

Proteolytic Activity and Trypsin Inhibiting Ability of Serum Fractions Obtained Chromatographically on Anion Exchange Sephadex

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The chromatographic separation of normal human serum by means of the anion exchanger DEAE-Sephadex in phosphate buffer at pH 6.6 is described. The presence of two trypsin inhibitors measured on gelatin is confirmed and the occurrence of activated plasmin in the last fraction with a high optical density is suggested. The proteolytic TAME-esterase activity is found and is assumed to show the presence of the complement factor C'1 in the non-adsorbed fraction.

In an earlier paper¹ the present author reported some studies on the arylesterase activity in normal human serum fractions. The separation was made on the cross-linked dextran gel, Sephadex G-25, introduced by Porath and Flodin². The presence of two arylesterases was indicated. The present communication gives some results obtained upon elution chromatography of normal human serum on the anion exchanger DEAE-Sephadex A-25 and, in this case, the proteolytic activity and the trypsin-inhibiting ability of the fractions were studied.

It was shown by Bergman and Fruton and their coworkers that proteolytic enzymes possess the ability to hydrolyze not only proteins but also certain synthetic peptides and even some related compounds. Neurath and coworkers³ found that the amino acid esters, α -benzoyl-L-arginine methyl ester (BAME) and α -toluenesulphonyl-L-arginine methyl ester (TAME), are hydrolyzed very rapidly by trypsin. The substrate TAME has been especially used for investigations of the proteolytic activity of serum and serum components, *cf.* among others, Sherry and Troll⁴ and Becker⁵. In this investigation two substrates were used: for the proteinase (endopeptidase) activity gelatin, and for the proteolytic esterase effect the synthetic substrate TAME.

MATERIAL AND METHODS

Serum from healthy blood donors was investigated. In some experiments, so-called inactivated serum was used, *i.e.* serum that had been kept at 56°C for 30 min.

Fractionation. The chromatographic column was set up as follows. Portions of 15 g DEAE-Sephadex A-25, medium, from Pharmacia, Uppsala, were treated according to the instructions and then suspended in 0.02 M phosphate buffer, pH 6.6. The column, 43 × 1.5 cm inner dimensions and furnished with a jacket, was cooled with water at + 12°C and the fractions were collected in 5 ml portions in a Radirac fraction collector from LKB, Stockholm, at an outflow rate of 90–110 ml/h. The elutions were made with increasing concentrations of NaCl (0.1, 0.2, 0.4, 0.6, and 1.0 M) in the same buffer. The optical density of the fractions was measured at 280 m μ in a Beckman spectrophotometer model DU.

Electrophoresis. Unfractionated serum and "peak fractions" were run electrophoretically on paper, model LKB, Stockholm, in 0.1 M "S:t Eriks" buffer *, at pH 8.6 for 17 h, 300 V and 12 mA, and then stained with naphthalene black 12 B.

Chemicals. The substrate TAME, HCl was obtained from Hoffman La Roche, Basel, the trypsin (N.B.C. 1:300) from Nutritional Biochemicals Corp., Cleveland, Ohio, the gelatin (U.S.P., granular) from Fisher Sc. and Co., New York, and the merthiolate from Schmitt-Jourdan, Boulogne-sur-Seine, France.

Determination of proteolytic activity. The determination of the gelatin-splitting activity was performed according to the viscosimetric method which has been suggested by Hultin ^{6,7} and developed to a high sensitivity by Hultin and Lundblad ⁸. The measurements were carried out at 35.5°C in Ostwald viscosimeters. The enzymic activities were calculated according to Hultin's formula ^{6,7}. The values so obtained were multiplied by 10⁹. It is suggested that the unit in which the values are thus expressed and for which the notation m μ A has been used previously should be called the Hultin unit (H.U.). The assay of the trypsin-inhibiting ability of the serum fractions was made as follows. Mixtures were made up of 0.50 ml of the various fractions and 0.50 ml of a trypsin solution (0.2 mg/ml in M/15 phosphate buffer, pH 7.25). The mixtures were preincubated for 30 min at + 20°C and then added to 3.00 ml of a 4.00 % solution of gelatin in the same buffer and with 0.01 % of merthiolate added as a sterilizing agent. Measurements were also carried out with buffer substituted for the serum fractions. The gelatinase activity of the fractions was determined in mixtures consisting of 0.50 ml of a fraction, either 0.50 ml of the buffer solution or 0.50 ml of a 0.1 M cysteine solution, and 3.00 ml of the gelatin solution.

