ISOLATION, CRYSTALLIZATION, AND PROPERTIES OF PEPSIN INHIBITOR

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When a solution of pepsinogen is acidified to pH 1.0–5.0, one or more reactions take place producing pepsin and certain polypeptides. It was previously noted by the writer that one of these polypeptides has a powerful inhibiting action on pepsin at pH 5.0–6.0 (1). The present paper describes the isolation, crystallization, and properties of this inhibitor of pepsin.

The decrease in milk clotting activity at pH 5.7 of a standard pepsin solution is used as a measure of inhibitor activity.

Preparation of the inhibitor consists of activation of the pepsinogen at pH 1.0–2.0 for a very short time followed by rapid alkali inactivation of the pepsin. Precipitation of the denatured pepsin is brought about with trichloracetic acid. The inhibitor remains dissolved in the trichloracetic acid filtrate. The total nitrogen in the trichloracetic acid filtrate is about 15 per cent of the original pepsinogen nitrogen; half of this non-protein nitrogen is inhibitor nitrogen. Separation of the inhibitor from the inert polypeptides has been accomplished by repeated fractional precipitation first with tungstic acid at pH 1.0–2.0 and second by magnesium sulfate in the presence of trichloracetate ion at about pH 3.0. When fractionation has brought the specific inhibiting activity, *i.e.* the inhibiting activity per milligram nitrogen $[\text{I.U.}]_{\text{mg. N}}$ to above 60 per cent of the value of the pure inhibitor, the preparation may be crystallized. Half saturated ammonium sulfate, pH 5.0, room temperature, and 3.0–5.0 mg. of inhibitor nitrogen per ml. are the conditions required for crystallization. The material first precipitates as clear spheroids which on standing form rosettes of fine needles, as may be seen in Fig. 1.

Fractional recrystallization and solubility experiments indicate the presence of not more than 20–25 per cent impurity in the material of highest specific activity.

The inhibitor is destroyed by pepsin between pH 2.0–5.0, with a rate maximum near pH 4.0.

The reversible combination of pepsin with the inhibitor follows quantitatively the simple mass law equation arranged for a similar reaction by Northrop (2).

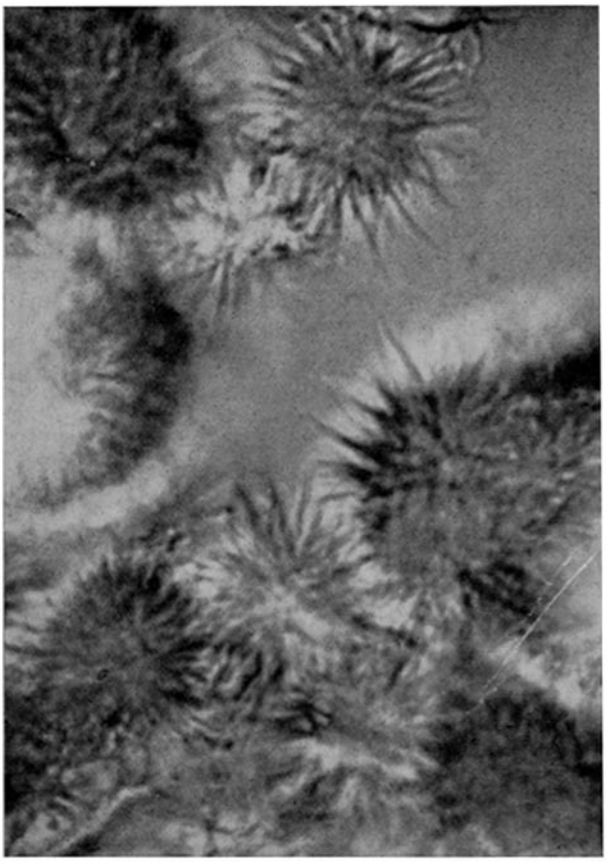

FIG. 1. Crystalline inhibitor of pepsin

The proteolytic action of pepsin as well as the milk clotting action is inhibited at pH 5.7. Dissociation of the pepsin inhibitor complex prevents tests at a more acid pH. There was no demonstrable effect of the inhibitor on crystalline trypsin as measured by the digestion of hemoglobin at pH 7.0–8.0, nor the milk clotting action of crystalline chymotrypsin or commercial rennet at pH 5.7. The crystalline trypsin inhibitor (3) had no effect on the milk clotting action of pepsin. This indicates a high degree of specificity among the inhibitors and is additional proof that the enzyme, rennet, is different from pepsin. An interesting result was obtained when

