ISOLATION OF PLASMA CO-FACTOR IN ADENOSINE DIPHOSPHATE-INDUCED AGGREGATION OF BOVINE PLATELETS

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1. Introduction

Recently the functions and structures of a platelet, which is the keystone of hemostasis, are being studied from the standpoint of biochemistry. Aggregation of platelets is induced by ADP in the presence of plasma which contains various kinds of proteins, lipids and metal ions, while the ADP-induced aggregation of platelets does not occur in an artificial medium such as saline or a buffer solution in place of plasma. Studies on plasma proteins participating in ADP-induced aggregation of platelets have been done by several workers; Born and Cross [1] reported a heat labile protein from pig plasma which may be present in euglobulin fraction and in a preparation of blood coagulation factor VIII, and Deykin et al. found two plasma proteins in human blood, fibrinogen and a heat stable protein [2]. Bang et al. [3] reported that fibrinogen, Hageman factor and gamma globulin in human blood are capable of restoring aggregation capability of washed human platelets. Ardlie et al. [4] demonstrated that ADP-induced aggregation of washed platelets from rabbit blood occurs in modified Tyrode's solution containing bovine serum albumin.

In the present study, authors isolate the plasma co-factor from bovine blood, which participates essentially in ADP-induced aggregation of bovine platelets. This plasma co-factor (mol. wt approx. 117 000, pI = 6.45) obtained in a homogeneous state has greater capability for ADP-induced aggregation of platelets than fibrinogen or serum albumin and is quite different in molecular weight and isoelectric point from the plasma proteins mentioned above.

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2. Materials and methods

Unwashed bovine platelets were prepared according to the method described previously [5]. Platelet aggregation was measured turbidmetrically with a Shimazu recording spectrophotometer SV-50. The buffer solution (Tris–ACD buffer solution) used for measuring aggregation of platelets consists of 5 vol of the mixture of 115 mM NaCl, 15 mM KCl, 25 mM Tris (pH 7.4) and 5 mM glucose and one vol of ACD solution [6]. The mixture of 5.0 ml of a sample protein solution and 0.1 ml of the platelet suspension ($1.6–2.0 \times 10^7$ cells/mm^3) was preincubated at 37°C for 20 min and then its optical density at 600 nm was recorded with time, stirring the reaction mixture with a stirrer (1000 rev/min). After one min, 0.1 ml of ADP with $CaCl_2$ was added to the mixture and the measurement of the optical density was continued at 37°C. The degree of aggregation was expressed conveniently in terms of the maximum decrease of absorbance at 600 nm.

3. Results and discussion

Isolation of the plasma co-factor participating in ADP-induced aggregation of platelets was carried out as follows. Step I. Ammonium sulfate precipitation; Ammonium sulfate was slowly added to one liter of bovine plasma (75 mg protein/ml) with ACD to give a final concentration of 0.3 saturation at pH 7.0. The precipitate was discarded after centrifugation, the so-

lution was brought to 0.6 saturation of ammonium sulfate, and the precipitate, after centrifugation and discarding of the supernatant solution was dissolved in Tris–ACD buffer solution and dialyzed against the same buffer solution. The protein solution (200 ml) obtained contains 62 mg protein/ml. Step II. Sephadex G-200 chromatography; the protein solution (50 ml) was passed through a Sephadex G-200 column (ϕ=5 cm, h=85 cm), eluted with Tris–ACD buffer and fractionated in each 14.5 ml. The protein solution eluted in fractions 46th through 49th was collected (260 ml, 3.3 mg protein/ml). The precipitate obtained by adding ammonium sulfate (0.9 saturation) to the protein solution was dissolved in 50 mM Tris-buffer (pH 8.5) and dialyzed against the same buffer solution. Step III. DEAE-cellulose chromatography; The dialyzed solution (5 ml) was passed through DEAE-cellulose column (ϕ=1.5 cm, h=42 cm) and eluted by 20 ml of 50 mM Tris buffer (pH 8.5) and eluted with a linear gradient (150 ml of 50 mM Tris buffer (pH 8.5) and an equal vol of the same solution containing 0.3 M NaCl). On the chromatogram obtained by fractionation (2.0 ml), the protein solution eluted with 0.03 M NaCl has a maximum activity of aggregation. This protein solution in these fractions were collected (80 ml, 0.89 mg protein/ml) and ammonium sulfate was added to the solution (pH 7.0) to give a concentration of 0.9 saturation. The precipitate was dissolved in 50 mM acetate buffer (pH 5.5) and then dialyzed. Step IV. CM-Sephadex chromatography; The dialyzed protein solution (2 ml) was passed through a CM-Sephadex column (ϕ=1.5 cm, h=4.2 cm) and eluted by 20 ml of 50 mM acetate buffer (pH 5.5), by 20 ml of 50 mM acetate buffer containing 0.15 M NaCl (pH 5.5) and finally eluted with a linear gradient (150 ml of 50 mM actate buffer containing 0.15 M NaCl and an equal volume of the same solution containing 0.5 M NaCl. The eluted solution was fractionated in each 4 ml, which is shown by fig.1. Curve A represents the degree of ADP-induced aggregation of platelets in the presence of plasma protein (0.2 mg protein/ml) in each fraction and curve B shows protein concentration. The peak position of the maximum protein concentration is in good agreement with that of the maximum aggregation capability. The protein solution eluted with 0.3 M NaCl in fractions 47th through 49th (60 ml, 0.14 mg protein/ml) were collected and stored at 0°C.

