PARTIAL PURIFICATION OF BOVINE PLASMA KALLIKREINOGEN, ITS ACTIVATION BY THE HAGEMAN FACTOR

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Received July 11, 1968

Kallikrein, which causes hypotensive responses by liberation of kinin from kininogen, is present in plasma as the inactive form, kallikreinogen (Werle et al., 1955). The mechanism by which kallikreinogen is activated in plasma is still largely unknown. The enzymatic activation of kallikreinogen was first noted by Werle et al. (1955) with the use of trypsin and unfractionated human, rat, dog, pig and bovine serums. Later, the activation of kallikreinogen by plasmin was proposed by Vogt (1964), but this process has still not been confirmed with purified enzymes.

On the other hand, it was proposed by Margolis (1960) that the Hageman factor (HF) will be required for the endogeneous activation of kallikreinogen in plasma. Other workers thought that HF activation of kallikreinogen proceeded via activation of prepermeability globulin (Becker and Kagen, 1964). This idea was mainly based on the observations that permeability globulin could be activated by the HF (Ratnoff and Miles, 1964), and that permeability globulin released kinin from unfractionated plasma. Recently, Webster (1968) isolated kallikrein, HF and permeability

globulin in their active forms from human plasma which had been treated with acetone. The present paper reports procedures for purification of bovine plasma kallikreinogen, and evidence for its direct activation by purified bovine HF.

MATERIALS AND METHODS

HF was purified by the method of Schoenmakers et al. (1965) from bovine plasma and shown to be free from other blood clotting factors. The activities of kallikrein were determined by the following methods: 1) The depression of the blood pressure of rabbits which had been anaesthetized with urethane; blood pressure was measured from the carotid artery with a mercury manometer after injection of samples into the femoral vein; synthetic bradykinin was used as a standard. 2) Arginine ester hydrolytic activity; this was determined with tosylarginine methyl ester (TAME) as substrate (Roberts, 1958). 3) Liberation of kinin from kininogen; kinin was estimated by its activity in causing contraction of guinea pig ileum (Rocha e Silva et al., 1949). Bovine kininogen-I, which is a specific substrate for bovine plasma kallikrein (Nagasawa et al., 1967), was obtained by the method of Yano et al. (1967). Trypsin and lima bean and soy bean trypsin inhibitors used were the products of Sigma Chemical Company. Trasylol was a gift of Bayer Pharmaceutical Company. Protein concentrations were determined by the measurements of absorbancy at 280 mμ.

RESULTS AND DISCUSSION

Preparation of bovine plasma kallikreinogen The pseudoglobulin fraction (Fuld and Spiro, 1900), precipitated between 30 and 50 per cent saturation with ammonium sulfate, was dissolved in deionized water and dialyzed against water at 4°C until the conductivity of the solution decreased to 6 mmho. The dialyzed fraction (total A_{280}=19.7 x 10^3, about 20 g of protein) from 1 L of plasma

was chromatographed on a DEAE-Sephadex A-50 column (4.5 x 39 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0. After washing the column with 300 ml of the buffer, kallikreinogen was eluted with a linear concentration gradient of NaCl from 0.02 M to 0.3 M NaCl in the buffer. For assay of kallikreinogen, 0.5 ml samples of each fraction of the eluate were activated by incubation with 2.5 μg of HF at 37°C for 30 minutes. The fractions in which TAME hydrolytic and hypotensive activities were induced by addition of purified HF were combined, diluted with water (conductivity, 3 mmho) and rechromatographed on a DEAE-Sephadex A-50 column (2 x 20 cm) equilibrated with 0.02 M Tris-lysine buffer, pH 8.5. On elution with a linear NaCl concentration gradient from 0.02 M to 0.1 M NaCl, the pre-enzyme with potential TAME hydrolytic and hypotensive activities was eluted at an NaCl concentration between 0.03 M and 0.06 M. When the kallikreinogen fraction (total A_{280} = 0.100) was activated by 16 μg of HF, it released kinin equivalent to 0.05 μg of synthetic bradykinin per minute from kininogen-I.

The kallikreinogen fraction was dialyzed over-night against saturated ammonium sulfate solution. The resulting precipitate was collected by centrifugation, dissolved in 10 ml of 0.02 M phosphate buffer, pH 6.0, and dialyzed against the same buffer, for 2 hours. Then it was chromatographed on a CM-Sephadex C-50 column. The kallikreinogen was eluted at an NaCl concentration between 0.18 M and 0.20 M (Fig. 1). Fractions 30 to 42 of Fig. 1 were combined and concentrated by ultrafiltration in a collodion bag. The bovine kallikreinogen, which had not previously been isolated as a protein entity, was thus obtained. This preparation showed only one main band on disc-, and immuno-electrophoreses, and was purified more than 80 fold from the first DEAE-Sephadex A-50 chromatography (Table I).

