The Plasmin Inhibitor of Human Plasma

IV. ITS ACTION ON PLASMIN, TRYPSIN, CHYMOTRYPSIN, AND THROMBIN*

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SUMMARY

Purified α1-proteolytic inhibitor from human plasma was shown to inhibit trypsin, chymotrypsin, plasmin, and thrombin. This inhibitor is probably identical with the inhibitor previously known as serum trypsin inhibitor, α1-antitrypsin, or antiplasmin. The enzymes were affected by the inhibitor via two different mechanisms. Trypsin and chymotrypsin reacted instantaneously in stoichiometric manner, and the enzyme substrate had no effect on the reaction. The inhibition of plasmin and thrombin was time-dependent and of nonstoichiometric nature, and it was retarded by the presence of enzyme substrate. Even so, kinetic studies failed to show a competition reaction between the substrate and the inhibitor on a single site of the enzyme molecule. It is therefore suggested that the inhibition mechanism might be an enzymatic inactivation of the proteolytic enzymes concerned. This inactivation was irreversible in the sense that no enzymatic activity could be recovered after acid dissociation of the enzyme-inhibitor complex. It was shown by differential titration that the inhibitory activity of the inhibitor towards all four enzymes is due to a single molecular entity; this conclusion is also suggested by the high purity of the inhibitor preparation. On the basis of its stoichiometric combination with trypsin, the α1-proteolytic inhibitor was estimated to have a molecular weight of 47,000 and to comprise about 2% of plasma proteins.

Trypsin (EC 3.4.4.4) inhibition by human and animal sera has been extensively studied during the last half century (1–7). Inhibition by these sera of other proteolytic enzymes, such as chymotrypsin (EC 3.4.4.5), plasmin (EC 3.4.4.14), and hirudin (EC 3.4.4.13), has also been reported (8–13). These findings pose the question whether such antiproteolytic activities of serum are exerted by a single, nonspecific component or by separate, specific inhibitors. The question is especially pertinent for enzymes with similar specificities, like plasmin and trypsin, on which conflicting observations have been reported. Some investigators (14–18) consider antiplasmin and antitrypsin to be different entities, while others (19–22) believe that the same plasma inhibitor acts on both trypsin and plasmin. In order to settle this problem, separation of the inhibitory activities in various plasma fractions has been attempted (14, 15, 17, 23, 24), but again with no conclusive results. The matter is further complicated by the presence in human plasma of several trypsin inhibitors (17, 25–28), some of which are reported to act solely on trypsin, and others to affect both trypsin and plasmin.

Moreover, plasmin itself is reportedly inhibited by two different mechanisms, suggesting that two different plasmin inhibitors exist in plasma (13, 29). One of these inhibitors was reported to react rapidly with plasmin and therefore was designated an “immediate” inhibitor, while the other was identified as a “slow” inhibitor because of its relatively slow reactivity rate.

On the basis of the foregoing findings, it seemed reasonable to use that investigation of a highly purified inhibitor would contribute to an understanding of the problem. Such an inhibitor has been reported in our previous work (30), and the purified antiplasmin was shown to be homogeneous α1-globulin with a molecular weight of about 55,000.

The present paper reports on and compares the actions of the purified inhibitor on plasmin, trypsin, chymotrypsin, and thrombin.

EXPERIMENTAL PROCEDURE

Proteolytic Enzymes

Human plasmin was obtained by activation of plasminogen that had been prepared from Fractions I and III of plasma by the method of Shamash et al. (31) and then activated with streptokinase (Varidase, Lederle). Four units of streptokinase were used per caseinolytic unit of plasminogen. The resulting plasmin was precipitated with 1 M NaCl at pH 2.0, redissolved in 0.1 M l-lysine hydrochloride, and adjusted to pH 7.4. Upon analysis, it contained about 10 caseinolytic units per mg of protein. A working solution containing 1 caseinolytic unit per ml was used.

