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The Multiple Nature of Crystalline Egg-White Lysozyme. (31697)

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Early in the course of our studies on radiation effects in egg-white lysozyme(1,2), it became necessary to determine the purity of commercial crystalline preparations of the enzyme. Two or three times recrystallized material as supplied commercially is usually prepared by the method of Alderton and Fevold(3). However, this crystalline lysozyme has already been shown to be composed of at least 3 fractions by chromatography on columns of Amberlite XE-64(4), calcium phosphate columns(5) and carboxymethylcellulose columns(6).

Our work required gram amounts of homogeneous enzyme to permit the detection of small amounts of products arising from irradiation. Initial purification attempts involved passing solutions of the crystalline material through large preparative columns of BioRex 70 prepared and eluted essentially according to the procedure of Tallan and Stein(4). The 3 components previously recognized could be easily observed, but the shape of the elution curve for the main component and rechromatography experiments indicated homogeneity had not been obtained. Modification of the eluting buffer system and a slower rate of elution increased resolution to the point that as many as 7 components can now be demonstrated in crystalline lysozyme.

Materials and methods. Preparative columns measuring 6.0 × 75.0-80.0 cm were packed with BioRex 70, 200-400 mesh (Bio-Rad Laboratories). This resin is a weakly acidic carboxylic cation exchanger similar in nature to the XE-64 resin employed by Tallan and Stein(4). The resin had been previously placed in the hydrogen form by stirring 1 kg resin with 4 liters 3 *N* HCl overnight at room temperature. The resin was washed with distilled water until the filtrate was at pH 6 and then was suspended in 0.2 *M* potassium phosphate buffer, pH 7.09. The pH of the suspension was adjusted to pH 7.09 by the addition of 20% potassium hydroxide with continuous stirring. After filtering off the resin it was washed with 0.2 *M* potassium phosphate, pH 7.09, 10 liters per kg original resin weight. Fines were removed by suspending the resin in buffer (4 liters per kg), letting settle 20 minutes, and decanting the supernatant. This step was repeated until no cloudiness was visible in the decanted solution. Then, the resin was suspended in an equal volume of buffer and the slurry poured into the columns. The bottom of the column was fitted with coarse fritted glass disc on top of which was packed a half-inch layer of glass wool to prevent plugging of the disc during column operation.

Buffer was permitted to drain from the column under gravity pressure until the buffer surface was 1 to 2 inches above the top of the resin bed. Then, another aliquot of slurry was added and this step repeated until the desired column height was achieved. Buffer was passed through the column until the eluate emerging from the bottom was at pH 7.09. Then, 3.0 to 10.0 g crystalline egg-white lysozyme (Worthington Biochemical Corp. or Pentex, Inc.) was dissolved in buffer

(10 ml per g) and applied to the column-top slowly so as not to disturb the resin surface. After entry of the sample solution into the column, the walls were washed down with two 10 ml aliquots of buffer and the column was eluted at room temperature (23-26°C) with 0.2 *M* potassium phosphate buffer, pH 7.09, at a rate of 1 ml/minute maintained by a Beckman solution metering pump, Model 746. No microbial growth was noted during the 5 days required for column elution. A newly packed column was used for each chromatographic run.

Protein concentrations in the eluate were calculated from optical density measurements at 280 mμ(7), assuming an optical density value of 2.64 for a solution containing 1 mg protein per ml in a 1.0 cm light path cell. If a fraction had an optical density greater than 1.6 at 280 mμ, the optical density at 300 mμ was determined to obtain a rapid assessment of peak position and height. Enzymic activity measurements employed lyophilized *Micrococcus lysodeikticus* cells (Worthington Biochemical Corp.) as substrate according to the procedure of Shugar (8) at a temperature of 30°C maintained in a Beckman Model DU spectrophotometer fitted with a thermospacer assembly.

