Protease Inhibitors

1. ASSAY AND NATURE OF SERUM ANTIPROTEASE

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The inhibition of trypsin by normal serum has been studied by many workers (Landsteiner, 1900; Fujimoto, 1918; Hussey & Northrop, 1922-3; Grob, 1943). The association of the trypsin inhibitor with the albumin fraction first noted by Landsteiner (1900) has been confirmed by subsequent workers using salt fractionation, and Smith & Lindsley (1939) have shown that on electrophoretic separation of the serum proteins the antitryptic power lay mostly in the albumin fraction. Several groups of workers (Schwartz, 1909; Bauer, 1910; Jobling & Petersen, 1914) have associated antitryptic activity in serum with lipids or fatty acids, and Ungar (1945) has found that it was removed by ether extraction and reconstituted by readdition of the ether extract to the aqueous residue. This association of antitryptic activity with lipids has been denied by many others (Meyer, 1909; Cobliner, 1910; Teale & Bach, 1919-20).

Studies by Christensen & MacLeod (1945) and MacFarlane & Pilling (1946) show that plasma trypsin (plasmin) is likewise inhibited by a constituent of the albumin fraction of plasma which the latter authors term antiplasmin. Beloff (1946) has shown that the skin protease studied by Beloff & Peters (1944, 1945, 1946) is also inhibited by the albumin fraction of serum. Her work clearly demonstrates that the inhibitory factor against skin protease is identical with the inhibiting factor for both trypsin and plasma trypsin, although she drew the opposite conclusion from a study of the literature. Schmitz (1937, 1938) claims to have isolated from serum a second antitryptic factor which he regards as a polypeptide similar in properties to that isolated from pancreas by Kunitz & Northrop (1936), and which he believes is normally present in combination with plasma trypsin, thereby inactivating it. The evidence brought forward by Schmitz is hardly sufficient to support his claims. Grob (1943) appears to have prepared a 'crystalline' serum antitrypsin by the methods of Schmitz. Many authors have described marked physiological and pathological variations in the antitrypsin activity of the sera of man and animals and a good summary is given by Grob (1943). The majority of the conditions reported showed a marked increase over the normal level, but no single factor appears to be

responsible, and the work is difficult to assess because of the wide variation in methods used, and the lack of any simple accurate technique for assaying serum antitrypsin.

The development by Swyer & Emmens (1947) of a satisfactory viscosimetric relationship (in the case of hyaluronidase) between enzyme activity and flow time, as measured in Ostwald viscosimeters, made it possible to assay serum antitrypsin viscosimetrically using gelatin as substrate and to restudy some of the findings of previous workers.

EXPERIMENTAL

Materials and methods

Gelatin solution. Gelatin 10% (w/v) in 0.85% (w/v) NaCl (saline) was autoclaved and stored in the cold in 100 ml. amounts. After warming, 10 ml. of 0.2M-phosphate buffer (pH 7.6) were added to every 90 ml. gelatin, and the mixture was then brought to pH 8.0 by addition of a few drops of 2N-NaOH. The solution was heated to nearly 100° and filtered through a Whatman no. 1 filter paper, the pH was adjusted to 7.6, and saline added so that the final relative viscosity after dilution with an equal volume of 0.5% (w/v) gum acacia in saline was about 2.3. The gelatin was stored in the cold until next day when it was melted at 37.5° and kept at that temperature during use. Merthiolate was added as a preservative.

Trypsin solution. Crystalline trypsin containing approximately 50% (w/v) MgSO₄ was dissolved at 0.1% (w/v) concentration in 0.01N-HCl and stored at 4°. Dilutions were made in 0.5% (w/v) gum acacia. As a standard of comparison, the enzyme was assigned the arbitrary value of 20,000 units/mg.

Estimation of enzyme and inhibitor. Equal volumes of enzyme and gelatin solution were mixed at 37°, and 2 ml. of the mixture transferred to Ostwald viscosimeters in a water bath at 37.5°. The viscosity of the mixture was measured 20 min. after mixing, and this was carried out more accurately by taking readings at 16, 20 and 24 min. respectively and interpolating when necessary. Mixtures of inhibitor and enzyme were incubated for 10 min. at 37° prior to mixing with gelatin. The viscosimeters used had a bulb capacity of a little more than 1 ml., a capillary of 0.5 mm. diam. and a flow time of 51 ± 3 sec.

