PROTEOLYTIC ENZYMES

III. Further Studies on Protein, Polypeptide, and Other Inhibitors of Serum Proteinase, Leucoproteinase, Trypsin, and Papain

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Proteolytic enzymes active at pH 7, including proteinases and peptidases, have been prepared from serum (1, 2), polymorphonuclear leucocytes (3), pancreas (4) and other tissues, various plants (including payaya), yeasts, molds, and bacteria (references cited in reference 5). Many of the proteinases are known to be inhibited by substances of biological importance. Trypsin is inhibited strongly by serum (6), by a crystalline polypeptide prepared from pancreas[1] (7), by a non-protein compound, perhaps a polypeptide, crystallized in small amounts from blood[1] (8, 9), by a mucoprotein prepared from egg white[1] (10), by a globulin crystallized from soy bean[1] (11), and by a pseudoglobulin prepared from lima bean[1] (12). Trypsin is also inhibited by high concentrations of oxidizing and reducing agents (5), heparin (13), and numerous other substances (references cited in reference 5). Serum proteinase is inhibited by serum (1, 14), by pancreatic (14, 15) and soy bean (16) inhibitors, and by heparin in sufficient concentration (17). Leucoproteinase is inhibited strongly by serum (3), and to a lesser extent by reducing agents, *p*-aminobenzoic acid, sulfonamides, and sulfones; but not by egg white inhibitor (5). Papain and most cathepsins are markedly inhibited by oxidizing agents and other sulfhydryl inhibitors, and activated by reducing agents (18). Papain is inhibited to a lesser extent by serum (5).

[1] Some properties of these inhibitors are summarized below (from references 7–12):

Source of inhibitor	Molecular weight	Nature of molecule	Reversibility of trypsin inhibition	Heat stability	Diffusibility through cellophane
Pancreas	6,000	Polypeptide (crystallized)	Reversible	Yes	Slowly
Blood	?	Polypeptide ?	?	Yes	Yes
Lima bean	10,000	Pseudoglobulin protein	?	?	?
Soy bean	24,000	Globulin protein (crystallized)	Irreversible	No	No
Egg white	29,000	Mucoprotein (ovomucoid)	Reversible	Yes	No

The inhibition of proteinolytic enzymes by serum has been the subject of numerous investigations, but the components of serum responsible for this inhibition have not yet been identified (9). In the studies to be reported here an effort has been made to characterize some of the proteinase-inhibitory components of the serum, and to compare their actions with those of proteinase inhibitors prepared from other sources. A study has been made of the inhibition of crystalline trypsin,[2] serum proteinase, leucoproteinase, and papain by: (*a*) Pancreatic,[2] soy bean,[2] lima bean,[3] and egg white[3] inhibitors. (*b*) Serum and plasma (normal human, pooled), and purified plasma protein fractions (human).[4] (*c*) Oxidizing and reducing agents, and mercurial sulfhydryl inhibitors. In addition, some observations have been made on the activation of serum proteinase.

Methods

Preparation of Serum Proteinase.—Serum proteinase was prepared by both chloroform and streptokinase activation of the inactive precursor present in serum (normal human, pooled), and in plasma protein fraction II + III.

Serum proteinase precursor was prepared by dialysis of serum for 24 hours against running tap water, followed by dilution with 2 volumes of distilled water and acidification with acetic acid to pH 5.3, centrifugation of resulting precipitate, and redissolving in a volume of 1/100 M phosphate-buffered normal saline at pH 7.4 equal to 1/10 of the original serum volume (19).

Streptokinase was prepared by Seitz filtration of an 18 hour culture of a "fibrinolytic" strain of group A β-hemolytic streptococci in beef-heart infusion containing 0.05 per cent glucose, precipitation of the streptokinase with alcohol, and redissolving in 1/100 M phosphate-buffered normal saline at pH 7.4 equal to 1/4 the volume of the infusion (20).

Chloroform-activated serum proteinase was prepared by shaking plasma (normal human, pooled) with 1/10 volume of chloroform for 2 minutes, and allowing the resulting mixture to stand at room temperature for a week. The denatured proteins were removed by centrifugation and the supernatant was then treated as in the preparation of serum proteinase precursor (2).

Preparation of Leucoproteinase.—The leucoproteinase preparations consisted of homogenates of polymorphonuclear leucocytes (rat and cat). These were obtained from sterile exudates produced by the pleural and peritoneal injection of 3 ml. (rat) or 10 ml. (cat) of 5 per cent aleuronat and 5 per cent gum tragacanth. The animals were killed after 24 hours by bleeding from the carotid artery, and the exudates were collected in citrated isotonic saline, centrifuged, and the sedimented leucocytes repeatedly washed with isotonic sodium chloride to remove the supernatant fluid, which

[2] Kindly provided by Dr. M. Kunitz, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

[3] Kindly provided by Dr. H. Lineweaver, Western Regional Research Laboratory, United States Department of Agriculture, Albany, California.

[4] Kindly provided by Dr. E. J. Cohn, Department of Physical Chemistry, Harvard Medical School.

contains substances of proteinase-inhibitory activity (3), presumably derived from the serum. Smears of the sediment showed over 90 per cent polymorphonuclear leucocytes. The leucoproteinase preparations were made by homogenizing the sediment in 10 times its volume of distilled water. No difference was observed in the proteinase activity of leucoproteinase derived from the rat and from the cat, or in the effect of various inhibitors on this activity.

Determination of Proteinase Activity.—Casein and denatured hemoglobin were the substrates employed. The initial proteinolytic change which rendered the protein molecules no longer precipitable by sulfosalicylic and trichloracetic acids was the index of proteinolytic activity.

Casein proteinolysis was determined by incubating a solution of 1.75 mg./ml. of casein[5] in M/25 Sørenson phosphate buffer[5] with the various enzyme preparations, at 37° C. and pH 7.6. The concentration of undigested protein was determined at intervals by precipitating 1 ml. of digest mixture with 3 ml. of 25 per cent HCl and 2 ml. of 20 per cent sulfosalicylic acid, and immediately[6] measuring the turbidity of the resulting suspension by the transmission of monochromatic light at 405 mμ wave length in a Coleman Junior photoelectric colorimeter.

The concentrations of proteinolytic enzymes that effected 50 per cent digestion of 1.75 mg./ml. of casein in approximately 4 hours were:

Trypsin[7] (crystalline): 1×10^{-9} M.

Chloroform-activated serum proteinase: 0.02 ml./ml.(0.015 mg./ml. protein N).

Serum proteinase precursor: 0.01 ml./ml. (0.018 mg./ml. protein N). This was activated by streptokinase, 0.01 ml./ml. (0.006 mg./ml. protein N), which was added to the precursor 20 minutes before addition of inhibitor.

Leucoproteinase: 0.02 ml./ml. (0.01 mg./ml. protein N).

Papain (crude, Difco): 0.003 mg./ml. (0.0002 mg./ml. protein N).

