DIRECT CRYSTALLIZATION OF LYSOZYME FROM EGG WHITE AND SOME CRYSTALLINE SALTS OF LYSOZYME

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A method of isolating and crystallizing lysozyme, the bacteriolytic substance in egg white, has been reported from this Laboratory (1). Crystallization was effected from acid solutions of purified material containing approximately 5 per cent salt, and it was also accomplished in the isoelectric region (pH 9.5 to 11.0) as a result of markedly lower solubility of the crystalline product in that region. Since that report was made, methods have been improved and lysozyme has been crystallized as the salt of several acids. In addition it has been crystallized directly from egg white by a method which markedly facilitates isolation in pure form.

Crystal Forms of Lysozyme

The previous report (1) states that the crystal form of lysozyme varied with the pH of crystallization and the acid radical with which it was combined. Table I presents briefly the results obtained when electrodialyzed, isoelectric lysozyme was crystallized after it had been dissolved to various pH values with hydrochloric acid in 5 per cent sodium chloride solution. Two forms of crystals were obtained. The form shown in Fig. 1 was obtained below pH 7.0; between pH 7.0 and 11 the type shown in Fig. 2 was obtained.[1] In appearance the crystals formed at any pH above 7.0 seem identical. They differ chemically, however, in that those formed in the isoelectric region are relatively insoluble in water, whereas those formed below pH 9.0 are increasingly soluble with decreasing pH. Apparently enough of the basic groups are combined with acid to make the product soluble, but the crystal form remains unchanged. Below pH 7.0, the form is markedly different and a form typical of lysozyme chloride is obtained, presumably because of combination of more and varied basic groups with acid.

Crystallization at the isoelectric point is readily accomplished by adjusting a 5 per cent solution of amorphous lysozyme chloride in 5 per cent sodium chloride solution to pH 10.5 with sodium hydroxide. No pre-

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[1] In some of the preparations at acid pH (4.8 or above) some needle-like crystals formed after standing for some time.

cipitation of amorphous material takes place, but on standing crystalline material begins to separate and continues to do so until the lysozyme in solution is reduced to approximately 0.1 per cent. The presence of increasing amounts of sodium chloride up to 5 per cent increases the amount

TABLE I

Effect of pH on Crystallization of Lysozyme

Original protein concentration, 4 per cent; salt concentration, 5.0 per cent.

pH	Degree of crystallization*	Crystal form
2.0	No crystals or ppt.	
3.5	Copious crystallization	Fig. 1
4.8	Moderate "	" 1
5.8	Very slight crystallization	" 1
7.2	" " "	" 2
8.2	Copious crystallization	" 2
9.2	" "	" 2
10.0	" "	" 2
11.3	" "	" 2

* Visual estimation after 48 hours of crystallization.

FIG. 1. Lysozyme hydrochloride; pH 4.5. × 80.

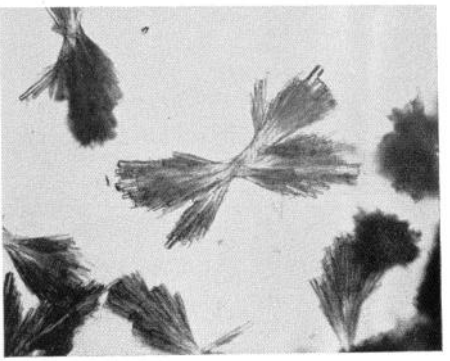

FIG. 2. Isoelectric lysozyme. × 80

of amorphous material that can be dissolved in the isoelectric region, but less crystalline material may be dissolved in 5 per cent salt than in lower salt concentrations.