pepsin from different species was tested with swine pepsin inhibitor. Bovine pepsin was inhibited to the same degree as swine pepsin but chicken pepsin was not inhibited at all. On the other hand, crude inhibitor solution prepared from chicken pepsinogen inhibited both swine and bovine pepsin but had no effect on the chicken pepsin.

Certain chemical and physical properties have been determined, such as the isoelectric point, optical rotation, elementary analysis, amino nitrogen, and rate of diffusion. A few amino acids making up the inhibitor have been roughly estimated as well as the number of peptide linkages. The indications are that the inhibitor has basic groups exposed since it is precipitated by many reagents used to precipitate basic substances, namely, tungstic, phosphotungstic, flavianic, picric, and picrolonic acids. The main basic amino acid is probably arginine. The molecular weight, as determined by diffusion and the tyrosine content and combining equivalence with pepsin lies somewhere between 4,000 and 10,000.

EXPERIMENTAL RESULTS

Preparation of Crystalline Inhibitor

Preparation of Crude Inhibitor Solution from Pepsinogen.—Purified swine pepsinogen as previously described (1) was first dialyzed; adjusted to 2 mg. P.N./ml. and titrated to pH 2.0, temperature = 20–25°C. for 1 minute, then brought to pH 11.0–12.0 with 5 N sodium hydroxide where it was allowed to remain for 5 minutes. Acidification to pH 2.0 was then brought about by the addition of 3.0 normal trichloracetic acid. These reagents were all added in fairly concentrated form to prevent too great a dilution and the amount required determined on an aliquot so that all solutions could be mixed rapidly. After standing about 20 minutes the precipitate was filtered off with suction and washed with 2.5 per cent trichloracetic acid. The filtrate and washings containing the inhibitor were combined and, as may be seen, solution No. 2 of Table I (analysis of an actual preparation) contains about 0.11 mg. inhibitor units $[I.U.]_{ml.}$ and 0.3 mg. total nitrogen per ml. Thus the specific $[I.U.]_{mg.\ N}$ is 0.3. From this it follows that one gets about 0.06 [I.U.] per milligram of original pepsinogen nitrogen.

Fractionation Procedures

Tungstic Acid.—The inhibitor is precipitated from the trichloracetic acid filtrate as follows: 0.2 ml. of 5 per cent sodium tungstate (0.16 M) is added for every 100 ml. of filtrate, the precipitate centrifuged or filtered, and discarded. To the supernatant is added 5 ml. of 5 per cent sodium tungstate per 100 ml. supernatant. This residue containing about 75 per cent of the activity is dissolved by titrating slowly until pink to phenolphthalein. The tungstate is precipitated by addition of an excess of barium chloride and the tungstate centrifuged off followed by removal of excess barium ion by acidification with dilute sulfuric acid and addition of sodium sulfate until no further precipitate of barium sulfate appears.

Magnesium Sulfate-Trichloracetate Ion.—After filtering off the barium sulfate and

