

On the Heterogeneity and Purification of Commercial Trypsin Preparations

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Three commercial preparations of crystalline trypsin were found to differ from one another in their specific activity as well as in the ratio between their activity and the STI-binding capacity. On gel filtration the trypsin preparations separated into two esterolytically active components differing from each other in molecular weight, activity in relation to STI-binding capacity and electrophoretic mobility. On electrophoresis in agarose the trypsin preparations separated into several enzymatically active fractions, differing in activity from fraction to fraction and from one preparation to another.

The use of crystalline soy-bean trypsin inhibitor, STI, for standardisation of trypsin preparations was introduced by Kunitz.¹ One mole of trypsin binds one mole of STI.¹⁻³ Judging from the molecular weight of trypsin and STI, the weight combining ratio (trypsin/STI) should be approximately 1.2.⁴ But the ratios found are usually higher and vary from one investigator to another.⁴⁻⁶ This has been ascribed to the presence of inert impurities in commercial preparations of crystalline trypsin.⁴ This assumption has been confirmed by ion-exchange chromatography of one trypsin preparation (Worthington), which was found to contain approximately 25 % inert protein and a small chymotrypsin-like activity.⁷⁻⁹ In an investigation of the activity of collagenmucoproteinase Banga¹⁰ found substantial differences between 10 commercial preparations of crystalline trypsin and concluded that not all of the impurities were inert. Personal preliminary investigations on the binding of trypsin to serum proteins corroborated Banga's conclusion. Jachan *et al.*¹¹ recently found that crystalline trypsin can be separated into 2 or 3 active fractions by paper electrophoresis at pH 4.9. They interpreted this as an effect of limited autolysis of the enzyme during isolation.

The purpose of the present investigation was to assess the degree of heterogeneity of different commercially available crystalline trypsin preparations and to devise a method for purifying such preparations.

EXPERIMENTAL

Reagents. Crystalline, lyophilized trypsin preparations from C. F. Boehringer & Soehne, Mannheim (preparation B), Novo A/S, Copenhagen (preparation N) and Worthington Biochemical Corporation, Freehold, New Jersey (preparation W) were used. The preparations were stored in the laboratory at +4°C. Solutions were prepared with cold solvents immediately before use.

Soy-bean trypsin inhibitor (3× cryst.) from Worthington Biochem. Corp. A 0.02 % solution in 0.0025 N HCl was prepared every week.

Benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA) from Nutritional Biochemical Corporation, Cleveland, Ohio. A supersaturated solution (0.003 M) was prepared daily by heating the substance to 85°C in distilled water.

Agarose l'Industrie Biologique Francaise S. A., Gennevilliers.

Polyacrylamide gel for gel filtration (Bio-Gel P 30). Bio Rad Laboratories, Richmond, Calif.

Methods. The esterolytic activity of trypsin was measured with BAPNA as a substrate according to a modification of the method of Erlanger *et al.*¹² The enzyme preparation was incubated at 25°C in a 0.05 M tris buffer, pH 8.2, containing 0.01 M CaCl₂. The volume incubated was 4.0 ml, the concentration of the substrate was 0.0015 M, and the incubation time was, as a rule, 10 min. The reaction was stopped by addition of 0.5 ml of 30 % acetic acid, after which the optical density was read at 410 m μ against the reagents as a blank.

The soy-bean trypsin inhibitor binding capacity (STIBC, mg STI/mg trypsin preparation) was determined in the following way. The preparation was incubated with STI slightly in excess. The excess amount of STI was measured by determining the inhibition of a subsequently added standard amount of trypsin (0.02 % Novotrypsin) the inhibition of which was directly proportional to the amount of free STI present (when the inhibition did not exceed 95 %). The approximate amount of inhibitor necessary for complete inhibition of the preparation was estimated from measurements of the activity against BAPNA in the presence of a varying amount of STI. The preparation was then incubated at pH 8.2 for 10 min with STI in 10–20 % excess. Trypsin (0.02 % Novotrypsin) in amount corresponding to about 150 % of the amount of STI was added, and incubation was continued for a further 10 min before addition of BAPNA and determination of the activity. For comparison the activity was estimated in an equal amount of trypsin to which no STI had been added. The values obtained in these tests were used for calculating the degree of inhibition, excess of STI, and the STIBC of the preparation.