Hemoglobin proteinolysis was determined after the method of Anson and Mirsky (21) by incubating a solution of 7.6 mg./ml. of denatured hemoglobin at pH 7.3 with the various enzyme preparations at 37°C. The concentration of digested protein was determined at intervals by precipitating unaltered protein with trichloracetic acid, and determining the tyrosine(-tryptophane-cysteine) equivalent of the filtrate with the phenol reagent. The above concentrations of enzymes effected 20 per cent digestion of 7.6 mg./ml. of denatured hemoglobin in approximately 8 hours.

Serum proteinase was inhibited by the high concentration of urea present in the hemoglobin substrate (21), so that for the estimation of hemoglobin proteinolysis by serum proteinase the substrate was prepared without urea by adjusting the pH of

[5] Stock solutions for preparation of digest mixture contained 0.5 per cent casein in 0.003 N NaOH (pH 8.5), and M/15 NaH_2PO_4-Na_2HPO_4 buffer (pH 7.4).

[6] Any delay in making the turbidity measurement was accompanied by flocculation of the precipitated casein. This occurred most rapidly in the absence of serum proteins, a small amount of which delayed the occurrence of flocculation.

[7] The preparation used contained 50 per cent $MgSO_4$. This could be removed by dialysis, resulting in some diminution in the activity of the preparation, but no alteration in the effects of the various inhibitors. The molecular weight of trypsin is 34,000 (4).

Anson's cathepsin substrate (22) to 7.8, and removing the undissolved hemoglobin. The concentration of denatured hemoglobin in the digest mixtures which did not contain urea was 2.5 mg./ml. None of the proteinases hydrolyzed native hemoglobin to any appreciable extent.

Smaller concentrations of the enzyme preparations than recorded above produced proportionately less proteinolysis; *i.e.*, in the range of enzyme concentrations employed the amount of casein or hemoglobin digested was a linear function of the concentration of free enzyme. This enabled calculation of the concentration of free enzyme from the determined amount of casein or hemoglobin proteinolysis, and hence the concentration of enzyme inhibited.

Solutions to be tested for inhibitory activity were buffered with phosphate and were exposed to the enzyme for at least 20 minutes at room temperature before addition of the substrate. The inhibitory activity of the purified inhibitors, and of serum and plasma protein fractions, against trypsin, serum proteinase, and papain was essentially the same when the substrate was denatured hemoglobin (with or without urea) as when the substrate was casein. Leucoproteinase, however, was inhibited to a lesser degree when the former substrate was employed. The results recorded were those obtained with casein substrate, but, with the above exception, comparable results were obtained with denatured hemoglobin. In the study of the influence of reducing and oxidizing agents on proteinase activity casein was the only substrate employed, as the phenol method of determining proteinolysis could not be used in the presence of these agents.

In the analysis of experimental findings the activity of serum proteinase, leucoproteinase, and papain has been expressed in terms of molar concentration of trypsin of equal proteinolytic activity against casein under the conditions of the experiment. As is evident from the different effects on these enzymes of the various inhibitors, and from their well known differences in substrate specificities and other properties, these enzymes are distinct, and it is not meant to imply that they have a common chemical constituent. Expression of the activity of serum proteinase, leucoproteinase, and papain in terms of concentration of trypsin of equal activity has merely provided a common measure of enzyme activity and has enabled easier comparison of the effects of the various inhibitors. In comparing the inhibitors the concentrations required to inhibit half of the enzyme activity present have been recorded (Tables II and III). At 50 per cent inhibition the slopes of the curves (Figs. 1 to 3) are greatest, and interpolation most accurate.

RESULTS

A. Proteinase Activity of Plasma Protein Fractions (Table I)

The proteinolytic activity of the various plasma protein fractions was determined prior to any treatment, and after treatment with streptokinase and chloroform. Untreated solutions of the plasma fractions had no appreciable proteinase activity against casein. Treatment with streptokinase or with chloroform resulted in the appearance of proteinase activity in fraction II + III, and to a lesser degree in fraction I. Fractions II, IV - 1, IV - 4, and V had little or no proteinolytic activity after this treatment. The proteinase activity of fraction II + III is believed to be due to fraction III.

The addition of streptokinase to fractions I or II + III resulted in approximately twice as much proteinolytic activity as did long standing in the presence of chloroform. Activation by streptokinase occurred within a few minutes, while activation by chloroform required several days. When sterile solutions

TABLE I

Proteinase Activity of Purified Plasma Fractions after Treatment with Chloroform or Addition of Streptokinase

Chloroform treatment consisted of adding 1/10 volume of chloroform to solutions of the plasma fractions, which were then shaken for 2 minutes, allowed to stand at 24°C. for 1 week, and the supernatant removed after centrifuging and added to the substrate. Streptokinase (0.03 ml./ml.) was added to other solutions of the plasma fractions 20 minutes before addition of the substrate.

Plasma fraction	Principle components*	Concentration in plasma*	Total plasma protein*	Concentration in digest mixture	Proteinase activity‡	
					After treatment with chloroform	After addition of streptokinase
		mg./ml.	*per cent*	*mg./ml.*		
I	Fibrinogen (61 per cent) β-Globulin (15 per cent) γ-Globulin (8 per cent)	3.4	5.2	0.5	0.30	0.64
II	γ-Globulin (97 per cent)	6.0	9.1	0.5	0.09	0.08
II + III	β-Globulin (48 per cent) γ-Globulin (37 per cent)	19.0	28.8	0.25	0.54	0.98
IV-1	α_1-Globulin (89 peer cent) β-Globulin (10 per cent)	5.0	7.7	0.33	0.06	0.10
IV-4	α_2-Globulin (46 per cent) β-Globulin (38 per cent)	5.8	8.8	0.25	0.03	0
V	Albumin (95 per cent)	31.0	47.7	0.5	0.04	0.02

* From the data of Cohn and his associates (23).

‡ Expressed in terms of concentration of trypsin ($M \times 10^{-9}$) of equal activity.

of the plasma protein fractions were allowed to stand for 1 week at room temperature, under aseptic conditions and in the absence of chloroform, some proteinolytic activity could be demonstrated in fraction II + III, but much less than appeared in the presence of chloroform.

B. Proteinase-Inhibitory Activity of Purified Inhibitors Obtained from Egg White, Pancreas, Soy Bean, and Lima Bean (Figs. 1 and 2, and Table II)

Study of the inhibition of *trypsin* by egg white, pancreas, soy bean, and lima bean inhibitors confirmed the reports of stoichiometric inhibition (10, 7, 11, 12).