TABLE I

Preparation of Pepsin Inhibitor

Procedures and materials	No.	Quantity	$[I.U.]_{ml.}$	Total [I.U.]	N/ml.	[I.U.]/N
		ml. or gm.			*mg.*	
Dialyzed freshly prepared pepsinogen.....	1	1032			5.2	
Solution No. 1 + 1 liter water + 80 ml. N/1 hydrochloric acid for 1 min. at 25°C. then + 150 ml. N/1 sodium hydroxide, pH = 11; allowed to stand 3 min. followed by 50 ml. 3 N trichloracetic acid, pH = 2; left 20 min., filtered and residue washed with 300 ml. 2.5 per cent trichloracetic acid. Residue discarded. Filtrate and washings.....	2	2665	0.11	290	0.33	0.3
No. 2 + 5 ml. of 5 per cent sodium tungstate solution, stirred + 5 gm. Filter-Cel, filtered and to the filtrate was added 150 ml. 5 per cent sodium tungstate, filtered with the aid of 10 gm. of Filter-Cel. Precipitate....	3P					
Filtrate.....	3F	2800	0.0032	9	0.075	0.043
No. 3P + 500 ml. water + 40 ml. N/1 sodium hydroxide to pH 9 (pink to phenolphthalein) then + 75 ml. M/1 barium chloride, let stand 10 min., filtered and washed precipitate twice with water. Filtrate + 75 ml. M/1 sodium sulfate + 0.5 N sulfuric acid to pH 3.5; filtered. Filtrate.....	4	725	0.3	220	0.74	0.4
No. 4 + 1450 ml. of the magnesium-trichloracetate solution (2750 ml. saturated magnesium sulfate + 200 ml. 3 N trichloracetic acid + 30 ml. 18 N sodium hydroxide) let stand 48 hrs. and filtered with the aid of 15 gm. Hyflo Super-Cel Precipitate....	5P					
Filtrate.....	5F	2200	0.015	33	0.087	0.17
Inhibitor dissolved out and separated from the Super-Cel Solution.....	6	158	1.15	182	2.0	0.57
No. 6 diluted to 1800 ml. with water then + 6 ml. 5 N hydrochloric acid to pH 2.0 followed by 90 ml. 5 per cent sodium tungstate with stirring. Filtered. Residue dissolved in water + N/1 sodium hydroxide until solution is pH 9.0; then + 20 ml. M/1 barium chloride, centrifuged and residue washed once with 10 ml. water. To the combined filtrate and washings 20 ml. M/1 sodium sulfate and sulfuric acid to pH 3.0 was added followed by filtration. Residue washed on funnel. Filtrate.....	7	134				
No. 7 + 200 ml. of the magnesium-trichloracetate solution (1.5 vol.) + 1 gm. of Hyflo Super-Cel, filtered Filtrate.....	8F	330	0.082	27	0.22	0.37
Precipitate....	8P					

TABLE I—*Concluded*

Procedures and materials	No.	Quantity	[I.U.]ml.	Total [I.U.]	N/ml.	[I.U.]/N
		ml. or gm.			*mg.*	
8P + water, filtered and residue washed	9	115	1.13	130	1.8	0.63
No. 9 diluted to 240 ml. with water then + 5 ml. 5 N hydrochloric acid to pH 2.0 and + 50 ml. 5 per cent sodium tungstate; centrifuged. Residue + 15 ml. 0.5 N sodium hydroxide + 1.5 ml. 5 N sodium hydroxide to pH 9.0 then + 10 ml. M/1 barium chloride let stand 0.5 hr., centrifuged and residue washed with 5 ml. water. Washings + supernatant + 6 ml. M/1 sodium sulfate + 0.5 N sulfuric acid to pH 5.0, cooled to 5°C., centrifuged, supernatant	10	52	1.95	100	3.6	0.54
No. 10 + 1.25 ml. 4 M pH 5.0 acetate + 16 gm. solid ammonium sulfate to 0.5 saturation, stirred at 25°C., solution clear for at least 0.5 hr. Left 20 hrs. at room temperature. Good crystals formed. Aliquot centrifuged. Supernatant	11					
Crystal precipitate dissolved	12					
Inhibitor in mother liquor No. 11 separated from ammonium sulfate by precipitation with an excess of sodium tungstate at pH 2.0 followed by removal of tungstate ion from residue by solution at pH 9.0 then addition of barium chloride and after filtration of barium tungstate, excess barium was removed from the filtrate by addition of excess sodium sulfate Solution	13		0.05		0.24	0.2
A similar treatment was used to separate the inhibitor from the ammonium sulfate in the solution of the crystals, No. 12	14		0.5		0.66	0.76

analyzing for inhibitor activity and nitrogen the inhibitor is next precipitated by the addition of 2 volumes (or if the inhibitor solution contains over 1 mg. nitrogen per ml. 1.5 volumes) of a reagent made up of 200 ml. 3.0 N trichloracetic acid + 30 ml. 18 N sodium hydroxide + 2750 ml. saturated magnesium sulfate. The residue is retained and the filtrate discarded. The residue dissolves readily in water.