Viscosimetric assay of enzyme and inhibitor

Enzyme. Although several investigators (Hussey & Northrop, 1922-3; Northrop & Kunitz, 1932-3; Smith & Lindsley, 1939; Christensen, 1945) have used viscosimetric

methods for the assay of proteolytic enzymes using gelatin as a substrate, it is doubtful if any of the formulae developed, which relate enzyme concentration to a rate of fall in viscosity, are entirely satisfactory. Swyer & Emmens (1947), in a study of hyaluronidase action, defined the flow-time index v as

$$\frac{(f_s - f_o)}{(f_s - f_o)} \times 1000,$$

where f_s = flow time of substrate and enzyme solution; f_o = flow time of solvents alone (these being in the same proportion as in the hydrolysis mixture); and f_e = the flow time of the enzyme substrate mixture after incubation for a given time such as 20 min. They showed that v gives a straight-line relationship when plotted against the logarithm of the enzyme concentration.

Since $f_s - f_o$ is the viscosity due to the substrate, and $f_s - f_e$ represents the reduction of viscosity caused by the action of the enzyme on the substrate, and since the curve relating flow time to substrate concentration is approximately linear at the concentrations of hyaluronic acid used,

Fig. 1. Relationship between concentration of gelatin and relative viscosity as determined in an Ostwald viscometer.

their finding evidently rests on the assumption that the flow-time index v is a measure of the fraction of the total substrate hydrolyzed in a given time. Between relative viscosities 2.7 and 1.6 the curve relating relative viscosity (or flow time) to gelatin concentration is likewise approximately linear (Fig. 1). The line does not pass through viscosity 1.0 as it would if a lower range of concentration were considered, but through a value, in this case 0.76, which depended on the batch of gelatin used and showed little tendency to vary. Working between initial and final relative viscosities of 2.5 and 1.6, an approximately linear relationship (Fig. 2) was found when v , the flow-time index, was plotted against the logarithm of the enzyme concentration for values of v between 150 and 730. The flow-time index can, therefore, be used as a measure of the amount of substrate hydrolyzed within a given range of viscosities, provided that these viscosities give a straight line when plotted against gelatin concentration and irrespective of where the line cuts the ordinate.

Gelatin as used in the assays increased slightly in viscosity during the first 3 hr. incubation at 37.5°, after which it underwent a slow loss amounting to between 0.01 and 0.02 unit relative viscosity/hr. Neither this slight fall nor the day-to-day variations encountered in making up the solutions had any significant effect on the value of v obtained provided the f_e values were redetermined at intervals.

Fig. 2. Relationship between flow-time index (v) and logarithm of enzyme concentration for crystalline trypsin and crystalline chymotrypsin. $\times$ — $\times$ Crystalline trypsin; $\circ$ — $\circ$ crystalline chymotrypsin.

Table 1 shows that even large variations in the initial viscosity of the gelatin from 2.5 to 1.96 altered v by only 40, there being a rise of about 7.4 in the flow-time index for each fall of 0.1 in the relative viscosity of the gelatin. In practice it was sufficient to adjust the viscosity each day to the same value, and to redetermine it at intervals on one

Table 1. Effect of change in initial viscosity of gelatin on the flow-time index v obtained with crystalline trypsin: each value an average of four to six determinations

Relative viscosity	v	
	Trypsin (100 units)	Trypsin (25 units)
2.50	563	263
2.20	590	288
1.96	604	304

viscosimeter, since the slope of the line relating the logarithm of the enzyme concentration to the flow-time index was identical at different viscosities, and for many months the flow-time index for 100 units of crystalline trypsin was constant at about 600. Slightly different values were obtained with other samples of gelatin. The slope of the line obtained with crude trypsin was identical, but that of crystalline chymotrypsin (Fig. 2) was different.

