Partial Purification of the Serum Trypsin Inhibitor*

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(Received for publication, December 3, 1957)

The serum trypsin inhibitor has been studied by many investigators since Lansteiner (1) associated it with the albumin fraction by salt fractionation as did Smith and Lindsley (2) by the electrophoretic separation of serum. Both Christensen and MacLeod (3), and MacFarlane and Pilling (4), also associated the trypsin inhibitor with the albumin fraction but differentiated it from the plasmin inhibitor in the globulin fraction. Recently, Jacobsson (5), with block filter paper electrophoresis, described the presence of two trypsin inhibitors in serum. The larger fraction, which was associated with the α1-globulin, inhibited both trypsin and plasmin. Partial purification of these fractions has been tried frequently. Schmitz (6), using the method of Northrup and Kunitz (7) for isolation of the pancreatic trypsin inhibitor, reported a substance which inhibited crystalline trypsin stoichiometrically. Duthie and Lorenz (8), using the method of Schmitz, obtained a yield of 0.02 per cent of the serum inhibitor. Peanasky and Laskowski (9) were unable to repeat this method.

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Boman (11), with an ion exchange resin for separation of serum, described a method which seemed applicable to separation of the serum trypsin inhibitor. The following study is based on this method.

MATERIALS AND METHODS

Trypsin Inhibition—Tryptar (Armour & Co., Lots 55409, 54011, and 55512) was used throughout. A modification of the assay method of Kunitz (12) with 2 per cent casein substrate was used. This provided a reproducible method with a coefficient of variation of 5.17 per cent when repeated on the same serum over a period of 1 week with different lots of trypsin and casein. Inhibitor values were expressed as micrograms of soybean inhibitor.

Soybean Trypsin Inhibitor—Soybean inhibitor (Nutritional Biochemicals Corporation) which is crystallized five times was used throughout for standardization.

Serum—Blood was obtained from women in labor since such patients have high levels of inhibitor (8). The blood was allowed to clot and the serum was removed and frozen until used.

Casein—Borden’s vitamin-free casein (The Borden Company) was prepared in a 10 per cent stock solution in 0.07 M NaOH and heated in a boiling water bath for 30 minutes. The final pH was adjusted to 7.6 with 0.1 M KH2PO₄ and 0.15 M NaCl. The stock solution was frozen and diluted to 4 per cent when used.

Anion Exchange Resin—Dowex 2-X, 200 to 400 mesh (Dow Chemical Co., Lot 3902-33) was used. The resin was used repeatedly for fractionation. After a run, the resin was removed from the glass column, washed twice with 250 ml. of 1 N NaCl to remove any serum contamination and then regenerated with two washings of 250 ml. of 1 N HCl. The column of resin was prepared as described by Boman (11), and measured 30 X 2.5 cm.

Buffer—Tris (Matheson Co., Inc., Lot 390271) was recrystallized in 100 per cent alcohol. A stock solution of 1 M concentration in water was made and the pH adjusted with 3 N HCl to pH 7.2 at 25°. Appropriate dilutions were made with distilled water and used as the eluent.

Fractionation of Serum—Whole serum was fractionated by precipitation by 40 per cent saturation with ammonium sulfate. The precipitate was centrifuged off and the supernatant fluid dialyzed against running tap water for 12 hours. The prepared anion exchange resin column was then equilibrated with 0.06 M Tris. Equilibration usually took 5 to 8 hours at 5° and required about 500 ml. of buffer. 8 ml. of serum were then placed on the column and developed through with three dilutions of Tris buffer, a flow rate of 1 ml. per minute at a temperature of 5° being maintained. The concentrations of buffer used were, in order, 0.06, 0.2, and 1.0 M. The eluate was collected in 12 ml. fractions and the optical density was then read on the Beckman ultraviolet spectrophotometer at 280 mµ in 1 cm. silica cells against the eluate of buffers of corresponding concentration from a blank column. Protein nitrogen was determined by a micro-Kjeldahl method.

Paper Electrophoresis—The method of Jencks et al. (13) was used for the identification of the fractions, with a barbital buffer at pH 8.6 and 0.075 M ionic strength.