These fractionation procedures were applied alternately. After each fractionation step both residue and filtrate were analyzed for inhibitor activity and total nitrogen before discarding either. Fractionation was continued by these two procedures until the activity nitrogen ratio was 0.6 or better.

Crystallization

The fractionation treatment prior to crystallization was a tungstate treatment leaving the inhibitor solution practically salt free. The solution, having approximately 5 mg.

nitrogen per ml. was then titrated to pH 5.0 with sodium acetate or acetic acid and solid ammonium sulfate added with care until the concentration was 0.5 saturated. This requires 31.5 gm. per 100 ml. solution. The solution should be clear after dissolving the ammonium sulfate but after a few minutes a precipitate appears composed of microscopic spheroids and on standing 12–24 hours the spheroids change to rosettes of tiny needles (Fig. 1). Recrystallization may be carried out by solution of the crystals in a small volume of M/10 pH 5.0 acetate followed by addition of an equal volume of saturated ammonium sulfate solution at room temperature.

Tests of Purity

Fractional Recrystallization.—An inhibitor preparation was fractionally recrystallized to see if there was any drift in properties indicating the presence of impurities. The results in Table II show no gross separation or drift indicating an inhibitor of higher activity. The differences are of the same order as the error of the experimental methods.

TABLE II

Fractional Recrystallization of Inhibitor

Fraction	Original material	Specific activity [I.U.]mg. N
	per cent	
First crystal cake	100	0.83
Second crystal cake	33	0.95
Mother liquor of second crystallization	16	0.95
Third crystal cake	13	0.95
Mother liquor of third crystallization	20	0.75

Solubility Curve.—The solubility curve shown in Fig. 2 indicates that the preparation having a specific inhibitory value of 0.9 probably contains not more than 25 per cent impurity.

Experimental Procedure

Fig. 2 represents the solubility of the amorphous form of the inhibitor at 23°C. approached from the supersaturated side of the equilibrium. The technique was for the most part similar to that described elsewhere (4). The solvent used in this experiment is composed of two solutions, first the dissolving solution, M/50 pH 4.0 acetate buffer, and the precipitating solution made up in the following way: to 2750 ml. of saturated magnesium sulfate, specific gravity 1.296, is added 200 ml. of 3.0 N trichloracetic acid and 30 ml. 18 N sodium hydroxide. The complete solvent is made up of 45 parts of the dissolving acetate and 55 parts of the precipitating magnesium sulfate-trichloracetate.

Inactivation of Inhibitor by Pepsin

It was soon found that whereas pepsin free inhibitor solutions are stable for long periods of time at acidities varying from pH 1.0–10.0 in the presence

of pepsin the inhibitor is rapidly inactivated with a pH maximum near pH 3.5, as may be seen in Fig. 3. There is an increase in amino nitrogen, as measured by Van Slyke's gasometric method, amounting to approximately 8 per cent of the total nitrogen or about 5 amino groups per molecule if we assume a likely molecular weight of 5,000 for the inhibitor.

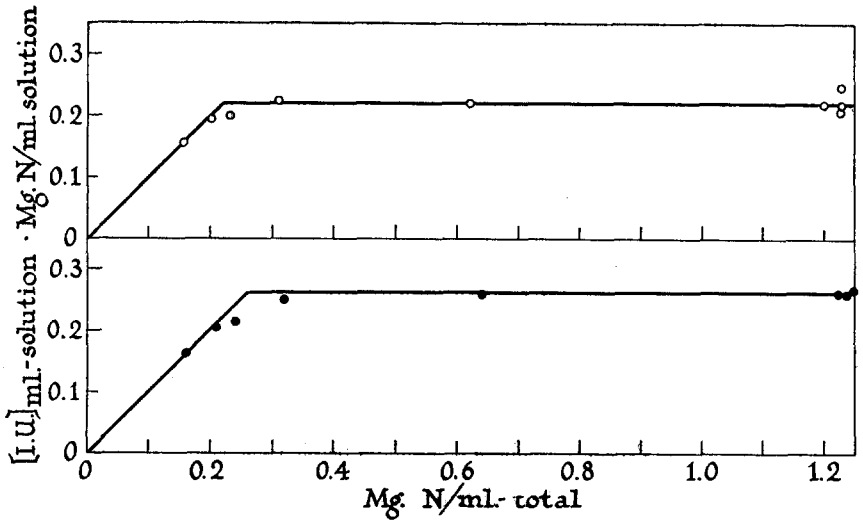

FIG. 2. Amorphous solubility curve of inhibitor in a magnesium sulfate-trichloracetate solution at 23°C.

