CRYSTALLIZATION OF SALT-FREE CHYMOTRYPSINOGEN AND CHYMOTRYPSIN FROM SOLUTION IN DILUTE ETHYL ALCOHOL

By M. KUNITZ

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

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The original methods developed for the isolation of crystalline chymotrypsinogen and chymotrypsin involve the use of ammonium sulfate (Kunitz and Northrop, 1935). It has recently been found possible to crystallize the purified preparations of chymotrypsinogen and chymotrypsin from dilute solutions of ethyl alcohol free of any salts. The crystals of both materials obtained in the presence of alcohol differ in form from those obtained in the presence of ammonium sulfate. The enzymatic properties of the new crystals, however, are identical with those of the crystals isolated from solution in the presence of ammonium sulfate.

Several enzymes and other proteins have recently been crystallized from solution in dilute alcohol in the presence or absence of salts. Ribonuclease has been crystallized from salt-free 50 per cent alcohol (Kunitz, 1940). Northrop (1946) reported the crystallization of pepsin from solution in 15 to 20 per cent alcohol in the presence of low concentrations of magnesium sulfate. Soybean trypsin inhibitor and also the trypsin-soybean inhibitor compound were found to crystallize readily from salt-free 20 per cent alcohol (Kunitz, 1946, 1947). Cohn, Hughes, and Weare (1947) employed alcohol in the presence of low concentrations of acetate buffer for the crystallization of human and bovine serum albumins.

The methods of crystallization of chymotrypsinogen and chymotrypsin from alcohol are as follows:—

1. Chymotrypsinogen
   
   (a) Preliminary Treatment.—Chymotrypsinogen is isolated from fresh beef pancreas and is recrystallized several times in the presence of ammonium sulfate, as described in the original publication on the isolation of chymotrypsinogen (Kunitz and Northrop, 1935).

   (b) Removal of Ammonium Sulfate by Dialysis.—Ten gm. of semidry filter-cake of crystals of chymotrypsinogen is stirred up with about 30 ml. of distilled water and is dissolved with the aid of several drops of 5 N sulfuric acid. The solution is then dialyzed in a collodion or cellophane bag against slowly running distilled water for 24 hours at about 5°C, preferably with stirring.

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1 Alcohol as an aid in crystallization of proteins was employed as early as 1882 by Hüffner and Otto (1882) for the isolation of methemoglobin.
(c) Crystallization from Alcohol.—The dialyzed solution of chymotrypsinogen is filtered clear and then made up with water to 50 ml. The pH of the solution is adjusted with dilute acid or alkali to about 4.0. The solution is cooled in an ice-salt bath to 1-3°C. and 12.5 ml. of ice cold 95 per cent alcohol is added slowly with stirring; the temperature of the solution is not allowed to rise above 5°C. during the addition of the alcohol. The pH of the solution is then adjusted with 1 N sodium hydroxide to about 5.0. A heavy amorphous precipitate forms at pH 5.0. The suspension is kept at 20 to 25°C. The precipitate gradually dissolves and is replaced within several hours by a crop of large, well formed crystals shown in Fig. 1. The crystallization is generally complete within 24 hours. The crystals are filtered with suction on hardened paper, washed with ice cold acetone, and dried in the room for 24 hours. The dried material is ground up in a mortar to a fine powder and is stored in a refrigerator.

The dry powder dissolves readily in dilute sulfuric acid at pH about 3.0 and yields crystalline chymotrypsin when treated as described in the original publication (Kunitz and Northrop, 1935).

A heavy suspension of the dried preparation of chymotrypsinogen in water at pH 5.0 and 20°C. gradually begins to yield long needles similar to those formed on crystallization of chymotrypsinogen in the presence of ammonium sulfate. The formation of the needles is readily demonstrable by preparing on a microscope slide under a cover slip a small drop of a thick suspension of the dry crystals in water. The needles begin to appear within 2 to 3 minutes and the progress of the formation and the growth of the needle crystals can thus be observed through a microscope.

2. Chymotrypsin

(a) Preliminary Treatment.—Chymotrypsin is prepared from crystalline chymotrypsinogen with the aid of trypsin as a catalyst, and is recrystallized once or twice in the presence of ammonium sulfate (Kunitz and Northrop, 1935).

(b) Removal of Salt by Dialysis.—Ten gm. of crystalline filter cake is dissolved in 30 ml. of cold water and then dialyzed against slowly running 0.005 N sulfuric acid at about 5°C. for about 24 hours, preferably with stirring.

(c) Crystallization from Alcohol.—The dialyzed solution is filtered clear, then made up with water to a volume of 50 ml., and cooled in an ice-salt bath to 2-3°C. The pH of the solution is adjusted to about 4.8 with the aid of several drops of 1 M sodium hydroxide. Ten ml. of ice cold 95 per cent alcohol is added slowly with stirring.

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2 The pH is tested by the drop method on a test plate using 0.1 M acetate buffer pH 5.0 as a standard and 0.01 per cent methyl red solution as an indicator.

3 The crystalline material on drying apparently changes into an amorphous material which evidently possesses a greater solubility in water at pH 5.0 than the needle crystals.

The crystallization of pure chymotrypsinogen in form of needles from a concentrated solution of the protein in water at pH 5.0, even in the absence of salt, has been recently observed and reported by Jacobsen (1947).
while the temperature of the solution is maintained at about 5°C. The solution is then titrated with 1 N sulfuric acid to pH 4.0 (light green to brom cresol green on test plate). A heavy amorphous precipitate is formed. The suspension is kept at about 5°C. The amorphous precipitate slowly changes into a paste of very fine needles and

Fig. 1. Chymotrypsinogen crystals in dilute alcohol. × 117
rosettes (Fig. 2). Seeding with a drop of a suspension of the crystals assures prompt crystallization within 24 hours. The paste of crystals is filtered with suction on hardened paper at about 5°C. The filtration generally takes several hours. The filter cake is dried on a watch glass placed for several days near the cooling coil in an
electric or gas refrigerator. The dry glassy material is ground in a mortar to a fine powder and stored in the refrigerator.  

The dry material dissolves readily in water at pH 3 to 4. It yields the usual rhombohedrons when recrystallized in the presence of ammonium sulfate at pH below 5.0.

The protease and milk-clotting activity per milligram protein of the dry salt-free powder is identical with those of the preparations of chymotrypsin crystallized in the presence of ammonium sulfate.

**SUMMARY**

Chymotrypsinogen and chymotrypsin crystallize readily from dilute solutions of ethyl alcohol in the absence of salts. The crystals formed in the presence of alcohol differ in appearance from those formed in the presence of ammonium sulfate. Chymotrypsinogen yields well formed polyhedrons instead of fine needles usually produced in ammonium sulfate solution. Chymotrypsin yields fine needles in the presence of alcohol and rhombohedrons in the presence of ammonium sulfate. The enzymatic properties of the crystals formed in the presence of alcohol are identical with those of the crystals isolated in the presence of ammonium sulfate.

**BIBLIOGRAPHY**


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4 The use of acetone as an aid in drying of the crystals is to be avoided because of the danger of partial denaturation of the relatively labile chymotrypsin.