

ISOLATION FROM BEEF PANCREAS OF CRYSTALLINE TRYPsinOGEN, TRYPsin, A TRYPsin INHIBITOR, AND AN INHIBITOR-TRYPsin COMPOUND

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The isolation of trypsin from active pancreatic extract (1) and of chymo-trypsinogen and chymo-trypsin (2)¹ from fresh inactive pancreas has been described in previous papers. Crystalline trypsinogen has also been obtained (3) from inactive cattle pancreas and transformed into active trypsin which may then be crystallized much more readily than by the earlier method. During the course of this work a polypeptide, which has a powerful inhibiting effect on trypsin, as well as a compound of this substance with trypsin, was also obtained in crystalline form (4). The present paper contains detailed descriptions of methods of preparing these substances and a brief description of their properties.

GENERAL PROPERTIES

Trypsinogen

Trypsinogen is obtained as small triangular prisms (Fig. 1). When these crystals are dissolved in neutral solution the trypsinogen is rapidly transformed into active trypsin and it has, therefore, been impossible so far to recrystallize trypsinogen. The original crystallization occurs without activation owing to the presence of the inhibitor and if inhibitor is added to a solution of trypsinogen recrystallization may be carried out without activation. Numerous attempts have been made to recrystallize inhibitor-free trypsinogen under conditions

¹ It has recently been found that poorly formed needle-shaped protein crystals, which have about the same activity as the usual chymo-trypsin crystals, may be obtained from the mother liquor of the chymo-trypsin crystallization. The properties of these new crystals are now being investigated.

which would not at the same time cause activation, but so far without success. Analyses and properties of this substance are therefore somewhat uncertain.

The transformation of trypsinogen into trypsin is accelerated by the addition of trypsin or enterokinase or concentrated solutions of magnesium sulfate or ammonium sulfate (5). The addition of inhibitor retards activation by all three methods and a large quantity of inhibitor will completely prevent activation. A solution of trypsinogen

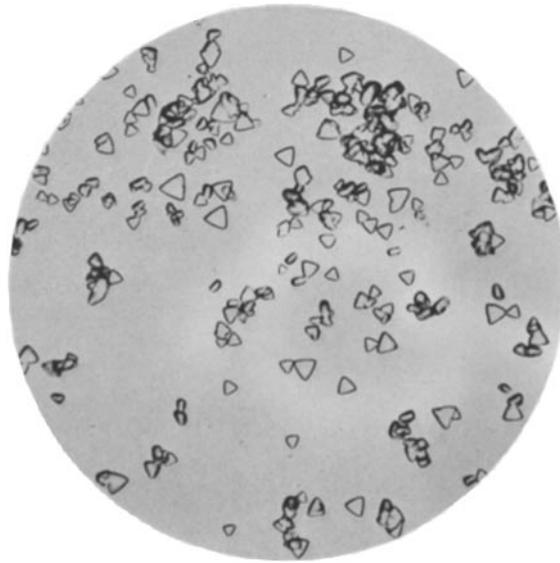


FIG. 1. Trypsinogen crystals.

to which inhibitor has been added behaves, therefore, just as the crude trypsinogen solution previously described (2). The fact that activation is accelerated by the addition of trypsin indicates that activation is autocatalytic and this is borne out by the kinetics of the reaction as shown in Fig. 2. Under these conditions the reaction follows quite closely that of a simple autocatalytic reaction. The rate of activation depends on the pH and is maximum at pH 7.0–8.0. It follows from this that if trypsinogen could be prepared completely free from active trypsin it would remain inactive. Owing to the extremely minute amounts of trypsin required to activate, however,

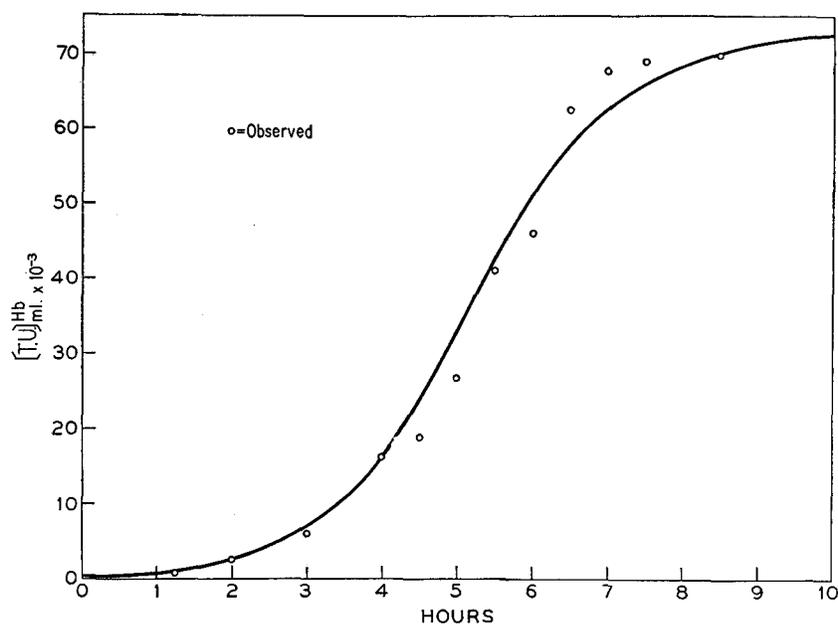


FIG. 2. Autocatalytic activation of crystalline trypsinogen. The smooth curve is calculated from the equation for a simple autocatalytic reaction