Twice crystallized trypsin (Worthington) was used to prepare a stock solution of 10 mg in 100 ml of 0.025 N HCl. Enzyme protein concentration was determined by measuring, with the
factor 0.685, the absorbance at 280 m\(\mu\) in a Beckman DU spectrophotometer (32). The activity of the enzyme was standardized against soybean inhibitor (32).

Three times crystallized \(\alpha\)-chymotrypsin (Worthington) was used to prepare a stock solution of 10 mg/100 ml in 0.025 N HCl. Enzyme protein concentration was determined as for trypsin, with the factor 0.50 (32).

Stock solutions of trypsin and chymotrypsin were diluted immediately before use with phosphate-sodium chloride buffer to contain an activity of 1 caseinolytic unit per ml. Trypsin, 3.2 \(\mu\)g, or chymotrypsin, 1.9 \(\mu\)g per ml, was needed to maintain such activity.

Bovine thrombin (Parke, Davis) was used. It was dissolved in phosphate-sodium chloride buffer to contain 500 NIH units per ml and was then frozen.

\section*{Inhibitors}

Plasma inhibitor was purified from human plasma as previously described (30, 33). This represented an 80-fold purification in terms of "slow" antiplasmin activity.

Five times crystallized soybean inhibitor (Mann) was used to prepare a stock solution of 10 mg/100 ml in 0.025 N HCl. The inhibitor protein concentration was determined as with trypsin, with the factor 1.10 (32).

\section*{Substrates}

Lyophilized Cohn's Fraction I of human fibrinogen with about 70\% clottable proteins was used to prepare a 1\% solution in phosphate-sodium chloride buffer.

Devitaminized casein from Sheffield Chemical Company was used. A 6\% solution was prepared as described elsewhere (31).

\section*{Buffers}

Phosphate-sodium chloride buffer was prepared by dissolving 13.4 g of K\(\text{H}_2\text{PO}_4\), 4.0 g of K\(\text{HPO}_4\), and 9.0 g of NaCl in 1000 ml of distilled water and adjusting the pH to 7.4.

Lysine buffer was prepared by dissolving 2.2 g of L-lysine hydrochloride in 100 ml of phosphate-sodium chloride buffer and readjusting the pH to 7.4.

\section*{Assay of Enzymatic Activities}

A modification of the caseinolytic method of Remmert and Cohen (34) was used for the activity assay of plasmin, trypsin, and chymotrypsin. To 1 ml of enzyme solution, 1 ml of phosphate-sodium chloride buffer and then 1 ml of casein solution were added, and the mixture was incubated at 37\(^\circ\). Samples of 0.5 ml were taken at zero time and after 1 hour of incubation and deproteinized by adding 1 ml of 15\% trichloroacetic acid; 15 min later, the mixture was centrifuged and the supernatant analyzed for acid-soluble tyrosine with the Folin reagent as described by Lowry et al. (35). One caseinolytic unit of activity was defined as the amount of enzyme capable of releasing 450 \(\mu\)g of acid-soluble tyrosine during 1 hour of incubation at 37\(^\circ\).

Thrombin activity was tested by its coagulative action on fibrinogen and by hydrolysis of benzoylarginine methyl ester as measured in a pH-stat. The coagulation test mixture comprised 0.5 ml of 1\% fibrinogen solution and 0.2 ml of thrombin solution containing 1 NIH unit. The mixture was incubated at 37\(^\circ\), and the coagulation time was recorded. The pH-stat tests were performed with a type TTT1a titrator from Radiometer. The reaction flask was maintained at 37\(^\circ\) and contained 50 NIH units of thrombin and 40 mg of BAmel in a total volume of 5 ml of water. The reaction proceeded at pH 7.4. Sodium hydroxide solution, 0.1 N, was used as titrating agent, and the base uptake was recorded.