RESULTS

Rate of reaction of the inhibitor on enzyme. The inhibition of crystalline trypsin by serum increases with the time of contact before the addition of

substrate (Fig. 3), the main part of the reaction being completed in the first 20 min. The 10 min. incubation period used in the course of the work was based on preliminary trials with crude trypsin, which showed very little increase in inhibition by serum after this time.

Fig. 3. Effect of time of contact of serum and trypsin solution at 37° before mixing with gelatin on the inhibition of enzyme.

Effect of concentration of inhibitor on degree of inhibition. The inhibitory action of varying concentrations of serum on given concentrations of crude and crystalline trypsin of equal activity is shown in Fig. 4, where the residual activity as

Fig. 4. Effect of addition of varying amounts of serum on flow-time index of 75 units/ml. of crude and crystalline trypsin (1 mg. crystalline trypsin = 20,000 units). Δ — Δ , crystalline trypsin + guinea pig serum; $\blacktriangle$ — $\blacktriangle$, crude trypsin + horse serum.

measured by v is plotted against volume of serum added. Apart from the fact that some ten times more serum was required to produce a comparable degree of inhibition in the case of crude trypsin, the

curves are identical. When the percentage inhibition of enzyme activity at each serum level is calculated by a comparison of Figs. 2 and 4, and this is plotted against the serum volumes added, a curve is obtained (Fig. 5) which is very similar to that obtained by

Fig. 5. Effect of increasing amounts of serum on a constant amount of enzyme (75 units/ml.) expressed as per cent enzyme inhibition (1 mg. crystalline trypsin = 20,000 units).

Fig. 6. Inhibition of trypsin by various sera as measured by the flow-time index; $\odot$, rabbit; $\square$, horse; $\triangle$, sheep; $\times$, normal human; $\boxtimes$, human (pregnancy); $\otimes$, human (tuberculosis).

Hussey & Northrop (1922-3), who studied the action of serum on crude trypsin, and of Kunitz (1947), who investigated the effect of soya-bean trypsin inhibitor on crystalline chymotrypsin. Kunitz has derived an equation, relating the degree of inhibition and the amount of soya-bean inhibitor added to a constant amount of trypsin, on the basis of this being a uni-molecular reversible reaction. The curve in Fig. 5 is similar to that derived from Kunitz's equation though the agreement was not exact.

A comparison of the inhibitory power of various sera on trypsin can be made by reference to the curves obtained when various amounts of serum were added to a given amount of enzyme, such as crystalline trypsin (Fig. 6). A comparison of the volumes of each serum (measured on the curves) required to produce the same degree of inhibition shows good agreement when measured at $v=500$ and $v=400$ (Table 2). Since eight samples of normal

Effect of fat solvents. The most marked destruction of the inhibitor was obtained by shaking and incubation with chloroform which acts as a protein denaturant (Table 4). Several attempts were made to repeat the findings of Ungar (1945), who claims that, whilst after ether extraction both the aqueous and ether fractions of normal guinea-pig serum have slight antitryptic action, their antitryptic power on recombination is equal to that of the original serum.

Table 2. Comparison of inhibitory power of various sera against crystalline trypsin for values of v in Fig. 6

Serum	$v=500$		$v=400$	
	Serum (ml.)	Volumes containing same activity relative to rabbit serum = 100	Serum (ml.)	Volumes containing same activity relative to rabbit serum = 100
Rabbit	0.66	100.0	1.22	100.0
Human	0.42	63.5	0.72	59.0
Horse	0.35	53.0	0.60	49.0
Sheep	0.20	30.0	0.40	32.8
Human (pregnancy)	0.19	28.5	0.37	30.0
Human (tuberculosis)	0.13	19.9	0.25	20.5

human serum taken on two successive days from four persons showed little or no difference in inhibitory power when compared in this manner, fresh normal human serum was used as the basis of comparisons carried out at different periods during the course of the work. Similar lack of variations in inhibitory power within individual species was encountered in the case of other normal animal sera.

Characteristics of the normal serum trypsin inhibitor

Stability of serum trypsin inhibitor. The inhibitor was more unstable in the acid than in the alkaline range and there was some species variation (Table 3).