$$KA_e t = 2.3 \log \frac{A(A_e - A_0)}{A_0(A_e - A)}$$

where

$$K = 14.6$$

$$A_e = 0.072 \text{ (T. U.)}^{\text{Hb}}$$

$$A_0 = 0.0003 \text{ (T. U.)}^{\text{Hb}}$$

Crystalline trypsinogen—10 gm. crystalline filter cake dissolved in 200 ml. N/400 hydrochloric acid, 200 ml. 5 per cent trichloroacetic acid added. Left at 20°C. for ½ hour. Filtered. Precipitate dissolved in 25 times its weight of N/50 hydrochloric acid. Ammonium sulfate added to 0.4 saturated ammonium sulfate. Filtered. Filter cake dissolved in 3 times its weight of N/200 hydrochloric acid and dialyzed against N/200 hydrochloric acid at 6°C. 2 ml. dialyzed solution plus 2 ml. M/1 pH 5.0 acetate buffer plus 16 ml. water at 8°C. 1 ml. samples removed at intervals and added to 9 ml. M/75 hydrochloric acid. Activity determined by the hemoglobin method.

Filtrate brought to 0.7 saturated ammonium sulfate and filtered.

it has so far been impossible to obtain such preparations. Activation by the addition of enterokinase or concentrated magnesium or ammonium sulfate is also autocatalytic in type (5). The mechanism whereby the activation is accelerated by kinase and neutral salts is still uncertain.

Trypsin

Crystalline trypsin obtained from inactive pancreatic extract (Fig. 3) has, as a rule, slightly lower activity than that obtained from

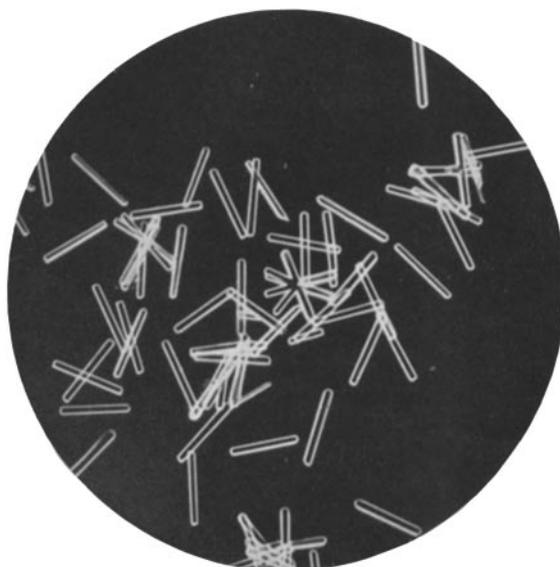


FIG. 3. Crystalline trypsin.

active extracts. This is due partly to the presence of small amounts of inactivated trypsinogen and partly to the presence of some inhibitor. The activity may be increased to the maximum value by repeated crystallization or more rapidly by precipitation with trichloroacetic acid. Either process results in trypsin having the same activity as that prepared from active pancreatic extract (see Table I). The composition, molecular weight, specific activity, etc. on various substrates are the same as previously described for trypsin from active pancreatic extract. These values are summarized in Table II.

The trypsin described above appears to be identical with the "proteinase" recently described by Waldschmidt-Leitz and Akabori (6). Proteinase was originally described (6) as having a lower activity on clupean than crystalline trypsin but according to Holter and the writers (7) both enzymes hydrolyze clupean at the same rate and to the same extent. This result has also been recently obtained by Professor Waldschmidt-Leitz (personal communication from Professor Waldschmidt-Leitz).

TABLE I
Activity of Various Preparations of Crystalline Trypsin

Preparation	[T. U.] _{mg. N} determined by		
	Gelatin viscosity pH 4.0	Hemoglobin	Casein formol
No. 50 prepared from crystalline trypsinogen—1 × crystallized	77	0.10	0.15
3 × "	73	0.12	0.15
5 × "	75	0.12	0.14
8 × "	90	0.14	0.15
3 × crystallized, precipitated + trichloroacetic acid (See Par. I-5)		0.16	
Crystals prepared from active pancreatic juice—1 × crystallized	98	0.17	
3 × "	120	0.15	
5 × "	{ 90 100	0.16	0.16

Inhibitor

It has long been known that pancreatic extracts contain some substance which inhibits trypsin and this was clearly brought out by Willstätter and Rhodewald (8). It has been suggested by Dyckerhoff, Miebler, and Tadsen (9) that trypsin exists in fresh pancreas as a compound with the inhibitor so that the activation of this inactive compound consists merely in the removal of the inhibitor and that no special inactive form of trypsin; *i.e.*, trypsinogen, exists. The actual isolation of trypsinogen, as described in the present paper, shows that this explanation is incomplete. The inhibitor, however, does play a very important part in regulating the activation of trypsinogen and there is no doubt that in partly activated pancreatic extracts more or less active trypsin occurs in the form of an inactive compound with