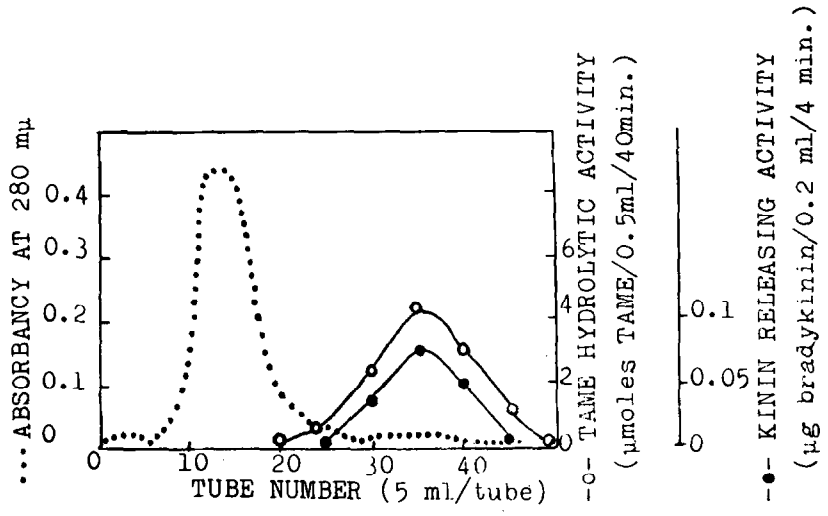

Fig. 1. Chromatography of kallikreinogen fraction. The sample obtained by rechromatography on DEAE-Sephadex A-50 was applied to a CM-Sephadex C-50 column (1.5 x 10 cm) equilibrated with 0.02 M phosphate buffer, pH 6.0. The dialyzed kallikreinogen fraction (absorbancy at 280 mμ = 9.75) was applied to the column. The column was eluted with a linear NaCl concentration gradient from 0 to 0.3 M NaCl in the buffer. Aliquots of fractions were activated with 2.5 μg of HF at 37°C for 30 minutes.

Table I
Procedures for Purification of Bovine Plasma Kallikreinogen

	Protein (total A at 280 mμ)	Hydrolytic Activity (total units of TAME)[a]	Specific Activity[b]
1st DEAE-Sephadex	674.30	62.4	0.09
2nd DEAE-Sephadex	11.55	15.1	1.31
CM-Sephadex	1.44	11.4	7.92

a) One unit of TAME hydrolytic activity is defined as the amount of enzyme which hydrolyzes 1 μmole of TAME per minute. TAME hydrolytic activity of kallikrein, developed on addition of HF, was assayed in the presence of lima bean trypsin inhibitor.
b) Specific activity was calculated as μmoles TAME hydrolyzed per unit absorbancy at 280 mμ.

When part of this preparation was activated by HF, kallikrein activity developed, as measured by release of kinin and by its effect on the blood pressure. This activated preparation did not show any caseinolytic activity and hydrolyzed 7.9 μmoles TAME per

unit absorbancy at 280 mμ per minute.

Activation of kallikreinogen by HF Addition of HF to kallikreinogen induced a gradual and steady increase in TAME hydrolytic ac-

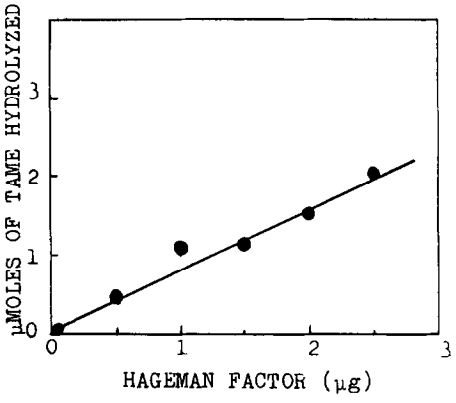

Fig. 2

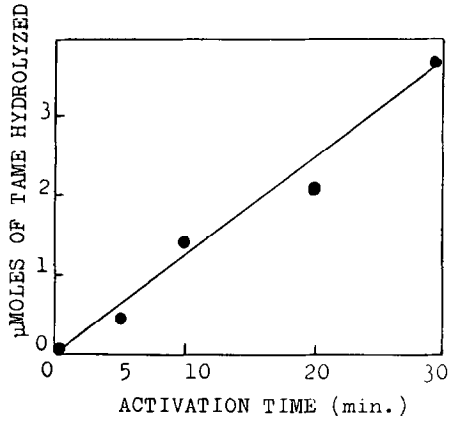

Fig. 3

Fig. 2. Activation of kallikreinogen as a function of HF concentration. Kallikreinogen preparation (absorbancy at 280 mμ = 0.024) and the indicated amounts of HF were incubated for 5 minutes at 37°C in 0.4 M Tris-Cl buffer, pH 8.5. To stop the action of HF, the incubation mixtures were mixed with 400 μg of lima bean trypsin inhibitor, and mixtures were incubated with 10 μmoles of TAME for 20 minutes at 37°C. After converting the residual TAME to its hydroxamate derivative (Roberts, 1958), the absorbancy at 500 mμ was estimated spectrophotometrically, and the amounts of TAME hydrolyzed were calculated.
Fig. 3. Time course of activation of kallikreinogen by HF. Kallikreinogen preparation (absorbancy at 280 mμ = 0.048) and 1 μg of HF were incubated for the indicated times at 37°C in 0.4 M Tris-Cl buffer, pH 8.5. To stop the action of HF, 200 μg of lima bean trypsin inhibitor were added to the incubation mixture. The amounts of TAME hydrolyzed were estimated as described in Fig. 2.

tivity and the rate of activation was dependent on the amount of HF added (Fig. 2, 3). A similar steady increase in hypotensive activity was observed on addition of HF. These results suggest that HF acts directly on kallikreinogen, and make it unnecessary to assume that HF causes activation of kallikreinogen by activation of some intermediate enzymatic step, such as that activating permeability globulin.

Kallikreinogen (ca. 10 μg) was activated completely by the incubation with trypsin (1 μg) for 10 min. at 37°C and the kinin releasing and TAME hydrolytic activities of the developed kallikrein were the same as those of kallikrein developed by HF.

The elution profile of potential TAME esterase from the chromatograms was always parallel with those of potential hypotensive, and kinin releasing activities.

Lima bean trypsin inhibitor, a specific inhibitor of HF (Schoenmakers et al., 1965), prevented the evolution of kallikrein activities by HF, but did not inhibit the activity of kallikrein once it had developed. Soy bean trypsin inhibitor (2 μg) and trasylol (10 units) prevented completely the kinin releasing activity of kallikrein (ca. 4 μg) developed by HF.

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