Prior to enzymic assays and molecular weight measurements, water and salts were removed from pooled column peak fractions. The most successful procedure used was that of Dixon(9) in which the protein solution was brought to a concentration of 5% in acetic acid by the addition of glacial acetic acid. To this solution was added a sufficient amount of BioRex 70, —400 mesh, hydrogen form, to absorb the protein as determined by optical density measurements at 280 mμ on the filtrate after 1 hour stirring in an ice bath. The resin had been previously equilibrated with 5% acetic acid prior to use. After adsorption of the protein, buffer salts were removed by washing repeatedly on a sintered glass funnel with 5% acetic acid until phosphate could no longer be detected in the filtrate, as judged by formation of a blue color with molybdate and 1-amino-2-naphthol-4-sulfonic acid reagents(10). Then, the protein was eluted from the resin with 50% acetic acid by stirring in an ice bath and filtering. Acetic acid and water were removed by lyophilization. After this desalting procedure, the dry protein from a given peak could be rechromatographed on BioRex 70 and 99% of the sample applied recovered as a single peak at the expected elution volume, as predicted from the initial purification column. The desalting procedure afforded 75-90% recovery of the protein present in pooled peak fractions.

Molecular weight values were calculated from osmotic pressure measurements and, in some cases, from retention volumes on columns of Sephadex G-100 (Pharmacia). Osmotic pressure (π) measurements were made on 4 to 6 concentrations (c) of a particular fraction ranging from 0.5 to 10.0 g% using the Mechrolab Model 503 low temperature (5°C) automatic membrane osmometer. The solvent used was 0.055 *M* sodium citrate, 6.0 *M* urea, adjusted to pH 3.75 with 1.0 *N* hydrochloric acid. The ratio π/c was plotted against c and extrapolated to $c = 0$. This extrapolated value of π/c was used in the equation $\pi/c = RT/M_n$ to calculate M_n, the number average molecular weight of a given fraction(11).

Retention volumes on Sephadex G-100 columns were obtained by a modification of the method of Whitaker(12). The gel was swollen in a 6.0 *M* urea solution containing 0.5 *M* sodium acetate and 0.1 *M* sodium chloride, pH 7.01. 4.0 × 120.0 cm columns of this material were packed and washed with the same solution for 6 days at 1 meter hydrostatic pressure. Ten to thirty mg of protein were dissolved in 2.0 ml of the solution, applied to the column and eluted with the urea solution described above. Complete details on protein standards and calculations of molecular weights are given by Whitaker (12).

Results and discussion. The major modification made in the procedure of Tallan and Stein(4) was the substitution of potassium for sodium as the cation in the 0.2 *M* phosphate buffer system. The pH of the eluting buffer was lowered about one-tenth of a unit; this increases the extent to which the various components are bound to the columns and,