Determination of TAME-esterase activity. The reaction mixtures were made up of 0.50 ml serum fraction, either 0.50 ml of M/15 phosphate buffer, pH 6.6 or 7.9, or 0.50 ml of a 0.1 M cysteine solution, and 1.00 ml of a 0.08 M solution of TAME in the corresponding buffer. The influence of trypsin on the activity of the serum fractions was measured with reaction mixtures made up as described with, however, a 0.02 mg/ml trypsin solution in the corresponding buffer substituted for the buffer solution. The samples were incubated at 35°C for 20 h and the hydrolysis was measured by titration of the liberated carboxyl groups in an automatic titrator, model TTT 1a from Radiometer, Copenhagen. The activity was expressed in ml of 0.01 N NaOH consumed. Blanks with buffer and without TAME were always run under the same conditions and corrections were made for the spontaneous hydrolysis of TAME when necessary.

RESULTS

When the proteolytic activity of the various fractions was measured viscosimetrically with gelatin, very low values were obtained, less than 50 H.U. The main assays of the proteolytic enzymes were therefore carried out by measuring not their ability to split gelatin but, instead, their ability to split TAME, as will be reported below. Gelatin, however, could be conveniently used for the measurement of the trypsin-inhibiting ability of the serum fractions.

* Na-veronal 18.2 g, N HCl 12 ml, Ba(Ac)₂ 0.96 g, ethylene glycol 90 ml, aq. dest. ad 1 000 ml.

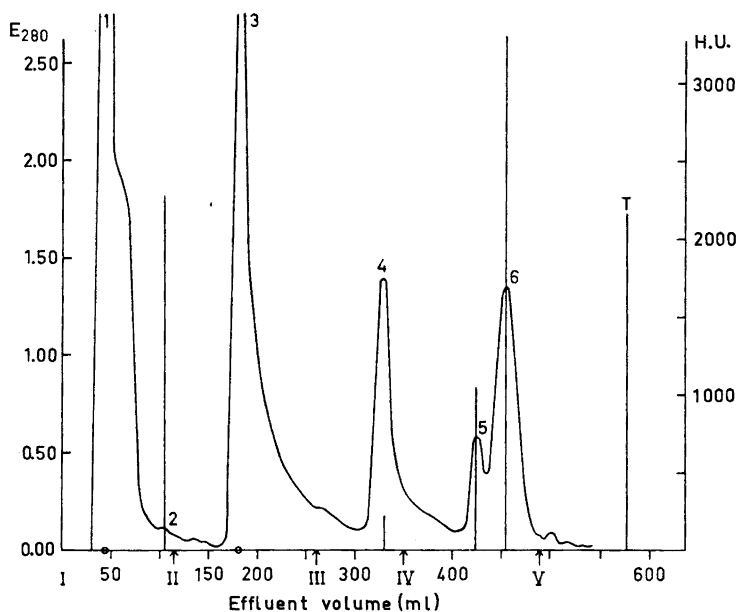


Fig. 1. Trypsin-inhibiting ability in a series of chromatographic fractions of human serum. Column, DEAE-Sephadex, 1.5 cm \times 43 cm. Eluent: I, 0.02 M phosphate buffer pH 6.6; II, 0.1 M NaCl; III, 0.2 M NaCl; IV, 0.4 M NaCl; V, 0.6 M NaCl and VI, 1.0 M NaCl, II–VI in buffer I. E_{280} = total optical density at 280 $m\mu$ (curve). The straight lines show the gelatin-splitting activity at pH 7.2 of trypsin and the serum fraction. T = gelatin-splitting activity of trypsin alone. The activity is expressed in viscosimetric Hultin units (H.U.).

The distribution of the trypsin-inhibiting ability in a series of fractions, obtained chromatographically, of normal human serum is shown in Fig. 1. Gelatin was used as substrate and the gelatin-splitting activity in the presence of trypsin in the different fractions can be compared with that of trypsin alone (T). In peaks 1 and 3 there is a complete inhibition, and also in peak 4 a very strong inhibition is found. In peak 5 there is 50% inhibition whereas in peak 2, where some inhibition might also be expected, there seems however to be a slight increase in activity as compared with the original trypsin activity (T). In peak 6 there is, in fact, an approximately 35% increase as compared with the gelatin-splitting activity of the trypsin solution.