Lysozyme has also been crystallized as the iodide (Fig. 3) and the bromide (Fig. 4) by dissolving 200 mg. samples of electrodialyzed isoelectric lysozyme in 10 ml. of solution of the appropriate acid at acid pH (bromide pH 6.0, iodide pH 4.0) and adding 5 per cent of the corresponding sodium

or potassium halide. On standing at room temperatures, crystals were deposited from both solutions. These crystals were needle-like, as shown, and thus were different in form from those obtained when hydrochloric acid was used at the same pH.[2]

Crystallization of lysozyme from a 1 per cent protein solution in nitric acid at pH 4.0 containing 5 per cent potassium nitrate resulted in the crystal form shown in Fig. 5. These crystals are different in form from any of the others.[3]

The most rapid crystallization of lysozyme was effected from bicarbonate solutions. Isoelectric lysozyme readily dissolves in carbonic acid and, when sodium bicarbonate (5 per cent) was added, crystallization began within a few minutes. The crystals were well defined, small needles (Fig. 6).

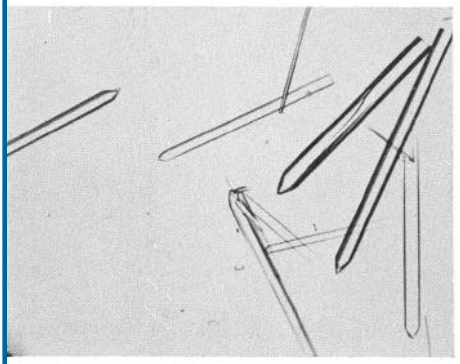

FIG. 3. Lysozyme hydroiodide. × 80

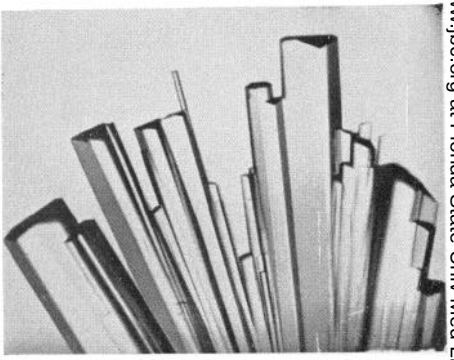

FIG. 4. Lysozyme hydrobromide; pH 6. × 80.

Attempts have been made to crystallize other salts of lysozyme, such as the acetate, sulfate, and tartrate, without success. The first crystalline lysozyme preparation was obtained from an acetate buffer on addition of ammonium sulfate but we have never been successful in obtaining crys-

[2] Determination of the combined chloride in the lysozyme hydrochloride crystals prepared at pH 4.3 and 8.2 gave values of 3.24 and 1.99 per cent chlorine, respectively. With 17,500 as the molecular weight for lysozyme, sixteen and ten basic groups are therefore combined with hydrochloric acid at pH 4.3 and 8.2, respectively. Similar analyses of the hydrobromide crystals at pH 4.0 gave 7.6 per cent bromine, or sixteen to seventeen combined groups. These values were arrived at by determining total halogen and sodium in the crystalline preparations and then calculating the combined halogen by subtracting the amount of halogen equivalent to the cation.

[3] Crystallographic characterization of the crystal forms will be reported by F. T. Jones of this Laboratory.

talline material from a system which contained only the sulfate or the acetate anion.

Direct Crystallization of Lysozyme from Egg White

The fact that 5 per cent solutions of lysozyme in 5 per cent sodium chloride at or near the isoelectric point readily yielded crystalline material, and that the crystalline material is only slightly soluble under these conditions, suggested the possibility that crystallization could be induced from egg white directly.

It was found in the initial experiments that egg white, freed of chalazae and homogenized, with adjustment to pH 10.8 and addition of 5 per cent sodium chloride, yielded no precipitate and no crystalline material when allowed to stand for several days. However, when a suspension of iso-