TABLE II
Analysis and General Properties

	Tryp- sinogen	Trypsin	Inhibitor-trypsin compound		Inhibitor
			Natural	Synthetic	
Elementary analysis per cent dry weight, calculated ash-free*	C	50.1	50.2	48.0	38.6
	H	6.9	6.6	6.9	7.6
	N	15.3	16.13	15.4	11.25
	S	1.1	1.1	—	—
	P	—	—	—	—
Protein nitrogen as per cent of total nitrogen		93	76	73	0
Amino nitrogen as per cent total nitrogen, formol					6.5
Van Slyke					6.5
Amino nitrogen as per cent total nitrogen after acid hydrolysis					80-90
Tyrosine + tryptophane: milliequivalents tyrosine per mg. total nitrogen		4.3×10^{-3}	3.7×10^{-3}	3.5×10^{-3}	2.1×10^{-3}
Optical rotation $[\alpha]^D$ per mg. nitrogen		-0.26°	-0.33°	-0.33°	-0.65°
		m/10 pH 5.5 acetate			In distilled water
Molecular weight by osmotic pressure in 0.25 saturated magnesium sulfate 6°C.		36,500	40,000		6,000
Specific activity [T. U.] per mg. protein nitrogen†	Substrate				
	Hemoglobin	—	0.17		
	Casein S	—	2.4		
	Casein F	—	0.16		
	Gelatin V	—	100.		
	Rennet	—	<0.1		
	Clot blood	—	1500.		
Sturin F	—	0.6			
Clupein F	—	0.8			
Clupean F	—	+			
Total digestion casein pH 8.0 ml. m/50 sodium hydroxide per 5 ml. 5 per cent casein		9-11			

* The trypsinogen and trypsin were prepared for analysis by precipitation with trichloroacetic acid followed by washing with trichloroacetic acid, distilled water, and alcohol and then dried with ether. The crystals of inhibitor-trypsin compound were washed with distilled water and alcohol and dried with ether. The inhibitor was dried in the form of filter cake with saturated magnesium sulfate. The analytical results are therefore of somewhat uncertain significance since they were not obtained upon the unchanged active preparations.

The elementary analyses were carried out by Dr. Elek in Dr. P. A. Levene's laboratory.

† Activity determinations were carried out as previously described (12). The sturin formol and clupein formol figures were obtained in exactly the same way as those for casein formol activity except that 5 cc. 5 per cent clupein or sturin solutions were used.

inhibitor. The inhibitor (Fig. 4) has the general properties of a polypeptide. It gives a faint biuret test and is precipitated by saturated magnesium sulfate or 0.7 saturated ammonium sulfate, but is not precipitated nor changed by 2.5 per cent trichloroacetic acid, either hot or cold, nor by boiling. It diffuses slowly through a collodion membrane and has a molecular weight, by osmotic pressure, of about 6,000. The carbon and nitrogen content (Table II) appear to be lower than usual for proteins. The amino nitrogen content is

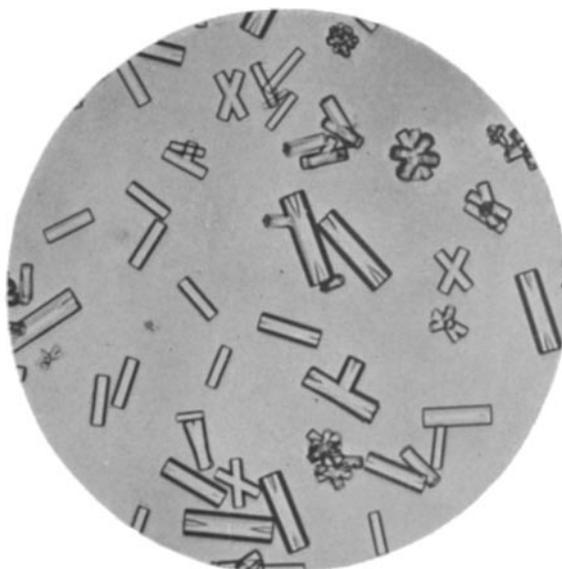


FIG. 4. Inhibitor crystals.

low but after acid hydrolysis amounts to 80–90 per cent of the total nitrogen so there is reason to believe that the substance is made up largely of amino acids.