RESULTS

The fractionation of a sample of serum from a pregnant woman in labor is shown in Fig. 1, in which the protein (optical density at 280 mµ) and the inhibitor activity of each fraction is recorded. Most of the protein is removed with 0.06 M Tris. That fraction removed with 0.2 M Tris contained a high concentration of inhibitor.

The subsequent yield and enrichment of these fractions is 1. The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane buffer.

* This work was supported by a grant from the American Heart Association and the section of Allergy and Infectious Diseases of the National Institutes of Health, United States Public Health Service, Bethesda, Maryland.

1 The abbreviation used is: Tris, tris(hydroxymethyl)aminomethane buffer.
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In order to identify the fraction containing the inhibitor, Eluates 16 and 17 were pooled and paper electrophoresis was performed (Fig. 2). When compared to normal serum it appeared that the fraction was composed of the α-globulin with a trace amount of albumin.

Effect of Temperature on the Inhibitor—To determine the heat stability of the isolated inhibitor fraction, 0.6 ml. of the fraction at a pH of 7.6 was placed in a water bath at 56°. After 10, 20, 30, and 60 minutes, 0.1 ml. was removed, diluted 1:25 and 1:50, and the activity of the inhibitor was determined in the usual way. For a control, 0.15 ml. was placed in a water bath at 37° for 1 hour, and the activity was then determined. The results are shown in Table II. The fraction used is not heat stable and is almost completely inactivated at 56° for 1 hour.

Effect of pH on the Inhibitor—To determine whether the activity of the partially isolated inhibitor is altered by changes in pH, phosphate buffers were made up as listed in Table III. An aliquot of 0.5 ml. of the fraction was added to 0.5 ml. buffer. This dilution was allowed to stand at room temperature for 1 hour. The activity of the fraction was then determined after adjustment of the pH to 7.6 with 2.5 ml. of buffer. The results are listed in Table III. It is evident that the activity is reduced when the pH falls below 5.

Trypsin Inhibition—The serum trypsin inhibitor inhibits trypsin in a stoichiometric fashion (14). The partially purified inhibitor reacts in a similar fashion, as shown in Fig. 3.

In this study the combining ratio, by weight, of the trypsin to the partially purified inhibitor varied between 1:3.8 at 20 per cent inhibition to 1:3.4 at 80 per cent inhibition. A simul-
TABLE II

Effect of temperature on the partially purified inhibitor

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity of inhibitor µg/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (37° for 1 hr.)</td>
<td>762</td>
</tr>
<tr>
<td>Control (37° for 1 hr.)</td>
<td>782</td>
</tr>
<tr>
<td>Control (37° for 1 hr.)</td>
<td>750</td>
</tr>
<tr>
<td>10 min. at 56°</td>
<td>487</td>
</tr>
<tr>
<td>10 min. at 56°</td>
<td>527</td>
</tr>
<tr>
<td>20 min. at 56°</td>
<td>367</td>
</tr>
<tr>
<td>20 min. at 56°</td>
<td>385</td>
</tr>
<tr>
<td>30 min. at 56°</td>
<td>189</td>
</tr>
<tr>
<td>30 min. at 56°</td>
<td>194</td>
</tr>
<tr>
<td>60 min. at 56°</td>
<td>82</td>
</tr>
<tr>
<td>60 min. at 56°</td>
<td>61</td>
</tr>
</tbody>
</table>

TABLE III

Effect of pH on the activity of the isolated inhibitor

<table>
<thead>
<tr>
<th>pH</th>
<th>Activity of inhibitor µg/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>63</td>
</tr>
<tr>
<td>4.5</td>
<td>85</td>
</tr>
<tr>
<td>5.01</td>
<td>104</td>
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<td>5.50</td>
<td>105</td>
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<td>5.59</td>
<td>106</td>
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<td>6.50</td>
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</tr>
<tr>
<td>7.50</td>
<td>106</td>
</tr>
<tr>
<td>8.02</td>
<td>106</td>
</tr>
<tr>
<td>8.55</td>
<td>106</td>
</tr>
<tr>
<td>8.99</td>
<td>106</td>
</tr>
</tbody>
</table>

DISCUSSION

In the techniques previously described for partial purification of the serum trypsin inhibitor, all investigators had employed a method of lowering the pH to levels which tend to inactivate the inhibitor. Peanasky and Laskowski (9) reported ammonium sulfate fractionation with a pH of 3.6 for a 12 hour period. Hayakawa (16) studied the effect of pH on the antitryptic activity of the inhibitor and stated that the loss of activity of the inhibitor at a low pH was dependent upon the length of time it was used. Schmitz (6) not only employed acid but heated the fraction to 80°.

