# A SENSITIVE ASSAY FOR EGG-WHITE AND BACTERIOPHAGE-INDUCED LYSOZYME

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The solubilization of bacterial cell walls has been used to assay the lysozyme produced after bacteriophage infection. Enzyme activity is ordinarily measured by the decrease in absorbancy at 490 mμ of purified cell wall suspensions after addition of enzyme.[1] The sensitivity of this assay is relatively low and requires a large number of infected bacteria to detect enzyme activity. This paper offers a new assay of high sensitivity, for lysozyme, which has been found suitable to detect phage-specific lysozyme produced in competent Bacillus subtilis infected with DNA from a virulent bacteriophage.

The assay consist in the quantitation of radioactivity rendered soluble in cold trichloroacetic acid when $H^3$-thymine labelled B. subtilis cells are used as the enzyme substrate in the presence of excess DNAse 1 and venom phosphodiesterase. In the presence of lysozyme the radioactive deoxyribonucleic acid is made available to nuclease digestion.

MATERIALS AND METHODS: Bacteria: Bacillus subtilis 168 $T^-$, [2], a thymine requiring auxotroph was used for the preparation of labelled substrate cells. B. subtilis 168 $P^-$, a proteinase negative mutant was provided by H. P. Rappaport and was employed for the preparation of extracts of phage-infected bacteria. Both strains were maintained on Difco brain-heart infusion agar plates.

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Abbreviations used: DNA, deoxyribonucleic acid; DNAse, deoxyribonuclease 1.

Bacteriophage: Phage 2C, a virulent B. subtilis bacteriophage[3] was used throughout this study. Phages were propagated and assayed with the general techniques described by Adams[4].

Preparation of Extracts: Extracts of infected B. subtilis 168 $P^-$ were prepared as described by Pène and Marmur[5] in 0.01 M Tris-0.01 M $MgCl_2$-0.01 M 2-mercaptoethanol, pH 9.

Preparation of Labelled Substrate Cells: B. subtilis 168 $T^-$ was grown for 24 hours at 37 C on solid minimal salts medium[6] supplemented with 0.02% casein hydrolysate, 0.05% yeast extract (Difco), 0.5% glycerol and 2 µg/ml of thymine. A small amount of cell paste was homogenized in saline phosphate buffer (SP; 0.15 M NaCl-0.02 M $K_2HPO_4$ pH 7.4) and transferred by spreading on a 35 mm petri dish containing 2.5 ml of the minimal medium described above. Unlabelled thymine was replaced by 0.25 mc/petri plate of $H^3$-thymine (New England Nuclear Corporation, 8.3 C/mM). The plate was incubated at 37 C for 13 hours. The cells were harvested by scraping the thin lawn with minimal salts medium containing 10 µg thymine per ml and washed 4 times with 15 ml of the same medium at 4 C by centrifugation at 6,000 rpm for 8 minutes in a Sorvall RC2 centrifuge. The bacteria were resuspended in 5 ml of minimal medium containing 10% glycerol (v/v) and frozen in an ethanol-dry ice bath in 0.5 ml samples in 10 x 75 mm test tubes. The tubes were allowed to stand in the ethanol-dry ice mixture for 20 minutes and then stored at -70 C. After freezing, a tube was melted at 37 C and used to determine the number of viable bacteria by serial dilution and surface plating on Difco brain-heart infusion plates.