The curves for inhibition of trypsin by egg white, pancreatic, and lima bean inhibitors were compatible with inhibition which was reversible under the conditions of the experiment, while the curve for soy bean inhibitor was compatible with equimolar irreversible inhibition. Soy bean inhibitor was most potent, perhaps because of the irreversible nature of the inhibition. The apparent dissociation constants[8] of the compounds formed by combination be-

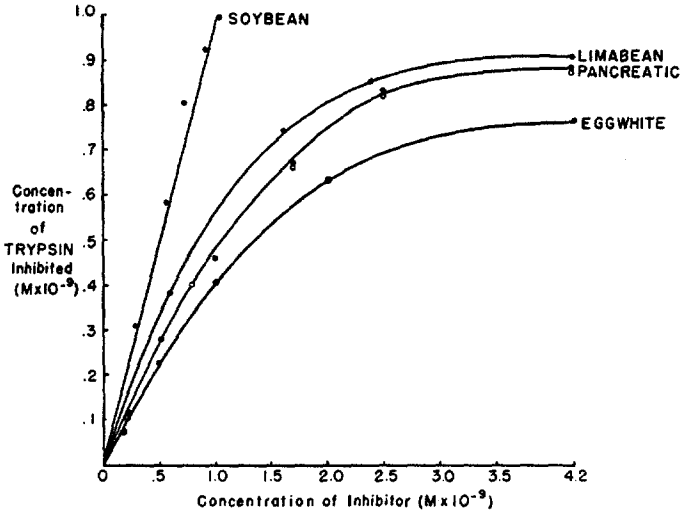

FIG. 1. (●) Inhibition of trypsin by soy bean, lima bean, pancreatic, and egg white inhibitors. The initial concentration of trypsin was 1×10^{-9} M.

(○) Inhibition of serum proteinase by pancreatic inhibitor. This was similar to the inhibition of trypsin by pancreatic inhibitor and is recorded in terms of concentration of trypsin of equal activity to the serum proteinase inhibited. The initial concentration of serum proteinase was equal in activity to 1×10^{-9} M trypsin.

tween trypsin and lima bean, pancreatic, and egg white inhibitors were, over most of the range of inhibitor concentration studied, approximately 0.4, 0.6, and 0.8×10^{-9}.

Serum proteinase and crystalline trypsin, in concentrations of equal activity, were equally inhibited by pancreatic inhibitor, the curve of inhibition being compatible with reversible inhibition. Serum proteinase was also inhibited by soy bean and lima bean inhibitors, but a somewhat higher concentration of

[8] $k = \frac{t(I-(T-t))}{T-t}$, where T = concentration of active plus inactive trypsin, t = concentration of active trypsin, and I = concentration of free plus combined inhibitor. Equation is from mass action equation $\left(\frac{k = t \times i}{ti}\right.$, where i = concentration of free inhibitor and ti = concentration of trypsin-inhibitor compound$\left.\right)$, following substitution for $i(=I-ti)$ and ti $(=T-t)$.

the former and a much higher concentration of the latter were required than was the case for pancreatic inhibitor. Serum proteinase was only slightly inhibited by relatively high concentrations of egg white inhibitor. The inhibi-

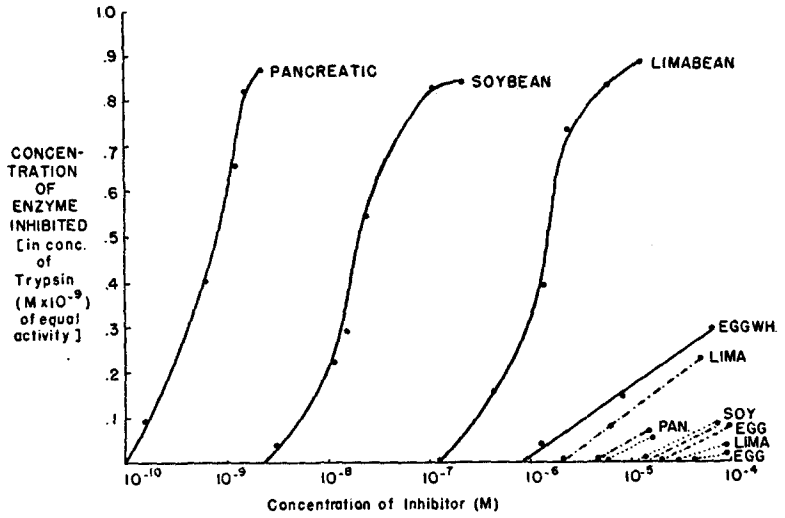

FIG. 2. Inhibition of serum proteinase (●————●), leucoproteinase (●—·—·—·—·—●), and papain (●.....●) by pancreatic, soy beanl lima bean, and egg white inhibitors. The initial concentration of each enzyme was equa, in activity to 1×10^{-9} M trypsin.

TABLE II

Proteinase-Inhibitory Activity of Purified Inhibitors Obtained from Pancreas, Soy Bean, Lima Bean, and Egg White

The initial concentration of enzyme was 1×10^{-9} M trypsin, and serum proteinase, leucoproteinase, and papain of equal activity.

Enzyme	Concentration of inhibitor (M) that inhibited 0.5×10^{-9} M trypsin and serum proteinase, leucoproteinase, and papain of equal activity			
	Pancreatic inhibitor	Soy bean inhibitor	Lima bean inhibitor	Egg white inhibitor
Trypsin	1.1×10^{-9}	0.5×10^{-9}	0.8×10^{-9}	1.3×10^{-9}
Serum proteinase	1.1×10^{-9}	4×10^{-8}	3×10^{-6}	$>10^{-4}$
Leucoproteinase	$>10^{-5}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
Papain	$>10^{-5}$	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$

tion of serum proteinase by any of the purified inhibitors was independent of the mode of activation of the serum proteinase (by chloroform or by streptokinase).

Leucoproteinase and papain were inhibited only very slightly by the purified inhibitors, even in relatively high concentrations. The egg white inhibitor had the least inhibitory activity.

