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GLASS-ACTIVATED PLASMA KALLIKREIN

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Abstract—Plasma kallikrein from horse plasma was purified by adsorption on glass-surface followed by chromatography on DEAE-cellulose. It is shown that kallikrein is activated, simultaneously adsorbed and afterwards eluted from glass surface in the same conditions as described by other authors to separate Hageman factor from plasma. Evidence is presented suggesting that glass-activated and acid-activated kallikrein are the same substance.

MARGOLIS[1] was the first to suggest that plasma prekallikrein was activated to kallikrein by activated Hageman factor (ac-HF). Since then ac-HF is usually regarded as indispensable in the activation of prekallikrein in plasma even when the activation is made by acetone,[2] acid[3] or chloroform.[4]

Several authors have suggested the presence of two kallikreins in plasma.[5–7] Vogt postulated that only one of them can be activated by glass (ac-HF), the other being activated by acid.

Hageman factor can be isolated by adsorption on to glass surface and subsequently eluted, as described by Schoenmakers, Kurstjens, Haanen and Zilliken.[8] In such experiments designed to study the role of Hageman factor in the activation of plasma kallikrein, we have shown that kallikrein itself is adsorbed on to glass and eluted under the same conditions as ac-HF.

In this paper we describe these experiments and a new method for the preparation of plasma kallikrein, and give some data which suggest that glass-activated and acid-activated kallikrein are the same substance.

MATERIAL AND METHODS

Bradykinin, synthetic, was kindly supplied by Sandoz, Ltd., Basle. Loss of bradykinin activity in the diluted solutions was prevented by the addition of oxalic acid[9] in the final concentration of 10^{-3}M.

Trypsin, crystalline, Sopfa, Czechoslovakia. The solution of trypsin was prepared immediately before use, in 1×10^{-3}M hydrochloric acid.

Substrate for the kininogenases. Fresh horse plasma was heated at 56° for 3 hr to remove prekallikrein and kallikrein[10] and then dialyzed against 0·9 per cent NaCl.

Kininogenase determination by bioassay of kinin released. The kinin released by the kininogenase preparations from heat-treated horse plasma was assayed on the isolated guinea pig ileum[11] and its activity expressed as bradykinin. The incubation was conducted either directly in the organ bath or in polyethylene tubes. In the latter case the

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reaction was stopped by boiling for 10 min after lowering the pH to 5·5 with 1 M HCl. Before assaying, the samples were neutralized with 1 M NaOH. The presence of 10^{-3} M *O*-phenantroline in all the incubations inhibited the action of kininase present.[12]

Protein was determined by the biuret reaction or according to Lowry, Rosebrough, Farr and Randall.[13]

Esterase activity on *p*-toluenesulfonyl-L-arginine methyl ester (TAME) was determined by the method of Brown.[14]

Plasma for the kallikrein preparations was obtained from horse blood collected over potassium oxalate in polyethylene containers (3g/l. blood). The plasma was separated by centrifugation at about 5000 rev./min in polyethylene tubes, immediately used or kept at 4–6° for 2–3 days in a closed polyethylene bottle in the presence of toluene. No loss of kallikrein was observed.

Glass powder P-3 BDH ballotini, 0·1 mm diameter was stirred with 4 M HCl, washed thoroughly with deionized water, stirred with 2 M NaOH and washed with deionized water until the pH of the supernatant was 6–7 and dried in an oven.

Glycine-saline buffer, pH 9·6, was prepared as recommended by Schoenmakers *et al.*[8]

Horse plasma kallikrein activated by acid was prepared as previously described[15] with the difference that pH 7·4 phosphate buffers were used for dialysis and chromatography on DEAE-cellulose.

RESULTS

Effect of the surface of glass ballotini on the degree of kallikrein adsorption

The following experiments were performed.

(1) Fresh horse plasma was shaken with 200 mg ballotini, 0·1 mm diameter, per ml of plasma, at room temperature, for 2 min. A fresh portion of 200 mg ballotini/ml of plasma was added to the decanted supernatant, the resulting mixture being shaken for 2 min. This procedure was repeated five times, on each occasion fresh ballotini was used. After decanting the plasma from ballotini the volume of plasma was restored to its original value by washing the ballotini with small amounts of 0·9 per cent NaCl. Each portion of ballotini was thoroughly washed with 0·9 per cent NaCl, three times (8 ml/g ballotini) and eluted twice with pH 9·6 glycine buffer by shaking the ballotini with 1 ml of buffer. The eluates were dialyzed against 1×5^{-3}M phosphate buffer, pH 7·4. Their activity was tested by incubation with heat-treated horse plasma and assayed using the guinea pig ileum.