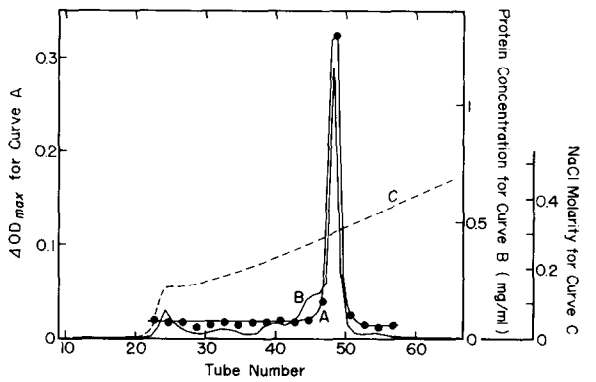

Fig. 1. Chromatogram of a protein solution with CM-Sephadex and ADP-induced aggregation capability in each fraction. Curve A; aggregation capability. Curve B; protein concentration. Curve C; NaCl molarity in the eluted solution. Concentrations, 4 μM ADP, 15 mM $CaCl_2$ and 4.0×10^5 platelet/mm^3.

A homogeneity of the plasma co-factor thus isolated was tested by disc electrophoresis, using acrylamide gel at pH 8.3, the gel with 1% sodium dodecylsulfate (SDS) at pH 7.2 and the gel with SDS after treating with β-mercaptoethanol. In each experiment, a single

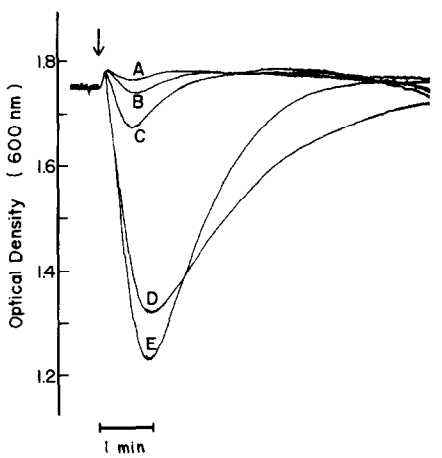

Fig. 2. ADP-induced aggregation of bovine platelets suspended in various media. ADP was added at a point indicated by the arrow. Curve A; Tris–ACD buffer solution. Curve B; Tris–ACD buffer solution containing 10 mg/ml of bovine serum albumin. Curve C; Tris–ACD buffer solution containing 10 mg/ml of fibrinogen. Curve D; Tris–ACD buffer solution containing 0.2 mg/ml of the plasma co-factor. Curve E; dialyzed whole plasma. Concentrations; 3 μM ADP, 15 mM $CaCl_2$ and 3.9×10^5 platelets/mm^3.

sharp band was obtained and the mol. wt was estimated to be 113 000. By passing the plasma co-factor through a Sephadex G-150 column (ϕ=3.2, h=50 cm) and eluting with Tris-ACD buffer solution, its mol. wt was determined to be 117 000. The isoelectric point was 6.45 ± 0.05, which was determined with an Ampholine LKB-8100.

ADP-induced aggregation of bovine platelets was measured with time in the presence of bovine serum albumin (10 mg/ml), bovine fibrinogen (10 mg/ml), the plasma co-factor (0.2 mg/ml) obtained in the present study, whole plasma or Tris–ACD buffer solution, which are shown in fig. 2. The ADP-induced aggregation of platelets does not take place in an artificial medium of Tris–ACD buffer solution (curve A). By adding bovine serum albumin (curve B) or bovine fibrinogen (curve C) in Tris–ACD buffer solution to the platelet suspension, the ADP-induced aggregation proceeds slightly. Addition of the plasma co-factor in Tris–ACD buffer solution to the platelet suspension causes the marked stimulation of ADP-induced aggregation of platelets (curve D), which is almost the same degree in aggregation capability as when whole plasma is added (curve E).

The plasma co-factor thus obtained, which is highly effective in ADP-induced aggregation of platelets, may have the therapeutic effect on hemostasis and thrombopathy.

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