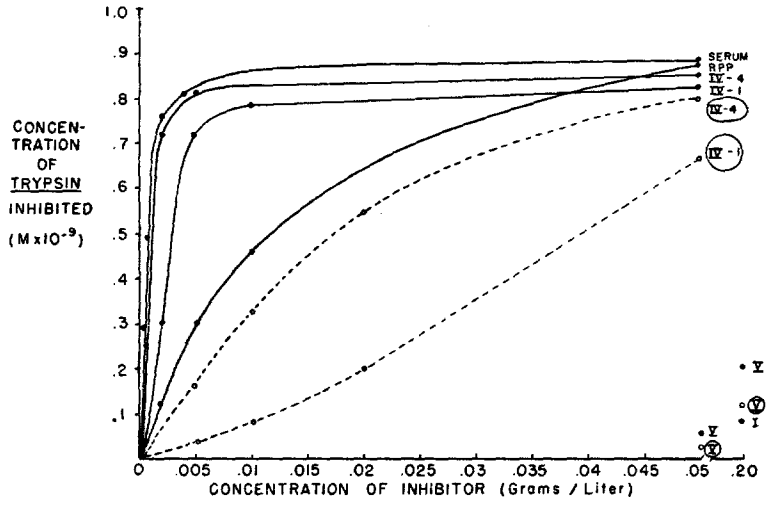

FIG. 3 *a*

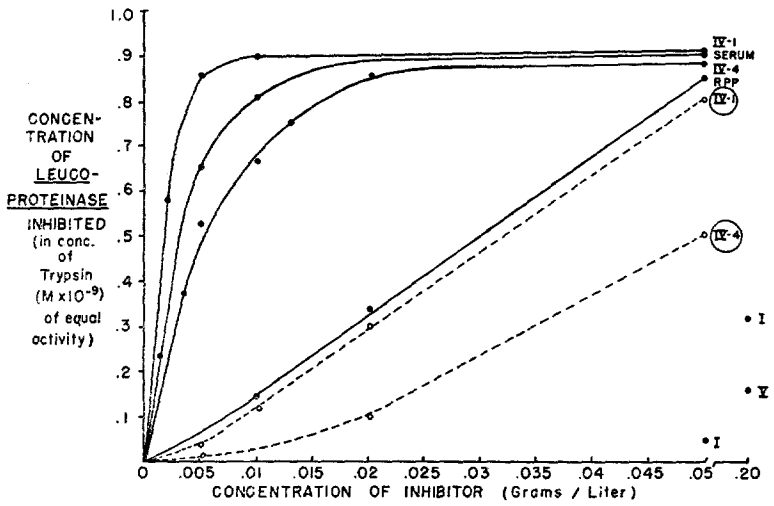

FIG. 3 *b*

FIGS. 3 *a—d*. ●————●, inhibition of trypsin (Fig. 3 *a*), leucoproteinase (Fig. 3 *b*), serum proteinase (Fig. 3 *c*), and papain (Fig. 3 *d*) by serum (or plasma), "reconstituted plasma proteins" (RPP), and plasma protein fractions I, II, II + III, IV - 1, IV - 4, and V. Where a protein fraction is not recorded the inhibitory activity was negligible. The concentration of serum is recorded as the concentration of total protein in the serum, which is almost the same concentration as that of the reconstituted plasma proteins.

O- - - - - - - -O, inhibition of the above enzymes by the concentration of plasma protein fraction (circled) present in the concentration of serum indicated by the abscissa.

The initial concentration of enzyme was 1×10^{-9} M trypsin, and concentrations of leucoproteinase, serum proteinase, and papain of equal activity.

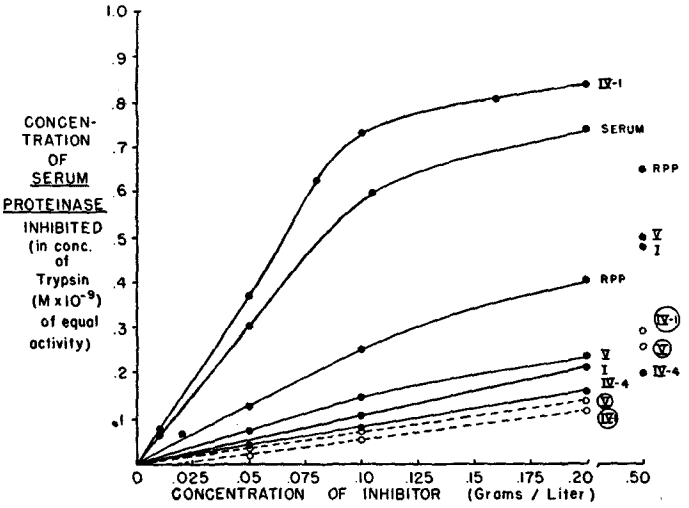

FIG. 3 *c*

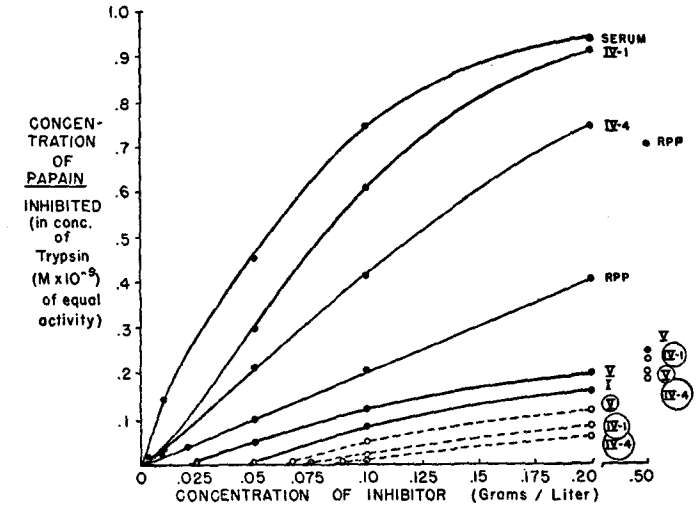

FIG. 3 *d*

C. Proteinase-Inhibitory Activity of Serum and of Purified Plasma Fractions (Figs. 3 a - d, and Table III)

Serum, even in high dilution, inhibited all the proteinases studied. Trypsin was inhibited by the lowest concentration of serum, while leucoproteinase re-

quired slightly more serum, and papain and serum proteinase considerably more. The inhibitory activity of plasma was equal to that of serum.

Plasma protein fractions IV - 1 and IV - 4, even in high dilution, inhibited all the proteinases studied, except that the inhibitory activity of fraction IV - 4 against serum proteinase was relatively slight. Much higher concentrations of fractions I and V were required to inhibit the proteinases, while fractions II and II + III had only slight inhibitory activity even in high concentration. Subfraction IV - 6 - 2 had nearly as much inhibitory activity against the proteinases as did its parent fraction IV - 4, while subfraction IV - 8 had considerably less. The curves for inhibition of any of the proteinases by serum and by

TABLE III

Proteinase-Inhibitory Activity of Serum and of Plasma Protein Fractions

The initial concentration of enzyme was 1×10^{-9} M trypsin and serum proteinase, leucoproteinase, and papain of equal activity.

Enzyme	Concentration of serum and of plasma protein fractions that inhibited 0.5×10^{-9} M trypsin and leucoproteinase, papain, and serum proteinase of equal activity						
	Plasma fraction				Reconstituted plasma proteins	Whole serum	
	I	IV-1	IV-4	V			
	Gm./l. $\times 10^{-3}$					Gm./l. of protein $\times 10^{-3}$	ml./ml. $\times 10^{-3}$
Trypsin	>500	3	1	500	12	1	0.01
Leucoproteinase	300	2	5	>500	36	4	0.06
Papain	>500	80	120	>500	300	58	0.83
Serum proteinase	500	65	>500	500	310	82	1.17

the plasma protein fractions were compatible with inhibition which was reversible under the conditions of the experiment.