The distribution of proteolytic TAME-esterase activity measured at pH 7.9 in a series of chromatographic fractions of serum is shown in Fig. 2. The original TAME-esterase activity is highest in peaks 1 and 3. With cysteine, a slight activation seems to have occurred in peaks 1, 2, and 3. When trypsin is added to the fractions, there is a rather strong activation in peak 1 whereas the activity in peaks 3–5 has about the same value as the sum of the activity of the trypsin solution and activity of the untreated fraction in question. In peak 2 an inactivation of the trypsin activity by about 35% was found.

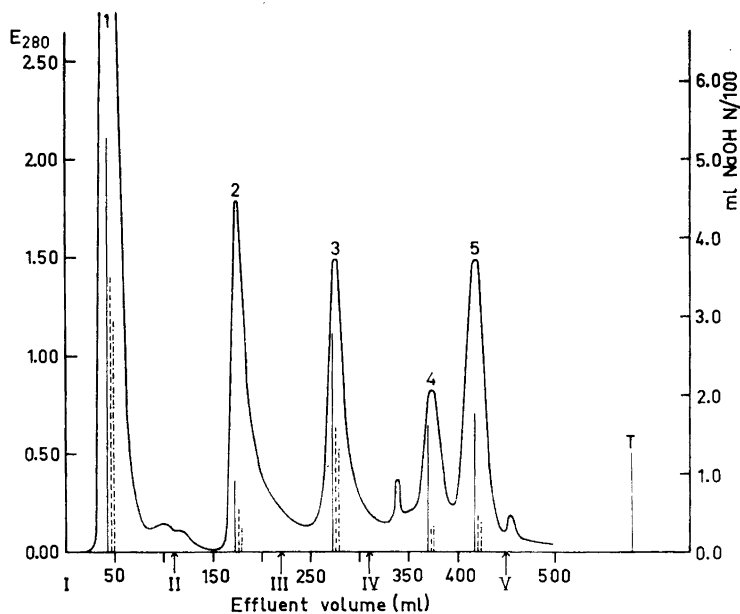


Fig. 2. Protolytic activity and trypsin-inhibiting ability in a series of chromatographic fractions of human serum. Column, DEAE-Sephadex, phosphate buffer pH 6.6. Elution as in Fig. 1. E_{280} = total optical density at 280 $m\mu$ (curve). Full lines, TAME-esterase activity in the presence of trypsin; broken lines, TAME-esterase activity in the presence of cysteine; dashed and dotted lines, TAME-esterase activity of fraction only. T = TAME-esterase activity of trypsin alone. The activity was measured in phosphate buffer at pH 7.9 and expressed as ml NaOH consumed.

The TAME-esterase activity was measured at pH 6.6 in two series of chromatographic serum fractions, and the result is shown in Fig. 3 A—B. Untreated normal human serum was used in one series (Fig. 3 A) and serum of the same batch, but heated however to 56°C and kept at this temperature for 30 min and then cooled, was used in the other series (Fig. 3 B). In Fig. 3 A, a high TAME-esterase activity is observed in peak 1. This activity is however considerably reduced in inactivated serum (Fig. 3 B). The activities of peaks 2—6 do not differ very much from each other and no significant difference can be found in the activities of the corresponding peaks in the two chromatograms. The presence of cysteine in the fractions has no effect upon the enzymatic activity. The addition of trypsin at this pH (6.6), as shown in Fig. 3 A—B, brings about no activation in the fraction studied. In the series with untreated serum (Fig. 3 A), the TAME-esterase activity in peak 1 is about the same as the sum of the activities of fraction and trypsin measured separately. In peaks 2 and 3 the addition of trypsin caused no change in activity and in peaks 4—6 the addition resulted only in a slight increase in activity. In the series with "inactivated" serum (Fig. 3 B), only slight increases in activity were observed.

