

ISOLATION OF A CRYSTALLINE PROTEIN COMPOUND OF TRYPSIN AND OF SOYBEAN TRYPSIN-INHIBITOR

BY M. KUNITZ

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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Trypsin combines with soybean trypsin-inhibitor to form a stable protein compound which has been isolated in crystalline form.

The compound consists approximately of equal parts by weight of the two components. The pure compound does not show either proteolytic or inhibitory activity. It exhibits, however, trypsin activity if it is partly denatured by heat in acid solution, and it shows also trypsin-inhibiting power if denatured in alkaline solution. The release of either trypsin or inhibitor is due to the difference in the rate of reversal of denaturation of these proteins.

Method of Crystallization of the Trypsin-Soy Inhibitor Compound

The compound crystallizes readily at pH 6-8 from a salt-free solution of a mixture of trypsin and soybean inhibitor. The crystallization is accelerated in the presence of about 20 per cent alcohol.

The details of isolation of the crystals of the new protein are as follows:

1. *Preliminary Step.*—1 gm. of dry soy inhibitor crystals is suspended in 40 ml. of distilled water at about 5°C. The mixture is titrated with 0.2 M NaOH to about pH 7.5. This brings about complete solution of the inhibitor crystals. 1 gm. of a preparation of dry crystalline trypsin (containing about 50 per cent anhydrous MgSO₄) is then added slowly with stirring. The mixture is thus made to contain an excess of inhibitor in order to avoid any proteolysis by trypsin.

The pH, if necessary, is readjusted to 7.5 with several drops of 0.1 M borate buffer pH 9.0. The solution, if turbid, is filtered with suction with the aid of 1 gm. of supercel on a small Büchner funnel. The residue on the funnel is washed with about 5 ml. of cold H₂O. The washings, if clear, are added to the main bulk of the filtrate which is titrated with 0.1 M HCl to about pH 6.0 and dialyzed overnight against slowly running distilled water at 5-10°C., preferably with stirring.

A granular precipitate gradually forms in the dialysis bag.

The dialyzed suspension is titrated with a few drops of 0.1 M HCl to pH about 5.4 (tested on a drop plate with 0.01 per cent solution of methyl red). The suspension is centrifuged. The residue yields the crystalline compound; the supernatant solution (designated as "first supernatant solution") contains the excess of soy inhibitor used which can be partly recovered.

2. *Crystallization of the Compound.*—The residue is suspended in about 20 ml. cold H₂O and recentrifuged. The washed residue is resuspended in 40 ml. of H₂O at about