On electrophoresis in cold agarose gel (1 %) use was made of an 0.07 M barbital buffer, pH 8.6, with an addition of 1.75 mM Ca²⁺. The voltage gradient was about 7 V/cm. Electrophoresis was run for about 5 h. The distance migrated by the fastest component was 10–15 cm. In the subsequent study of the enzymatic activity 40 μ g of trypsin was generally used and for protein staining 500 μ g per gel. The trypsin was dissolved in the above-mentioned barbital buffer. For determination of the activity of the electrophoretic fractions against BAPNA the gel was cut transversely in 2 mm wide parallel segments, which were eluted in 2.5 ml tris buffer at pH 8.2 by shaking over night at +4°C in a flask shaker.

Gel filtration was done in a column (6.15 cm² × 35 cm) filled with Bio-Gel P 30, which was equilibrated with 0.01 M citrate buffer pH 3.5. To the column was applied 2 ml of 1 % trypsin dissolved in the above-mentioned buffer. Elution was performed at a rate of 16 ml/h. Filtration was done at +4°C.

The protein content of the preparations was determined with the Kjeldahl technique or expressed as optical density at 280 m μ .

To locate the proteolytic activity in the agar gels a slight modification of the method of van Veurooij¹³ was used which utilizes the ability of trypsin to digest a gelatin film. Exposed, developed, fixed and rinsed roentgen film (Gevaert or Ferrania) was placed for about 1 min in 0.1 M tris buffer, pH 9.2, after which excess fluid was removed with a filter paper. The agarose gel was then placed on the moist film, care being taken to avoid the occurrence of air pockets between the gel and the film, and incubated in a moist chamber at room temperature or at 35°C. The incubation period was varied between one minute and several hours according to the activity to be studied. On marked digestion the gelatin film in the areas covered by the fractions was disintegrated entirely. When digestion was less strong, the surface of the film lost its lustre after the gel had been

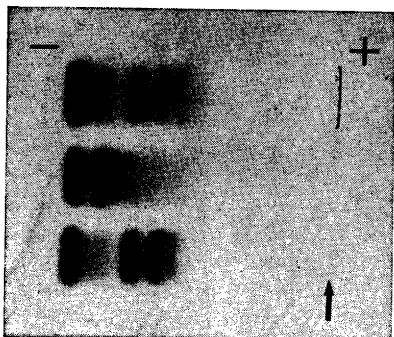


Fig. 1. Pattern of proteins separated by electrophoresis in agarose gel. Arrow indicates site of application. Anode to the right. From above preparations B, N, and W.

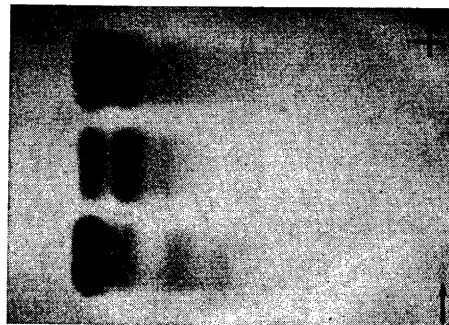


Fig. 2. Tryptic activity of trypsin preparations separated by electrophoresis and assessed by film technique. Arrow indicates site of application. Anode to the right. From above preparations B, N, and W.

removed as the film dried. This dullness of the surface was best seen in reflected light as a distinct intensification of the blackening (Fig. 2). The technique enabled good appraisal of the relative activity of the various fractions.

RESULTS

Agarose gel electrophoresis. When the crystalline trypsin preparations were separated electrophoretically in agarose gel and stained for protein, a number of distinct bands appeared on the cathode side of the origin (Fig. 1). Five components were distinguished. They showed corresponding rates of migration in the electrophoretic patterns of all three preparations. In two (B and W) of the preparations the pattern was dominated by the faster component; in the third (N), by the two fastest, which were of roughly equal size. The pattern of the proteolytic activity obtained with the film technique resembled that of the proteins (Fig. 2), but in all three preparations the second fastest component appeared to possess a higher activity for the amount of protein. When the four most active bands were cut out of the gel and placed in separate gels, subsequent re-electrophoresis and examination by the film technique showed only one active component. The electrophoretic mobility of each component was the same as that found on primary electrophoresis.