\section*{Assay of Inhibitory Activities}

Inhibition of plasmin was assayed as previously described (33, 36). The "immediate" type of inhibition was estimated by measuring the caseinolytic activity, as described above, in a mixture containing inhibitor. The difference in caseinolytic activity between such a mixture and a control mixture without inhibitor represented the value of the immediate inhibition. The "slow" type of inhibition was estimated by measuring, over specified time intervals, the caseinolytic activity of samples from a mixture of plasmin and inhibitor preincubated at 37\(^\circ\). Inhibitor of trypsin and chymotrypsin was estimated from the difference in caseinolytic activity between a control containing casein and enzyme alone and a mixture to which inhibitor was added.

The unit of inhibitory activity was defined as the micrograms of protein necessary to inhibit 1 caseinolytic unit of each of these enzymes. This was usually calculated from the value of 50\% inhibition of plasmin or inhibitor, or both, on clot formation and lysis was investigated by adding 0.2 ml (1 caseinolytic unit) of plasmin or 0.1 ml (40 \(\mu\)g) of inhibitor, or both, to the fibrinogen-thrombin mixture.

\section*{RESULTS}

\section*{Action of Inhibitor on Plasmin}

The kinetics of plasmin inhibition with respect to time and inhibitor concentrations is shown in Figs. 1 and 2. It is evident from these figures that the inhibition reaction is time-dependent and shows a deviation from linearity with increasing amounts of inhibitor. As calculated from Fig. 2, 24 \(\mu\)g of inhibitor represents 1 unit of antiplasmin activity. At the inhibitor concentrations indicated—namely, up to 20 \(\mu\)g of protein—only a negligible effect of the so-called immediate inhibitor could be detected.

This type of activity was apparently exhibited when a large excess of the inhibitor (more than 100 \(\mu\)g) was applied to plasmin in the presence of casein. The activity in the latter case, however, was more likely due to the slow action of excessive amounts of inhibitor, since it was observed that, in the presence of these amounts of inhibitor, caseinolytic activity as a function of time deviated from linearity (Fig. 3). Determination of immediate inhibition, on the other hand, is based on the assumption that casein stops the action of slow inhibition and consequently that the caseinolytic curve plotted against time ought to be linear.

The influence of substrate on plasmin inhibition can be deduced by comparing Figs. 1 and 3. With 12 \(\mu\)g of inhibitor, 33\% of the plasmin was inhibited within 1 hour of incubation in the absence of casein, compared to about 3\% inhibition in the

\footnote{The abbreviation used is: BAmel, benzoylarginine methyl ester.}
Fig. 1. Inhibition of plasmin by the purified plasma inhibitor as a function of time. Plasmin was preincubated with the inhibitor at 37°. Samples of 1 ml were withdrawn from this mixture after various time intervals and assayed for their caseinolytic activities (ordinate).

Fig. 2. Inhibition of plasmin by the purified plasma inhibitor as a function of inhibitor concentration. Plasmin was preincubated with various amounts of inhibitor at 37° for 120 min and then assayed for its caseinolytic activity.

Fig. 3. Inhibition of plasmin by the purified plasma inhibitor in the presence of 2% casein. Caseinolytic activity was determined in a mixture of plasmin, inhibitor, and casein at various time intervals during 100 min of incubation at 37°.

Fig. 4. The effect of enzyme substrate (casein) on the enzymatic activity of plasmin with and without inhibitor. The reaction mixture consisted of 20 μg of purified inhibitor, 0.5 caseinolytic unit of plasmin, and various concentrations of casein (0.05 to 0.2%). The same amount of inhibitor, when incubated with plasmin for 2 hours at 37° in the absence of casein, resulted in 75% inhibition.

The possible recovery of enzymatic activity from the enzyme-inhibitor complex was tested. A mixture containing 24 μg of inhibitor per caseinolytic unit of plasmin was incubated for 2 hours at 37°. Under these conditions about 85% inhibition was recorded. The mixture was then acidified with 0.1 N HCl to pH 2.0 and reincubated for 4 hours at 37°. Despite complete destruction of the inhibitory activity under these conditions, as shown by a control experiment, no enzymatic activity could be recovered from the acidified plasmin-inhibitor complex.