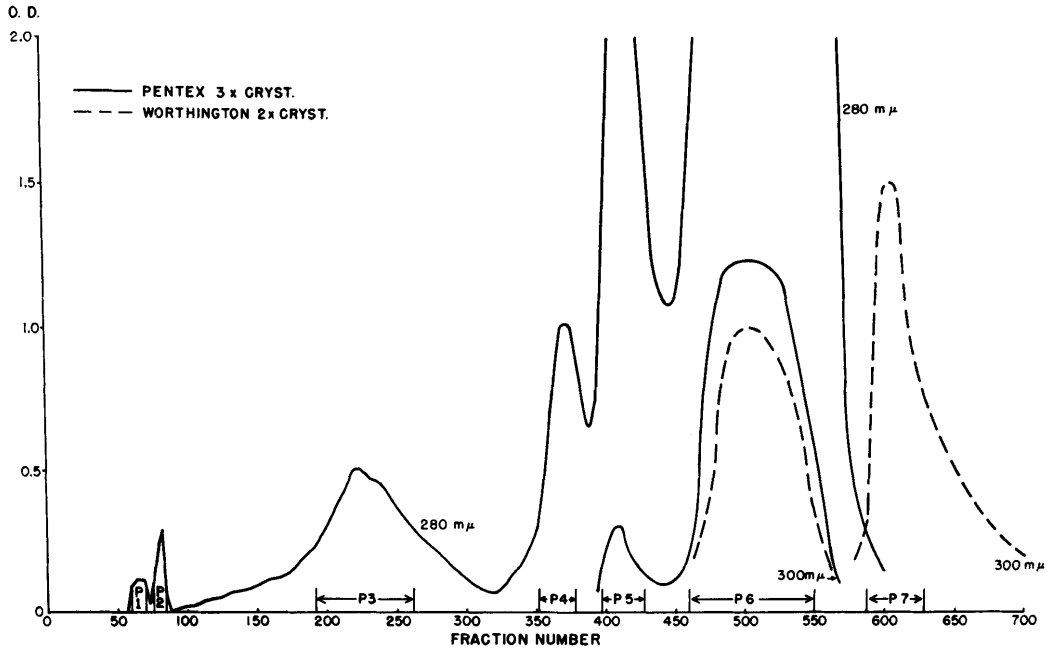

FIG. 1. Chromatographic fractionation of crystalline egg-white lysozyme. 10.0 g was applied to a 6.0 x 80.0 cm BioRex 70 column. Fractions pooled and desalted to obtain the specified fractions are indicated.

hence, increases their elution time and separation from each other. Also, the elution rate was lowered considerably considering the large volume of the preparative columns used. Changes in pH and elution rate made with 0.2 *M* sodium phosphate buffers actually did not give any better resolution of P-4, P-5 and P-6 (Fig. 1) than reported by Tallan and Stein who, in fact, did not resolve these materials. Also, reproducibility from column to column was poorer using sodium phosphate buffers. Our best resolution was obtained under the conditions given in Fig. 1. Though 10 g may seem to be an extraordinarily large column load, resolution at lower loads down to 1 g is not significantly better. The major component, P-6, is well-resolved from other peaks.

In this elution pattern, six components are clearly evident in the 3X crystallized preparation of egg-white lysozyme obtained from Pentex. Such patterns were consistently obtained during the elution of 26 preparative columns performed as described but varying in column load. With the exception of component P-4, 50 mg samples of desalted pooled peak fractions showed only one peak upon rechromatography on 2.0 × 50.0 columns of BioRex 70 equilibrated and eluted with 0.2 *M* potassium phosphate, pH 7.09. Disc electrophoresis at pH values of 4.3 and 6.6(13) showed only one band, except in the case of P-4 where a minor band did appear.

Elution patterns similar to that shown in Fig. 1 were obtained with 2X crystallized preparations of egg-white lysozyme obtained from Worthington Biochemical Corp., with one notable exception. P-1 through P-5 were present in essentially the same amounts as indicated in Table I. However, P-6 accounted for only about 60% of the crystalline material. Approximately 30% of the applied sample was eluted as another peak appearing soon after but well separated from P-6 under the conditions described in Fig. 1. Such elution patterns have been obtained from 3 different production batches of the enzyme from the same commercial source during the course of running eleven columns.

In Table I, the sum of the entries in the "% of sample" column is less than 100.0 since some protein between peaks was discarded in

TABLE I. Occurrence, Activity and Molecular Weight of Chromatographic Species in 3× Crystallized Egg-White Lysozyme.*

Species†	% of sample‡	Specific enzymic activity‡	Molecular wt: From gel filtration§	Molecular wt: From osmotic pressure‖
P-1	.19	.002	14,500	17,400
P-2	.10	.222	—	—
P-3	1.25	.904	16,780	14,900
P-4	.84	.937	14,870	14,000
P-5	2.26	.772	14,580	15,700
P-6	93.10	1.010	14,600	14,500
3× cryst. lysozyme	—	1.000	14,300	14,750

* Pentex lot EZ 1962.

† See Fig. 1 for designations.

‡ Average values obtained from 11 columns. Individual deviation from the average was no more than 3.0% of the average value.

§ Average values obtained from at least 2 gel filtration experiments where individual deviation did not exceed 5.0% of the average value.