Table 3. Stability of serum trypsin inhibitor under various conditions

(In all experiments except the last, the serum was first diluted to 10% (v/v) in saline.)

Time	Temp. (°)	pH	Loss in inhibitory power (%)	
			Sheep	Horse
2 hr.	37	4.6	27	80
2 hr.	37	9.6	Nil	Nil
30 min.	60	7.4	20	46
30 min.	70	7.4	33	70
30 min.	80	7.4	95	70
12 months	4	—	Nil	Nil

There was a considerable loss above 60° in both horse and sheep sera. The loss on storage at 4° in both sera was very small, and there was even evidence of some increase.

No details are given by Ungar, but in the present studies neither prolonged shaking nor reflux distillation for 20 min. of both guinea-pig and horse sera with ether removed more than a small fraction (often none) of the total activity, and this slight loss could

Table 4. Stability of sheep serum inhibitor on exposure to fat solvents

(10% (v/v) solvent added, shaken 10 min. and incubated 5 hr. at 37°; activity measured at final serum dilution 1/1600 (v/v) against 100 units crystalline trypsin.)

	v	Loss in inhibitory power (%)
Trypsin alone	575	—
Trypsin + serum treated with saline	163	—
" " " " acetone	290	32
" " " " ethanol	188	Nil
" " " " ether	157	Nil
" " " " chloroform	476	75

not be restored by readdition of the extract. Ether extraction at -20° resulted in a 20% loss which was not restored by readdition of the ether soluble fraction. The inhibitor could not be extracted by acetone or chloroform.

Precipitation by trichloroacetic acid and dialysis. The antitryptic factor was precipitated with a loss of about 50% by the addition of an equal volume of 5% (w/v) trichloroacetic acid to sheep serum. The supernatant fluid was without activity. Sera diluted 1:10 and brought to various pH values were pressure-dialyzed through cellophan membranes, but the filtrates were without activity.

Salt fractionation. Serum treated with ammonium sulphate showed a marked loss in inhibitory power unless it was first buffered with sodium glycerophosphate, 7 % (w/v). Under these conditions at least 90 % of the total activity of sheep serum could be recovered (Table 5). A lower final yield was

Table 5. *Comparison of inhibitory activity of serum fractions against crystalline trypsin after salting out*

Fraction ...	Inhibitory activity as % of original serum		
	Albumin	Globulin	Total, reconstituted
Sheep serum once fractionated with $(\text{NH}_4)_2\text{SO}_4$	65	20	80
Horse serum once fractionated with Na_2SO_4	32	13	65

obtained on fractionation by 18 % sodium sulphate for 1 hr. at 37° using horse serum. The inhibitor was present in both albumin and globulin fractions although there was a marked reduction in the inhibitory power of either fraction on subsequent precipitation. Separated electrophoretically both fractions were inhibitory, the globulin being about twice as active on a weight basis.

A similar fractionation to that made by Beloff (1946) with ammonium sulphate showed that about two thirds of the total inhibitory activity of the albumin fraction lay in the more soluble part (Table 6), that is the fraction precipitated by full

Serum antitrypsin factor of Schmitz. Schmitz (1937, 1938) claimed to have separated an antitryptic factor from serum, which appeared to resemble a polypeptide, and which he believed served as a bound inactivator of the plasma trypsin. The antitryptic factor was obtained in acetone extracts of human plasma and by fractional precipitation of ox blood with ammonium sulphate following the addition of 2 vol. of 0.5 N-sulphuric acid. In the present study no inhibitory activity was found in acetone extracts of human plasma following Schmitz's directions. The fractional precipitation of 1.5 l. of ox blood gave approximately 180 mg. of material, representing a total inhibitory activity of only some 0.02 % of the original 1.5 l. Two thirds of the product was ammonium sulphate, and this cannot be removed according to Schmitz since the inhibitor itself is dialyzable. A similar product was obtained from human blood which was not destroyed by the addition of streptokinase as might have been expected from its supposed nature.

Variation in antitryptic level

(a) *Normal animals.* Although the antitrypsin levels of different animal species varied considerably there was little or no variation between individual members of the same species.