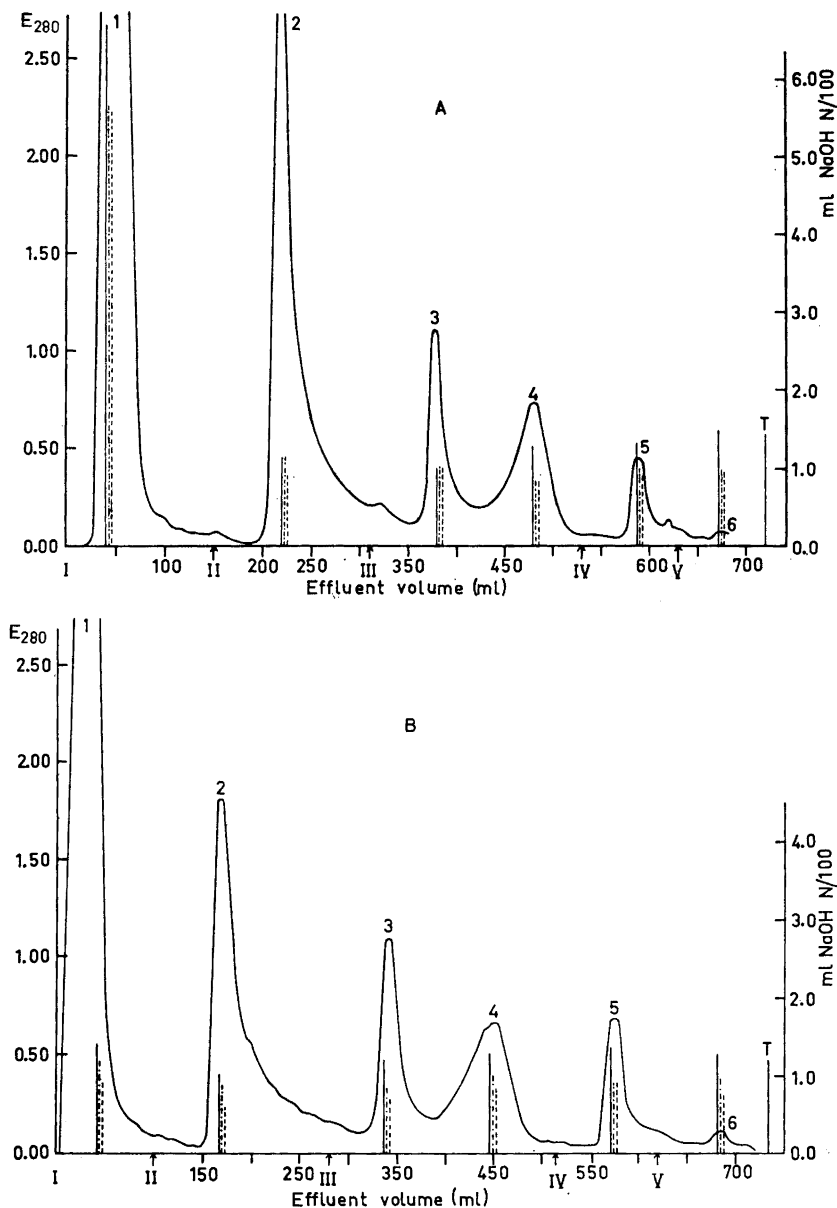


Fig. 3 A—B. TAME-esterase activity and trypsin-inhibiting ability in a series of chromatographic fractions of human serum. A, Untreated serum; B, Inactivated serum, *i.e.* kept at 56°C for 30 min. Column, DEAE-Sephadex in phosphate buffer, pH 6.6. Elution as in Fig. 1. Full lines, TAME-esterase activity of fractions in the presence of trypsin; broken lines, TAME-esterase activity in the presence of cysteine; dashed and dotted lines, TAME-esterase activity of fraction only. T = TAME-esterase activity of trypsin alone. The activity was measured in phosphate buffer at pH 6.6 and expressed in ml NaOH consumed.

