CRYSTALLINE SOYBEAN TRYPsin INHIBITOR

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1. Method of Isolation

In a recent publication (1) the writer reported the isolation from soybean meal of a crystalline protein of the globulin type which inhibits the proteolytic action of trypsin.

The method of isolation of the inhibitor crystals consists essentially in the following operations:

Commercial soybean meal, defatted by a solvent extraction method by the manufacturer, is washed in the laboratory with 80 per cent ethyl alcohol. This washing facilitates the filtrations throughout the subsequent operations. The inhibitor, which is stable in acid, is then extracted from the meal with 0.25 N sulfuric acid. The acid extract is treated first with a small amount of bentonite which adsorbs some of the inert protein and is rejected, and then with a larger amount of bentonite—enough to adsorb all the inhibitor protein, leaving behind other materials in the acid extract.

The inhibitor is eluted from the bentonite by means of a dilute aqueous pyridine solution, and the pyridine is removed by dialysis.

The dialyzed solution is partly purified by adjusting the pH to 5.3 and filtering off the precipitate of inert material formed. The inhibitor protein is then precipitated from solution by adjusting the pH to 4.65 at 5-10°C. Finally a concentrated solution of the amorphous material is adjusted with acid to pH 5.1 and left at 36°. Crystals in the form of fine needles and thin hexagonal plates gradually appear (Fig. 1). The crystallization is generally complete in 5 to 6 hours. The inhibitor crystals can be recrystallized from solution by addition of alcohol (Fig. 2). The crystals are then filtered, washed with cold acetone, and dried in the air at room temperature. The dried material retains its crystalline structure, is doubly refractive, and has the same inhibiting power on trypsin as the non-dried crystals isolated in the absence of alcohol.

The details of the method of isolation are as follows:

1. Washing with 80 Per Cent Alcohol.—1000 gm. cold-processed, defatted soybean meal1 is added to a mixture of 2400 ml. 95 per cent alcohol cooled to

1 Soybean meal, Nutrisoy XXX, in the form of flakes, supplied by the Archer-Daniels-Midland Co., Chicago, Illinois, was used throughout this work. According to the manufacturer's statement, "the flakes were processed by the solvent extraction method of manufacture and no heat was used."
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Fig. 1. Soybean trypsin inhibitor crystallized in H₂O. × 225.

5°C. and of 450 ml. distilled water. The suspension is stirred well and left at
20–25°C. for 30 minutes. It is then filtered with suction on a 32 cm. Büchner
funnel through filter cloth.² The filtrate is rejected.

² Filter cloth, 146 T W, supplied by the Filter Media Corporation, Irvington-on-
Hudson, N. Y.
2. Extraction in 0.25 N Sulfuric Acid.—The semidry meal is resuspended in 5000 ml. 0.25 N sulfuric acid (7 ml. concentrated H₂SO₄ per liter of water) at 20–25°C. and left for 1 hour at room temperature with occasional stirring. The suspension is refiltered with suction on the same filter cloth. The meal residue is rejected.
3. Removal of Inert Protein by Means of Bentonite.—20 gm. of a stock mixture of one part by weight of bentonite (U.S.P. Powder, Amend Drug and Chemical Co., New York) and an equal part of hyflo super cel (Johns-Manville Corporation) is added to the acid filtrate and stirred for 10 minutes. The suspension is filtered with suction on 32 cm. No. 303 E & D filter paper (The Eaton-Dikeman Co., Mt. Holly Springs, Pennsylvania). The residue on the paper is washed twice with portions of 125 ml. of water. The residue is rejected.

4. Adsorption of the Inhibitor on Bentonite.—100 gm. of the stock of bentonite-super cel mixture is added gradually to the combined filtrate and washings from step No. 3. The solution is stirred gently while the bentonite mixture is added and the stirring is continued for 10 minutes. The suspension is filtered and the residue is washed, as described in step No. 3. The filtrate and washings are rejected.

5. Elution with Pyridine and Dialysis.—The bentonite residue is stirred up with 270 ml. of water. At this stage the suspension can be stored in the refrigerator overnight. The suspension is warmed to 25° C. and 30 ml. of pyridine (Stock No. 214, Eastman Kodak Co.) is added with stirring. The thick suspension is filtered with suction on 24 cm. No. 303 E & D filter paper in a hood. The filtration generally requires several hours. The residue on the funnel is washed once with 200 ml. 5 per cent pyridine in water. The combined filtrate and washing is dialyzed overnight in 12 inch long cellophane tubings placed in a tall jar with running tap water, in the hood, if possible.

6. Removal of Inert Material at pH 5.3.—The dialyzed solution, free of any gummy residue adhering to the dialysis tubes, is adjusted to pH 5.3 with the aid of about 2 ml. of 1 N HCl. (The pH is tested by the drop method on a plate using 0.1 M acetate buffers as standards and 0.01 per cent methyl red as an indicator.) 4 gm. of bentonite-super cel mixture is stirred into the solution which is then filtered with suction on a 15 cm. No. 303 E & D filter paper and the residue is washed several times with 15 ml. portions of water. The washings, if not clear, are refiltered. The residue is rejected.

7. First Precipitation of the Inhibitor at pH 4.65.—The combined filtrate and washings of step No. 6 is cooled to 5°C. and then titrated with 1 N HCl to pH 4.65 (tested carefully with 0.05 per cent bromcresol green on a drop plate). A heavy precipitate is formed which is filtered off at 5–8°C. on 15 cm. No. 303 E & D filter paper on a Büchner funnel without suction. The filtration is completed with very light suction. Weight of filter cake 10 to 12 gm. The filtrate is rejected.