Reaction between Inhibitor and Trypsin

When a solution of the inhibitor is mixed with a solution of trypsin of equal molecular strength at pH 7.0 the activity of the mixture decreases rapidly with time and after about $\frac{1}{2}$ hour at 6°C. it is completely inactive as measured by the digestion of hemoglobin. If the

solution is allowed to stand in the pH range of 7.0–3.0 it remains inactive upon addition to hemoglobin digestive mixture (10), but if titrated to pH 1.0 before addition to hemoglobin the activity rapidly reappears and in about $\frac{1}{2}$ hour will have completely returned. The cycle may be repeated indefinitely. The inhibitor evidently reacts with trypsin to form an addition compound which dissociates in acid

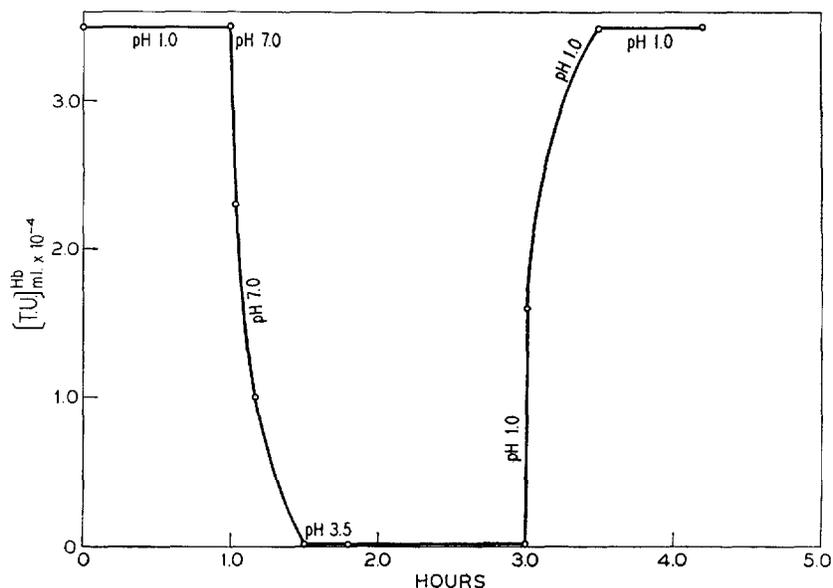


FIG. 5. Effect of standing at 6°C. and various pH on the activity of inhibitor-trypsin compound.

Solution of inhibitor-trypsin compound pH 5.0. 1.3 mg. P. N./ml. diluted 1/500 with 0.0025 N hydrochloric acid. pH changed as indicated. 1 ml. samples taken at various time intervals, added immediately to standard hemoglobin solution, and the amount of digestion determined after 10 minutes.

solution. Both dissociation and combination require measurable time intervals so that the reaction does not appear to be ionic. An experiment illustrating this inactivation and reactivation is shown in Fig. 5. This inactivating effect is also apparent when the activity is measured by the digestion of casein, clotting of blood, digestion of

sturin, or by the activation of chymo-trypsinogen or trypsinogen in the presence of salt. The substance also inhibits chymo-trypsin but to a less marked extent.

Inhibitor-Trypsin Compound

This substance is obtained in the form of hexagonal, many-faced crystals (Fig. 6). It consists of one molecule of inhibitor combined with one molecule of trypsin (Table III). These may be separated

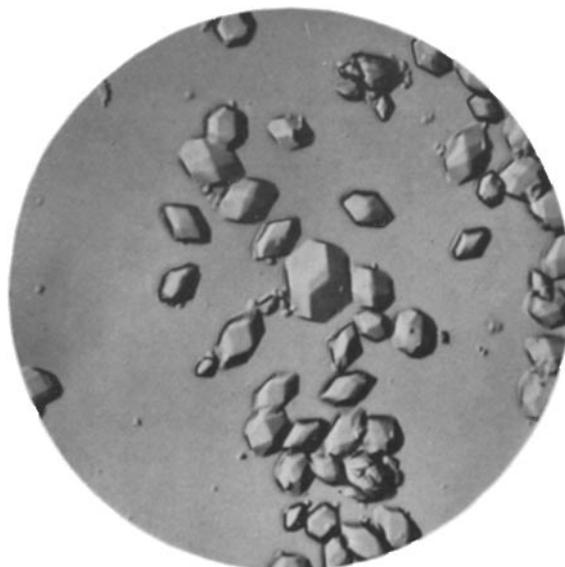


FIG. 6. Crystals of inhibitor-trypsin compound.

by precipitation with trichloroacetic acid which precipitates the trypsin and leaves the inhibitor in solution. As described under "inhibitor," the compound when dissolved in acid solution and added to protein solutions possesses the full tryptic activity but if allowed to stand for a short time at pH 7.0 and then added to the protein solution it is completely inactive. It differs in one marked respect from trypsin in that it is not adsorbed by egg albumin under the conditions described by Waldschmidt-Leitz (11), whereas trypsin itself is adsorbed under these conditions. The molecular weight by osmotic pressure is 40,000.