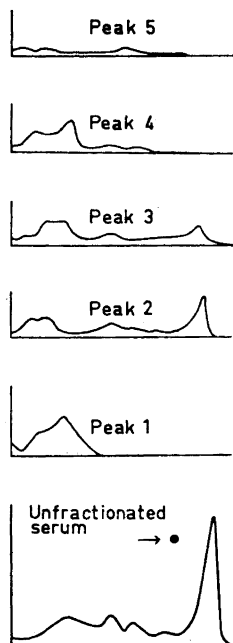


Fig. 4. Electrophoretic diagram of unfractionated serum and of peaks 1–5 in Fig. 2. These fractions are comparable, respectively, with peaks 1 and 3–6 in Fig. 1. Paper electrophoresis in "S:t Eriks" buffer pH 8.6, ionic strength 0.1, 300 V, 12 mA, for 17 h. Protein dye: naphthalene black 12 B.

It may finally be mentioned in this section that unfractionated normal serum and samples corresponding to the five main peaks in the chromatographic separation of the normal serum were taken out for paper electrophoresis. The results are shown in Fig. 4. It can be concluded from this figure that albumin is present in peaks 2 and 3 but not in noticeable quantities in peaks 1, 4, and 5.

DISCUSSION

A comparison between gelatin and TAME as substrates for the chromatographic fractions might be of interest both concerning the original proteolytic activity of the fractions and the activity of mixtures of trypsin and the fractions. When gelatin is used as substrate and the activity is measured at pH 7.2, trypsin inhibition occurs in several peaks (Fig. 1). When, however, TAME is used as substrate and the activity is measured at pH 7.9, an apparent inhibition of trypsin occurs only in the second peak (Fig. 2). When the activity is measured at pH 6.6, there is an apparent trypsin inhibition in all the fractions investigated (Fig. 3 A–B).

The influence of serum fractions on trypsin, as shown in Fig. 1, reveals the complete separation of two trypsin inhibitors by the chromatographic procedure since there is a complete inhibition of the trypsin activity in peak 1, no inhibition or only a slight increase in activity in peak 2, and again a complete inactivation in peak 3. The existence of two trypsin inhibitors in serum

has been demonstrated and reviewed by Jacobsson⁹. The increase in gelatin-splitting activity seen in peak 6, Fig. 1, and of TAME-esterase activity in the presence of trypsin as shown in peak 1, Fig. 2, seems to be due to the well-known ability of trypsin to activate proenzymes as reported by several authors: for trypsinogen and chymotrypsinogen by Northrop and Kunitz¹⁰, for plasminogen by Alkjaerrig *et al.*¹¹, and for prothrombin by Schultze and Schwick¹². The intravenous injection of trypsin in rabbits resulted in the circulation of a measurable TAME-esterase activity for a short time¹³.

The influence of cysteine upon the TAME-esterase activity, as shown in Fig. 2, reveals a slight activation in the first peak. The activation decreases continuously in the subsequent peak fractions. Since none of the proteolytic enzymes of serum with esterase activity (*i.e.* plasmin and thrombin) needs sulfhydryl groups for its activity, complement components might be involved. It has been reported by Pillemer, Lepow and coworkers^{14,15} and Becker⁵ that the complement factor C'1 is a TAME-esterase and it was shown by Cushman, Becker and Wirtz¹⁶ that sulfhydryl compounds inhibit immune hemolysis.

The strong esterase activity found in untreated serum (peak 1, Fig. 3 A) was greatly reduced when the serum was "inactivated" (peak 1, Fig. 3 B). It has been long known that the complement factors C'1 and C'2 are completely inactivated when serum is kept at 56°C for 30 min.

Now the question arises about the role of thrombin and plasmin in this connection. It seems unlikely that the activity in peak 1, Fig. 3 A, should be derived from thrombin since calcium was not added but is necessary in higher concentrations than in normal serum for the conversion of prothrombin to thrombin. Furthermore no α_2 - β -globulin is present in this fraction (peak 1) as can be seen in Fig. 4. As regards plasmin the activation by trypsin seen in Fig. 1 is localized in peak 6 and is probably derived from activated plasminogen. The heat-labile TAME-esterase in peak 1, Fig. 3, cannot belong to the plasmin system since the plasmin system is much more heat stable according to Troll and Sherry¹⁷. It thus seems reasonable to assume that part of the TAME-esterase activity in peak 1 may be derived from the complement factor C'1.

Acknowledgements. The author wishes to express his thanks to Dr. E. Hultin for valuable discussions and to Mrs. E. von Zeipel, Miss I. Augustsson, Mr. H. Ryttman and Mr. I. Eriksson for skilful technical assistance.

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Received November 21, 1961.