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Distinction of Serum Inhibitor of Activator-Induced Clot Lysis from α_1-Antitrypsin (38148)

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A fraction of human serum prolongs the euglobulin clot lysis time (1) and inhibits plasminogen activation by the activator derived from the human vascular trees (2) as well as the activators from other sources (urokinase from human urine and tissue activator from human heart) (3). This fraction, designated as antiactivator fraction (2), exerts a little antiplasmin activity and is biochemically distinguished from a major antiplasmin fraction (α_2-macroglobulin) which does strongly inhibit plasmin but exerts little effect on activator-induced clot lysis (2). However, distinction of this antiactivator from another antiplasmin namely α_1-antitrypsin or α_1-antiplasmin was not established, although the location of the peak of antiactivator activity on the gel chromatography was different from that of α_1-antitrypsin (2). Furthermore, the question as to whether antiplasmin activity observed in antiactivator fraction is inherent to the antiactivator or is entirely or partly attributable to the coexisting α_1-antitrypsin has not been determined. To answer these questions the present study was undertaken with the use of antiserum to α_1-antitrypsin.

Materials and Methods. Reagents. Human urokinase (7000 CTA units per mg protein, Mochida Pharm. Co., Tokyo), bovine thrombin (Mochida Pharm. Co., Tokyo), human fibrinogen containing plasminogen (Green Cross Corp., Osaka), trypsin (crystallized, Worthington Bioch. Corp., Freehold, N.J.), casein (Hammersten grade, E. Merck, Darmstadt) purified by repeated acid precipitation according to Muellertz (4). Plasmin spontaneously activated in 50% glycerol as described (3). Sephadex G-200 and Sepharose 4-B (Pharmacia Fine Chemicals, Uppsala). Lysine-coupled Sepharose was prepared as described by Deutsh and Mertz (5).

IgG-coupled Sepharose. Rabbit antisera to human α_1-antitrypsin (Behring Werke, Marburg). IgG fraction of the antisera was prepared by DEAE-cellulose chromatography as described by Cambell *et al.* (6). A trace amount of plasminogen contaminating the IgG fraction was removed by passing the fraction through a column of lysine-Sepharose. Three ml of IgG fraction (1.3 mg/ml) thus obtained were coupled to 2 ml of Sepharose 4-B according to the method described for other ligands by Cuatrecasas *et al.* (7, 8). The coupling was done at pH 9.0. The coupled-Sepharose was washed with 1 *M* ethanolamine (pH 9.0) and subsequently with Tris buffered saline (pH 7.4).

Serum antiactivator concentrate. Five ml of fresh human serum were passed through a column of 3 ml of lysine-Sepharose equilibrated with barbital buffered saline (0.005 *M* barbital 0.15 *M* NaCl, pH 7.4) to remove plasminogen. The first 2 ml of the effluent was discarded and the following 6 ml which contained most of the protein were collected. Five ml of this plasminogen-free serum was chromatographed on Sephadex G-200 and antiactivator concentrate was obtained as described previously (3).

Affinity chromatography. One and one half ml of antiactivator concentrate (13.8 mg/ml) were applied on a column of 2 ml of IgG-coupled Sepharose which had been washed with barbital buffered saline. When approximately 1 ml of the sample entered the column, the flow was stopped by closing the outlet. The sample and the Sepharose were gently mixed in the column by a small wooden rod, and they were kept at room temperature for 30 min and then at 4° for 3 hr. During this period the sample and the Sepharose were mixed frequently. The flow was resumed by opening the outlet and more barbital buffered

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saline was applied on the top of the column. After 5 ml of the effluent were collected, the elution was started with 5 M $MgCl_2$ which had been adjusted to pH 5.4 with 1 *M* NaOH. Each 1 ml fraction of the eluate was dialyzed immediately against phosphate buffer (0.15 *M*, pH 7.5) in cold to avoid inactivation of α_1-antitrypsin by acidic environment in $MgCl_2$ solution. The dialysate was changed every 30 min for the first 3 hr, and then dialysis was continued for 14 hr using Tris buffered saline (0.05 *M* Tris 0.15 *M* NaCl, pH 7.4) as the dialysate. Each fraction of the effluent was analyzed for protein, antitrypsin, antiplasmin and antiactivator activity. The sepharose column was washed with 1 *M* acetic acid followed by Tris buffered saline and was ready for reuse.

Assay methods. Protein was determined by the method of Lowry *et al.* (9) with bovine crystalized serum albumin (Armour Pharm. Co., Kankakee, Ill.) as a standard. Plasmin activity was assayed by the caseinolytic method (10). As a modification the incubation time of the test sample with casein was extended to 60 min and the activity was expressed in uits defined by Remmert and Cohen (11). For the assay of antiplasmin activity 0.2 ml of plasmin solution (5 units per ml) was added to 0.4 ml of the test sample and the total volume was adjusted to 3 ml with phosphate buffer (0.15 *M*, pH 7.5). The mixture was left at 25° for 60 min, and then remaining plasmin activity was assayed. The control was run replacing the test sample with buffered saline. The difference between the control measurement and the test run, expressed in caseinolytic plasmin unit, divided by the volume (0.2 ml) of the test sample represents the antiplasmin activity units per ml test sample. Antitrypsin activity was measured as described previously (2) with casein as substrate. Antiurokinase activity was assayed by the fibrinolytic antiactivator assay described previously (2, 3) using human urokinase and human fibrinogen.