TABLE III
Composition of Inhibitor-Trypsin Compound (Corrected for Ash)

	Trypsin	Inhibitor	Inhibitor-trypsin compound	
	Observed	Observed	Observed	Calculated, assuming 1 molecule trypsin (36,500) + 1 molecule inhibitor (6,000)
C	50.2	38.6	48.0	48.6
H	6.60	7.62	6.90	6.70
N	16.13	11.25	15.40	15.40
O	27.1	42.50	29.70	29.30

Molecular weights

	Observed	Observed	Observed
	36,500 ± 2,000	6,500 ± 1,000	40,000
		Calculated* from per cent composition of trypsin, inhibitor, and inhibitor-trypsin compound, assuming molecular weight of trypsin 36,500	Calculated from molecular weight trypsin + inhibitor
		X	42,500
		C 8,500	
		H (15,000)	
		N 6,400	
		O 7,400	

* X = gm. inhibitor in 1 gm. mol inhibitor-trypsin compound

C_c = per cent carbon in compound

C_t = " " " " trypsin

C_i = " " " " inhibitor

$$\text{Then } X = \frac{36,500 C_t - C_c}{100 C_c - C_i}$$

X is calculated from the percentages of the other elements in the same way as shown above for carbon.

EXPERIMENTAL METHODS

The methods of preparing these compounds are given in the following sections. The yields reported represent average figures and may vary considerably in individual preparations but the proportion of precipitate to solvent specified in the text must be accurately adhered to. Weights of precipitates refer to the weight of the filter cake that is removed from the Büchner funnel. It is essential

for the success of the preparation that these filtrations be as complete as possible. Large Büchner funnels must be used, the filter cake pressed with a spatula so as to fill all the cracks, and the filtration continued until little or no foam is drawn through the funnel. As a rule, the dry precipitate should form a layer not more than 2–3 mm. thick. The preparations are all quite stable in the form of a moist filter cake if kept in the ice box. Permanent dry preparations may be obtained by allowing the filter cake from saturated magnesium sulfate to stand near the coils of a mechanical refrigerator. Under these conditions the water evaporates and a mixture of the dry crystals with the anhydrous salt remains. Such dry preparations keep indefinitely.

pH determinations were made on a test plate by mixing 1 drop of the Clark indicator with 1 drop of the solution. Standards were prepared by mixing 1 drop of the indicator with 1 drop of the standard buffer. This method, of course, gives only apparent pH values which may be considerably removed from the true pH of the solution. The method, however, is perfectly adequate for reproducing the necessary conditions.

The saturated magnesium sulfate and ammonium sulfate solutions were prepared at 20°C.

The method of preparation varies somewhat depending upon the desired product. The first method describes a complete fractionation whereby all of the substances may be obtained starting from fresh inactive cattle pancreas. This is the best method for obtaining chymo-trypsinogen and fair yields of trypsinogen but does not always give good yields of inhibitor-trypsin compound. The second method is the most convenient and reproducible method for obtaining trypsin and inhibitor-trypsin compound but yields no trypsinogen. The third method is the most efficient for the preparation of inactive trypsinogen. The fourth method describes the conversion of trypsinogen into active trypsin and the fifth method describes the isolation and crystallization of trypsin from active pancreas.

I. Isolation of Crystalline Chymo-Trypsinogen, Trypsinogen, and Trypsin Inhibitor from Fresh Cattle Pancreas

1. Preliminary Purification and Concentration²

Remove pancreas from cattle immediately after slaughter and immerse at once in enough ice cold 0.25 N sulfuric acid to cover the glands. Remove fat and connective tissue and mince in a meat chopper within a few hours. Suspend 3 liters

²The method is the same as that already described for the preparation of chymo-trypsinogen (2) up to the crystallization of this compound.

of minced pancreas in 6 liters of 0.25 N sulfuric acid at 5°C. and allow suspension to stand at about 5°C. for 18–24 hours. Strain the suspension through gauze, resuspend the residue in an equal volume of cold 0.25 N sulfuric acid, and strain through gauze immediately. Reject residue. Dissolve 242 gm. of solid ammonium sulfate in each liter of combined filtrate and washings. Filter through fluted paper (S. and S. No. 1450 $\frac{1}{2}$) in cold room. Reject precipitate. Dissolve 205 gm. of solid ammonium sulfate in each liter of filtrate. Heavy precipitate forms. Remove foam and allow to settle for 2 days at 5°C. Decant supernatant solution and filter residue with suction through hardened paper (S. and S. No. 575) on a large funnel. Yield about 100 gm. precipitate. Reject filtrate. Dissolve each 100 gm. precipitate in 300 ml. water, add 200 ml. saturated ammonium sulfate, stir in 5 gm. Standard Super Cel (Celite Corporation)³ and filter *with suction* through soft paper (S. and S. No. 1450 $\frac{1}{2}$). Reject precipitate. Add slowly 205 gm. solid ammonium sulfate to each liter of filtrate and filter with suction through hardened paper (S. and S. No. 575). *Precipitate A* about 90 gm. (mixture of crude chymo-trypsinogen, trypsinogen, and inhibitor). Reject filtrate.

2. Crystallization of Chymo-Trypsinogen

Dissolve each 90 gm. of precipitate A in 135 ml. water, add 45 ml. saturated ammonium sulfate, then adjust to pH 5.0 (brick red color with 0.01 per cent methyl red solution on test plate) by addition drop by drop of about 2 ml. 5 N sodium hydroxide. Allow to stand for 2 days at 20–25°C. A heavy crop of chymo-trypsinogen crystals gradually forms. Filter with suction through hardened paper (*Filtrate Tg*). Wash crystalline filter cake with 0.25 saturated ammonium sulfate and finally with saturated ammonium sulfate, and store at 5°C. Yield about 25 gm. For recrystallization and activation see (2).

