Identification of a Component of Crystalline Egg Albumin Bactericidal for Thermophilic Aerobic Sporeformers

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During an investigation of the effect of basic and acidic proteins on the growth of thermophilic aerobic sporeformers, crystalline egg albumin was found to be strongly bactericidal. This finding was uncharacteristic of acidic proteins. The bactericidal fraction was heat sensitive and separated from the non-bactericidal albumin fraction during gel filtration on Sephadex G-75. Cells of Micrococcus lysodeiticus and Bacillus stearothermophilus were lysed rapidly by the bactericidal component, leading to its tentative identification as lysozyme. The bactericidal substance possessed an electrophoretic mobility on polyacrylamide gel containing sodium dodecyl sulfate identical to that of crystalline egg white lysozyme. Users of crystalline egg albumin are cautioned that commercial preparations may be contaminated with lysozyme. Destruction of the thermophilic aerobes by lysozyme should be considered when performing counts on egg products.

The presence in natural products of inhibitors and bactericidal substances for a variety of microorganisms has been well documented (1–4, 6, 8–10). During an investigation of the inhibition of Bacillus stearothermophilus by milk, the effect of a variety of proteins on the growth of this organism was studied (1). The results suggested that basic proteins such as transferrin, protamine, histone, and poly l-lysine were potent inhibitors, whereas acidic proteins such as bovine serum albumin, pepsin, and β-lactoglobulin failed to inhibit. An exception to this rule was found in crystalline egg albumin, which has an isoelectric point of 4.6 but prevented growth of B. stearothermophilus at extremely low concentrations. The purpose of this study was, therefore, to determine the nature of the bactericidal substance for thermophilic sporeformers in crystalline egg albumin.

MATERIALS AND METHODS

Test organisms. The B. stearothermophilus strains used were obtained as follows: NCA 1518S from M. L. Fields, University of Missouri, Columbia; C953 from J. E. Auclair, INRA, Jouy-en-Josas, France; LLC from L. L. Campbell, University of Delaware, Newark; and NRC 2814 from H. Koffler, Purdue University, Lafayette, Ind. Bacillus coagulans strains T7 and T12 were obtained from I. R. Forrester, Lincoln College, Canterbury, New Zealand. Stock cultures were maintained on nutrient agar slants incubated at 55 C for 48 h and stored at 4 C until needed.

Bactericidal assay. The assay for the bactericidal substance was carried out with membrane fil-
ter-sterilized samples in either dextrose tryptone agar (DTA) or nutrient broth (NB). In the DTA assay, petri plates were inoculated with 0.1 ml of the appropriate dilution of an 18-h NB culture of the test organism to result in a colony count of 150 to 200. Increasing concentrations of the test sample in 1 ml of 0.1 M phosphate buffer, pH 6.2, were added to a series of inoculated plates and mixed uniformly with the addition of 10 ml of DTA. Incubation was at 55 C for 48 h. The bactericidal activity was determined from the colony counts. In the NB assay, the fraction being tested and sterile distilled water in a total volume of 1 and 0.1 ml (approximately 105 cells) of an 18-h NB culture of the test organism were added to tubes containing 10 ml of sterile broth. The tubes were incubated at 55 C for 18 h. Growth was determined by absorbance at 600 nm.

Fractionation of crystalline egg albumin. Egg albumin (2× crystallized, Worthington, Califio-chem; 2× and 5× crystallized, Nutritional Biochemicals) was dissolved in 0.1 M phosphate buffer, pH 6.2, applied to a column (2.5 by 40 cm) of Sepha-
dex G-75, and eluted with 0.1 M phosphate buffer, pH 6.2. The fractions (3 ml) collected were assayed for the ability to prevent growth of B. stearothermophilus and B. coagulans and for lysozyme activity and protein concentration.

Lysozyme assay. Lysozyme activity was assayed as described by Parry et al. (10). The method involved measurement of the change in absorbance of a suspension of ultraviolet-killed cells of Micrococcus lysodeiticus (Difco) at 540 nm. Measurements were taken at 1-s intervals over a 2-min period, and the change in absorbance per minute was determined from the portion of the assay during which clearance was linear. Protein concentrations were determined spectrophotometrically (7). Crystalline lysozyme (Calbiochem, 3×) was used as a standard.

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Acrylamide gel electrophoresis. Crystalline egg white lysozyme (Calbiochem) was dissolved (4 mg/ml) in 0.1 M phosphate buffer, pH 6.2. β-Mercaptoethanol and sodium dodecyl sulfate were added to the protein solution to give a final concentration of 1% (wt/wt) for each. The bactericidal fraction of crystalline egg albumin eluted from Sephadex G-75 was treated in a similar manner. The protein samples were applied (20 μl) to acrylamide gels, and electrophoresis was carried out as described by Weber and Osborn (12).