**Action of Inhibitor on Trypsin and Chymotrypsin**

Incubating various amounts of purified inhibitor with trypsin for various periods of time revealed that these ingredients re-
acted instantaneously and stoichiometrically, regardless of the presence or absence of the enzyme substrate. Fig. 5 illustrates this type of reaction. The unit of antitryptic activity was found to be 6.25 μg of inhibitor, and a combining ratio by weight of inhibitor to trypsin (standardized by soybean inhibitor) of 1.95:1.0 was calculated. Results were the same whether residual enzymatic activity was measured after 1 min or after 30 min of incubation with the inhibitor. On the basis of our finding that 100 μg of plasma proteins were capable of inactivating 1 μg of trypsin, the purified inhibitor represents a 50-fold purification with regard to its antitryptic activity. The possible recovery of enzymatic activity from the enzyme-inhibitor complex was tested as with plasmin. Inhibitor, 6.2 μg, was mixed with trypsin, 3.2 μg, and the mixture was acidified as described above. In this case, again, no enzymatic activity could be recovered from the acidified trypsin-inhibitor complex.

The action of purified inhibitor on chymotrypsin is shown in Fig. 5. Just as in the case of trypsin, the inhibitory reaction was instantaneous, of stoichiometric nature, and independent of the enzyme substrate. The inhibitory activity unit was found to be 26.5 μg, and a combining ratio of 1.4 μg of inhibitor to 1 μg of α-chymotrypsin was calculated. Taking into account that 90 μg of plasma proteins inactivated 1 μg of α-chymotrypsin under the test conditions, the purified inhibitor represents a 64-fold purification in terms of antichymotryptic activity.

**Action of Inhibitor on Thrombin**

Thrombin exposed to the action of the inhibitor lost its ability to clot fibrinogen and to hydrolyze BAE. One NIH unit of bovine thrombin was completely inactive on fibrinogen after 2 hours of incubation at 37° with 30 μg of purified inhibitor. The inactivation of thrombin was followed with a pH-stat with the use of BAE as substrate. Fig. 6 reveals an immediate drop in activity at zero time followed by a progressive type of reaction. Fifty per cent inhibition was attained when 50 NIH units of bovine thrombin were incubated at 37° for 50 min with 400 μg of inhibitor.

**Effect of Inhibitor on Clot Lysis**

The effect of inhibitor on the fibrinogenolytic and fibrinolytic activity of plasmin was studied by varying the time and the order of mixing the reagents, as indicated in Table I. It was found that the inhibitor does not prevent clot lysis when it is added to the mixture simultaneously with the other ingredients. On the other hand, incubation of plasmin and inhibitor for 2 hours at 37° before fibrinogen and thrombin were added resulted in marked inactivation of plasmin and a high increase in clot lysis time. It was also noted that plasmin inactivation by the in-

![Fig. 5. Inhibition of trypsin and of chymotrypsin by purified plasma inhibitor as a function of inhibitor concentration.](image-url)

![Fig. 6. Inhibition of thrombin by purified plasma inhibitor as a function of time. Thrombin was preincubated with the inhibitor for the specified time periods and then assayed in the pH-stat with the use of BAE as substrate. The ordinate presents the micromoles of 0.1 N NaOH consumed per min.](image-url)