After electrophoresis of the trypsin preparations the gels were sometimes cut into narrow parallel segments, from which the activity was eluted. The results obtained on subsequent quantitative estimation of the activity of the components with BAPNA agreed well with those found with the film technique (Fig. 3).

In order to check the possibility of autolysis as a source of error the three trypsin preparations were dissolved in a STI solution containing inhibitor in excess. On gel electrophoresis the enzyme-inhibitor complex thereby formed separated into various fractions coinciding in number, relative position and size with what was obtained on electrophoresis of trypsin preparations alone.

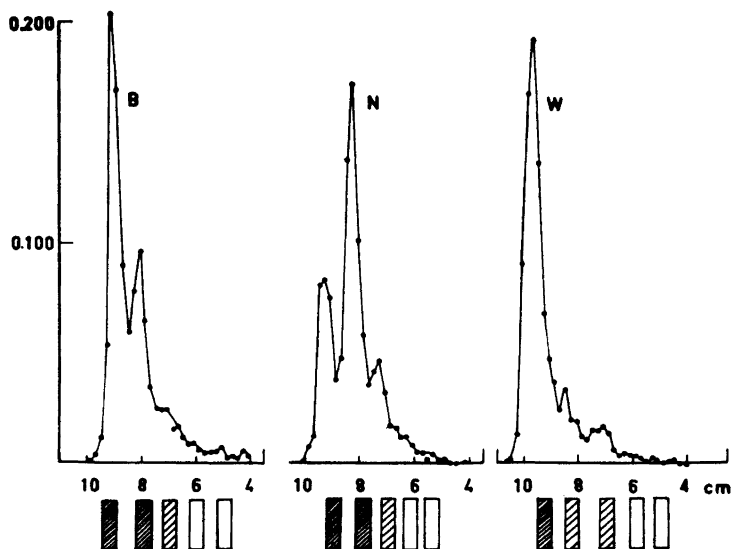


Fig. 3. Tryptic activity of trypsin preparations separated electrophoretically in agarose gel. From left: Boehringer, Novo, and Worthington preparations. Electrophoretic separation of all three preparations was run simultaneously under identical conditions with 40 μ g trypsin, after which the agarose gels were cut into parallel segments and the active components were eluted, Abscissa: cathodal migration in cm. Ordinate: esterolytic activity (BAPNA) expressed as optical density at 410 $m\mu$ after correction for blank. Incubation time: 20 min for Novotrypsin and 40 min for Boehringer and Worthington. The blocks below the diagram indicate the sites of the trypsin activity located by the film technique in simultaneous experiments.

Gel filtration in polyacrylamide. On filtration through Bio-Gel P 30 all three preparations were separated, though not completely, into two components (Fig. 4). In two (B and W) of the preparations, the first component, which was also of higher molecular weight, was found to represent about one fourth of the entire protein content, while the remaining preparation (N) the first component represented a much smaller proportion. The R_F -values of the two components were largely the same in each of the preparations.

The pattern of the esterolytic activity resembled that of the protein, a minor peak followed by a major one (Fig. 4). In preparation N the smaller component of higher molecular weight showed no esterolytic activity at all. The specific esterolytic activity, *i.e.*, activity per unit of weight of protein, of the high molecular component of preparations B and W was much lower than in the larger component of lower molecular weight.

On electrophoresis and determination of the activity with the film technique the main fraction obtained on gel filtration of the three preparations showed largely the same picture as the original preparation. The first (of high molecular weight) component showed quite a different picture with four activity bands, one immediately cathodal to the origin, one main component with a mobility corresponding to about 1/6 of that of the main component in