3. Crystallization of Trypsinogen

Adjust filtrate and washings from chymo-trypsinogen crystallization (*Filtrate Tg*) to pH 3.0 (pink with 0.01 per cent methyl orange on test plate) with about 1 ml. 5 N sulfuric acid per 100 ml. filtrate. Dissolve 30.4 gm. of solid ammonium sulfate in each 100 ml. of filtrate and filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (40 gm.) in 120 ml. water, add 80 ml. saturated ammonium sulfate and 2 gm. filter cell, and filter with suction through soft paper. Wash paper with 0.4 saturated ammonium sulfate. Reject precipitate. Add slowly 100 ml. saturated ammonium sulfate to each 100 ml. of combined filtrate and washings. Remove foam and filter with suction through hardened paper, size 18.5 cm. or larger. Reject filtrate. Wash precipitate on funnel with saturated magnesium sulfate in 0.02 N sulfuric acid to remove excess of ammonium sulfate. The washing with saturated magnesium sulfate must be done rapidly, otherwise the precipitate is partly dissolved. Saturated magnesium sulfate is

³ This useful material was suggested by Dr. M. L. Anson.

poured on the precipitate to a height of about 5 mm., allowed to filter for a few minutes, then the excess of saturated magnesium sulfate is decanted, and filtration is continued until complete. Dissolve precipitate (30 gm.) in 30 ml. 0.4 M borate buffer pH 9.0 at 2–5°C. (in an ice water bath), add more borate buffer drop by drop to pH 8.0, measure solution, and add equal volume of saturated magnesium sulfate, mix and allow solution to stand in ice box at about 5°C. (*Solution B*). Short triangular prisms of trypsinogen appear in the course of 2–3 days. If the solution is inoculated with crystals of trypsinogen crystallization is much more rapid, but the crystals are not so well formed. (If crystallization is delayed more than 4–5 days, or if the material has become partly active during the preparation crystals of trypsin may appear.)

Filter the crystals with suction at 5°C. (*Filtrate C*). The precipitate (about 10 gm.) is washed on the funnel several times with cold 0.5 saturated magnesium sulfate made up in 0.1 M borate buffer pH 8.0 and finally with saturated magnesium sulfate made up in 0.1 N sulfuric acid at room temperature. The crystals are then dried in an electric refrigerator at 5°C. and stored in the ice box. The dried material generally contains about 40 per cent of trypsinogen protein and 60 per cent magnesium sulfate. For further purification of trypsinogen by means of trichloroacetic acid see legend to Fig. 2. For activation into trypsin see Section IV.

4. Crystallization of Inhibitor-Trypsin Compound

Combine Filtrates C and washings from several trypsinogen crystallizations (1000 ml.). Adjust to pH 3.0 with 5 N sulfuric acid and saturate with magnesium sulfate by stirring for 15 minutes at 25°C. with an excess of crystals of magnesium sulfate. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (150 gm.) in 750 ml. water and add 750 ml. 5 per cent trichloroacetic acid. Heat the mixture at 80°C. for 5 minutes, cool to 25°C., and filter with suction through hardened paper. Reject precipitate. Adjust filtrate to pH 3.0 with 5 M sodium hydroxide (about 3 ml. per 100 ml. of solution). Saturate with crystals of magnesium sulfate. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (15 gm.) in 45 ml. 0.02 M hydrochloric acid, add 1.5 gm. of crystalline trypsin, allow to stand until the trypsin is dissolved, adjust to pH 8.0 by addition of 0.4 M borate pH 9.0. Allow to stand for 1 hour at 5°C. Adjust to pH 5.5 by addition of 5 N sulfuric acid, saturate with crystals of magnesium sulfate at 25°C., allow to stand for 2 days at 20–25°C. Hexagonal crystals of inhibitor-trypsin compound gradually appear mixed with amorphous precipitate. Filter with suction. Wash precipitate on paper with 0.5 saturated magnesium sulfate. This dissolves the amorphous precipitate. Residue on paper—crystals of inhibitor-trypsin compound. Yield about 5 gm. Filtrate and washings, when saturated with crystals of magnesium sulfate, on standing may yield more crystals of inhibitor-trypsin compound.

Recrystallization of the Inhibitor-Trypsin Compound.—Dissolve the filter cake

of crystals (5 gm.) in 50 ml. *m*/10 acetate buffer pH 5.5, filter through Whatman No. 42 fluted paper. Saturate with crystals of magnesium sulfate and allow to stand 1 day at 20–25°C. Hexagonal crystals of the inhibitor-trypsin compound rapidly appear. Yield about 3 gm. filter cake.

5. *Isolation of Crystalline Trypsin Inhibitor and of Crystalline Trypsin from a Solution of Crystalline Inhibitor-Trypsin Compound*

Dissolve 1 gm. crystalline filter cake (of 3 times recrystallized inhibitor-trypsin compound) in 10 ml. water and add 10 ml. 5 per cent trichloroacetic acid; allow to stand at 20°C. for 30 minutes until precipitation is about complete. Filter with suction (*Filtrate In*). The *precipitate (Ts)* is worked up for trypsin, as described below.