Activated kallikrein was found in the first and second supernatant plasmas but no kininogenase activity could be detected in the third supernatant plasma.

Table 1 shows that all the first six eluates were active, the specific activity increases from the first to the fourth eluate and decreases in the sixth. The seventh eluate was inactive in the conditions of our test for kininogenase. The specific activity of the fourth and fifth eluates was 11·4 μg Br/mg protein, showing a high degree of purification after a single operation.

When activated by hydrochloric acid, as previously described,[15] the original plasma and the supernatant after the seventh adsorption showed a kallikrein activity of 0·94 and 0·18 μg Br/ml plasma, respectively. The kininogenase activity of the acid-activated plasma and supernatant plasma increased significantly after one or more days standing in the refrigerator.

TABLE 1. ACTIVITY OF HORSE PLASMA KALLIKREIN ELUTED FROM GLASS BALLOTINI

Eluates	Specific activity (μg Br/mg protein)	Total activity from 20 ml plasma (μg Br)
1	3·8	4·3
2	3·8	5·1
3	8·2	4·7
4	11·4	6·1
5	11·4	5·0
6	5·7	1·2
7	—	—
	Total	26·4

Horse plasma was repeatedly shaken with fresh portions of ballotini (200 mg/ml plasma), the ballotini were then thoroughly washed with saline and eluted with pH 9·6 glycinate NaCl buffer. The eluates were dialyzed against pH 7·4 phosphate buffer and the kinin released was tested by incubation with heat-treated horse plasma and assayed with the ileum. The kinin activity is expressed as bradykinin (Br).

(2) A second experiment was performed increasing the proportion of ballotini to plasma to 1 g/ml. The third eluate was already inactive, thus indicating that two grams of ballotini were sufficient to adsorb all the active or potentially active kininogenase present in one ml of plasma.

No activity could be detected in the acid activated supernatant plasma from this experiment if it were tested as soon as dialysis was complete. An easily detectable activity appeared during the following days if the acid-activated supernatant was left in the refrigerator.

Influence of the time of contact of the adsorbed protein with glass ballotini on the kininogenase activity of the eluates

Samples of plasma were shaken once with ballotini (1 g ballotini/ml plasma) and separate plasmas were left in the cold in contact with ballotini for 0, 24, 48 and 72 hr, in an atmosphere of toluene (a small tube containing toluene was inserted in the flask with ballotini and plasma). The ballotini were then washed and eluted as in the previous experiments. The dialyzed eluates obtained after 24 and 48 hr of contact with ballotini had about 20 per cent of the activity of the eluate which was made immediately after adsorption. The eluate which had 72-hr contact with ballotini was completely inactive.

The same experiment was repeated in which the ballotini was washed immediately after 2 min shaking, and the washing fluid decanted until a thin layer was left covering the ballotini. The tubes were left standing in the cold, as above, for the same periods of time. Table 2 shows that after 24 hr contact the eluate was twice as active as the one obtained immediately after adsorption, 30 per cent of the activity was found after 48 hr, and about 20 per cent after 72 hr.

Later experiments on columns showed that shorter periods of contact were sufficient to obtain the maximum activation of the kininogenase.

Purification of plasma kallikrein by glass adsorption and chromatography on DEAE-cellulose

Horse plasma was percolated over a column of dry glass ballotini, 0·1 mm diameter, 1 g of glass ballotini being used for each ml of plasma, as no higher specific activity or yield was found using 2 g ballotini/ml plasma in the conditions of this experiment. After percolation the column was washed with 0·9 per cent sodium chloride until the absorbance of the washing fluid at 280 mμ was negligible. The column was then closed, leaving a thin layer of the washing fluid on top of the glass powder, and left for 16–20 hr in the cold. Kallikrein was eluted from the glass powder column with pH 9·6 glycine buffer containing NaCl. The eluates were separately dialyzed against 1×5^{-3} M phosphate buffer, pH 7·4, and their protein and kininogenase activities were determined (Fig. 1). All the operations described here were carried in the cold room (4–6°).