‖ Average values obtained from at least 2 osmotic pressure experiments where individual deviation did not exceed 2.0% of the average value.

order to obtain well-resolved material. With the exception of component P-1, all components possess enzymic activity. P-3 and P-4 are very nearly as active as P-6, the major component, whereas P-2 and P-5 possess less specific activity. The seventh peak found in 2X crystallized egg-while lysozyme possessed the same specific activity as P-6 in that material or P-6 in 3X crystallized lysozyme.

Molecular weight values found for P-6 and crystalline lysozyme lie very close to the theoretical value of 14,307 known from the well-established structure of lysozyme(14). The molecular weights of P-3, P-4 and P-5 are not markedly different from those values found for P-6 whether using osmotic pressure measurements or retention volumes on Sephadex columns for calculation of molecular weight values. Only small amounts of P-1 and P-2 were available, so thorough study of their molecular weights with this method was not possible. The value obtained for P-1 is higher than that for P-6, but this component has practically no enzymic activity and may only be extraneous inactive egg-white protein occluded during the crystallization of lysozyme. P-2, P-3, P-4 and P-5 may differ from P-6 in the number of amide groups per molecule as proposed by Jolles(15). This would readily explain the chromatographic behavior of these components emerging earlier than P-6 since more carboxyl groups in the enzyme should lower its net positive charge at near neutral pH values, and hence, its affinity for the cation exchange resin, BioRex 70. Deamidation of glutamine and asparagine residues could be expected during the alkaline conditions (up to 10) used in crystallization of the enzyme(3).

Another possible explanation, which cannot be discounted by available information, is that some rearrangement of disulfide bonds in "native" lysozyme may have occurred during exposure to alkaline conditions in the crystallization process. The results of Anfinsen's group(16) indicate that active lysozyme molecules result from only one arrangement of four disulfide bonds from the eight half-cystine residues present in the peptide chain of lysozyme. There exists still the possibility that less than 4 of the native disulfide bonds are really required for enzymic activity, and other than "native" arrangements in "non-essential" disulfide bridges could account for the existence and chromatographic behavior of the minor components in crystalline egg-white lysozyme. Intended studies of peptides from partial enzymic hydrolysates of these components should clarify these points.

Our experience with different commercial preparations of the same crystalline enzyme clearly indicates that caution is warranted in the use of such preparations of even such a well-characterized small molecular weight enzyme as lysozyme for purposes of standards in peptide chromatographic patterns, studies

of active sites, physico-chemical studies of secondary and tertiary structure, amino acid sequence studies, to mention only a few possibly dangerous instances. In our studies(1, 2) where small amounts of active and inactive radiation products from egg-white lysozyme must be isolated, the necessity for enzyme homogeneity is immediately obvious. Our studies of radiation effects, at present have been limited to those on the major chromatographic component from crystalline egg-white lysozyme. The availability of the minor chromatographic components in gram amounts from our many preparative columns now affords the opportunity to study radiation effects in these materials with a view to assessing the radiation lability of closely related protein structures.

Summary. Seven components have been demonstrated chromatographically in crystalline preparations of egg-white lysozyme. This was effected with a chromatographic procedure employing BioRex 70 columns eluted with 0.2 *M* potassium phosphate buffer, pH 7.09. Six components possess enzymic activity and 3 have molecular weights very close to that of the major chromatographic component. The potential danger of assuming homogeneity in crystalline lysozyme preparations is indicated, particularly in regard to the study of radiation products.

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Anti-Alcohol Properties of Metronidazole in Rats.* (31698)

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It is known that certain laboratory animals will freely imbibe weak solutions of ethyl alcohol in preference to water, such preference varying from species to species(1), and within species according to genetic constitution(2-5), nutritional state(6-11), metabolic factors(12) and conditions of stress(13-18); there is even some evidence(19) that individual personality traits (*i.e.* timidity) may determine an animal's drinking pattern.

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The most thoroughly studied species in this regard is the rat. It has been found that some rats naturally prefer alcohol solutions to water. If they do not, a preference may be induced by simply restricting their fluid intake to an aqueous solution of alcohol for an extended period(20); the different degrees of preference which develop appear to be a function of the length of time the animals consume alcohol, and not of the particular concentration consumed(21). Preferences for