Assay Procedure: Assays were performed in 13 x 100 mm tubes. Each assay contained 2 to 5 x $10^5$ $H^3$ -labelled B. subtilis 168 $T^-$, 10 µg of deoxyribonuclease 1 and 1 µg of venom phosphodiesterase (Worthington Biochemical Co.; dissolved in saline phosphate buffer) and SP buffer containing 0.005 M $MgCl_2$ to a final volume of 0.5 ml. The tubes were incubated at 37 C for 20 minutes with shaking. At the end of the incubation period

carrier salmon sperm DNA (0.25 mg; Nutritional Biochemicals Corporation) and cold trichloroacetic acid (final concentration 5%) were added to each assay. The tubes were chilled to 0 C and centrifuged in an International refrigerated centrifuge for 10 minutes at 2,500 rpm, 2 C. Samples of the supernatant liquid (0.5 to 0.8 ml) were withdrawn and counted in 20 ml of Bray's scintillator[7] in an Ansitron liquid scintillation counter. Labelled substrate cell preparations were characterized by determining the trichloroacetic acid-precipitable and soluble radioactivity in the fraction used in the enzyme assay. The stability of the cells during the incubation period was determined by measuring the trichloroacetic acid-soluble radioactivity before and after the incubation period in the presence of nucleases but in the absence of extract from phage-infected cells or lysozyme (purchased from Worthington Biochemical Co.). The blank or the amount of trichloroacetic acid-soluble radioactivity in the absence of lysozyme at the end of the incubation period (1 to 5% of the trichloroacetic acid-precipitable radioactivity) was subtracted from the value in assay tubes.

RESULTS AND DISCUSSION. The requirements of the lysozyme assay are presented in Table I. In the absence of lysozyme or extract from phage-infected bacteria, approximately 1% of the radioactivity was rendered trichloroacetic acid-soluble at the end of the incubation at 37 C. In the presence of 20 μg of egg-white lysozyme all of the radioactivity was made trichloroacetic acid-soluble in 20 minutes. The reaction was completely dependent on the presence of deoxyribonuclease and venom phosphodiesterase. In the presence of crude extract from phage-infected bacteria corresponding to $1 \times 10^6$ infected centers 48% of the radioactivity was made soluble in trichloroacetic acid. After incubation of the radioactive cells with extract from uninfected bacteria or boiled extract from infected bacteria approximately 1% of the radioactivity was made trichloroacetic acid-soluble in 20 minutes at 37 C.

TABLE I

Requirements for Assay of Crystalline Egg-white and Phage-induced lysozyme.

| Additions | TCA soluble cpm in 20 min. | TCA soluble cpm, % of input |
|---|---|---|
| Complete system + 20 μg egg-white lysozyme | 87,984 | 100 |
| Complete system + 1 μg egg-white lysozyme | 18,100 | 20.6 |
| omit lysozyme | 970 | 1.1 |
| omit nucleases | 935 | 1.07 |
| Complete system + extract from phage-infected B. subtilis | 41,966 | 48 |
| Complete system + extract from uninfected B. subtilis | 987 | 1.13 |
| Complete system + boiled extract from infected B. subtilis | 996 | 1.14 |

The complete system consisted of 2 x $10^5$ $H^3$-thymine labelled B. subtilis 168 $T^-$, 10 μg DNAse 1, 1 μg of venom phosphodiesterase in final volume of 0.5 ml made up with SP buffer containing 5 mMolar $MgCl_2$. Addition of extract from phage infected bacteria represented approximately $10^6$ infected bacteria.

The relationship between the amount of crystalline egg-white lysozyme and the acid soluble radioactivity released from labelled substrate cells is presented in figure 1. Under the conditions described above acid soluble radioactivity detected was proportional to the amount of lysozyme in the range of 0.001 μg to 1 μg of purified enzyme. The results of a similar experiment with lysozyme present in phage-infected bacterial extracts are presented in figure 2. The number of phage 2C - infected bacteria was calculated from the multiplicity of infection. With phage-induced lysozyme, acid soluble radioactivity was proportional to the amount of lysozyme with-

