with DEAE-cellulose. It was strongly adsorbed on P-cellulose in 5 mM sodium phosphate buffer, pH 7·4, being eluted with a linear gradient obtained by the addition of 1 M NaCl to the same buffer. The active fractions started to be eluted when the NaCl concentration reached 0·32 M. Slower gradients were tried but activity was lost.

Chromatography on P-cellulose, using 5 mM phosphate buffer at pH 6·0, and elution with the NaCl gradient did not lead to purification of the kallikrein. One attempt to purify the enzyme by chromatography on QAE-Sephadex (in 50 mM Tris–HCl buffer, pH 8·7) was unsuccessful.

With the data obtained above, a choice was made for the steps of purification to be followed after elution and dialysis of the glass-activated kallikrein. In one of the experiments exclusion chromatography on DEAE-cellulose was followed by chromatography on P-cellulose (Table 2). The fraction containing the peak of kallikrein activity had a kininogenase specific activity of 375 and the pool of the most active fractions (sp. act. 214) gave a yield of 56 per cent in relation to the initial bead eluates.

In another experiment chromatography on DEAE-cellulose was followed by chromatography on SP-Sephadex, using the same conditions as the chromatography on P-cellulose (Table 2, Fig. 1). The fraction containing the peak of activity had a kininogenase specific activity of 460 and esterase specific activity of 27·4 (on TAME). The yield of the most active fractions (sp. act. 286) was 23 per cent in relation to the initial bead eluates.

It should be noted that for this study we used plasma kallikrein (glass eluates) with different initial specific activities, depending upon the preparation available. This fact could give a misleading picture of the purification found. However, in the case of the columns of SP-Sephadex and P-cellulose, it is clear that as well as leading to a greater purification (see factor in Table 2), P-cellulose columns gave a much better yield of purified kallikrein. Therefore the data suggest that the sequence glass beads–DEAE-cellulose–P-cellulose is the best. More work is being done to confirm this finding.

DISCUSSION

Optimal conditions for plasma kallikrein activation and adsorption onto a glass surface were studied previously [8]. We found that the period of contact with glass should be very short; 30 sec contact gave an

Table 2. Chromatography of glass-activated kallikrein on DEAE-cellulose followed by chromatography on SP-Sephadex or P-cellulose

Fraction	Prot. obtained (mg)	Kininogenase specific activity				Purification factor (over previous stage)	
		Peak	(yield %)	Pool act. fs.	(yield %)	Peak	Pool act. fs.
Glass bead eluates	11·3	—	—	21·7	(100)	1	1
DEAE-cellulose column*	5·8	53	(75)	35	(86)	2·4	1·6
SP-Sephadex column†	0·19	460	(19)	286	(23)	13	8
Glass bead eluates	12	—	—	6·4	(100)	—	1
DEAE-cellulose column*	5·0	—	—	15	(96)	—	2·3
P-cellulose column†	0·2	375	(27)	214	(56)	25	14

* 1·4 × 14 cm.
† 1·5 × 15 cm.
Columns were developed as described in Table 1. SP-Sephadex column appears in Fig. 1.

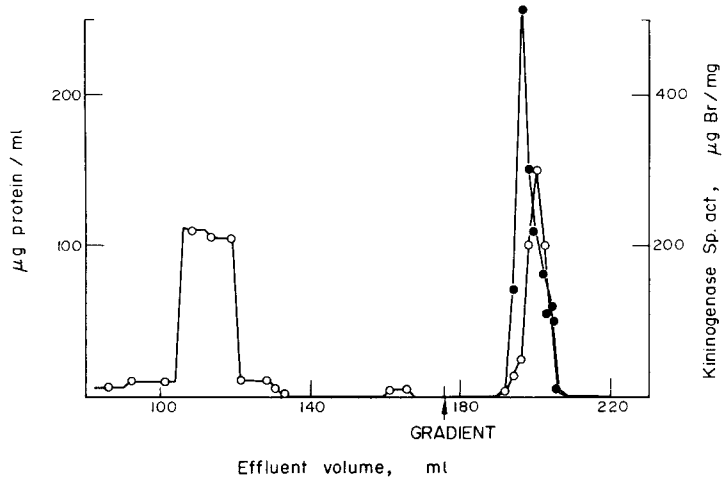

Fig. 1. Elution pattern of plasma kallikrein from a SP-Sephadex column. Kallikrein eluates from a DEAE-cellulose column (see Table 1) were loaded on a column (1·5 × 15 cm) of SP-Sephadex equilibrated with 5 mM sodium phosphate buffer, pH 7·4. Elution was carried out using a linear gradient of 0–1 M NaCl. ○, protein; ●, kininogenase activity.

initial kallikrein preparation with a high specific activity (kinin-releasing and esterase). Longer periods of shaking led to gradual inactivation of the enzyme. This finding is in disagreement with the observation of Wendel *et al.* [7] when using quartz powder as the contact surface. These authors found that the activity was maximal after 2·5 min stirring with quartz and remained constant during stirring for a further 15 min. They found, however, a very low kininogenase specific activity in their eluates, 2·2 μg bradykinin-equivalents/mg protein while in our preparations the specific activity of the glass eluates was on average always greater than 20, reaching values as high as 60 in the first eluates. We only obtained kallikrein with such a low kininogenase activity as the one found by Wendel *et al.* [7] when the plasma was shaken with glass beads for periods exceeding 10 min [8]. The observation that long periods of stirring lead to gradual inactivation of kallikrein suggested that it was possible that human plasma contains a kallikrein degrading enzyme similar to that previously described in rat serum [13]. This hypothesis has been confirmed by experiments with human supernatant plasma from glass beads in which we detected a 'kallikreinase' (results in publication). It is therefore important to observe rigorously the period of shaking and to separate and wash the beads with the adsorbed enzyme, as soon as possible, to prevent inactivation of kallikrein by the enzyme present in the supernatant plasma.

The method described in this paper results in a preparation of highly active human plasma kallikrein, with kininogenase and esterase specific activities as high as or higher than the ones of the most active preparations of plasma kallikrein so far described in the literature [1, 7]. This method has the advantage of consisting of only three steps: activation by and adsorption on glass beads, chromatography on DEAE-cellulose followed by chromatography on SP-Sephadex or P-cellulose. One of the preparations described in this paper had a kininogenase specific activity of 460 (kinin released expressed in μg bradykinin) and an esterase specific activity (TAME) of 27·4 (μM/min per mg protein). Habermann and Klett [1] obtained a highly purified kallikrein from porcine plasma with a kininogenase specific activity of 360 and a TAMEase specific activity of 22·2. The results might not be exactly comparable, as their value for kininogenase activity was found after incubation of purified plasma kininogen with the kallikrein while we used heated plasma as substrate. It has to be pointed out, however, that they used a very time-consuming procedure (seven steps), including three precipitations and four column chromatography steps. The method described by Wendel *et al.* [7] is also more laborious than ours and leads to a less active human plasma kallikrein than the one described here, perhaps due to using quartz eluates with very low kininogenase specific activity as starting material.

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