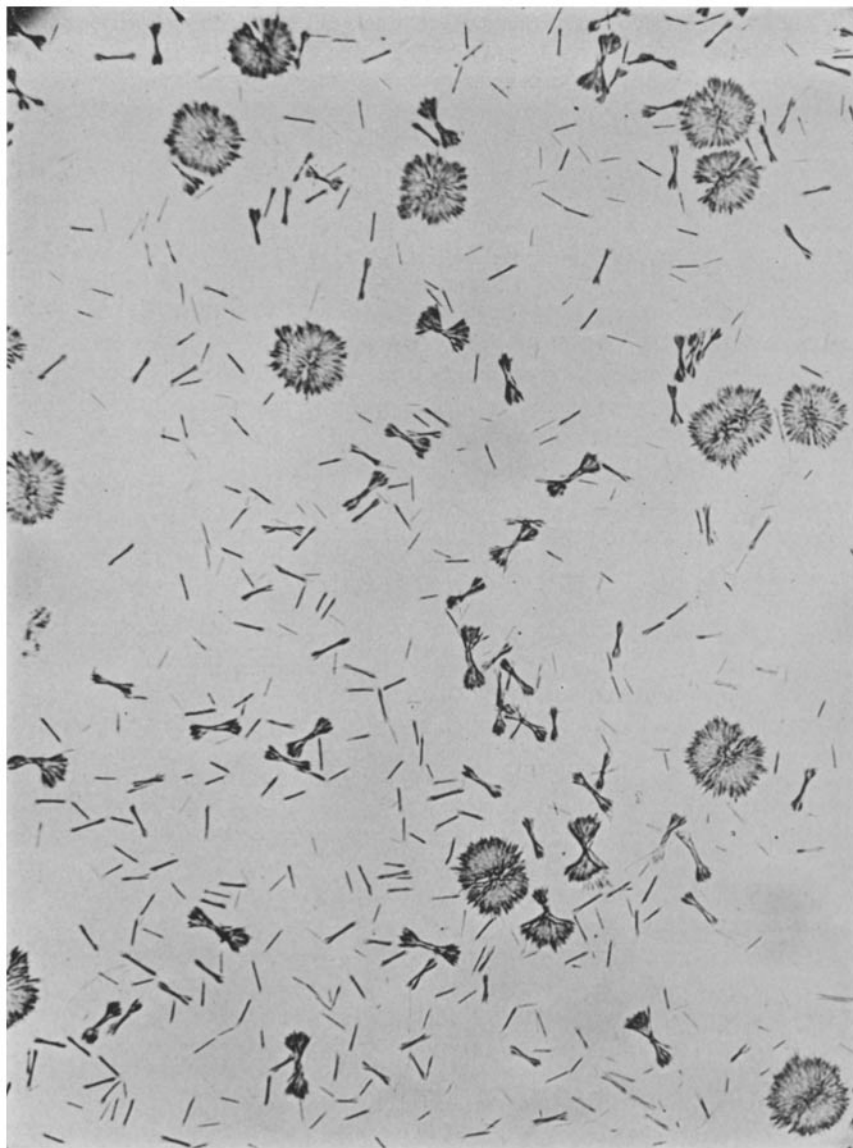


FIG. 1. Compound of trypsin and of soybean inhibitor crystallized in water. $\times 220$.

5°C. and titrated drop by drop with 0.2 or 0.5 M NaOH to pH 9.0 (pink to 0.1 per cent phenolphthalein on test plate), when complete solution generally occurs. The solution is then titrated with a few drops of 0.2 M HCl to very slight opalescence and stored at about 20°C. Fine crystals in the form of small rosettes or bundles of needles and plates gradually appear (Fig. 1). The suspension of crystals is centrifuged after a

day or so; several drops of 0.1 M HCl are added to the supernatant solution until a slight turbidity is formed. The solution is stored for several hours at 20°C. A second crop of fine crystals generally appears which is centrifuged on the top of the first crop of crystals. More acid is added to the supernatant solution and the process is repeated until pH 5.8 is reached, or until the final acidified supernatant solution no longer yields crystals. It is rejected or is combined with the "first supernatant solution" to be worked up for soy inhibitor, as described in step 7.

3. *Recrystallization of the Compound.*—The crystals are suspended in about 20 volumes of cold water and titrated with several drops of 0.5 M NaOH to incipient clearing. The solution is allowed to stand for 5 to 10 minutes at 5°C. and then filtered, if turbid, on fluted No. 3 paper moistened with cold water pH 9.0. The filter paper is washed once with cold water. The clear filtrate and washing is titrated with several drops of 0.2 M HCl to very slight opalescence. It is seeded and left at 20°C. Crystallization is generally complete within 24 hours. The crystals are centrifuged. The supernatant solution is titrated with 0.2 M HCl to slight turbidity and left at 20°C. for several hours. A second crop of crystals is obtained which is centrifuged in the flask containing the first crop of crystals. The operation is repeated several times until no further yield of crystals is obtainable. The supernatant solution is treated as described in step 5.

4. *Drying of Crystals.*—The combined crystals are resuspended in a small amount of distilled water and filtered with suction on hardened paper. The crystals are dried for 24 hours in a mechanical refrigerator at about 5°C. and then in a desiccator over anhydrous CaSO₄ (drierite) at 20°C. The dried material is ground fine in a mortar and stored in a refrigerator.

5. *Crystallization in Dilute Alcohol.*—The final supernatant solution in step 3 may further yield crystals if it is cooled to 5°C., $\frac{1}{4}$ of its volume of cold 95 per cent alcohol is added, and the pH of the solution is adjusted with 0.2 M HCl to 5.8. A precipitate forms which, when left at 20°C., changes gradually into rosettes of fine plates (Fig. 2). The crystals are filtered on hardened paper and dried first in a refrigerator and then in a desiccator over anhydrous CaSO₄ (drierite).

6. *Recrystallization in Dilute Alcohol.*—The dry crystalline powder is suspended in about 50 times its weight of water. (The centrifuged residue of crystals, not dried, is suspended in 25 times its volume of water.) The suspension is titrated with several drops of 0.5 M NaOH to pH 9.0. The crystals gradually dissolve when left for about 10 minutes at 5°C. The solution, if turbid, is filtered and then $\frac{1}{4}$ of its volume of cold 95 per cent alcohol is added. The pH of the solution is adjusted to about 5.8. A heavy precipitate is formed which changes into crystals on storing for a day or two at 20°C.