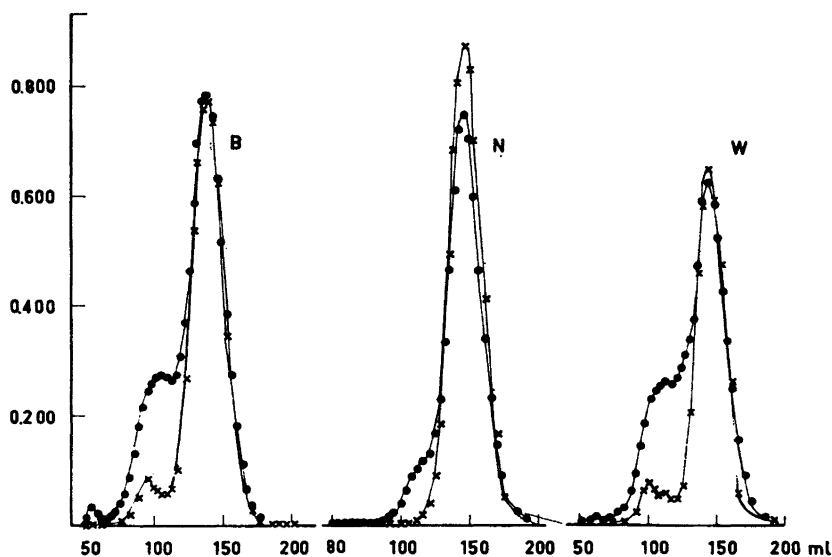


Fig. 4. Distribution of proteins and enzymatic activity of trypsin preparations separated by gel filtration. From left to right: Boehringer, Novo, and Worthington trypsin. Filtration on same column (Bio Gel P 30) with 20 mg trypsin in each experiment.

- Protein content of fractions expressed as optical density at 280 $m\mu$.
 - × Esterolytic activity (BAPNA) expressed as optical density at 410 $m\mu$.
- Abscissa: volume (ml) used for elution.

the original preparation and two narrow bands corresponding roughly to the two slowest bands in the original preparation. The action on the film was very small in relation to the amount of activity used, measured with BAPNA.

Determination of the specific activity and inhibition with STI. The specific activity, measured as activity against BAPNA per μg nitrogen according to the Kjeldahl analysis, varied substantially from one preparation to another. The values are given in arbitrary units (Table 1). The specific activity also varied, though less markedly, when measured as the ratio between the esterolytic activity and STIBC (Table 2).

Table 1. Esterolytic activity/ μg nitrogen in the trypsin preparations expressed in relative values, where the value of Novotrypsin was taken as 1.00.

Trypsin preparation	Specific esterolytic activity
Boehringer	0.65
Novo	1.00
Worthington	0.52

After electrophoresis of the various trypsin preparations in agarose gel, the active fractions were located with the film technique, after which the most mobile (I) and next mobile (II) ones were cut out separately, while the other were taken together (III). The fractions were eluted and their activity with BAPNA and STIBC was determined (Table 2). In two (B and N) of the preparations fraction II had a higher specific activity (esterolytic activity/STIBC) than fraction I. In the third preparation (W), which had a relatively small fraction II, the specific activity of fraction I was somewhat higher. In all three preparations fraction III showed the lowest specific activity.

In some experiments the ratio was determined between the esterolytic activity and STIBC in two fractions separated by gel filtration (Table 3).

Table 2. Esterolytic activity/STIBC in the trypsin studied and in electrophoretic fractions thereof (see text) expressed in relative values, where the value for Novotrypsin was taken as 1.00.

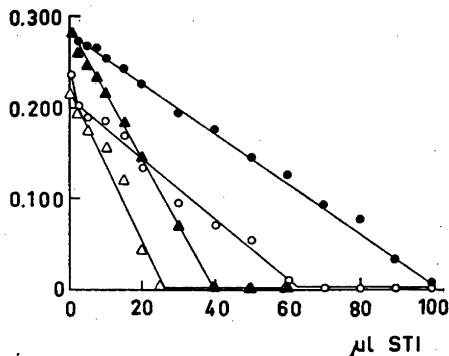
Trypsin preparation	$\frac{\text{Esterolytic activity}}{\text{STIBC}}$
Boehringer	
Original preparation	0.90
Fraction I	0.87
Fraction II	0.92
Fraction III	0.73
Novo	
Original preparation	1.00
Fraction I	0.85
Fraction II	1.00
Fraction III	0.84
Worthington	
Original preparation	0.83
Fraction I	0.87
Fraction II	0.84
Fraction III	0.68

Table 3. Esterolytic activity/STIBC in fractions after gel filtration of trypsin (see text) expressed in relative values, where the value for Novo crystallised trypsin was set as 1.00.

