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A NOVEL THROMBIN ENHANCEMENT FACTOR IN HUMAN PLASMA

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A protein has been isolated from human plasma by gel filtration followed by affinity chromatography with a derivative of wheat germ agglutinin and ion exchange chromatography. This protein showed one peak in high performance liquid chromatography but in gel electrophoresis, in the presence of sodium dodecyl sulfate and β-mercaptoethanol, revealed two major components of 74 kDa and 55 kDa. These results indicate that the protein probably exists as a complex of the two polypeptides. This protein complex enhanced platelet aggregation by thrombin while aggregation induced by ADP was not significantly affected. Similarly, the rate of thrombin action on fibrinogen and N-benzoylarginine ethyl ester as measured in a spectrophotometer was increased in the presence of this plasma protein. These results suggest the presence of a protein complex in human plasma which can directly interact with thrombin and enhance its reactivity.

Thrombin is the central enzyme in blood coagulation and converts soluble fibrinogen to insoluble fibrin. This enzyme is also a potent and physiologic activator of blood platelets (1). The generation of thrombin in the blood coagulation cascade is controlled by such proteins as factor VIII, factor V and protein C which are in turn activated by thrombin. Thrombin, once generated, may be inhibited by antithrombin and α_2-macroglobulin. In addition, proteins located on the surface of cells such as platelets and endothelial cells can also neutralize thrombin. Thus, thrombin's action in blood coagulation may be controlled by autoregulation of its production and by inhibitors of the activated enzymes in the coagulation cascade (2). In spite of these constraints, thrombin functions in the physiological system with remarkable accuracy and reproducibility. The exact mechanism by which blood coagulation may be regulated has remained unclear. The possibility needs to be entertained that there may be proteins present in plasma which are capable of directly modulating thrombin activity. The presence of such a protein has recently been reported in platelets

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(3). We report the detection and some properties of a similar protein in human plasma which may enhance thrombin activity on several substrates.

MATERIALS AND METHODS

Details of the procedures utilized in this study have been described in details in a number of papers (3-5). The thrombin enhancement factor was isolated by sequential fractionation of fresh human plasma through Sephacryl 300, wheat germ agglutinin derivative coupled to Affigel 10 and anion exchanger DE 52. The material was finally eluted from the ion exchange column with 0.5M Tris, pH 7.8. Samples were analyzed in a Waters 600 solvent delivery system with a U6K injector, Schoeffel variable wavelength detector and a Hewlet Packard 3380A integrator utilizing Altec Spherogel TSK colums. The protein was analyzed by slab gel electrophoresis with or without sodium dodecyl sulfate (SDS) (3). Platelet aggregation studies were carried out in a dual channel Payton aggregometer (3,4). The inducer was preincubated with the plasma protein complex in a total volume of 50 µl for 5 min at room temperature and this mixture was then added to the cuvette which contained 450 µl of the platelet suspension. The interaction of thrombin with fibrinogen was measured in a Hitachi Perkin Elmer spectrophotometer (7). Initially, all measurements were made at 605 nm and showed the rate of fibrin polymerization. But later determinations were done at 280 nm where greater sensitivity could be obtained. The procedure for esterolytic assay with N-benzoylarginine ethyl ester has been described (8).

RESULTS AND DISCUSSION

When human plasma was fractionated through a column of Sephacryl 300, the typical three peaks were observed. The first peak in the void volume contained fibrinogen, the second peak contained IgG and the third peak had albumin. The fractions that eluted after IgG were pooled. An aliquot of this pool was then applied to an affinity column with a derivative of wheat germ agglutinin bound to Affigel (4). The column was extensively washed to remove the unbound proteins until the optical density at 280 nm was lower than 0.01. The material bound to the lectin derivative was eluted with 0.1M N-acetylglucosamine. This eluate contained mainly the thrombin enhancement factor and occasionally, a trace of IgG which was removed by anion exchange chromatography. The final protein isolate showed a major peak in HPLC under different conditions suggesting apparent homogeneity of the material (Fig. 1a). The position of the peak varied between between IgG and albumin standards possibly due to a slight interaction of the protein with the column matrix. However, when the same material was analyzed by gel electrophoresis in the presence of SDS, two major components with apparent molecular weights of 74,000 and 55,000 were always observed (Fig. 1b). Two faint bands corresponding to 27 kDa and 20 kDa were occasionally noted. Thus, it