The crystallization in alcohol is more rapid and the yield of crystals is greater than in the absence of alcohol. There is also the advantage that the alcohol keeps the solution sterile. There is however the possibility that alcohol causes slight denaturation of the protein.

7. *Partial Recovery of Excess of Inhibitor.*—"The first supernatant solution," of step 1, is titrated with 0.5 M HCl to pH 4.65 at 5°C. and centrifuged at about the same temperature. The supernatant solution is rejected. The residue is suspended in about 5 volumes of cold water and is titrated with 0.2 M NaOH to pH 5.2. Any



FIG. 2. Compound crystallized in 20 per cent alcohol. $\times 239$.

precipitate left undissolved is centrifuged off and is rejected (or worked up for compound as described in step 2).

The supernatant solution is cooled to 5°C. and $\frac{1}{4}$ of its volume of 95 per cent alcohol of 5°C. is added. The solution is adjusted with several drops of 0.1 M HCl to pH

5.0, is seeded with soy inhibitor crystals and left at 30°C. Crystals of inhibitor gradually form. The crystals are filtered after several hours, washed with cold acetone, and dried in the room. Yield 0.1 to 0.2 gm. of dry soy inhibitor crystals.

Some of the Properties of the New Crystalline Compound

Isoelectric Point.—The compound is a globulin and is least soluble at about pH 5.2.

The isoelectric point of the compound is at about pH 5.0, as tested by cataphoresis.

Stability.—The compound is stable over a wide range of pH if kept at a temperature below 30°C. and is practically free of either proteolytic or inhibitor activity.

Reversible Heat Denaturation at pH 3.0. Release of Trypsin.—Heating of a solution of the compound at pH 3.0 results in the denaturation of the compound. The denaturation is accompanied by an apparent release of the two components, both in denatured form. On cooling there is a reversal of denaturation of both components, the rate of reversal however being greater for trypsin than for the soy inhibitor, so that the reversed material acts like a mixture of native trypsin and denatured soy inhibitor protein. This results in rapid autolysis if the solution is made slightly alkaline soon after cooling.

Experimental Procedure.—

(a) 10 ml. of 0.25 per cent solution of crystalline compound in 0.0025 M HCl (final pH about 3.0) was heated for 5 minutes in boiling water, then placed for 2 minutes at -5°C. 1 ml. 0.5 M phosphate buffer pH 7.4 was added and the mixture was transferred to 36°C. Samples of 1 ml. were taken at various times, added to 5 ml. 5 per cent trichloroacetic acid, and heated for 10 minutes at 85°C. The mixtures were centrifuged after standing in the room for about 1 hour. The protein content of the supernatant solution was then determined spectrophotometrically at 280 m μ as described elsewhere(1).

(b) The same experiment was repeated with a similar solution of the compound which had not been heated in boiling water.

(c) A solution of 0.25 per cent crystalline trypsin in 0.0025 M HCl was heated in boiling water and then treated as in (a).

(d) A similar solution of crystalline trypsin was not heated but otherwise treated as in (a).

The results are shown in Fig. 3.

There is a striking difference in the rate of autolysis of the heated compound as compared with the autolysis of the non-heated sample. The heated sample is autolyzed initially at a rate approximately equal to that of the same concentration of trypsin. The autolysis of the compound gradually slows down and it stops when about 70 per cent of the total protein is digested. This is probably caused by the gradual reversal of the denatured inhibitor part of the mole-

cule. There is no striking difference in the rate of autolysis of heated and non-heated trypsin, since the heated trypsin is rapidly reversed to its native state on cooling (2).

The gradual release of free trypsin on heating of the compound at 60°C. in acid solution is demonstrated in the following experiment.

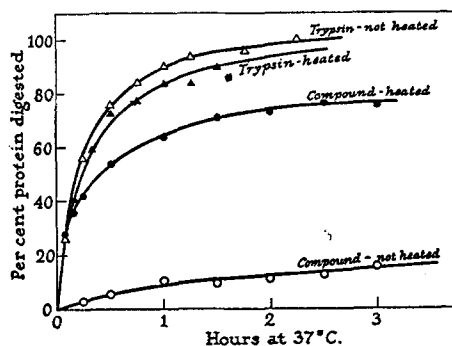


FIG. 3. Autolysis of heat-denatured compound compared with autolysis of trypsin.