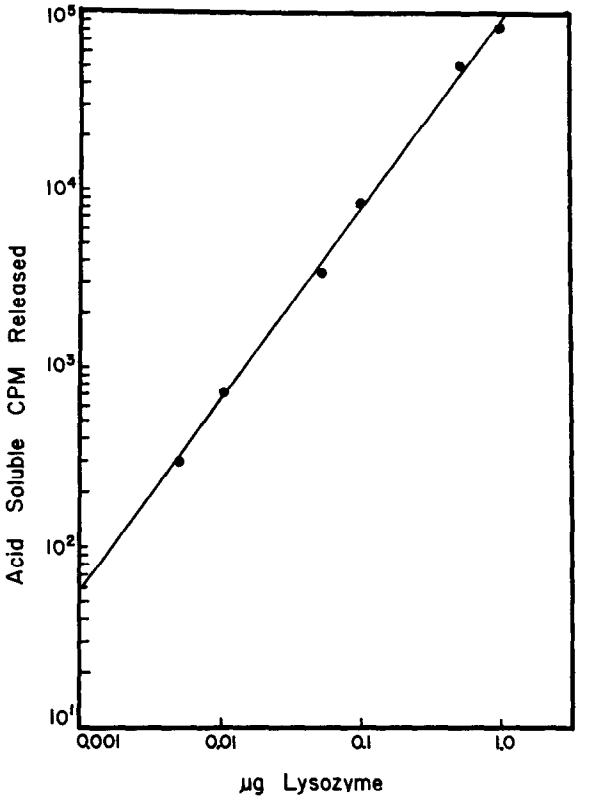


Figure 1. Relationship between acid soluble radioactivity released from $H^3$ -thymine labelled B. subtilis and lysozyme concentration. The assay was performed as described in the text.

in the range of enzyme produced by 5 x $10^3$ to 1 x $10^6$ infected bacteria. The activity of phage-induced lysozyme can therefore be described in terms of the activity of known amounts of crystalline egg-white lysozyme. One unit of phage lysozyme is defined as the activity corresponding to 1 µg of egg-white lysozyme.

The stability of the labelled substrate cells used in this assay decreases on storage at -70 C. As can be seen from the data in figure 3 the percentage of the input radioactivity made soluble in 5% trichloroacetic acid after incubation of substrate cells in the absence of lysozyme

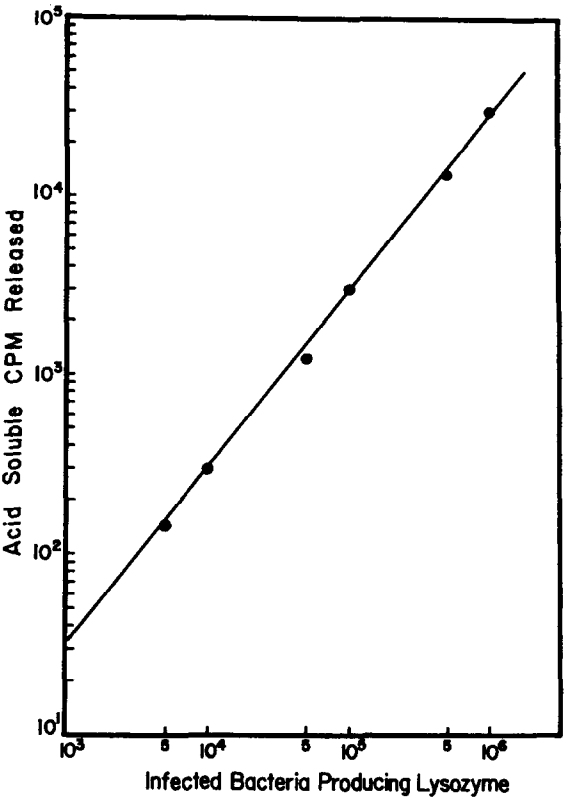


Figure 2. Relationship between acid soluble radioactivity released from $H^3$ -thymine labelled B. subtilis and phage-induced lysozyme concentration. Extracts were prepared 25 minutes after phage addition (moi = 5) as described in the text. From the number of infected cells in the total extract the number infected bacterial equivalents producing lysozyme in the dilution of extract added was calculated.