(a) *Crystallization of Trypsin Inhibitor*.—Heat the trichloroacetic acid filtrate (*In*) for 5 minutes at 80°C., cool, and filter through fluted Whatman No. 42 paper. Reject precipitate. Adjust filtrate to pH 3.0 with 5 *m* sodium hydroxide. Dissolve 5.6 gm. of solid ammonium sulfate in every 10 ml. of filtrate. Filter with suction. Reject filtrate. Dissolve precipitate (0.25 gm.) in 2.5 ml. water, adjust to pH 5.5 with 0.4 *m* borate pH 9.0. Add equal volume of saturated ammonium sulfate. Filter through No. 42 filter paper. Wash paper with 0.5 saturated ammonium sulfate. Add more saturated ammonium sulfate to filtrate and washings combined until slight precipitate forms. The amorphous precipitate gradually changes into long hexagonal prisms. Allow to stand for 2 days at 20°C. Filter with suction. Filter cake 0.15 gm. of inhibitor crystals. Wash filter cake with saturated magnesium sulfate if it is desired to have the crystals free from ammonium salt, and recrystallize with magnesium sulfate.

Recrystallization.—Dissolve crystals (0.15 gm.) in 1.5 ml. of *m*/10 acetate buffer pH 5.5. Add 7.5 ml. saturated ammonium sulfate (or 7.5 ml. saturated magnesium sulfate plus a few crystals of solid magnesium sulfate). Allow to stand at 20°C. for 1 day. Crystals of inhibitor gradually appear. Yield about 0.1 gm. filter cake.

(b) *Crystallization of Trypsin*.—Wash the trichloroacetic acid precipitate (*Ts*) on the filter paper with water to remove the free acid. Dissolve precipitate (0.7 gm.) in 20 ml. 0.02 *m* hydrochloric acid. Allow to stand 30 minutes at 20°C. Add 5 gm. solid ammonium sulfate. Filter through fluted Whatman No. 42 paper until clear. Dissolve 5 gm. of solid ammonium sulfate in the filtrate and filter with suction through 5 $\frac{1}{2}$ cm. hardened paper. Reject filtrate. Wash precipitate on paper with saturated magnesium sulfate in 0.02 *N* sulfuric acid. Dissolve precipitate (0.5 gm.) in 0.25 ml. water. Cool to 5°C., add about 0.5 ml. borate buffer pH 9.0 until the solution reaches pH 8.0 (pink to 0.01 per cent phenol red but not to 0.01 per cent cresol red on test plate). Add 0.5 ml. saturated magnesium sulfate. Allow to stand at 5°C. Square prismatic crystals of trypsin rapidly appear. Filter with suction. Yield about 0.25 gm.

II. Isolation of Crystalline Trypsin and Inhibitor Trypsin Compound from Chymo-Trypsinogen Free Activated Pancreatic Extract

Preliminary purification and concentration is the same as described under I for preparation of Solution B (Section I, 3).

1. Crystallization of Trypsin

Inoculate Solution B (about 100 ml.) with trypsin crystals, allow to stand at 5°C. for several days. Precipitate of very small crystals of trypsin gradually forms. Occasionally a few triangular trypsinogen crystals may also appear. Filter with suction at 5°C. *Filtrate E*. Filter cake is washed on paper several times with 0.5 saturated magnesium sulfate at 5°C. and finally with saturated magnesium sulfate in 0.1 N sulfuric acid at room temperature. Yield 8 gm. of filter cake.

2. Recrystallization of Trypsin

Dissolve filter cake (8 gm.) in 6 ml. 0.02 N sulfuric acid. Add a few drops of 5 N sulfuric acid if solution is incomplete. Cool to 5°C., add 12 ml. saturated magnesium sulfate and 6 ml. 0.4 M borate pH 9.0. Adjust to pH 8.0 with saturated potassium bicarbonate or 5 N sulfuric acid if necessary. Inoculate. Allow to stand for 1 day at 5°C. Yield 3 gm. filter cake.

3. Purification of Trypsin by Trichloroacetic Acid

When first crystallized trypsin sometimes has a slightly low specific activity due partly to the presence of some inhibitor or trypsinogen. The activity may be raised to the maximum value by repeated recrystallization or, more easily, by precipitation with trichloroacetic acid followed by crystallization. The procedure for the latter method is the same as that described under Section I, 5 for the preparation of trypsin from inhibitor-trypsin compound except that the starting material is trypsin instead of inhibitor-trypsin compound.