(b) *Anaphylaxis.* Burdon (1942) claimed that there was a 100 % increase in the antitryptic power of the sera of guinea pigs sensitized with egg albumin which fell rapidly to normal following injection of a shocking dose. None of these changes could be confirmed.

Table 6. *Comparison of inhibitory activity of various sheep serum protein fractions following ammonium sulphate precipitation*

	Globulin	Total albumin (fraction A)	Albumin precipitated by 50 % saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 4.2 (fraction B)	Albumin precipitated by 100 % saturation with $(\text{NH}_4)_2\text{SO}_4$ at pH 4.2 (fraction C ₁)
Activity recovered expressed as percentage total activity of serum	20	65	12	45
Concentration of fraction in serum (%)	3.0	3.5	1.92	1.17
Activity	6.6	18.5	6	38
Concentration				

saturation with ammonium sulphate at pH 4.2, and that on a weight basis this was about six times as active as either the less soluble fraction precipitated at pH 4.2 and 50 % (w/v) saturation or the globulin fraction. A further precipitation of the more soluble fraction corresponding to Beloff's C₁ showed a loss of 50 % on a weight basis. The figures given are very approximate, but serve to illustrate the wide difference in inhibitory power of the different fractions.

(c) *Immunity.* Grob (1943) claimed that the daily intramuscular injection of crude trypsin in rabbits over 4 weeks resulted in a rise of between 70 and 250 % in the antitrypsin titre of the animals, and that a similar, though less constant, rise was obtained in 4-8 days by the daily oral administration of the same product. In the present studies there was a rise of some 15 % in the antitrypsin activity of the serum of a rabbit injected daily subcutaneously for 4 weeks with crude trypsin, but no

rise was observed following oral administration as claimed by Grob.

(d) *Alterations in disease.* Many observers have noted an altered level of serum antitrypsin activity in various pathological conditions in man and animals, usually associated with a considerable degree of cachexia. The sera of seven persons all showing marked cachexia were examined. An increase of about 150 % was found in the antitryptic titre of the serum of three cases of tuberculosis. The remaining four (two tubercular and two non-tubercular) were normal.

(e) *Pregnancy.* The sera of eight women all in the terminal stages of pregnancy showed a uniform increase in the antitrypsin level of about 100 %.

DISCUSSION

The exact nature of the serum factor responsible for trypsin inhibition is still in doubt. Although associated with all fractions obtained by fractional salting out, it may yet be a single factor which is adsorbed on other proteins during precipitation. All attempts to separate it from albumin either by solvent extraction, adsorption or dialysis, under various conditions, have been unsuccessful, thus confirming the findings of other authors. Recently, Lineweaver & Murray (1947) have shown that the trypsin-inhibitory property of egg white is due to ovomucoid, and it is possible that the antitryptic action of serum is associated with a similar small molecular compound such as serum mucoid or seroglycoid (Hewitt, 1937). Unfortunately, the preparation of either of these substances, if they are indeed distinct (Meyer, 1938; Rimington, 1940), is carried out by methods likely to impair antitryptic properties, such as successive fractionation and crystallization at the isoelectric point (Hewitt, 1937), or by removal of the other proteins by boiling (Rimington, 1940). It will, therefore, be necessary to devise other methods for their isolation if this matter is to be tested.

A comparison of these studies with those of Beloff (1946) makes it clear that the serum inhibitor of skin protease and serum antitrypsin are identical. Serum trypsin is likewise inhibited by the same factor (Christensen, 1946). Beloff failed, however, to find any inhibitory activity against skin protease in the globulin fraction of serum, as did other authors who used pancreatic trypsin. In the present studies inhibitory activity was present in the globulin after two precipitations with ammonium or sodium sulphate, although it was much less than the activity after one precipitation. Globulin separated electrophoretically was about half as inhibitory on a weight basis as was albumin when measured against crystalline trypsin.