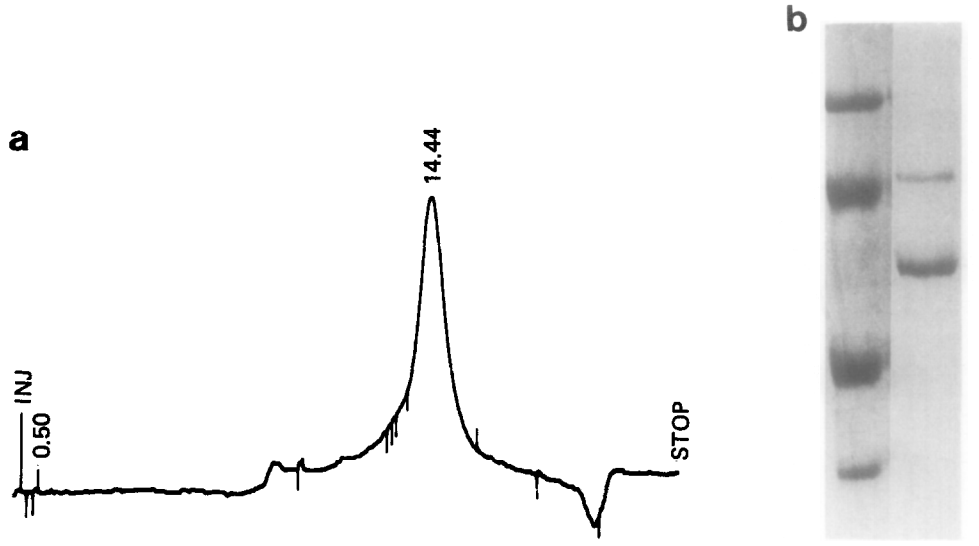

Fig. 1a: High performance liquid chromatography pattern of the plasma protein through a spherogel TSK 3000SW column (0.75 cm x 30 cm). The buffer used was 0.1M potassium phosphate - 0.1M NaCl, pH 6.8 and the flow rate was maintained at 0.6ml/min.

Fig. 1b: Slab gel electrophoretic pattern of the plasma protein complex in the presence of sodium dodecyl sulfate and β-mercaptoethanol (right lane). The two main components have molecular masses of 74 kDa and 55 kDa. Molecular weight markers phosphorylase a (94,000), bovine serum albumin (68,000), ovalbumin (43,000) and carbonic anhydrase (30,000) are shown (left lane).

appears that the plasma protein, like the thrombin-reactive platelet protein (3), exists as a complex of at least two major polypeptides of 74 kDa and 55 kDa.

In order to delineate the possible physiologic function of this protein complex, its effect on platelet aggregation by different agents was tested. The plasma protein enhanced platelet aggregation by thrombin while aggregation by ADP was not significantly affected (Fig. 2). Purified albumin, N-acetylglucosamine or IgG did not affect platelet aggregation by thrombin. To explore substrate specificity, the effect of the plasma protein on the action of thrombin on fibrinogen was studied in a spectrophotometer. The protein enhanced the rate of action of thrombin on fibrinogen while the maximum extent of the reaction was similar to the control (Fig. 3). The results presented in this figure is representative of at least ten different determinations. Further confirmation of the above results was obtained in experiments in which the hydrolysis of the synthetic substrate, N-benzoylarginine ethyl ester, by thrombin was measured.