4. Crystallization of Inhibitor-Trypsin Compound

Adjust Filtrate E (Section II, 1) to pH 3.0 with 5 N sulfuric acid and saturate with crystals of magnesium sulfate at 25°C. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (10 gm.) in 50 ml. M/16 hydrochloric acid and pour solution with stirring into a large beaker containing 250 ml. M/16 hydrochloric acid at 90°C. Cool after 1 minute in running cold water to 25°C. (use coils described elsewhere (1) if large quantities of solutions are used). Dissolve 24.2 gm. solid ammonium sulfate in each 100 ml. of solution, filter through fluted paper; dissolve 20.5 gm. of solid ammonium sulfate in each 100 ml. of filtrate, refilter with suction. Dissolve filter cake (3 gm.) in 12 ml. water, cool in ice water, add about 3 ml. 0.4 M borate pH 9.0 in order to bring the solution to pH

8.0, and pour with stirring into a large beaker containing 75 ml. boiling distilled water. Heavy precipitate forms. Cool after 1 minute in running cold water to 25°C. Dissolve 24.2 gm. of solid ammonium sulfate in each 100 ml. of suspension and filter with suction through hardened paper. Reject precipitate. Adjust filtrate to pH 3.0 by addition of several drops of 5 N sulfuric acid and then dissolve 20.5 gm. of solid ammonium sulfate in each 100 ml. of solution. Filter with suction on a large funnel. Reject filtrate. Wash precipitate with saturated magnesium sulfate.

Dissolve precipitate (1 gm.) in 5 ml. M/10 acetate buffer pH 5.5. Adjust to pH 5.5 with about 1 ml. 0.4 M borate pH 9.0. Filter through Whatman's No. 42 paper into a flask containing enough crystals of magnesium sulfate to saturate the solution. Wash filter paper with 4 ml. M/10 acetate buffer pH 5.5. Stir the solution after completion of filtration. Hexagonal crystals of inhibitor-trypsin compound rapidly appear. Allow to stand for 1 day at 20°C. to complete crystallization. Yield about 0.25 gm. filter cake. For recrystallization see Section I, 4.

III. Isolation of Trypsinogen from Fresh Beef Pancreas

Preliminary purification and concentration the same as described for preparation of Precipitate A (Section I, 1).

Wash Precipitate A with saturated magnesium sulfate on the filter paper. Dissolve precipitate (80 gm.) in 80 ml. 0.4 M borate buffer pH 9.0 at 5°C., add 136 ml. saturated magnesium sulfate. Adjust to pH 8.0 with a few drops of saturated potassium bicarbonate or 5 N sulfuric acid, if necessary. Inoculate with trypsinogen crystals. Allow to stand 2-3 days at 5°C. Filter with suction and wash crystals of trypsinogen with magnesium sulfate as described in Section I, 3. Yield about 10 gm. filter cake. Adjust filtrate to pH 3.0, saturate with magnesium sulfate at 25°C., filter with suction, wash with saturated ammonium sulfate, and proceed for isolation of chymo-trypsinogen (partly active), trypsin, and inhibitor-trypsin compound as described in Section I, 2, 3, up to preparation of Solution B and then in Section II.

IV. Conversion of Trypsinogen into Active Trypsin and Crystallization of Trypsin

If the trypsinogen is in the form of semi-dry filter cake then it is treated exactly as in the procedure described for recrystallization of trypsin (Section II, 2). Dried trypsinogen containing dry magnesium sulfate is treated as follows: Dissolve 10 gm. in about 50 ml. of water. Add a few drops of 5 N sulfuric acid if solution is incomplete. Saturate with excess of crystals of magnesium sulfate by stirring for 15 minutes at 25°C., filter with suction. Dissolve precipitate (20 gm.) in 20 ml. 0.4 M borate buffer pH 9.0 at 5°C. Add 34 ml. saturated magnesium sulfate. Adjust to pH 8.0 with a few drops of saturated potassium bicarbonate or 5 N sulfuric acid, if necessary. Inoculate with trypsin crystals. Allow to

stand 2-3 days at 5°C., then filter, and recrystallize for trypsin. Yield about 5 gm. filter cake.

V. Isolation of Crystalline Trypsin from Active Pancreatic Extracts

The starting point for this method may be either frozen pancreas which has been stored for some time until it is active or fresh pancreas which has been allowed to stand in the cold for several days until the extracts are active. The active pancreas is minced and extracted with two volumes of 0.25 N hydrochloric or sulfuric acid for 18-24 hours at 5°C. The acid extract is filtered and the filtrate is treated as described previously in Paragraph 2 of Table IV (1) for the preparation of Precipitate 3 and the procedure followed until Precipitate 5 is obtained. Precipitate 5 is then washed with saturated magnesium sulfate in 0.02 M sulfuric acid. Each 30 gm. of precipitate is dissolved in 30 ml. 0.4 M borate buffer pH 9.0 at 2-5°C., the solution titrated to pH 8.0 by the addition of borate buffer, and an equal volume of saturated magnesium sulfate added. This solution corresponds to Solution B (II, 1) and is then treated as described under II, 1 for the crystallization of trypsin from Solution B.

Most of the activity determinations and some of the preparative work described in this paper were carried out by Miss Margaret R. McDonald.

SUMMARY

Methods are described for the isolation and crystallization of trypsinogen, trypsin, a substance which inhibits trypsin, and an inhibitor-trypsin compound. Analyses and some of the properties of these compounds are given.

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