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increases at the rate of one per cent per day of storage at -70 C. Labelled substrate cells are therefore usable for a limited period of time, and must be freshly prepared when used to assay extracts of low activity. Since labelled substrate cells are prepared by growing the bacteria on high levels of $H^3$ -thymine it is possible that the observed increase of the blanks on storage at -70 C reflects radiation damage near or at the cell membrane which affects the permeability of the cells to the exogenous nucleases added. Labelled substrate cells are, however, easily and con-

veniently prepared. The stability of substrate cells can be improved by growing the bacteria on minimal medium supplemented with non-radioactive thymine.

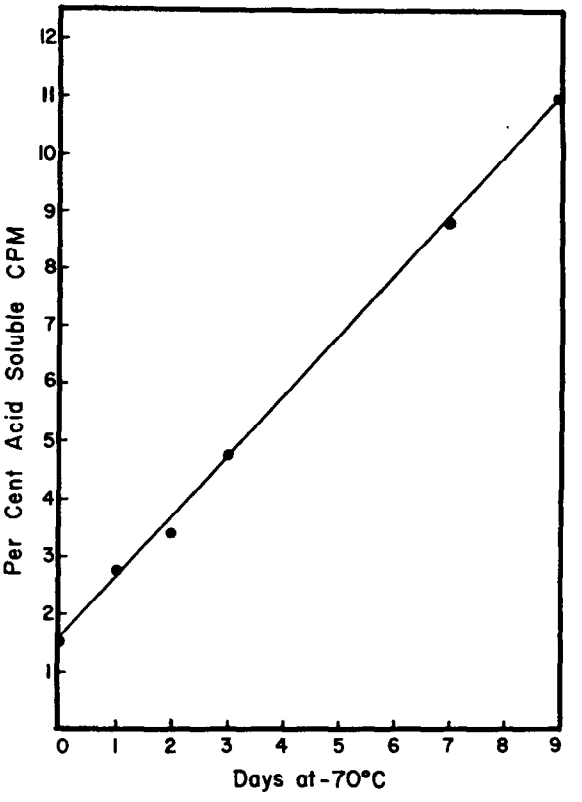


Figure 3. Stability of $H^3$ -thymine labelled B. subtilis substrate cells on storage at -70 C.

The blanks or per cent of radioactivity rendered TCA soluble in the presence of nucleases but in the absence of lysozyme is plotted against days of storage of labelled cells at -70 C.

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The sensitivity of this assay has allowed the detection of phage-specific lysozyme produced after infection of competent B. subtilis with phenol-extracted phage deoxyribonucleic acid. B. subtilis 168-2,[8], was made competent according to the procedure described by Spizizen[6]. Phenol extracted phage DNA free of plaque forming units was added at a concentration of 15 µg/ml. Under these conditions the cells begin to release phage

70 minutes after DNA addition. Lysozyme activity was measured at various times after DNA addition. The results of this experiment are presented in Table II. Phage-specific lysozyme was detected 50 minutes after DNA addition. No enzyme activity above the blank was measured in extracts prepared from competent bacteria infected with DNA which had been previously treated with DNAse.

TABLE II

Formation of Lysozyme in Competent B. subtilis infected with phage DNA.

| Minutes after Phage DNA addition | Units of lysozyme/$10^9$ competent cells |
|---|---|
| 30 | 0.18 |
| 45 | 0.19 |
| 50 | 0.7 |
| 55 | 1.25 |
| 60 | 2.8 |
| 65 | 5 |

Phenol extracted phage DNA (15 µg/ml) was added to maximally competent B. subtilis 168-2. Samples of transfected cells (50 ml) were collected by centrifugation at various times after DNA addition. Extracts were prepared by crushing the cells in a French pressure cell in 0.01 M Tris-0.01 M $MgCl_2$-0.01 M 2-mercaptoethanol, pH 9. Lysozyme was measured as described in the text.

The sensitivity of the assay of phage-induced lysozyme offers the possibility to determine the effects of in vitro modification of phage DNA on the synthesis of the enzyme produced in transfected cells.

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