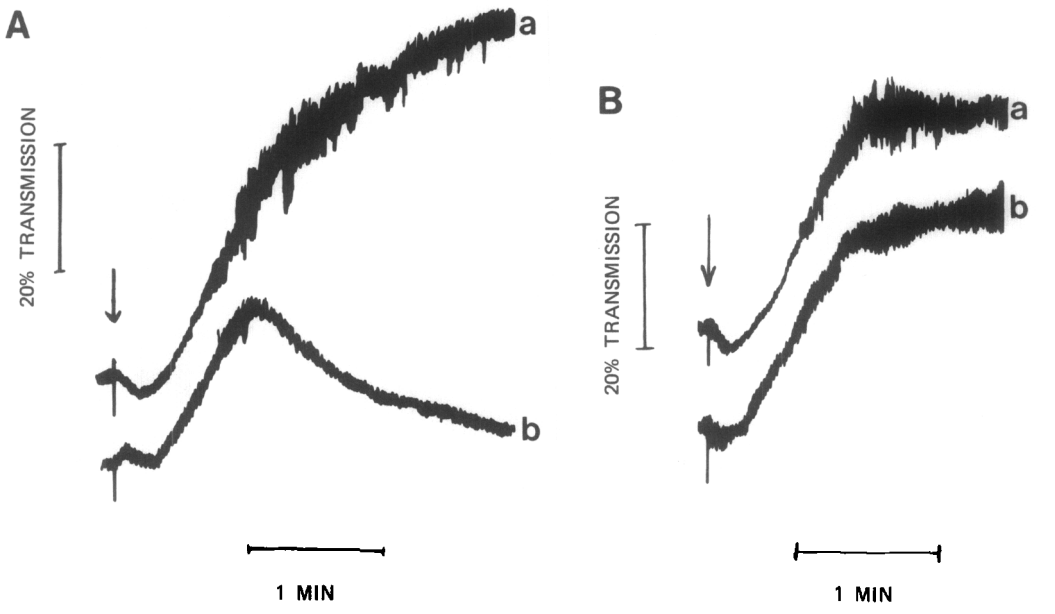

Fig. 2: A. Enhancement of thrombin-induced platelet aggregation by the plasma protein complex. Thrombin (60 milliunits ≈ 1.2 nM) was incubated with 84 ng of the protein for 5 min at room temperature. The reaction was initiated by adding the thrombin-protein mixture (arrowed) to 450 µl of the platelet suspension. The change in light transmission was followed versus time. (a) thrombin plus protein; (b) control in which the protein was replaced by buffer. B. Lack of effect of the plasma protein complex on platelet aggregation induced by ADP. This experiment was carried out in the same manner as in Fig. 2A except that ADP (10 µM) was used instead of thrombin. (a) control with an equal volume of buffer; (b) ADP plus protein (40 ng). Similar results were obtained with other ADP and protein concentrations.

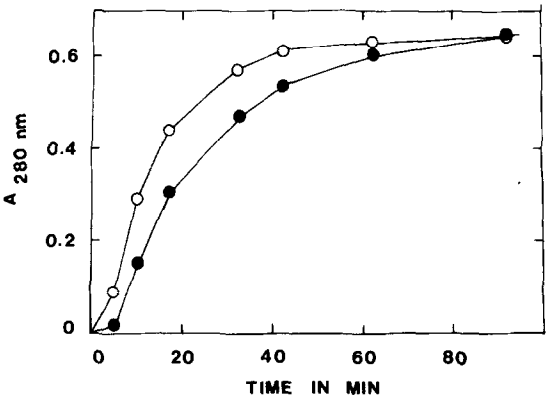

Fig. 3: Effect of the plasma protein complex on thrombin-induced clotting of fibrinogen. Thrombin (100 mU) was incubated with buffer (control ●——●) or with 470 ng of the protein (O——O) for 5 min at room temperature and was then added to 3 ml (final) of 0.59 µM fibrinogen in 25 mM Tris-HCl, pH 7.4 plus 125 mM NaCl and 10 mM $CaCl_2$.

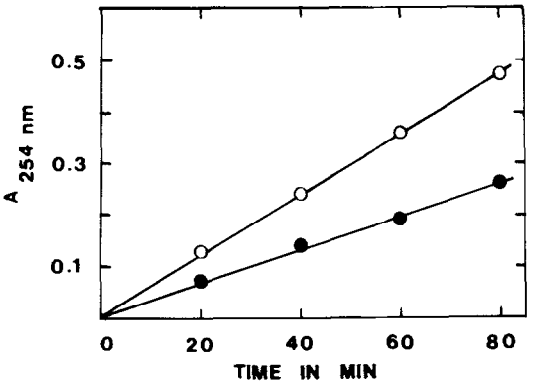

Fig. 4: Influence of the plasma protein complex on the esterolytic activity of thrombin. Thrombin (200 mU) was incubated as in Fig. 3 with 470 ng of the protein (O——O) or with buffer (●——●) and then added to 3 ml (final) of 0.58 nM N-benzoylarginine ethyl ester. Absorbance at 254 nm was monitored with time.

Compared to the control, thrombin activity on the ester was significantly enhanced in the presence of the protein (Fig. 4). These results suggest that the plasma protein directly interacts with thrombin and enhances its reactivity without substrate specificity.

We previously reported the presence of a thrombin-reactive protein complex in human platelets (3). This protein complex consists of two major subunits of 74 kDa and 55 kDa each of which specifically blocked platelet activation by thrombin while aggregation induced by trypsin, ADP, collagen or ristocetin was not significantly affected. However, when these two proteins are preincubated together, instead of inhibiting, they enhanced thrombin-induced platelet aggregation. Similar enhancement was observed with the intact protein complex as isolated without dissociation of the polypeptide components (3). The properties of the plasma protein complex described in this study are in many ways similar to the platelet protein complex. Further studies will be necessary to establish the identity of this plasma protein complex and its relationship with the platelet protein. The isolated plasma protein complex enhanced the action of thrombin on platelets, fibrinogen as well as on N-benzoylarginine ethyl ester. It appears reasonable to conclude that the protein acts directly on thrombin and not on a particular substrate. The detection of such a protein in plasma which can directly enhance thrombin reactivity raises the possibility that this protein may play an important role in the regulation human blood coagulation.

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