The sensitivity of the proteinases to inhibition by fractions IV - 1 and IV - 4 was in general similar to their sensitivity to inhibition by whole serum: trypsin and leucoproteinase were inhibited by smaller concentrations of these fractions than were papain and serum proteinase. The concentration of fraction IV - 1 or IV - 4 required for inhibition of any of the proteinases was roughly equal to the concentration of total protein in serum that gave equal inhibition, except for fraction IV - 4 against serum proteinase.

The sum of the proteinase-inhibitory activities of the plasma protein fractions, in concentrations similar to those present in plasma, was less than the inhibitory activity of whole serum (or plasma). When the plasma protein fractions were mixed in proportions similar to those present in plasma the inhibitory activity of the resulting "reconstituted plasma proteins" against any

of the proteinases was approximately equal to the sum of the inhibitory activity of the constituent plasma fractions, but was only $\frac{1}{12}$ to $\frac{1}{4}$ that of whole serum (or plasma) containing an equal concentration of proteins. The contributions of the various plasma fractions to the inhibitory activity of the "reconstituted plasma proteins" varied with each enzyme. Fractions IV - 1 and IV - 4 had over 100 times more inhibitory activity against trypsin and leucoproteinase than did fraction V, so that the inhibitory activity of the plasma proteins against these enzymes was due almost entirely to fractions IV - 1 and IV - 4, even though the concentration of fraction V in the serum (or plasma) is 3 times that of fractions IV - 1 and IV - 4 combined. With respect to papain and serum proteinase, on the other hand, fractions IV - 1 and IV - 4 had less than 10 times the inhibitory activity of fraction V, so that fraction V made a slightly greater contribution to the inhibitory activity of the plasma proteins against these enzymes than did fraction IV - 1 or IV - 4. Fractions I, II, and III did not contribute significantly to the inhibitory activity of the plasma proteins against any of the proteinases.

The inhibition of serum proteinase by serum or by any of the plasma protein fractions was independent of the enzyme precursor employed (serum proteinase precursor (19), or fraction II + III), or of its mode of activation (by chloroform or streptokinase). The degree of inhibition of serum proteinase by serum (or plasma) did not increase when the concentration of substrate was decreased from 1.75 mg. per cent to 1.0 and 0.5 mg. per cent, which suggests that over this limited range of substrate concentration the components of serum that inhibited serum proteinase did not compete with the substrate for the enzyme.

D. Effect of Heat on Polypeptide and Protein Proteinase Inhibitors

Pancreatic, serum "polypeptide," and egg white inhibitors, when tested against trypsin, have been found to be relatively heat stable at neutral pH (6, 9, 10), while soy bean inhibitor has been found to be heat labile above 90°C. (11). Lima bean inhibitor resembled pancreatic and egg white inhibitors in being relatively heat stable when tested against trypsin and serum proteinase after being heated at 100°C. for 10 minutes, while soy bean inhibitor was heat labile when tested against these enzymes (Table IV).

Serum, "reconstituted plasma proteins," and fractions IV - 1 and IV - 4 were heat labile: heating at 100°C. for 10 minutes greatly diminished (but did not entirely destroy) their inhibitory activity against any of the proteinases (Table IV). It is of interest that the addition of a small amount of serum or serum albumin to pancreatic and serum "polypeptide" inhibitors caused the latter substances to lose their heat stability as inhibitors of trypsin (9) and (for pancreatic inhibitor) of serum proteinase (Figs. 4 *a* and 4 *b*).

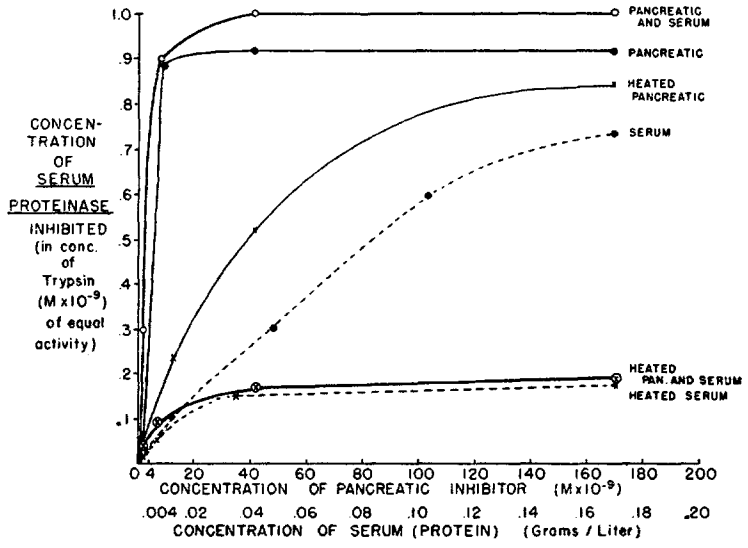

FIG. 4 *a*

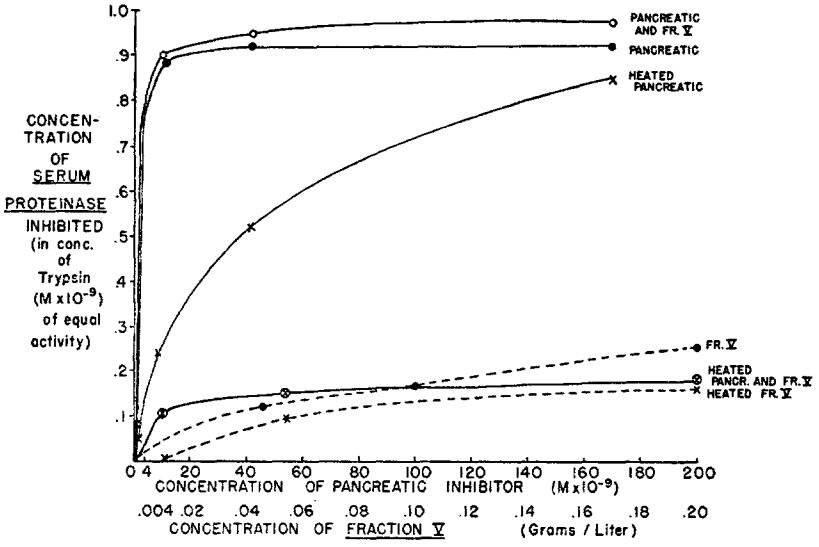

FIG. 4 *b*

FIGS. 4 *a* and *b*. Increase in the heat lability of pancreatic inhibitor (tested against serum proteinase) by serum (Fig. 4 *a*), and by fraction V (Fig. 4 *b*). The initial concentration of serum proteinase was equal in activity to 1×10^{-9} trypsin.

●————●, inhibition of serum proteinase by pancreatic inhibitor.

x————x, inhibition of serum proteinase by heated* pancreatic inhibitor.

●- - - - - - -●, inhibition of serum proteinase by serum (Fig. 4 *a*) or fraction V (Fig. 4 *b*).

x- - - - - - -x, inhibition of serum proteinase by heated* serum (Fig. 4 *a*) or fraction V (Fig. 4 *b*).