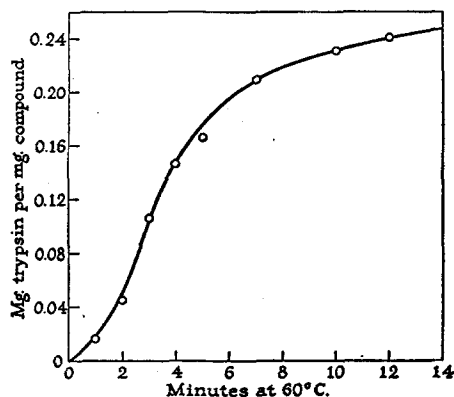


FIG. 4. Release of trypsin from compound on heating at 60°C.

Experimental Procedure.—A set of 1 ml. samples of 0.1 per cent solution of compound in 0.0025 M HCl was placed at 60°C. in order to bring about gradual denaturation. Samples were mixed at various times with 5 ml. 0.06 M acetate buffer pH 4.5 which had been cooled to -5°C . The addition of buffer pH 4.5 prevents the reversal of denaturation of the inhibitor part of the molecule but does not interfere with the reversal of denaturation of the trypsin part of the molecule. The tryptic activity of the acetate mixtures was then measured by the effect on digestion of casein (1).

The results are shown in Fig. 4. The gradual appearance of free trypsin is due to the gradual denaturation of the compound on heating at 60°C. The

release of free trypsin reaches however only 50 per cent of the theoretical value. This may be due to partial reversal of denaturation of the inhibitor in the dilute casein solution at pH 7.6.

Release of Inhibitor from Compound on Denaturation in NaOH at 36°C.

It has been shown (3) that trypsin is rapidly and irreversibly denatured at pH above 12.0. Soy inhibitor is likewise denatured in alkaline solution but the denaturation of the inhibitor is reversible if the treatment in alkali is not prolonged. It is thus possible to isolate free inhibitor by exposing the compound to the action in 0.1 M NaOH for about 5 minutes at 36°C. and then neutralizing

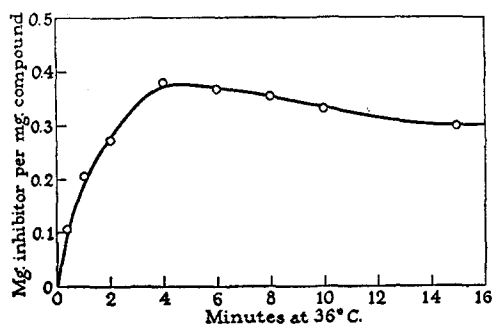


FIG. 5. Release of inhibitor from compound on denaturation in 0.09 M NaOH at 36°C.

the solution. The appearance of free inhibitor in a solution of the compound in 0.09 M NaOH is demonstrated in the following experiment.

Experimental Procedure.—1 ml. of 0.5 per cent solution of crystalline compound was mixed with 9 ml. 0.1 M NaOH and placed at 36°C. in order to bring about gradual denaturation of the compound. Samples of 1 ml. were mixed at various times with 9 ml. 0.01 M HCl. The neutralized mixtures were left for 18 hours at 10°C. to bring about the complete reversal of denaturation of the inhibitor part of the compound. The solutions were then tested for inhibiting activity as described elsewhere (1). The results are shown in Fig. 5. There is a rapid release of inhibitor, the maximum being reached in about 4 minutes at 36°C. The gradual falling off in the later part of the curve is evidently due to the secondary reaction, namely, the irreversible denaturation of the inhibitor on long exposure to the alkali treatment.

In the experiment just described, the speed with which free inhibitor was formed depended on the rate of denaturation of the compound in the alkaline solution at 36°C. In the following procedure the compound was rapidly and completely denatured by heating a slightly alkaline solution of the material for 2 minutes at 90°C. The solution, after acidification, was left at 25°C. The

denatured inhibitor rapidly regained its activity. The rate of formation of free inhibitor in this case was dependent entirely on the rate of the reversal of denaturation of the inhibitor fraction of the compound.

Experimental Procedure.—2 ml. of stock of 0.1 per cent solution of crystalline compound in 0.0025 M HCl was adjusted with 0.4 ml. 0.02 M NaOH to pH about 8.0. The clear solution was heated for 2 minutes at 90°C. and then mixed with 35 ml. of ice cold 0.0025 M HCl. The mixture was left at 25°C. Samples of 1 ml. were tested at various times for inhibiting activity. The results are shown in Fig. 6. There is a rapid and complete gain in free inhibitor on standing at 25°C.