Finally there is no evidence that the inhibitor or portion of the inhibitor can be extracted with ether as claimed by Ungar (1945), nor is there any real evidence that serum contains a polypeptide inhibitor similar in its properties to the pancreatic trypsin inhibitor isolated by Kunitz & Northrop (1936), as is claimed by Schmitz (1937, 1938). The treatment of whole blood with 0.5 N-sulphuric acid followed by fractionation between 40 and 70 % saturation with ammonium sulphate undoubtedly gives a product with weak inhibitory powers. The hypothesis that this product is a specific inactivator for plasma trypsin, similar to Northrop's inhibitor for pancreatic trypsin, is very much open to doubt, since it rests on nothing more than a comparison of the stoichiometric properties of the two substances. Schmitz was unable to free his inhibitor from considerable amounts of ammonium sulphate, and his estimate of enzyme inhibition was based on the increase in non-protein nitrogen following relatively short hydrolysis of casein by trypsin. If the substance obtained by this method from human plasma is the specific inactivator for plasma trypsin, it would be expected to lose activity in the presence of streptokinase, which was not the case. From the method of fractionation employed it might reasonably be concluded that the substance is part of the serum albumin which has escaped precipitation with the blood, but is partly denatured.

SUMMARY

1. Trypsin and trypsin inhibitors can be assayed viscosimetrically with gelatin as a substrate, using the flow-time index relationship developed by Swyer & Emmens (1947) for the assay of hyaluronidase. Some of the principles involved are discussed.

2. The serum trypsin inhibitor is relatively stable and following fractional precipitation or electrophoresis it is associated with all fractions though mainly with the most soluble albumin fraction. It cannot be removed or destroyed by ether extraction, but is easily damaged by chloroform. It is sensitive to increased hydrogen-ion concentration and is lost slowly, if at all, on storage.

3. A small amount of inhibitory material was obtained from human and sheep blood using the method described by Schmitz (1938). The human material was not affected by the addition of streptokinase and it is doubtful if it has the role assigned to it by this author.

4. There is a marked species variation between the antitryptic power of different animal sera, but little variation between the sera of individual members of the same species.

5. No increase was found in the antitrypsin level of rabbit sera following feeding of trypsin and a slight increase after repeated daily injections of trypsin. No change was observed in the inhibitory power of guinea pig sera following protein sensitization either before or after the production of anaphylaxis.

6. The sera of certain persons suffering from tuberculosis showed a marked increase in inhibitory

power as did the sera of all women examined in the terminal stages of pregnancy.

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Protease Inhibitors

2. BACTERIAL PROTEASES AND THEIR INHIBITORS

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Inhibition of bacterial proteases by normal or immune sera has been studied by various authors, although the results are not always in agreement, probably because of the failure to recognize that the filtrates might contain several proteases, only one of which was inhibited by normal serum. In addition, sensitivity to inhibitor might vary according to the organism and type of culture used. von Dungern (1898) found that the hydrolysis of gelatin by filtrates of *Bacillus anthracis*, *Vibrio cholerae* and *Staphylococcus aureus* was inhibited by immune but not by normal serum. Bertiau (1914) found that normal human serum had no inhibitory action on the proteases of *Bacillus subtilis* and *Pseudomonas pyocyanea*, although specific immune serum inhibited. Blanc & Pozerski (1920) showed that gelatin hydrolysis by filtrates of *Clostridium sporogenes* was inhibited by normal horse serum,

which had no action on filtrates of *Cl. histolyticum*. Pozerski & Guelin (1938) showed that raw egg white had similar properties with regard to these filtrates. A more comprehensive study by Maschmann (1937*a, b*, 1938*a, b, c, d, e*) showed that at least three extracellular proteases could be recognized, and that a bacterial filtrate might contain one or more of these depending on the organism and method of preparation. A summary of Maschmann's findings is given in Table 1.

Smith & Lindsley (1939), in a study of the hydrolysis of gelatin by filtrates of various Clostridia, showed that, whilst normal serum inhibits proteases of non-pathogenic bacteria, it has no action on those of pathogens such as *Cl. histolyticum*, *Cl. welchii* and *Cl. oedematiens*. Filtrates of a rough strain of *Cl. histolyticum* were, however, inhibited by rabbit serum, but experiments showed that,