○————○, inhibition of serum proteinase by pancreatic inhibitor and serum (Fig. 4 *a*) or fraction V (Fig. 4 *b*).

⊗———— ⊗, inhibition of serum proteinase by pancreatic inhibitor heated* in the presence of serum (Fig. 4 *a*) or fraction V (Fig. 4 *b*).

* Heated in solution at 100°C. for 10 minutes.

TABLE IV

The Influence of Heat on Protein and Polypeptide Proteinase Inhibitors (Heated in Solution at 100°C. for 10 Minutes)

Inhibitor	Concentration of inhibitor	Trypsin* Inhibited (M × 10^{-9})		Leucoproteinase* Inhibited		Concentration of inhibitor	Serum proteinase* Inhibited		Papain* Inhibited	
		By unheated inhibitor	By heated inhibitor	By unheated inhibitor	By heated inhibitor		By unheated inhibitor	By heated inhibitor	By unheated inhibitor	By heated inhibitor
Pancreatic inhibitor	10^{-8}M	0.93	0.80			10^{-8}M	0.92	0.80		
Soy bean inhibitor	"	1.00	0.16			10^{-6}M	0.83	0.30		
Lima bean inhibitor	"	0.95	0.93			10^{-5}M	0.88	0.60		
Egg white inhibitor	"	0.93	0.53							
Serum‡	0.05 gm./l.	0.88	0.08	0.90	0.25	0.20 gm./l.	0.73	0.17	0.92	0.24
Reconstituted plasma proteins	"	0.87	0.07	0.85	0.20	"	0.40	0.10	0.40	0.15
Fraction IV-1	"	0.82	0.18	0.91	0.33	"	0.84	0.05	0.91	0.30
Fraction IV-4	"	0.85	0.09	0.88	0.30	"	0.15	0.03	0.74	0.20

* Initial concentration of enzyme was 1 × 10^{-9} M trypsin, and leucoproteinase, serum proteinase, and papain of equal activity. Concentration of the latter enzymes is recorded as concentration of trypsin (M × 10^{-9}) of equal activity.

‡ Concentration of serum is recorded in terms of concentration of serum protein.

E. Proteinase-Inhibitory Activity of Reducing and Oxidizing Agents

Reducing and oxidizing agents, in very high concentration, inhibit trypsin and leucoproteinase (5). For trypsin (M × 10^{-9}) the molar ratio of reducing or oxidizing agents to enzyme necessary to diminish enzyme activity by 50 per cent was approximately 10^7:1. Similar high concentrations of reducing and oxidizing agents were required to inhibit serum proteinase activity, as indicated by casein proteinolysis, while lower concentrations had no appreciable effect (Table V). The concentrations of oxidizing agents that were inhibitory were sufficient to produce protein denaturation, but the reducing agents that were studied are not protein denaturants (24), so that some observations have been made to attempt to elucidate their possible mode of action.

There was no correlation between the inhibition of serum proteinase activity by different reducing agents and their ability to reduce a given oxidizing agent (*e.g.* sodium 2,6-dichlorophenol indophenol, Fig. 5). On the other hand there did appear to be a rough correlation between the degree of inhibition by some reducing agents and the level of EMF measured potentiometrically (Fig. 5).

However, a direct relation between inhibition of serum proteinase activity by these reducing agents and the level of EMF is not proved, particularly since under the conditions of the experiment the reducing agents are not components

TABLE V

Inhibitory Effect of High Concentrations of Reducing and Oxidizing Agents on the Activity of Serum Proteinase (Chloroform-Activated)

Serum proteinase activity is expressed as concentration of trypsin (M $\times 10^{-9}$) of equal activity. The initial concentration was equal in activity to 1×10^{-9} M trypsin.

Agent	Concentration of agent (M $\times 10^{-3}$)	Serum proteinase inhibited
Glutathione	1.0	0
	2.8	0.18
	4.9	0.38
	7.3	0.54
	9.8	0.58
	13.2	0.70
Cysteine	2.0	0
	4.1	0.26
	6.3	0.44
	8.3	0.65
Ascorbic acid	4.5	0.05
	9.0	0.30
	12.3	0.36
Sodium thioglycollate	35.1	0.16
	70.2	0.40
	94.7	0.46
Sodium cyanide	4.1	0.39
Sodium hypochlorite	0.1	0
	1.0	0.70
Hydrogen peroxide	4.4	0
	8.8	0.10
	27.0	0.42

of strictly reversible oxidation-reduction systems. This is emphasized by the finding that high concentrations of sodium cyanide, a reducing agent in a different category than the others, inhibited serum proteinase activity even though this compound did not alter the level of EMF of the digest mixture and did not reduce sodium 2,6-dichlorophenol indophenol.

The degree of inhibition of serum proteinase by reducing agents (*e.g.* glu-

tathione) did not increase when the concentration of casein was decreased. Although this suggests that, in the range of concentration studied, the reducing

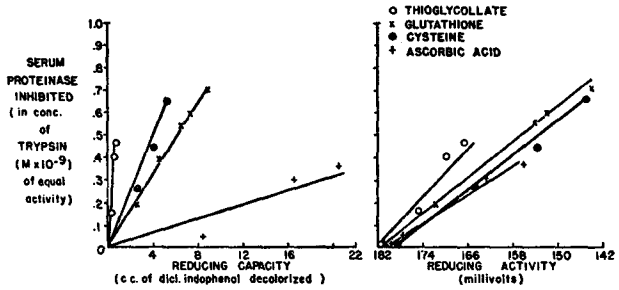

FIG. 5. *Left.* Lack of correlation between the inhibition of serum proteinase activity by different reducing agents and their ability to reduce a given oxidizing agent. (This "reducing capacity" is expressed as cubic centimeters of 0.0005 M sodium 2,6-dichlorophenol indophenol declorized by 1 cc. of digest mixture at pH 7.4 within 15 seconds.)

Right. Rough correlation between the inhibition of serum proteinase activity by some reducing agents and their level of EMF measured potentiometrically in millivolts by the Cambridge electron ray pH meter ("reducing activity").

The initial concentration of serum proteinase was equal in activity to 1×10^{-9} M trypsin. Straight lines have been drawn through or near the various points for purposes of comparison, although S-shaped curves would be more appropriate.

TABLE VI

Prevention or Reversal of Glutathione Inhibition of Serum Proteinase by the Addition of Potassium Ferricyanide 2 Hours after the Onset of Digestion

The initial concentration of glutathione was 7.3×10^{-3}M. Serum proteinase activity is expressed as concentration of trypsin (M $\times 10^{-9}$) of equal activity. The initial concentration of serum proteinase was equal in activity to 1×10^{-9}M trypsin. K ferricyanide, in the concentrations employed, did not accelerate proteinolysis in the absence of glutathione.