<table>
<thead>
<tr>
<th>Test mixture components and order of mixing</th>
<th>Clot lysis time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmin + fibrinogen + thrombin</td>
<td>10 min</td>
</tr>
<tr>
<td>Plasmin + inhibitor + fibrinogen + thrombin</td>
<td>10 min</td>
</tr>
<tr>
<td>Plasmin + inhibitor, 2 hrs incubation; fibrinogen + thrombin</td>
<td>180 min</td>
</tr>
<tr>
<td>Inhibitor + fibrinogen + thrombin</td>
<td>No lysis</td>
</tr>
<tr>
<td>Plasmin, 2 hrs incubation; fibrinogen + thrombin</td>
<td>12 min</td>
</tr>
<tr>
<td>Fibrinogen + plasmin, 2 hrs incubation; thrombin</td>
<td>No clot</td>
</tr>
<tr>
<td>Fibrinogen + plasmin + inhibitor, 2 hrs incubation; thrombin</td>
<td>No clot</td>
</tr>
<tr>
<td>Fibrinogen, 2 hrs incubation; thrombin</td>
<td>No lysis</td>
</tr>
<tr>
<td>Fibrinogen + inhibitor, 2 hrs incubation; thrombin</td>
<td>No lysis</td>
</tr>
<tr>
<td>Fibrinogen + inhibitor + thrombin</td>
<td>No lysis</td>
</tr>
</tbody>
</table>
hibitor was prevented by the presence of fibrinogen and that fibrinogenolysis took place in the presence, as well as in the absence, of the inhibitor.

**Differential Titration of Inhibitory Activities**

The activity of the inhibitor towards trypsin, chymotrypsin, plasmin, and thrombin was studied after its complete or partial neutralization by each of the enzymes in this group.

A sample of inhibitor was titrated with trypsin to the point of equivalence and was then tested for its antiplasmin, antithrombin, and antichymotrypsin activities. None of these activities could be demonstrated. Similarly, no antitrypsin, antithrombin, or antichymotrypsin activity was demonstrated with an inhibitor that was neutralized by chymotrypsin.

Other attempts were made to evaluate the antitrypsin and antichymotrypsin activities of the inhibitor after partial neutralization by either plasmin or thrombin. Indeed, conditions could be selected under which inhibitory activities toward these enzymes decreased after partial neutralization; yet, owing to the nature of the reaction as explained above, a clear-cut interpretation of the results was made difficult by the presence in the system of active uncombined reactants.

**DISCUSSION**

Proteolytic inhibitors from human, bovine, or ovine serum have been purified and studied by several investigators. Table II summarizes the reported specificities of these inhibitors. Although it is difficult to correlate such controversial findings, the following general remarks may be made.

1. Purified and characterized trypsin-inhibiting preparations have shown that the antitryptic potential of plasma resides in the &alpha;1-globulin. The inhibitors of Shulman (25) and of Heide et al. (28) account for only a small part of plasma antitryptic capacity.

2. Species variations could be demonstrated in the &alpha;1 inhibitor.

<table>
<thead>
<tr>
<th>Inhibitory material</th>
<th>Mol wt</th>
<th>Inhibitory activity* on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trypsin</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antifibrinolysin (37)</td>
<td>16,700</td>
<td>+</td>
</tr>
<tr>
<td>Proteolytic inhibitor (25)</td>
<td>16,700</td>
<td>+</td>
</tr>
<tr>
<td>α inhibitor (17)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>α inhibitor (19)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>α-antiplasmin (29)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>α-Trypsin inhibitor (38)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>α-Trypsin inhibitor (26)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>α-Antitrypsin (C) (30)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>Inter-α-trypsin inhibitor (32)</td>
<td>45,000</td>
<td>+</td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α crystalline trypsin inhibitor (39)</td>
<td>70,000</td>
<td>+</td>
</tr>
<tr>
<td>Proteolytic inhibitor (40)</td>
<td>70,000</td>
<td>+</td>
</tr>
<tr>
<td>Ovine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor (41)</td>
<td>40,600</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, activity present; -, activity absent; 0, activity not tested.

Homogeneous preparations from bovine and ovine sera inhibited both trypsin and plasmin. Inhibition of the latter was difficult to effect in preparations of human origin. Moll et al. (38) and Schulte et al. (27) did not find antiplasmin activity in their highly purified preparations of &alpha;1 inhibitor.

