Correlation of Antiplasmin to Antitrypsin and Antichymotrypsin in Human Plasma*

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The numbers of antiplasmin, antitrypsin and antichymotrypsin in human plasma and mutual relationship among them are discussed in this paper.

Human blood plasma was divided into 2 major fractions by adding ammonium sulfate, after desalting by sephadex, the fractions being further subfractionated by using column chromatographic procedure on DEAE-cellulose. The determination of antiproteolytic activities and electrophoretic analysis were performed on the subfractions eluated by the following order of solvents; (1) pH 7.8, 0.0175 M potassium phosphate buffer (KPB), (2) pH 7.1, 0.04 M KPB, (3) pH 6.64, 0.1 M KPB and (4) 0.0025 M hydrochloric acid containing 1 M sodium chloride. Three chromatographically different antiplasmin fractions which were capable of both antitrypsin and antichymotrypsin activities, and another antitrypsin fraction which showed antichymotrypsin, but was devoid of antiplasmin activity were detected in human plasma.

The numbers of antiplasmin, antitrypsin and antichymotrypsin in the human blood plasma, and the mutual relationship among them have not as yet been completely clarified. It was formerly thought that the majority of antiplasmin activity of the blood was found in the albumin fraction. Ratnoff, Lepow and Pillemer reported that there were at least 3 inhibitors of plasmin present in plasma. One plasma component was heat labile at 56°C for 30 minutes, and others, which are heat stable, can be separated further into 2 inhibitors from the stability test to ammonia or primary amine. Grob fractionated plasma by Cohn method 6, and found both plasmin and trypsin inhibitors in Fraction IV, and plasmin inhibitor largely free from trypsin inhibitor in Fraction V. Jacobsson noted that antiplasmin activity largely resided in the alpha-2 globulin, whereas the greatest part of antitrypsin was found in the alpha-1 globulin fraction which was devoid of antiplasmin activity. Shulman concluded by using his differential titration method that some 10% of the plasma trypsin
inhibitor was also capable of inhibiting plasmin. Norman\(^6\)\(^7\) found 2 antiplasmins in human plasma; one inhibitor, heat labile, belonged to alpha-1 globulin and combined non-dissociably with plasmin at a slow rate depending on temperature, the second, heat stable, resided in alpha-2 globulin and combined dissociably with plasmin independently of temperature. Wu and Laskowski\(^8\) prepared from bovine plasma crystalline a trypsin inhibitor which was mucoprotein nature. Gray, Priest, Blatt, Westphal and Jensen\(^9\) obtained from bovine plasma an essentially homogeneous preparation having both antiplasmin and antichymotrypsin activities. Martin\(^10\) noted the inhibition of trypsin, chymotrypsin and plasmin by an inhibitor isolated from sheep serum. The author reported that plasma contained at least 2 inhibitors to both plasmin and trypsin and one inhibitor to trypsin, lacking in antiplasmin activity\(^11\).

In this paper, plasma divided into 2 fractions by adding ammonium sulfate was further subfractionated on DEAE-cellulose column chromatograph, and antiproteolytic activities and some physical properties are discussed and compared with previously reported inhibitors.

**MATERIALS AND METHODS**

1. **Materials**

Samples of blood were collected from 4 healthy male adults, 4 pregnant women and 2 patients with cancer of the cervix. The plasma was obtained by centrifuging a mixture of 9 vol. of venous blood and 1 vol. of 3.8% sodium citrate.

2. **Fractionation of plasma by ammonium sulfate**

Ten ml. of plasma were divided into 2 major fractions, supernate and precipitate, by adjusting to 50 or 55% saturation with saturated ammonium sulfate solution. The precipitate was dissolved in 10 ml. of potassium phosphate buffer (KPB) of pH 7.8, 0.0175 M.

3. **Desalting procedure by sephadex column chromatography**

Ammonium sulfate and low molecular substances in the above 2 fractions were removed by column chromatography of sephadex. The details of the procedure were described in the previous report\(^11\).

4. **Fractionation of ammonium sulfate-divided plasma on DEAE-cellulose column chromatograph**

The stepwise elution was performed on the desalted plasma fractions by the previous method using the following order of solvents; (1) pH 7.8, 0.0175 M KPB, (2) pH 7.1, 0.04 M KPB, (3) pH 6.64, 0.1M KPB and (4) 0.0025 M hydrochloric acid containing 1M sodium chloride\(^11\).