The loss of activity of the inhibitor at high temperatures and at a low pH was subsequently confirmed, as shown under "Material and Methods." This gives further reason to control carefully the conditions for isolation.

Attempts in this laboratory to repeat the salting out method (9) were unsuccessful and the failure was believed to be related to the pH changes. The anion exchange resin method of extraction obviates these wide changes in pH.

It is apparent from the work of Jacobsson (5) that there are two electrophoretic fractions in serum which inhibit trypsin. From the electrophoretic analysis in the study reported herein, only the α 1 fraction has been separated by the anion exchange method and this fraction failed to inhibit plasmin. Whenever trace amounts of the α 2-globulin were present, no antitrypsin activity could be demonstrated.

Since the calculated values of yield and enrichment are based upon total trypsin inhibition, such data are inaccurate in view of simultaneous study of trypsin and soybean inhibitor had a combining ratio of 1:0.5.

Effect of Anions—To determine the effect of anions on the activity of the partially purified inhibitor 0.01 and 0.005 M concentrations of calcium chloride, magnesium chloride, and manganese chloride and 0.01 and 0.02 M concentrations of potassium chloride were studied. The inhibitory activity was not significantly altered from control values determined in the phosphate buffer.

Effect of Other Enzymes—The α 1 inhibitor had no effect against serum plasmin activated by streptokinase or against hyaluronidase as measured by the turbidometric technique (15). A report by Shulman (17), who employed a differential titration, that 10 per cent of the trypsin inhibition of serum is due to plasmin inhibitor. Serum from pregnant women was used for fractionation in this study because of its high inhibitor titer but the ratio of the α 1 to α 2 inhibitor in such serum is not known. Jacobsson (5) states that in all patients studied, with the exception of the nephrotic, an increase in the trypsin inhibition was due almost entirely to an increase of the α 1-trypsin inhibitor. Therefore, it is likely that the yield may be somewhat higher than recorded. Peanasky and Laskowski (9) described a 5 per cent yield. Jacobsson (5) reported between 71 and 93 per cent recovery of total inhibitory activity from the eluted fractions from the paper electrophoretic technique.

Serum trypsin inhibitor has been shown by several investigators (5, 14, 18) to react stoichiometrically with trypsin. The serum inhibitor after fractionation described herein reacted in a similar fashion.

There was little change in the combining ratio of the trypsin and inhibitor between 20 and 80 per cent inhibition despite the absence of calcium ion. Viswanatha and Liener (19) have...
demonstrated the stabilizing effect of calcium ion on the combining ratio when the inhibitor and substrate are preincubated. However, in the method used in this study, the enzyme was kept at pH 3.5 until combined with the inhibitor at pH 7.6 and then added to the substrate. At a pH of 3.5 the inactivation of trypsin does not occur (20), and the addition of calcium in borate buffer did not produce significant changes in the ratio of inhibitor to trypsin.

The physiological importance of this serum inhibitor is unknown. However, the serum level is elevated in most inflammatory states, in pregnancy, and after the administration of cortisone (21). Ungar and Damgaard (22) have described the presence of a proteolytic enzyme in tissues following an allergic reaction and preceding the liberation of histamine and heparin. Therefore, the inhibitor may represent a mechanism of defense against tissue destruction or may be a product of it. However, the partial purification of the inhibitor will permit further studies on its physiological function.

**SUMMARY**

A method is described for the partial purification of the serum trypsin inhibitor using an ion exchange resin. There was a 37 per cent yield and a 12-fold enrichment. The material was shown to be an α-globulin, to react stoichiometrically with trypsin, to be heat labile, and to be partially inactivated at a pH below 5.

**REFERENCES**

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