3. Jacobsson (17) reported that no antiplasmin activity was found in the electrophoretically defined &alpha;1 region. Norman and Hill (29), on the other hand, contradicted this finding by showing that "slow" antiplasmin activity has been separated as &alpha;1-globulin.

4. Chymotrypsin was found to be inhibited by all preparations tested.

The similarity of the &alpha;1 inhibitor studied here to those investigated by Moll et al. (38), by Bundy and Mehl (26), and by Schulte et al. (27) has already been mentioned (30). Yet the inhibition of plasmin by our preparation is an outstanding feature that merits further comment. The failure of other &alpha;1 preparations of human origin to show antiplasmin activity might be due to an inadequacy of the methods applied, since the roles of time and substrate in the slow type of plasmin inhibition have not been fully considered. As a result of his test for slow inhibition (13), Norman (29) reported antitryptic activity in the &alpha;1-globulin. To the extent that "immediate" antiplasmin activity has been demonstrated in human serum, it has always been associated with &alpha;1-globulin (17, 29, 42). Our purified &alpha;1 preparation was shown to be devoid of such type of activity.

Two types of reaction were observed between the enzymes tested and the inhibitor. Trypsin and chymotrypsin combined instantaneously and stoichiometrically with the inhibitor, without being affected by the enzyme substrate. The other type of reaction—observed with plasmin and thrombin—was time-dependent and did not occur in the presence of the enzyme substrate. Yet the substrate inhibitor relation could not be attributed to competition on a single site of the enzyme molecule, as deduced from the curve of 1/V with respect to 1/S. The situation was better explained by assuming that the inhibitor itself is a proteolytic enzyme and that, in the presence of large excess of another protein (casein), the chances that it will combine with plasmin are very small. These findings, as well as those from clot lysis study (Table I), support Norman's suggestion (13) that inhibitory activity is strongly retarded in the presence of plasmin substrate (e.g. fibrinogen); this suggestion would explain how plasmin can function in the blood stream in the presence of an overwhelming amount of inhibitor.

Enzyme inactivation by the inhibitor should be considered irreversible, since no activity was recovered from an inhibitor-enzyme complex upon destroying the inhibitory activity by treatment with acid.

From the combining ratio by weight of 1.95 (inhibitor) to 1.0 (trypsin), a molecular weight of 46,800 is calculated for the inhibitor, assuming an equimolar combination and a trypsin molecular weight of 24,000. This figure is not extremely far from the molecular weight of 55,000 determined by sedimentation-diffusion methods and is a further indication of the purity of the inhibitor. The combining ratio hereby presented is the smallest reported for &alpha;1 trypsin inhibitor from human serum.

The finding that 1 ml of plasma inhibited 0.70 to 0.75 mg of trypsin placed the estimate of the amount of &alpha;1 inhibitor in plasma at about 1.3 mg per ml, i.e. 1.8% of the total plasma proteins. This estimation is based on the combining ratio (see
above) and on the assumption that 90% of plasma antitryptic activity is due to the α1 inhibitor (17). This also means that the purification presently calculated for antitryptin, namely, 50-fold that from plasma, is within the range of maximum purity, since the theoretical possible purification is 55-fold as deduced from the ratio of 70 mg of plasma proteins to 1.3 mg of α1 inhibitor.

Calculations based on stoichiometric combination of the inhibitor and chymotrypsin gave a rather low value: 1.4 parts inhibitor combined with 1.0 part chymotrypsin by weight, and the exact activity of chymotrypsin per mg of protein is not known, the reliability of this value is rather doubtful.

In view of the homogeneity of the inhibitor by ultracentrifugation, immunoelectrophoresis, and starch gel electrophoresis, and of the fact that its purity is within the range of the theoretical value, it is rather unlikely that this preparation is a mixture of several specified inhibitors. That it is a single molecular entity with multiple activities seems to fit better with the experimental data. Further support in favor of this concept is lent by differential titrations, which show that complete or partial neutralization of inhibitory activity on other enzymes.

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REFERENCES