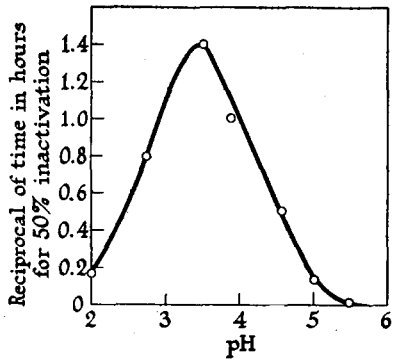

FIG. 3. pH-inactivation curve of inhibitor in the presence of pepsin

These experiments are strong evidence that the inactivation of the inhibitor is in fact a hydrolysis catalyzed by pepsin. In this connection it might be pointed out that the pH maximum at pH 3.5–4.0 shown in Fig. 3 is close to that found by Fruton and Bergmann for the hydrolysis of their synthetic substrates by pepsin (5).

Experimental Procedure

To 2 ml. of a pepsin solution at approximately the desired pH containing 250 rennet units per ml. were added 2 ml. of a crude inhibitor solution containing 250 inhibitor units per ml. and 0.5 ml. of M/10 acetate buffer of the desired pH. The temperature was 35°C. At certain time intervals samples were taken and diluted 0.1/10 in M/10 pH 5.7 acetate. After standing 30 minutes the milk clotting activity was determined and the amount of inhibitor calculated. Curves were plotted and the times for 50 per cent destruction were read off the curves.

For the amino nitrogen analyses a highly purified sample of inhibitor of specific activity = 1.0 was used. To a solution containing 1.6 mg. inhibitor nitrogen at pH 3.5 was added a dialyzed pepsin solution to bring the pepsin protein nitrogen to 0.2 mg. per ml. Immediately after mixing and again in 60 hours samples were analyzed for inhibitor action and Van Slyke amino nitrogen.

Application of the Mass Law to the Combination Reaction of Pepsin and Inhibitor

Some evidence has been obtained to indicate that the combination of pepsin with the inhibitor is a simple reversible dissociation as illustrated in equation I

$$\text{Pepsin} + \text{inhibitor} \rightleftharpoons \text{inhibitor pepsin compound} \tag{I}$$

This type reaction should follow the mass law which in its simplest form is equation II

$$\frac{[\text{Pepsin}]\quad[\text{inhibitor}]}{[\text{Pepsin-inhibitor compound}]} = \text{constant} \tag{II}$$

where the values within brackets are concentrations. In using the simplest form it is assumed that one molecule of pepsin reacts with one molecule of inhibitor.

Equation II cannot be used as such but may be rearranged as was done by Northrop (2) so that it will contain terms that are easily measurable. The equation used in the present work is the same as that used by Northrop with a few minor changes in symbols and is equation III,

$$P_f = \pm\sqrt{\left(\frac{I_t - P_t + K}{2}\right)^2 + KP_t} - \frac{I_t - P_t + K}{2} \tag{III}$$

where P_f = free pepsin; P_t = total pepsin; I_t = total inhibitor expressed in terms of pepsin units; and K = the constant for the equation.

In order to use this equation, the total inhibitor concentration used must be constant and the total pepsin concentration varied. The free or uncombined pepsin is determined and the combined pepsin obtained by the difference between the total and free pepsin. It is also necessary to make

certain assumptions which, as nearly as could be tested, are valid namely, that the pepsin inhibitor compound has no activity and that on addition of the compound into the Klim solution there is no appreciable dissociation due to dilution before the Klim is clotted.