5. **Determination of protein concentration**

The protein concentration of the eluates was determined spectrophotometrically at 275 m\(\mu\).
6. Determination of antitrypsin and antichymotrypsin activities

For the purpose of determination of antitrypsin and antichymotrypsin activities, the standard enzyme solutions were prepared by dissolving crystalline trypsin (Mochida Co. 10,000 H.U.M.) and crystalline chymotrypsin (Eisai Co. 25 ch. u) in 75 ml. and 100 ml. of physiological saline solution, respectively. Before the test, the fractions eluated by the final solvent were dialyzed to 0.04 M, pH 7.1 KPB through a cellophane bag in an ice chest for 13 hours in order to eliminate the salt and pH influence of the reaction. The activities were determined on 0.5 ml of the eluates by the previous method with a preincubation time of 30 minutes\textsuperscript{12}. The activities were simply expressed by the difference of optical density at 275 m\textmu\textit{M} between the standard and the test.

7. Determination of antiplasmin activity

i. Determination of immediate inhibitor: As standard plasmin solution, Actase (Ortho Co. 25 casein units, 45,000 casein activator units, 1,000,000 fibrinolytic units in 1 vial) was dissolved in distilled water and the activity was controlled to dissolve completely 0.1% clotted fibrinogen for approximately 10 minutes under the conditions.

A test tube containing 0.5 ml of eluates and 0.2 ml of standard plasmin was

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\textbf{Table I. Abbreviations of Fractions and Eluates}

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Fractionated by 50% saturation of ammonium sulfate</th>
<th>Fractionated by 55% saturation of ammonium sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernate (A)</td>
<td>(A\textsubscript{1}) (A\textsubscript{2}) (A\textsubscript{3}) (A\textsubscript{4})</td>
<td>(B\textsubscript{1}) (B\textsubscript{2}) (B\textsubscript{3}) (B\textsubscript{4})</td>
</tr>
<tr>
<td>Precipitate (B)</td>
<td>(C\textsubscript{1}) (C\textsubscript{2}) (C\textsubscript{3}) (C\textsubscript{4})</td>
<td>(D\textsubscript{1}) (D\textsubscript{2}) (D\textsubscript{3}) (D\textsubscript{4})</td>
</tr>
</tbody>
</table>

A\textsubscript{1}, A\textsubscript{2}, A\textsubscript{3} and A\textsubscript{4} express the fractions having the protein peak which appeared in the eluates of 0.0175 M KPB, 0.04 M KPB, 0.1 M KPB and 0.0025 M hydrochloric acid containing 1 M sodium chloride, respectively. B\textsubscript{1-4}, C\textsubscript{1-4} and D\textsubscript{1-4} are expressed by the same fashion.

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\textbf{Table II. Electrophoretical Components of Fractions}

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Electrophoretical components detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A\textsubscript{1}</td>
<td>alpha-2 and beta globulin</td>
</tr>
<tr>
<td>A\textsubscript{2}</td>
<td>not detected</td>
</tr>
<tr>
<td>A\textsubscript{3}</td>
<td>albumin and alpha-1 globulin</td>
</tr>
<tr>
<td>A\textsubscript{4}</td>
<td>not detected</td>
</tr>
<tr>
<td>B\textsubscript{1}</td>
<td>alpha-2, beta and gamma gloubulin</td>
</tr>
<tr>
<td>B\textsubscript{2}</td>
<td>alpha-1 and alpha-2 gloublin</td>
</tr>
<tr>
<td>B\textsubscript{3}</td>
<td>albumin and alpha-1 gloublin</td>
</tr>
<tr>
<td>B\textsubscript{4}</td>
<td>alpha-1 and alpha-2 globulin</td>
</tr>
</tbody>
</table>
incubated for 5 minutes at 37°C, and then 0.1 ml. of thrombin and 1 ml. of cooled 0.1% fibrinogen were added. The activity was expressed by the difference between the time required for complete dissolution of the fibrin clot in the test and the standard.

ii. Determination of slow inhibitor: Slow inhibitor of Norman was determined by 2 hours preincubation of a mixture of 0.5 ml. of eluates, 0.2 ml. of standard plasmin and 0.1 ml. of 1 M ethylamine hydrochloride in phosphate buffer (Sorensen, pH 7.4). After that, 0.1 ml. of thrombin and 1 ml. of 0.1% fibrinogen were added, and the activity was measured in a similar way to the immediate inhibitor assay. The addition of ethylamine was made to stabilize the plasmin during preincubation.