Enzyme fraction	$\frac{\text{Esterolytic activity}}{\text{STIBC}}$
Boehringer	
Low molecular	0.91
High molecular	0.53
Worthington	
Low molecular	0.91
High molecular	0.53

Fig. 5. Inhibition with STI of enzyme fractions after gel filtration of crystalline trypsin. The ordinate gives the esterolytic activity (BAPNA) expressed as optical density at $410\text{ m}\mu$ the abscissa the amount of STI added.

- Fraction of high molecular weight (Worthington).
- ▲ Fraction of low molecular weight (Worthington).
- Fraction of high molecular weight (Boehringer).
- △ Fraction of low molecular weight (Boehringer).



The specific activity of the first component in each of the two preparations studied (B and W) was about half of that in the other component. The specific activity in the other component of lower molecular weight of one (B) of the preparations was slightly higher than that in the original preparation (B). In the other preparation (W) the specific activity in the second component was clearly higher. The different activity of the fractions in relation to STIBC was reflected well by the varying inhibition of the fractions with STI. In one experiment an increasing amount of STI was added to a constant of each fraction obtained by gel filtration, after which the activity against BAPNA was determined. Inhibition of a given activity of the first component to the same extent as the second required about twice as much STI (Fig. 5).

DISCUSSION

The inhibition curves obtained on addition of an increasing amount of STI to a heterogeneous trypsin were linear up to inhibition of about 85 %, after which they gradually became asymptotic. This suggests the occurrence of protein with some affinity for STI, but with little or no enzymatic activity (damaged trypsin molecules?). Curves of similar appearance can be obtained also with other trypsin inhibitors.^{4,14} The total STIBC of the preparations could not be calculated with any degree of accuracy from these inhibition curves. Therefore, the method for determining STIBC described above was used. The results obtained by the method proved reproducible and included all STI bound to the preparation so strongly that subsequently added excess of active trypsin was unable to detach and bind the inhibitor during the period studied. If STI were also bound to the preparation so loosely that it could be rapidly transferred to active trypsin, it would be of less interest because then it would not be able to disturb standardisation of the trypsin preparation with STI. The determinations did not include such loosely bound inhibitor.

Attempts have not been made to characterise or identify the various fractions of the crystalline trypsin preparations. The molecular weight of the first, smaller fraction obtained by gel filtration probably lies between that of trypsin, 24 000, and the exclusion limit, which according to the manu-

facturers of the gel, is 30 000, provided that none of the fractions were bound to the gel, for then, the fraction could consist either of trypsin bound to some other component (possibly partial inhibitor from pancreas) or of some other pancreatic enzyme. The size of the fraction of higher molecular weight found in the present investigation is compatible with findings made by previous investigators using Worthington trypsin.^{4,7-9} The fraction was not homogeneous and the bulk of the protein may have been enzymatically inactive.

It is not known whether the active electrophoretic fractions were naturally occurring isoenzymes, or artefacts, *e.g.*, slightly damaged trypsin molecules, and if so which fraction should be regarded as representing the natural enzyme.

It is clear from the results that commercial crystalline trypsin is a mixture of various enzymatically active components differing from one another in specific activity, molecular weight and electric charge. The three preparations as well as their fractions also differed in the ratio between the activity and STIBC. This is of interest because the inhibition of STI by trypsin is generally used for standardisation of trypsin.

It might be mentioned that in some experiments in a related study with a partially purified α_1 -antitrypsin preparation and with the trypsin inhibitor Trasylol (Bayer), we found analogously varying ratios between activity and inhibitor binding capacity. In the investigation of such a complicated system as the binding of trypsin to proteins in serum the components of the trypsin preparations with low enzymatic activity in relation to the α_1 -antitrypsin binding capacity, *i.e.*, activity relatively difficult to inhibit, are inclined to concentrate in the system, because readily inhibited components are bound to the inhibitor. This may lead to the erroneous conclusion that the activity in question is stabilised against α_1 -antitrypsin by specific factors in the serum.

Commercial crystalline trypsin should be checked for purity and if necessary, purified before use in analytic studies. A fair degree of purification can be obtained by gel filtration. If only minute quantities are required, a high degree of purification can be achieved by electrophoresis in agarose.

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