When the amount of total pepsin is plotted against the combined pepsin we get a smooth curve, as seen in Fig. 4, which approaches a limiting value depending on the amount of inhibitor used. Since the above equation

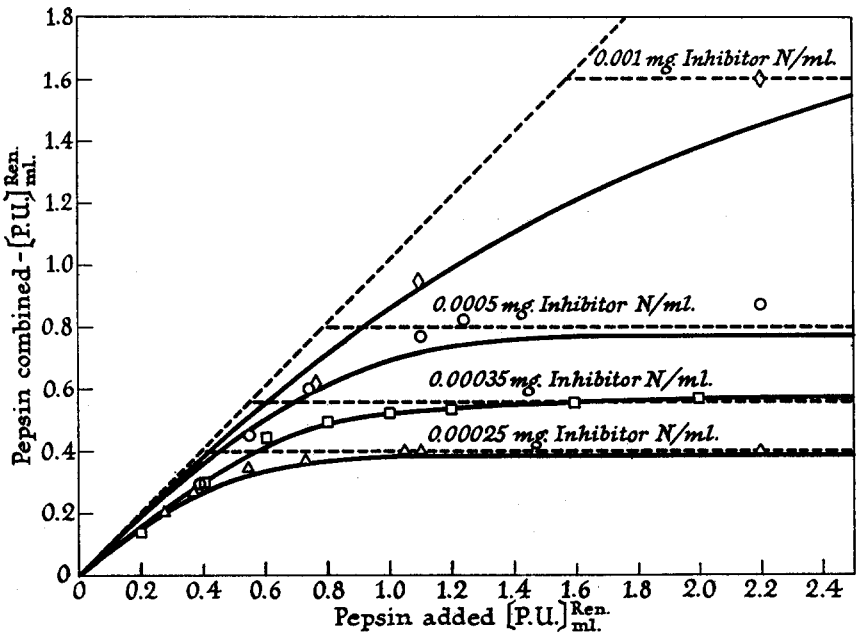

FIG. 4. Effect of increasing amounts of pepsin and inhibitor on the amount of pepsin bound by the inhibitor at pH 5.7. The points are the determined values. The solid lines are the theoretical curves calculated from the mass law as indicated in the text. The broken lines indicate the course if the reaction were stoichiometric.

(III) calls for the inhibitor in terms of pepsin units, this limiting value or maximum value of pepsin to combine with the indicated amount of inhibitor can be substituted for the inhibitor concentration. In other words, the total inhibitor is expressed as that amount of pepsin with which the inhibitor will combine when there is a large excess of pepsin.

In the present instance the pepsin equivalent value for the inhibitor was obtained only from curve I; *i.e.*, 0.00025 mg. inhibitor nitrogen is equivalent to 0.4 pepsin rennet units. The pepsin equivalent values for the inhibitor in curves II, III, and IV were calculated from curve I. This was possible

since the amount of inhibitor nitrogen used in these curves was predetermined.

The solid lines are the calculated curves obtained by calculating back with the equation using an average value of the constant K.

It may be seen in Fig. 4 that the experimental points show a reasonable approach to the calculated curves.

If one takes the figures obtained from the above experiment, namely that 0.00025 mg. inhibitor nitrogen is equivalent to 0.4 rennet units of pepsin or approximately 0.0012 mg. pepsin nitrogen, one may then calculate the molecular weight of the inhibitor. Such a calculation has been performed and it indicates a molecular weight of about 7,000.

TABLE III

Reaction of Inhibitors from Different Species with Different Pepsins

Source of inhibitor	Enzyme	Substrate	pH	Inhibiting action
Swine pepsinogen	Swine pepsin	Klim (milk clotting action)	5.7	+
" "	" "	Denatured pepsin	5.7	+
" "	" "	" hemoglobin	5.7	+
" "	Bovine "	Klim (milk clotting action)	5.7	+
" "	Chicken "	" " " "	5.7	−
Chicken "	Swine "	" " " "	5.7	+
" "	Bovine "	" " " "	5.7	+
" "	Chicken "	" " " "	5.7	−
Bovine trypsin inhibitor	Swine "	" " " "	5.7	−
Swine pepsinogen	Bovine chymotrypsin	" " " "	5.7	−
" "	" rennet	" " " "	5.7	−
" "	" trypsin	Denatured hemoglobin	7.6	−