8. Electrophoresis of the eluates on filter paper

In order to carry out electrophoresis, each eluate containing protein peak was concentrated by dialysis against 50% arabic gum in veronal buffer (pH 8.6, $\mu_{v}$

![Chromatograms of fraction A.](image-url)
0.05) through a celophane membrane. The proteins in the eluates, concentrated to 0.5–2%, were separated by electrophoresis on filter paper.

RESULTS

The following abbreviations, represented in parentheses, were conventionally used for the fractions and eluates (Table I). The protein components found in the electrophoretical analysis on filter paper are presented in Table II. From the Table, it can be concluded that there were no distinct differences in the components between corresponding subfractions, for example between A₁ and B₁. Accordingly, it would be natural to assume that the antiproteolytic activities appearing in such subfractions as A₁–A₄ might be similar to those of B₁–B₄. The results of electrophoretical analysis were essentially similar to those of Abelson and

![Fraction B Chromatograms](image)
Rawson\textsuperscript{13}, who studied serum by DEAE-cellulose column chromatography, using the increasing molar concentration and decreasing pH of sodium phosphate buffers.

The typical chromatograms of protein concentration and antiproteolytic activities of normal plasma are presented in Figs. 1, 2, 3 and 4 which represent those of fractions A, B, C and D, respectively. A marked difference of chromatograms was noted between fractions A and C. The former gave 4 protein peaks by each solvent, while the latter essentially failed to produce protein peaks by the solvents of 0.0175 M and 0.04 M KPB. This might have resulted from the difference in solubility of the plasma proteins. The chromatograms of fractions B and D showed almost similar patterns of protein concentration and antiproteolytic activities. There were no significant differences among the patterns of plasma chromatography obtained from pregnant women, patients with cancer of the

\begin{center}
\textbf{Fraction C}
\end{center}

\begin{center}
\begin{tabular}{c}
\textbf{Protein concentration} \\
\hline
0.0 & 0.5 & 1.0 \\
\hline
\textbf{Antitrypsin} \\
\textbf{Antichymotrypsin} \\
\textbf{Antiplasmin} \\
\end{tabular}
\end{center}

\begin{center}
\begin{tikzpicture}
\begin{axis}[
xtick={0,10,20,30,40},
xticklabels={0,10,20,30,40},
width=0.9\textwidth,
height=0.6\textwidth,
]
\addplot [mark=none, black, line width=1pt] table [x=Fraction No., y=OD] {data.csv};
\end{axis}
\end{tikzpicture}
\end{center}

Fig. 3. Chromatograms of fraction C.
cervix and normal controls. However, a marked loss of antiproteolytic activities was noted in 2 of 10 cases. This might be due to spontaneous destruction of antiproteolytic activities during the experiment.

For the purpose of determination of the heat stability of the antiproteolytic subfractions, B₁–B₄, were heated at 56°C for 30 minutes, following which immediate acting antiplasmin was examined. The results are shown in Table III. A newly appearing antifibrinolytic activity in the fraction B₁, seemed to be an inhibitor which had been masked by the significant amounts of plasminogen in the fraction. The plasminogen was destroyed by heating, and then the antifibrinolytic activity became detectable. The presence of this plasminogen was naturally considered from the previous experiment of fractionation of the euglobulin on DEAE-cellulose column chromatography¹⁴. In addition, fraction B₁ possessed
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TABLE III. Inhibitory Action of Fractions on Plasmin and Heat Stability

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Fraction</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediate inhibitor</td>
<td></td>
<td>8'</td>
<td>5'</td>
<td>30'</td>
<td>20'</td>
<td>10'10''</td>
</tr>
<tr>
<td>Slow inhibitor</td>
<td></td>
<td>12'20'</td>
<td>60'</td>
<td>9'30'</td>
<td>40'</td>
<td>15'10''</td>
</tr>
<tr>
<td>Immediate inhibitor</td>
<td>120'</td>
<td>13'</td>
<td>11'22'</td>
<td>11'</td>
<td>10'10''</td>
<td></td>
</tr>
<tr>
<td>after heating</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

potent antiproteolytic activities to trypsin and chymotrypsin. The fact that the antifibrinolytic activities in fractions B₂ and B₄ were enhanced by the preincubation with ethylamine suggested that they would be equal to the slow acting inhibitor of Norman. In the previous experiment¹¹, the presence of inhibitor in the fraction B₁ had not been noted because the heat stability test was not carried out.