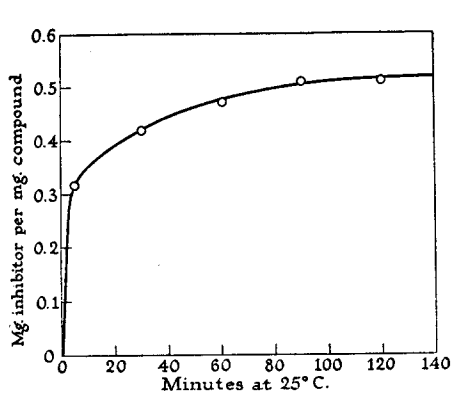


FIG. 6

FIG. 6. Release of inhibitor from denatured compound on reversal of denaturation.

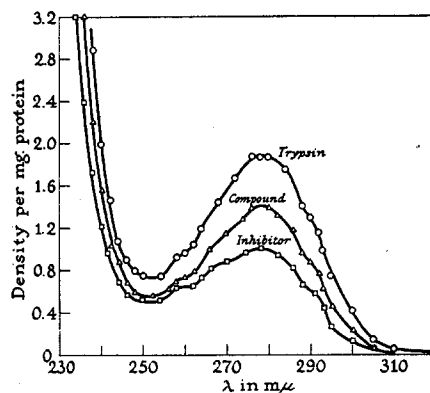


FIG. 7

FIG. 7. Ultraviolet light absorption spectra of trypsin, soy inhibitor, and compound.

The Combining Weight of Trypsin and Soy Inhibitor

It has been shown in the preceding paper (1) that pure crystalline soy bean inhibitor counteracts the proteolytic activity of about an equal weight of pure crystalline trypsin. The reaction results in the formation of the inert compound. The crystalline compound isolated from a mixture of trypsin and the soy inhibitor does apparently consist of about equal weights of the two components. This is shown by an analysis of some of its "extensive" properties such as tyrosine and tryptophane contents, ultraviolet light absorption, and optical rotation per unit weight of the compound and also of trypsin and the soy inhibitor, as given in Table I. The values of these properties per unit weight of compound are approximately equal to the average values of the properties in the two components. The formula for the value of C of any "extensive" property per unit weight of the compound in terms of the values a and b of the property per unit weight of trypsin and of the inhibitor respec-

tively, is $C = \frac{a + rb}{1 + r}$ where r is the ratio of weight of inhibitor to that of trypsin in the compound.

When $r = 1$

$$C = \frac{a + b}{2} = \text{average}$$

Molecular Composition of the Compound

No satisfactory data are available at present on the molecular weight of the

TABLE I
Comparison of Some of the Properties of Trypsin, Soybean Inhibitor, and of Trypsin-Soy Inhibitor Compound

	Tyrosine*	Tryptophane†	Optical rotation $[\alpha]_D^{25}$	Optical density at 280 mμ‡	Free amino nitrogen by formal titration (§)
	per cent protein	per cent protein	per gm. per ml.	per mg. per ml.	per cent total N
Trypsin.....	7.8	3.65	-45	1.88	6.90
Soy inhibitor.....	4.0	2.23	-105	1.05	3.95
Average.....	5.9	2.94	-75	1.47	5.43
Compound.....	6.1	2.89	-72	1.40	4.30

* Kindly determined by Miss Jean Grantham in the laboratory of Dr. E. Brand in the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, following NaOH hydrolysis by the method of Brand, E., and Kassell, B., *J. Biol. Chem.*, 1939, **131**, 489.

† Tryptophane was determined by the method of R. W. Bates (4).

‡ See Fig. 7.

compound and no definite conclusion can be drawn as yet on the molecular composition of the compound in terms of moles of trypsin and inhibitor.

The Nature of the Reaction between Trypsin and the Soy Inhibitor

It has been pointed out in the preceding paper that the reaction between trypsin and the inhibitor is apparently instantaneous and that it resembles the ordinary acid-base neutralization. An approximate estimation of the free amino nitrogen of the three materials indicates a slight loss in free amino nitrogen on formation of the compound. (See last column of Table I.) The reaction between trypsin and the soybean inhibitor consists apparently in neutralization of free amino groups of trypsin by free carboxyl groups of the soy

inhibitor protein. Further analysis of the amino acid composition of the three materials may elucidate this phase of the problem.

SUMMARY

A crystalline protein compound has been isolated from a solution containing crystalline trypsin and crystalline soybean inhibitor. The protein consists of about equal weights of trypsin and of the inhibitor. Denaturation by heat or by alkali resolves the compound into its components.

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