8. Second precipitation at pH 4.65.—The filter cake is suspended in 100 ml. of water cooled to 5°C., the water being added gradually to the precipitate and incorporated thoroughly with a porcelain spatula. 1 N NaOH is added

27/32 "Nu Jax Visking Cellulose Sausage Casing," manufactured by The Visking Corporation, Chicago, Illinois.
drop by drop with stirring until the precipitate is dissolved. Care should be taken, however, not to raise the pH of the solution above 6.4. The clear solution is warmed to 25°C and titrated slowly with 1 N HCl until a slight permanent precipitate is formed. Two gm. of standard super cel is stirred into the solution which is then filtered with suction on 7 to 11 cm. No. 303 E & D filter paper. The residue on the paper is washed with several milliliters of water. The combined filtrate and washing is cooled to 5°C and titrated to pH 4.65. It is filtered with light suction on 15 to 18 cm. No. 612 E & D filter paper at 5–8°C. Yield about 8 to 10 gm. of filter cake which is stored in refrigerator. The filtrate is rejected.

9. Crystallization. The filter cake of step No. 8 (about 10 gm.) is ground up to a uniform suspension with 10 ml. of cold water and then warmed to about 35°C. 0.5 N NaOH is added drop by drop with careful stirring until the precipitate is almost completely dissolved and the pH of the solution is about 5.2. The clear solution is decanted into a 50 ml centrifuge tube. Any residue in the beaker is stirred up with 1 to 2 ml. of cold water, dissolved with the aid of a drop of 0.1 N NaOH, and added to the main bulk of solution in the centrifuge tube which is then placed at 35–37°C for crystallization. A heavy sediment of crystals is obtained within 5 to 6 hours. Inoculation with a few crystals greatly facilitates the process of crystallization. The suspension is centrifuged for 10 minutes at about 3000 R.P.M. The residue is stored in the refrigerator, while the supernatant liquid is either stored or, if time permits, titrated with a few drops of 0.2 N HCl to pH 5.1 at 36–37°C, inoculated, and left at that temperature. Another crop of crystals is gradually formed which is centrifuged off after several hours and added to the first crop of crystals. The supernatant liquid is rejected.

It is preferable to begin step No. 9 in the morning, so as to be able to centrifuge before the end of the day. The crystals, as well as supernatant solutions, should be stored overnight in the refrigerator.

10. Recrystallization.—The combined crystal residues of step No. 9 (about 7 ml.) are stirred up with twice the volume of cold water and titrated with 0.5 N NaOH to clearing, the final pH being about 6.0. The clear solution is warmed to 35°C and titrated with 0.5 N HCl to pH 5.1 when a slight permanent precipitate is formed. The solution is mixed with 2 gm. standard super cel and filtered clear with suction on a small No. 303 E & D filter paper. The filtrate is inoculated and left at 36–37°C. A heavy suspension of crystals forms gradually and is centrifuged after 5 to 6 hours. The residue is stored at 5°C. The pH of the supernatant liquid is readjusted with 1 to 2 drops of 0.2 N HCl to pH 5.1, inoculated, and left for several hours longer at 36–37°C when another crop of crystals is formed which is centrifuged off and added

4 It is advisable to begin the crystallization (step No. 9) with at least 50 gm. of amorphous precipitate collected from several preparations.
11. Crystallization in Dilute Alcohol.—Centrifuged crystals are stirred up with five times the volume of cold water, and 0.5 N NaOH is added drop by drop until the crystals are all dissolved. The pH of the solution is not allowed, however, to rise above 6.6. The clear solution is titrated with 0.2 N HCl to pH 5.2. Any precipitate formed is filtered off with suction on No. 303 filter paper with the aid of 4 gm. of standard super cel per 100 ml. of solution. The residue on the funnel is washed with several milliliters of water. The volume of the filtrate and washings is measured and the solution is then cooled in an ice-water bath to about 5°C. A quarter of its volume of 95 per cent alcohol, cooled to 5°C., is added slowly to the cold solution. A heavy precipitate is formed. The pH of the mixture is adjusted with 0.2 N HCl to 5.0 and left at 30°C. The amorphous precipitate changes within 2 hours into well formed hexagonal and rhomboid crystals and plates (Fig. 2) which settle rapidly to the bottom of the vessel. The supernatant solution is decanted every hour, adjusted with 0.2 N, or more dilute HCl to pH 5.0, and returned to the original vessel containing the settled crystals. This is continued for several hours until no formation of precipitate is noticed on adjusting the pH of the supernatant solution to 5.0. The crystallization mixture is then allowed to stand at 30°C. for 30 minutes longer and then filtered with suction on hardened paper, washed on the funnel several times with cold acetone, and allowed to dry in the room for 24 hours. It is stored in the refrigerator.

12. Recrystallization in Alcohol.—The dry crystals are suspended in thirty times their weight of cold water, allowed to soak for 5 to 10 minutes, and then treated exactly as in step No. 11.

Yield

The yield of trypsin inhibitor crystals varies considerably with the stock of soybean meal used. Nutrisoy XXX generally yields about 1 gm. of four times crystallized inhibitor per 1000 gm. of meal.

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BIBLIOGRAPHY