Experimental Procedure

To a 1 ml. amount of inhibitor solution (the concentration of which is stated in Fig. 4) in a series of tubes was added a 1 ml. of pepsin of various concentrations dissolved in M/10 pH 5.7 acetate. These solutions were left at 35°C. for 30 minutes after which the milk clotting activity was determined in the usual way. The inhibitor had a specific inhibiting value per milligram of nitrogen of 0.9 while the pepsin was a 2 times crystallized Cudahy pepsin preparation of $[P.U.]^{Hb}_{mg.P.N.} = 0.3$ and $[P.U.]^{Ren.}_{mg.P.N.} = 300$.

Comparisons of Inhibitors from Various Sources on Different Enzymes

It was of interest to see whether the pepsin inhibitor had any action on other proteolytic enzymes than pepsin and to see if other inhibitors affected

pepsin. In the experiments, the results of which are summarized in Table III, all concentrations of inhibitor were equal to or greater than that used in the normal pepsin estimation so that one might easily expect to detect any appreciable action of the inhibitor.

The results show that the high degree of specificity usually associated with enzymes also exists among some inhibitors of enzymes. This is shown clearly in the fact that rennet from calves' stomachs is not inhibited whereas the milk clotting activity of bovine pepsin is inhibited to exactly the same degree as swine pepsin. On the other hand, chicken pepsin is not inhibited but the inhibitor prepared from activated chicken pepsinogen inhibits swine and bovine pepsin but not the homologous chicken pepsin.

Chemical and Physical Properties

Some of the chemical and physical properties of the purified inhibitor $[\text{I.U.}]_{\text{mg. N}} = 0.95$, have been collected together in Table IV.

In Table IV *B* are a few amino acid analyses along with certain other analyses and certain values deduced from them. For instance, assuming the molecular weight to be 5,000 there are then 57 atoms of nitrogen per molecule of inhibitor. In the intact inhibitor there are 8 free amino nitrogens while after acid hydrolysis there are 38. It follows therefore that there has been an increase of 30 amino groups but 3–4 of this increase of amino groups was found to be the amide nitrogen which on acid hydrolysis yields ammonia. Therefore there are a possible 26 peptide linkages. There must also be some 19 non-amino nitrogen. The arginine content of 31 per cent represents about 7–8 molecules of arginine per inhibitor. Since 3 of the 4 nitrogens in the arginine molecule are non-amino all of the non-amino nitrogen can be explained by the arginine content. The tyrosine content is so low that a fairly exact molecular weight can be obtained. The analysis by the Folin phenol test after acid hydrolysis of the inhibitor and comparison with a solution of standard tyrosine yields 0.32 per cent which is 1 tyrosine per molecule of a 4400 molecular weight inhibitor or 2 per molecule of 8,800 molecular weight. The tryptophane test of May and Rose is negative.

It seems very likely that the inhibitor has a number of strongly basic groups exposed for it is precipitated from dilute solution practically quantitatively by tungstic, phosphotungstic, flavianic, picric, and picrolonic acids, all of which are supposed to precipitate basic substances. The relatively high content of arginine would tend to account for this.

Experimental Methods

Pepsin Milk Clotting Activity Measurement.—This measurement was carried out exactly as described in the experimental methods of a previous paper (1). The rennet unit of pepsin activity is also defined.

Inhibitor Activity Measurements.—A. Pepsin Control.—1 ml. of a solution of pepsin containing 1.0 rennet units, 1.0 [R.U.] in M/10 pH 5.7 acetate was added to 1 ml. of M/10

TABLE IV

Chemical and Physical Properties

A

Property	Method of analysis	Per cent of moisture free material
C		48.07
H		8.07
N	Dumas	16.65
N	Kjeldahl	16.7
Ash		0.5
$[\alpha]_D^{pH\ 7}$ gm.		−104°
Diffusion constant	Northrop and Anson	0.09 cm.²/day
I. E. P.	Cataphoresis of collodion particles	pH 3.7
Molecular weight	Diffusion	8,000
Molecular weight	Tyrosine content	$(5,000)_n$