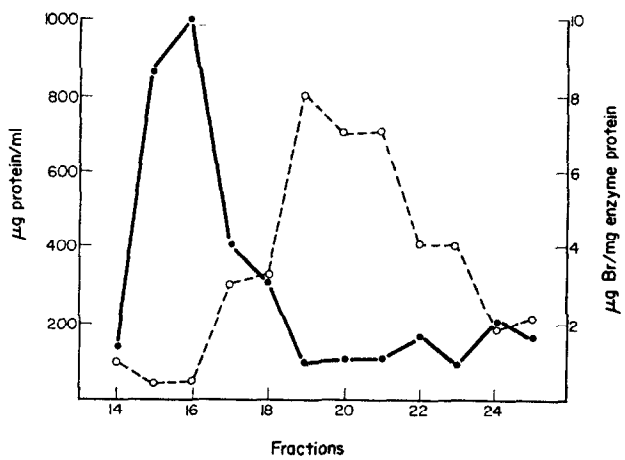

FIG. 1. Elution of plasma protein from glass ballotini. Column dimensions 60 × 2 cm. Protein was eluted by 0·1 M glycine buffer containing 50 g NaCl/l. ● — ● protein; ○ — ○ kininogenase activity expressed as bradykinin (Br).

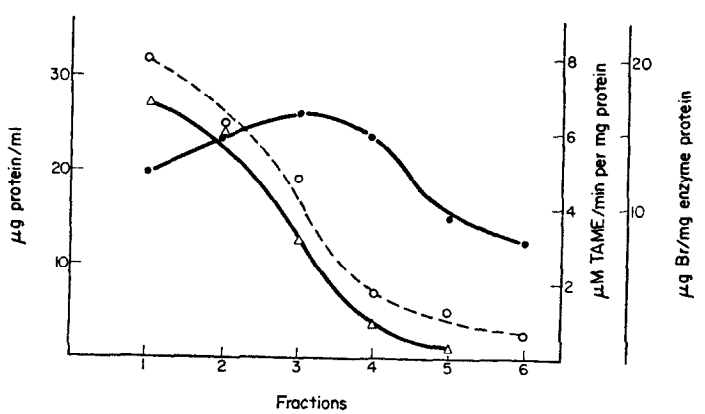

FIG. 2. Elution chromatography of plasma glass-activated kallikrein on DEAE-cellulose. Column dimensions 2 cm × 10 cm. Protein eluted with 0·02 M phosphate buffer (pH 7·4) ● — ● protein; ○ — ○ kininogenase activity; △ — △ esterase activity.

The fractions containing the peak of activity (19, 20 and 21 in Fig. 1) were chromatographed on a DEAE-cellulose column, as previously described for the acid-activated kallikrein.[15] The curve of kininogenase and TAMEase activity of the fractions obtained and its protein content are shown in Fig. 2. It can be seen that after these two operations a kallikrein with a specific activity of 20 (1 mg of protein of fraction 1 released 20 μg of kinin, expressed as Br, from heat-treated plasma, as tested on the guinea pig ileum, incubated in the bath) is obtained. Figure 2 also shows that the esterase activity accompanies the kininogenase activity in the fractions. Disk electrophoresis of the fraction 1 showed that, apart from the main band of protein, there were 2–3 feeble bands of protein with different mobilities.

TABLE 2. INFLUENCE OF TIME OF CONTACT OF THE ADSORBED KININOGENASE WITH GLASS BALLOTINI

Time of contact (hr)	Specific activity (μg Br/mg protein)
0	4·3
24	9·0
48	2·5
72	2·0
96	0·25

Ballotini which had been shaken with plasma (1g/ml), thoroughly washed with saline and kept in the cold for the specified periods of time, were then eluted and tested as described in Table 1.

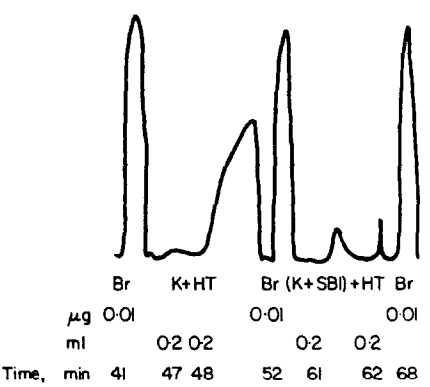

FIG. 3. Glass-activated kallikrein solution (0·3 ml) was incubated with 500 μg of soy-bean trypsin inhibitor (SBI), in presence of *o*-phenantroline, for 10 min. 0·2 ml of the incubate were added to ileum, followed by 0·2 ml of heat-treated plasma (HT). K: same incubation mixture without SBI.