Concentration of K ferricyanide (M $\times 10^{-3}$)	Calculated active glutathione after addition of K ferricyanide (M $\times 10^{-4}$)	Serum proteinase inhibited
0	7.3	0.54
0.9	6.4	0.44
1.8	5.5	0.30
3.1	4.2	0.22

agents did not compete with the substrate for the enzyme, it does not exclude alteration of the substrate by the reducing agents. As would be expected, heating the reducing agents at 100°C. for 10 minutes did not alter their inhibitory activity, reducing capacity, or reducing potential. Reducing agents had ap-

proximately the same inhibitory effect on streptokinase-activated serum proteinase as on the chloroform-activated enzyme, but oxidizing agents had a greater effect on the former. This may have been due to the inhibitory action of oxidizing agents on streptokinase (25) before maximal activation of serum proteinase precursor had occurred.

The inhibition of serum proteinase activity by reducing agents (*e.g.* glutathione) could be prevented or reversed by oxidizing agents (*e.g.* potassium ferricyanide) (Table VI). The suppression of glutathione effect by ferricyanide appeared to be a stoichiometric reaction.

In contrast to the inhibition by high concentrations of reducing agents of serum proteinase, leucoproteinase, and trypsin is the activation of papain by these substances (even in low concentration) (18). The activation of papain is believed to be due to the reduction of inactive disulfide to active sulfhydryl groups (18), though the mechanism of action of cyanide in this regard is not clearly understood (26). Organic mercurial compounds that are potent inhibitors of sulfhydryl enzymes such as papain (18), *e.g.* benzyl mercuric chloride and p-tolyl mercuric chloride, in concentration of 1×10^{-5}M, had no effect on the activity of serum proteinase. They were likewise without significant effect on the activity of trypsin or leucoproteinase (5).

DISCUSSION

Serum Proteinase

Serum proteinase normally exists in an inactive precursor state and can be activated slowly by chloroform, other organic solvents and other agents, and rapidly by streptokinase (14). The mechanism of activation is not yet clearly understood. Evidence exists that activation by streptokinase is catalytic, and that the slower activation by chloroform may be due to the removal by the chloroform of serum proteinase inhibitors, followed by the autocatalytic activation of the precursor (14, 27).

Proteinase activity has previously been demonstrated in plasma protein fractions I, II + III (especially III - 2), and IV - 1 after treatment with chloroform (28, 29). In the experiments described above even greater proteinase activity resulted from the activation of fractions I and II + III by streptokinase than by chloroform. The lesser activity of the chloroform-activated fractions may have been due to incomplete activation, autodigestion of enzyme, or denaturation of some of the enzyme or its precursor by chloroform.

Fraction II + III did not have appreciable inhibitory activity against serum proteinase. Therefore, the inactivity (prior to activation) of the serum proteinase precursor present in this fraction was not due to the presence of free proteinase inhibitors, and the activation by streptokinase and chloroform of the enzyme in this fraction was not due to the removal of such inhibitors. Although

some spontaneous activation of the proteinase precursor in fraction II + III could be demonstrated after long standing under aseptic conditions, this activation was much less than occurred in the presence of chloroform. This suggests that chloroform may not only permit the spontaneous activation of serum proteinase precursor by removing proteinase inhibitors when present (14), but may also increase this activation in an as yet undefined way.

Proteinase Inhibitors in the Serum

The crude "albumin" fraction of the serum, as prepared by ammonium sulfate precipitation, has long been associated with the inhibitory activity of the serum against trypsin (30) and against serum proteinase (14). Study of purified protein fractions revealed that protein fractions IV - 1 and IV - 4 had greatest inhibitory activity against the proteinases studied, fractions V (albumin) and I had much less inhibitory activity, and fractions II and II + III had little or none. Fractions IV - 1 and IV - 4 were especially potent as inhibitors of trypsin and leucoproteinase, and were responsible for almost all the inhibitory activity of the plasma proteins against these enzymes. The inhibitory activity of fractions IV - 1 and IV - 4 against serum proteinase and papain was less marked, although, except for the slight activity of fraction IV - 4 against serum proteinase, greater than that of fractions V and I. Because of its relatively high concentration in the serum fraction V made a slightly greater contribution to the inhibitory activity of the plasma proteins against serum proteinase and papain than did fraction IV - 1 or IV - 4. Fractions I, II, and III were relatively unimportant with respect to proteinase-inhibitory activity exerted by the plasma proteins.

The components of fractions IV - 1 and IV - 4 responsible for their proteinase-inhibitory activity have not been determined. According to the studies of Oncley, Scatchard, and Brown (31) 89 per cent of fraction IV - 1 consists of α_1-globulin (lipoprotein) of molecular weight 200,000 and 10 per cent β-globulin of molecular weight 90,000, while 46 per cent of fraction IV - 4 consists of α_2-globulin of molecular weight 300,000 and 38 per cent β-globulin of molecular weight 90,000. If the molecular weight of the components of these fractions responsible for their proteinase-inhibitory activity is 90,000 or above, the molar concentration that inhibited 0.5×10^{-9} M trypsin would be less than 1×10^{-9} M of protein of fraction IV - 4 and less than 3×10^{-9} M of protein of fraction IV - 1. This suggests the possibility of stoichiometric reaction between components of these fractions and trypsin.

The plasma proteins, as "reconstituted" from the purified plasma protein fractions, had approximately 8 to 25 per cent of the proteinase-inhibitory activity of whole serum (or plasma) containing an equal concentration of proteins. The remaining proteinase-inhibitory activity of the serum may be due to non-protein inhibitory substances in the serum, and to any loss of inhibitory

activity of the plasma protein fractions that may have occurred during the process of fractionation and purification. There is evidence for the presence of non-protein proteinase inhibitors in the serum, the most potent being a crystallizable compound which may be a polypeptide (8, 9). Lesser degrees of inhibitory activity are exerted by α-tocopheryl phosphate (32) and even less by reducing substances, such as ascorbic acid, cysteine, and glutathione and other cysteine peptides. Heparin does not contribute significantly to the proteinase-inhibitory activity of the serum (9).

The possibility exists that the plasma protein fractions may have contained small amounts of the serum "polypeptide" inhibitor. However, if this substance were responsible for all the trypsin-inhibitory activity of fractions IV - 1 and IV - 4, it would have to constitute approximately 5 per cent of these protein fractions. In addition, there is a striking difference between the action of the serum "polypeptide" inhibitor and of whole serum against at least one enzyme (chymotrypsin) (8), indicating that the "polypeptide" is not the only important inhibitor in the serum.

The heat lability of the proteinase-inhibitory activity of the serum is due, at least in part, to the heat lability of the proteinase-inhibitory activity of the serum proteins, but is in contrast to the heat stability of the purified serum "polypeptide" inhibitor (9). A parallel situation exists with respect to dialyzability. However, the presence of serum albumin or of whole serum alters the properties of the serum "polypeptide" inhibitor (as it does the pancreatic polypeptide inhibitor) in such a way as to make it heat labile (9). It is of interest in this regard that plasma polypeptides are said to be normally adsorbed to plasma proteins (33).