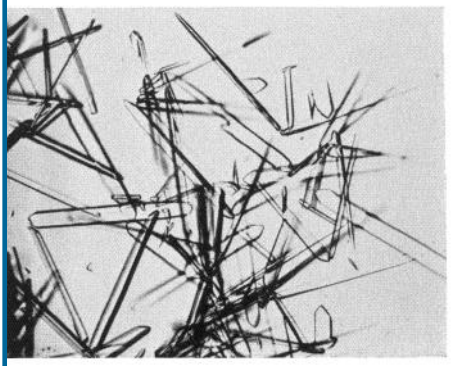

FIG. 5. Lysozyme nitrate. × 80

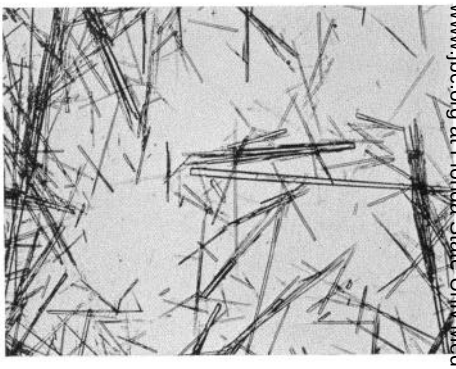

FIG. 6. Lysozyme carbonate. × 80

electric crystalline lysozyme was added to the egg white solution under the same conditions, crystallization was induced and 50 per cent of the lysozyme of the egg white was obtained as fine needle-like crystals.

In an attempt to improve the yield, a series of experiments was carried out to determine the effect on crystallization of pH in the isoelectric region, salt concentration, and temperature. The alkalinity was adjusted as desired by addition of 1 N potassium hydroxide. The results (Table II) show that the yield of crystalline material was markedly affected by the temperature of crystallization. Very low yields were obtained at −8° and 23°, as compared with 4°. Also, a pH of 9 to 9.5 resulted in more rapid crystallization than when higher alkalinities were used.

Yields of 60 to 80 per cent of the total lysozyme contained in egg white were regularly obtained after egg white had been allowed to stand for 3 to 4 days at 4°. The egg white, after adjustment to pH 9.5 and addition of

5 per cent sodium chloride, must be seeded with small amounts of crystalline lysozyme. Occasionally crystallization will set in without seeding but not in most instances. The crystalline product thus obtained is redissolved in acetic acid (pH 4 to 6) and any insoluble material is removed by centrifugation and discarded. The soluble material is then recrystallized after addition of 5 per cent sodium chloride and adjustment to pH 9.5 to 11.0. This recrystallization can also be quickly accomplished by making the acid solution of the crystals 5 per cent with respect to sodium bicarbonate (pH 8.0 to 8.5).

TABLE II

Effect of Salt Concentration, pH, Temperature, and Time on Crystallization of Lysozyme from Egg White

Effect of salt concentration (pH 10.5, 4°)			Effect of pH (salt concentration 5 per cent, 4°)			Effect of temperature (pH 10.5, salt concentration 5 per cent)		
Salt	Yield*		pH	Yield*		Temperature	Yield*	
	20 hrs.	72 hrs.		20 hrs.	72 hrs.		20 hrs.	72 hrs.
per cent	*per cent of total*	*per cent of total*		*per cent of total*	*per cent of total*	°C.	*per cent of total*	*per cent of total*
0	0	5	9.0	72	86	−8	5	23
1	2	7	9.5	76	88	4	12	75
2	5	11	10.0	62	84	23	5	12
4	16	62	10.5	18	75			
5	18	75	11.0	10	64			
6	10	74						
8	6	72						

* Determined by the assay method of Boasson (2).

SUMMARY

Lysozyme has been crystallized over the pH range of 3 to 11 from 5 per cent sodium chloride solutions. Two distinct crystal forms were obtained, one below pH 7.0 and the other at basic reactions. The chloride, bromide, iodide, nitrate, and carbonate of lysozyme have been prepared in crystalline form.

A method of crystallizing lysozyme from egg white directly has been described. Yields of 60 to 80 per cent were obtained.

We are indebted to F. T. Jones for photomicrographs of the crystalline material, and to L. M. White and C. M. Johnson for the halogen and sodium analyses.

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2. Boasson, E. H., *J. Immunol.*, **34**, 281 (1938).