RESULTS AND DISCUSSION

Growth of strains NCA 1518S, C953, LLC, and NRC 2814 of B. stearothermophilus and strains T7 and T12 of B. coagulans was prevented by commercially available crystalline egg albumin. Because B. stearothermophilus NCA 1518S exhibited typical growth failure patterns in agar and broth, it was chosen for further study. An example of the bactericidal response for B. stearothermophilus NCA 1518S in DTA is presented in Table 1. Growth was prevented completely by a concentration of 100 μg of 2x-crystallized egg albumin per ml of DTA. Growth was affected to a greater extent by the albumin in NB, being prevented by a concentration of 1 to 10 μg/ml of broth. This greater potency in broth is not unusual. The ability of agar to interfere with the activities of antimicrobial agents is well documented (11).

The bactericidal substance in crystalline egg albumin was heat sensitive, being destroyed completely at 80 C in 10 min. The bactericidal substance was non-dialyzable and was not overcome by the addition to the medium of iron as FeSO₄ in concentrations (0.1 to 2.0 mM) that did not themselves inhibit the test organism. Therefore, bactericidal activity was not due to conalbumin (3), a constituent of eggs known to chelate iron which is an essential element for the growth of B. stearothermophilus (5).

Solutions of crystalline egg albumin were assayed for lysozyme activity using ultraviolet-killed suspensions of M. lysodeikticus cells or suspensions of B. stearothermophilus cells. Crystalline egg albumin was found to contain lysozyme activity. On a protein weight basis, 2x-crystallized albumin possessed 4 × 10⁻² times the activity of crystalline lysozyme.

Lysozyme was separated from crystalline egg albumin by gel filtration on Sephadex G-75 (Fig. 1). The albumin fraction eluted in a major 280-nm absorbing peak in the first 60- to 65-ml column exclusion volume. There was no lysozyme or B. stearothermophilus bactericidal activity in this peak. A second minor 280-nm absorbing peak eluted in the 70- to 150-ml volume range. Protein in this peak had minimal ability to absorb at 280 nm but possessed lysozyme and B. stearothermophilus and B. coagulans bactericidal activities equivalent to crystalline lysozyme at the same concentration. Approximately 86% of the lysozyme activity in the albumin sample applied to the column was recovered in the bactericidal peak.

The fractions containing lysozyme and B. stearothermophilus bactericidal activity from several Sephadex G-75 separation trials were combined and concentrated by ultrafiltration using Diaflo UM-1 membranes (Amicon) to contain approximately 4 mg of protein per ml of solution. A solution of crystalline egg white lysozyme was prepared to contain 4 mg/ml also. A portion of the lysozyme preparation was mixed 1:1 with the concentrated bactericidal fraction. The three preparations were subjected to electrophoresis on polyacrylamide gel (Fig. 2). The crystalline lysozyme, the Sephadex G-75 bactericidal fraction, and the mixture of crystalline

| Albumin source | μg of albumin/ml of medium |
|---------------|---------------------------|
| 0             | 0                         |
| 10            | 5                         |
| 25            | 0                         |
| 50            | 0                         |
| 100           | 0                         |
| 200           | 0                         |

Table 1. Colony count of B. stearothermophilus NCA 1518S in DTA in the presence of added crystalline egg albumin

**FIG. 1.** Elution pattern showing absorbance at 280 nm (solid line) and lysozyme activity (broken line) of fractions of crystalline egg albumin obtained during gel filtration on Sephadex G-75.
lysozyme and bactericidal fraction all resulted in patterns suggestive of single proteins of identical molecular weight. These observations confirm that the prevention of growth of *B. stearothermophilus* and *B. coagulans* by commercially available 2×- and 5×-crystallized egg albumin is due to the presence of lysozyme in the egg albumin. This minor contaminant may be of no consequence for many applications. However, those requiring egg albumin of absolute purity are cautioned that the commercially available 2×- and 5×-crystallized varieties may contain small amounts of lysozyme. In addition, those involved in the enumeration of thermophilic aerobes in egg products should consider the bactericidal effects of lysozyme. In the case of spore counts, treatments at 80 or 100 C for 10 min would be adequate to inactivate the lysozyme without adversely affecting the spores. In the case of total thermophilic aerobic counts, the lysozyme may have inactivated all vegetative cells in the sample. However, separation of bacterial cells and lysozyme can be effected by such sample pretreatments as centrifugation or membrane filtration.

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