Immunodiffusion. Double diffusion was carried out by the micro-Ouchterlony slide method (12) using 1% agarose in buffered saline.

SDS acrylamide gel disc electrophoresis. SDS disc electrophoresis was performed according to Weber and Osborn (13). The reference proteins used for estimation of mol wt by SDS electrophoresis were albumin, ovalbumin, cytochrome C, chymotrysinogen and myoglobin (all purchased from Mann Research Lab., New York).

Results. The typical elution pattern of the affinity chromatography is given in Fig. 1. The sample applied to the column contained 21 mg protein, 270 μm antiurokinase, 5 μm antiplasmin and 250 μm antitrypsin. Most of

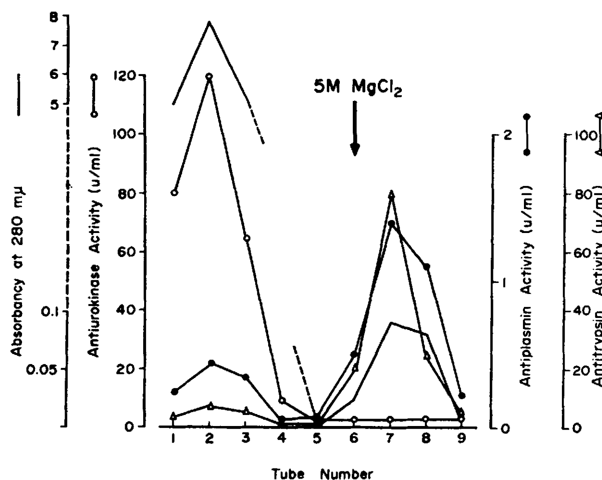

FIG. 1. Affinity chromatography of serum antiactivator concentrate using Sepharose coupled with IgG of antiserum to α_1-antitrypsin. Each fraction 1 ml. The scale of absorbancy was divided into the two regions of low and high absorbancy. The arrow indicates the start of elution with magnesium chloride solution. For details see the text.

the protein applied was not retained and passed through the column. This unretained fraction contained all of the antiurokinase activity originally present in the antiactivator concentrate applied to the column. However, only 20% and less than 10% of antiplasmin and antitrypsin activity respectively were recovered in this fraction. Most of antiplasmin and antitrypsin activity were bound to the column and were subsequently eluted with 5 *M* $MgCl_2$. Further elution of the column with 1 *M* acetic acid yielded only a trace amount of protein which has no activity. The total recovery of antiplasmin and antitrypsin activity was 84% and 61% respectively. The peak portion of the effluent were subjected to double immunodiffusion with antiserum to α_1-antitrypsin. No precipitin line was detected with the unretained fraction, although a distinct precipitin line was observed with the $MgCl_2$-eluted fraction. The peak portion of the eluted fraction was analyzed by SDS disc electrophoresis. Twenty μl (approximately 14 μg protein) of the fraction was applied to the electrophoresis, and the electrophoretic pattern revealed a major protein band with mol wt of 54,000 which is the same as reported for α_1-antitrypsin (14) and a faint band which corresponds to albumin.

Discussion. Evidence has accumulated for the existence of plasma constituents capable of inhibiting the activation of plasminogen (15, 16). The existence of such naturally occurring substances (antiactivators) distinct from naturally occurring antiplasmins was suggested by the several clinical observations (17–21), especially by the report on the individuals who have no or little inhibitory activity of serum to urokinase in spite of normal content of antiplasmins in serum (22).

The results presented previously (2) and in this paper indicate that serum antiactivator activity, which inhibits activator-induced clot lysis, is separate from major antiplasmins and that most of the antiplasmin activity present in the antiactivator fraction (2) is due to α_1-antitrypsin contaminating the fraction and can be removed by affinity chromatography on Sepharose coupled with IgG of antiserum to α_1-antitrypsin. Alpha$_1$-antitrypsin thus obtained exhibited strong antiplasmin activity as well as antitrypsin activity. This supports the claim (23, 24) that progressive α_1-antiplasmin is identical with α_1-antitrypsin. The interesting finding is that α_1-antiplasmin or α_1-antitrypsin did not contribute to any antiactivator activity, and this observation is concordant with the fact that the total antiactivator activity remained in full after α_1-antitrypsin was removed from the fraction. The weak antiplasmin and antitrypsin activity still present in the fraction after the removal of α_1-antitrypsin appears not to be due to remaining α_1-antiplasmin or α_1-antitrypsin, since the fraction did not exhibit a precipitin band on immunodiffusion with antiserum to α_1-antitrypsin and the antiplasmin activity was not removed with an additional treatment with the IgG-coupled Sepharose. The question as to whether or not this weak antiprotease activity remaining after the removal of α_1-antitrypsin is an intrinsic function of the antiactivator needs further investigation, including the isolation of the antiactivator in a pure form.

Summary. Antiactivator activity of human serum, which inhibits activator-induced clot lysis, was separated from α_1-antitrypsin by affinity chromatography on Sepharose coupled with IgG of antiserum to α_1-antitrypsin. It was found that most of antiplasmin activity formerly found in the antiactivator fraction is actually due to α_1-antitrypsin contaminating the fraction. Antiactivator itself has no or little antiplasmin activity. On the other hand, α_1-antitrypsin possesses antiplasmin activity but has no antiactivator activity.

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