The glass-activated kallikrein was inhibited by trypsin soy-bean inhibitor, as shown in Fig. 3.

Adsorption of acid-activated kallikrein on glass ballotini

Acid-activated kallikrein purified by adsorption on a DEAE-cellulose column and consequently dissolved in 0·02 M phosphate, pH 7·4, was adsorbed on a glass column

in the same conditions as described above. No activity was found in the filtrate or washings but no significant increase of specific activity was observed in the dialyzed eluates. The most active fraction had a specific activity of 5·7 while the specific activity of the initial solution was 5·0. About 80 per cent of the total activity was recovered.

DISCUSSION

Margolis[16, 17] suggested that the activation of Hageman Factor by exposure of blood plasma to glass surface initiates a series of reactions leading to the conversion of prekallikrein to kallikrein. Other authors, studying kinin-release after activation of plasma by glass surface or glass beads coated with plasma protein, also support this point of view.[18–20] This hypothesis was strengthened by the findings of Nossel *et al.*,[21] who reported that hexadimethrine, a substance which had been shown by Eisen[18] to block the conversion of prekallikrein to kallikrein, inhibits the activation of Hageman Factor by glass.

Kallikrein and Hageman Factor could however be only different names for the same substance. Our results showing that kallikrein as described for Hageman Factor is adsorbed and eluted by glass under the same experimental conditions, could be taken to indicate the identity of the two substances. That this is not so is shown by the fact that lima-bean trypsin inhibitor inhibits the activity of Hageman Factor,[22] without affecting that of kallikrein,[23] whereas soy-bean trypsin inhibitor and Trasylol inhibit plasma kallikrein,[23, 24] having no inhibitory effect on Hageman Factor.[25]

Nevertheless the similarity of the adsorption and elution behaviour of the two substances reported in this paper is compatible with the hypothesis that the activation of both substances, although having the common feature of being caused by adsorption on glass, might be due to the development of two independent processes. This hypothesis can only be settled when prekallikrein is isolated in pure state.

Hageman-deficient plasma is unable to render glass beads capable of activating the prekallikrein present in the γ-globulin fraction, isolated from guinea pig serum.[20] This contrasts with the activation observed with glass beads previously shaken with normal plasma,[20] which is a strong evidence that Hageman factor is implicated in the activation of prekallikrein.

Recently Nagasawa *et al.*[23] prepared glass-activated kininogenase from the pseudoglobulin fraction of bovine plasma. They suggest that the glass-activated kininogenase should be different from the acid-activated kallikrein. Our observation that purified acid-activated kallikrein was completely adsorbed on, and eluted from glass surface, in the same conditions of the glass-activated enzyme, is a strong evidence that we are dealing with the same kallikrein. Also the behaviour of the glass-activated kallikrein on DEAE-cellulose ion-exchanger was exactly the same as that of acid-activated kallikrein. Both preparations are equally inhibited by soy-bean trypsin inhibitor.

The method of preparation of kallikrein described in this paper is simpler than the one described previously[15] and the kallikrein obtained had a much higher specific activity (20 μg Br/mg enzyme protein). It is also simpler than the one described by Nagasawa *et al.*[23] who prepared glass-activated kallikrein from the pseudoglobulin fraction of bovine plasma. One mg of the enzyme described by these authors hydrolyzed 4 μg TAME/min, while ours hydrolyses 6·8 μg TAME/min.

Although this method does not lead to a preparation as pure as the one described

by Habermann and Klett,[24] it is extremely simple and permits a degree of purification sufficient for many experimental purposes.

It was noted by Alkjaersig, Fletcher and Sherry[26] and also Burdon and Iverson[27] that temporary acidification of plasma results in increased caseinolytic activity. Therefore the increase of activity (kininogenase) observed in the acid activated supernatants from ballotini which appeared when these activated plasmas were left for 1 day or longer in the refrigerator, might be explained by activation of plasmin. However further work should be done to verify this hypothesis.

The disappearance of plasma kallikrein observed when plasma was kept in contact with glass ballotini for 24 hr or longer, would suggest that horse plasma might contain a degrading enzyme for plasma kallikrein similar to the one described by Werle and Schmal[28] in rats plasma, for pancreatic kallikrein.

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