From these observations, it can be presumed that there were at least 3 chromatographically different antiplasmin fractions which were capable of both antitrypsin and antichymotrypsin activities, and another antitrypsin fraction which showed antichymotrypsin but was devoid of antiplasmin activity in the human plasma separated by the chromatographic procedure.

DISCUSSION

The method applied in this assay has some disadvantage, because the fractionation is not distinctly clear. Therefore, even divided subfractions were mixtures of various plasma proteins. The method, however, seems to have an advantage in that the activities of plasma components can be caught with less overlooking than is possible by other fractionation methods.

From the literature which has been reported and from our experimental observations, the inhibitors found in the fractions can be classified and summarized as in Table IV. The reports of Shulman and Jacobsson that antiplasmin resided in alpha-2 globulin were substantiated by our experiment. However, they overlooked the presence of antiplasmin in alpha-1 of Norman which reacts slowly with plasmin. The inhibitors to trypsin seem to be similar to those of chymotrypsin. Of course, it is possible to presume that the antiproteolytic activities in the fractions are the simple mixture of individual inhibitor to plasmin, trypsin or chymotrypsin. It is, therefore, necessary to prepare homogeneous preparations of inhibitor for the final conclusion.

The antitrypsin assay has been substituted for the antiplasmin determination in our experiments¹⁵⁻¹⁷. Though, there was statistically close relationship between fibrinolysis and antitrypsin activity¹⁵, the antitrypsin cannot completely represent the antiplasmin from the view point of the above experiment. This finding brought us to the necessity of preparing the new antiplasmin assay method.
TABLE IV. Some Properties of Inhibitors in Fractions and Their Comparison with Already Reported Antiplasmins

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Inhibitor eluted by 0.0175 M KPB</th>
<th>Inhibitor eluted by 0.04 M PKB</th>
<th>Inhibitor eluted by 0.1 M KPB</th>
<th>Inhibitor eluted by 0.0025 M HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor to plasmin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>trypsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>chymotrypsin</td>
<td>+</td>
<td>+</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Heat stability</td>
<td>stable</td>
<td>labile</td>
<td>/</td>
<td>labile</td>
</tr>
<tr>
<td>Behavior of acting (slow or immediate)</td>
<td>immediate</td>
<td>slow</td>
<td>/</td>
<td>slow</td>
</tr>
<tr>
<td>Possible electrophoretical properties of inhibitor</td>
<td>alpha-2 globulin</td>
<td>alpha-1 globulin</td>
<td>alpha-1 globulin</td>
<td>alpha-1 or alpha-2 globulin</td>
</tr>
<tr>
<td>Comparison with Ratnoff’s</td>
<td>heat stable antiplasmin</td>
<td>heat labile antiplasmin</td>
<td>heat labile antiplasmin</td>
<td>slow inhibitor</td>
</tr>
<tr>
<td>Norman’s</td>
<td>immediate inhibitor</td>
<td>slow inhibitor</td>
<td>slow inhibitor</td>
<td></td>
</tr>
<tr>
<td>Shulman’s</td>
<td>alpha-2 globulin antiplasmin</td>
<td>alpha-2 globulin antiplasmin</td>
<td>alpha-2 globulin antiplasmin</td>
<td></td>
</tr>
<tr>
<td>Jacobsson’s</td>
<td>alpha-2 globulin antiplasmin</td>
<td>alpha-1 globulin antiplasmin</td>
<td>alpha-1 globulin antiplasmin</td>
<td></td>
</tr>
</tbody>
</table>

Regarding the other antiplasmin, Muellertz\(^8\) reported that fibrin is *per se* an inhibitor of plasmin by adsorbing plasmin or plasminogen from the circulation. Platelet contains antiplasmin which reacts with plasmin almost immediately\(^10-21\). On the other hand, blood also contains an inhibitor of plasminogen activation\(^22\). They will be described later.

References

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