B

		Per cent of total nitrogen	No./molecule*
Primary amino N	Van Slyke gasometric	18	8–9
Amide N	Alkaline distillation after acid hydrolysis	6.5	3
Non-amino N	Difference between total N and NH_2N after acid hydrolysis	23	19
Peptide linkages	Difference between total NH_2-N after hydrolysis and original NH_2-N plus amide N		27
Arginine	Sakaguchi	31	8
Tryptophane	May and Rose	0	0
Tyrosine	Folin phenol	0.4	1

* Assuming a molecular weight of 5,000.

pH 5.7 acetate buffer; left 10 minutes at 35°C. 0.5 ml. of this solution is then pipetted into 5 ml. of 20 per cent Klim in M/10 pH 5.0 acetate at 35°C. and the time of clotting noted. It should be very close to 2 minutes.

B. Inhibitor.—1 ml. of the same pepsin solution as used in *A* is added to 1 ml. of a dilute (0.0001–0.0003 inhibitor units, [I.U.] per ml.) solution of inhibitor in M/10 pH 5.7 acetate and placed at 35°C. for 10 minutes after which 0.5 ml. is put into Klim and the time required for clotting noted.

C. Calculation of Inhibitor Activity and the Units.—The per cent inhibited is obtained by substituting the two clotting time values in the formula

$$\text{Per cent inhibited} = 100\left[1 - \left(\frac{\text{clotting time of inhibited solution}}{\text{clotting time of control solution}}\right)\right]$$

Having the per cent inhibited, one reads off directly from the curve in Fig. 5 the inhibitor units [I.U.] or the equivalent amount of pure inhibitor nitrogen. The unit inhibitor activity [I.U.] is the inhibiting activity of 1 mg. of N of the purest inhibitor such as that used in obtaining the curve in Fig. 5 which was crystalline and nearly solubility pure. Expressing it in another way, 2×10^{-4} [I.U.] will cause 50 per cent inhibition of the standard pepsin solution when treated as described above. This may be seen by examining Fig. 5. The specific inhibiting activity is merely the inhibiting activity per milligram of nitrogen $[\text{I.U.}]_{\text{mg.N}}$. When the inhibitor is pure the $[\text{I.U.}]_{\text{mg.N}}$ will, of course, then be 1.0.

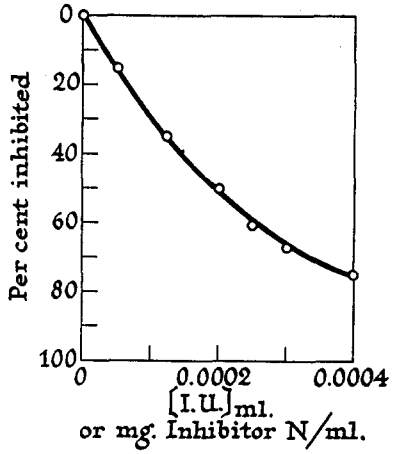

FIG. 5. Inhibitor-activity calibration curve

pH.—Unless otherwise stated all pH determinations were carried out with the aid of Clark and Lubs indicators. The pH values given are those of standard buffer solutions giving the same color with a proportional amount of indicator.

Nitrogen.—Nitrogen estimation was by the micro-Kjeldahl as previously described.

Pepsin.—The pepsin used in the estimation of inhibitor was a glycerinated 2 times crystalline Cudahy preparation having 300 rennet units per milligram protein nitrogen.

Pepsinogen.—The pepsinogen used to prepare the inhibitor was prepared exactly as previously described (1).

SUMMARY

A method has been described for the isolation and crystallization of swine pepsin inhibitor from swine pepsinogen.

Solubility experiments and fractional recrystallization show no drift in specific activity.

The reversible combination of pepsin with the inhibitor was found to obey the mass law.

The inhibitor is quite specific, failing to act on other proteolytic and milk clotting enzymes. The inhibitor is destroyed by pepsin at pH 3.5.

Chemical and physical studies indicate that the inhibitor is a polypeptide of approximately 5,000 molecular weight with an isoelectric point at pH 3.7. It contains arginine, tyrosine, but no tryptophane and has basic groups in its structure.

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