Comparative Activities of the Proteinase Inhibitors

Comparison between the proteinase-inhibitory effects of the inhibitors studied is made difficult by inability to determine the molar concentration of impure substances of unknown molecular weight, such as serum proteinase, leucoproteinase, crude papain, and the proteinase inhibitors in the various plasma fractions. However, expressing the concentrations of the above enzyme preparations in terms of molar concentration of trypsin of equal activity has enabled some comparison to be made between the effects of the purified inhibitors on these enzymes (Table II). If the molecular weight of the plasma protein inhibitors is assumed to be 90,000 or above, their molar concentrations ($\times 10^{-9}$) would be approximately equal to, or less than, those indicated by the figures in Table III, and some comparison may also be possible between the inhibitory activities of the plasma protein inhibitors (Table III) and of the purified inhibitors (Table II).

Of the enzymes studied, trypsin was the most sensitive to inhibition, being inhibited (stoichiometrically) by low concentrations of soy bean, pancreatic,

lima bean, and egg white inhibitors, by low concentrations of fractions IV - 1 and IV - 4 (possibly stoichiometrically) and serum, and by higher concentrations of fraction V. Serum proteinase was inhibited by low concentrations of pancreatic inhibitor, and by higher concentrations of soy bean inhibitor, serum, fractions IV -1, V, and I, and lima bean inhibitor. Leucoproteinase was inhibited by low concentrations of serum, and fractions IV - 1 and IV - 4, and by higher concentrations of fraction I. Papain was inhibited by low concentrations of mercurial sulfhydryl inhibitors, and by higher concentrations of serum and fractions IV - 1 and IV - 4.

Of the inhibitors studied, serum and fraction IV - 1 were the least specific, inhibiting all four proteinases. Egg white inhibitor was the most specific, inhibiting only trypsin. The reducing and oxidizing agents required such high concentrations for their inhibitory activity against trypsin, serum proteinase, and leucoproteinase that their inhibitory activity appears to be non-specific, and perhaps the result of action on substrate rather than on the enzymes. Inhibition by mercurial sulfhydryl inhibitors was limited to papain, which is thus seen to be the only enzyme of the proteinases studied which contains free sulfhydryl groups essential for its activity.

Of the enzymes studied, only trypsin was in pure form. The other enzyme preparations may be mixtures of enzymes, and may contain inhibitors and activators. It is therefore necessary to interpret the results as applying to the enzyme trypsin, and to the crude enzyme preparations of serum proteinase, leucoproteinase, and papain. In addition, the method employed furnished information with respect to the inhibition of the proteinase activity of these enzymes on the substrates casein and denatured hemoglobin. Information concerning inhibition of their proteinase activity on other substrates, and of their peptidase activity, awaits further study. Finally, only in the case of the inhibition of trypsin by pancreatic, egg white, soy bean and lima bean inhibitors has a direct effect of inhibitor on enzyme been proved (7, 10–12). In the other instances this can only be inferred from the observed effect of the inhibitors on enzyme activity.

Physiological Significance of the Proteinases and Their Inhibitors

Serum proteinase is capable of exerting a thromboplastic action which may be important in blood coagulation (34). Coagulation is inhibited by several inhibitors of serum proteinase, including serum "polypeptide" inhibitor (9), pancreatic inhibitor (9, 15), soy bean inhibitor (16), fraction IV - 1 (but also IV - 4) (35), and high concentrations of reducing and oxidizing agents (36). Serum proteinase may play a part in fibrinolysis and clot retraction, which are also inhibited by pancreatic and soy bean inhibitors (14, 37). Serum proteinase may play a rôle in the release of protein-bound histamine and heparin in anaphylactic and peptone shock (17, 38), which are said to be inhibited by soy

bean inhibitor (39). Alterations in the proteinase and proteinase-inhibitory activity of the serum have been reported in a variety of clinical conditions, including anaphylaxis, high fever, anemias, pregnancy, many chronic debilitating diseases (references cited in reference 9), and malignant tumors (40).

Leucoproteinase plays an important part in the dynamics of inflammatory processes (3). The products of the hydrolysis of protein by leucoproteinase or trypsin have been found to stimulate bacterial growth and to directly and indirectly inhibit sulfonamide action. Bacterial growth is inhibited by pancreatic trypsin inhibitor even in the absence of non-bacterial proteolytic enzymes, perhaps as a result of inhibition of bacterial proteinases (5, 9).

Trypsin is important in intestinal proteolysis. The release of trypsin into the peritoneal cavity in pancreatitis may account for many of the lesions that are observed in this disease. Trypsin and papain have both been extensively used in the preparation of protein digests for oral and parenteral administration, for bacterial media, and for other medical and industrial purposes (41).

There are numerous other proteolytic enzymes and enzyme systems of physiological and biological importance. These include the cathepsins, serum and tissue peptidases, skin protease (42), necrosin (a proteolytic enzyme important in inflammation) (43), and bacterial and mold proteases. The blood pressure regulatory mechanism is thought by many to include a kidney proteinase (renin) and a serum protease (a polypeptidase known as hypertensinase) (44). Study of the effect on these enzymes of serum, plasma protein fractions, and purified trypsin inhibitors may prove of interest.

SUMMARY

1. Serum proteinase precursor was found in plasma protein fractions I and III of Cohn. Inhibitors of serum proteinase, leucoproteinase, trypsin, and papain were found in fractions IV - 1 and IV - 4, and to a lesser extent in fractions V and I.

2. Pancreatic, soy bean, lima bean, and egg white inhibitors inhibited trypsin stoichiometrically. Pancreatic inhibitor had comparable inhibitory activity against serum proteinase; soy bean inhibitor had somewhat less, lima bean inhibitor even less, and egg white inhibitor very little. None of these inhibitors appreciably inhibited leucoproteinase or papain.

3. Serum and fractions IV - 1 and IV - 4 had marked inhibitory activity against trypsin and leucoproteinase, and somewhat less against serum proteinase and papain. The inhibitory activity of the plasma proteins against trypsin and leucoproteinase was due almost entirely to fractions IV - 1 and IV - 4; against serum proteinase and papain fraction V was slightly more important. The "reconstituted plasma proteins" accounted for 8 to 25 per cent of the proteinase-inhibitory activity of whole serum or plasma.

4. The proteinase-inhibitory activity of serum, plasma protein fractions,

and soy bean inhibitor was heat labile, while that of pancreatic, lima bean, and egg white inhibitors was relatively heat stable.

5. Reducing and oxidizing agents, in very high concentration, inhibited serum proteinase, as well as trypsin and leucoproteinase. These proteinases were not influenced by mercurial sulfhydryl inhibitors, indicating that free sulfhydryl groups do not play an important part in their activity.

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