THE ISOLATION OF AN INHIBITOR OF T-EVEN PHAGE LYSOZYME FROM *E. COLI B* CELLS

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1. Introduction

In the course of performing assays for T2 lysozyme in an *in vitro* protein synthesis system [1] we found that the activity of purified T4D lysozyme was reduced when the enzyme was added to an aliquot of the *in vitro* system mixture before assay. This suggested the presence of an inhibitor of lysozyme in the S-30 fraction. In the work reported here we have carried out a partial purification of the inhibiting agent which is an acidic polysaccharide.

2. Methods and materials

2.1. *Preparation of the inhibitor*

The inhibitor was prepared from *E. coli B* cells. The cells were grown at 37° in 10 l of M9 medium supplemented with 0.2% glucose, to a density of $6-7 \times 10^8$ cells/ml and were harvested in the Sharples continuous flow centrifuge. A broken cell-free extract in Tris/Mg buffer (0.01 M MgAc, 0.01 M Tris/HCl pH 7.6) was prepared by grinding with alumina. The final volume of the centrifuged supernatant after dialysis against the Tris/Mg buffer for 4 hr was 30 ml. The inhibitor was then purified according to the following steps:

(1) RNase and DNase treatment to remove nucleic acids.

(2) Boiling of the extract for 2 min to precipitate the bulk of the proteins.

(3) Alcohol precipitation of the boiled extract supernatant.

(4) Removal of remaining protein impurities from the resuspended alcohol precipitate by the Sevag procedure [2] (repeated shaking with chloroform/isoamyl alcohol).

(5) Precipitation with cetyl pyridinium chloride (a specific precipitating agent for acidic polysaccharides).

The resulting inhibitor material in the acid polysaccharide fraction (which gave a positive Molisch test) had an inhibitory effect 340 times that of the original extract per mg dry weight.

2.2. *Lysozyme assay*

The T4D lysozyme used in the experiments was purified by a method based on the chitin column procedure described by Jensen and Kleppe [3].

Two methods for the assay of lysozyme have been used.

(a) Turbidimetric assay. The substrate consisted of *E. coli B* cells sensitised to the action of lysozyme by treatment with chloroform [4]. The cells were suspended in 3 ml 0.05 M Tris/HCl pH 7.6, adjusted to an O.D. 450 mμ of 0.7. Enzyme activity at 37° was followed in a Spectronic 600 E recording spectrophotometer by measurement of the linear decrease in turbidity at 450 mμ.

(b) Hexosamine determination. To four 1 ml volumes of lysozyme substrate (containing approx. 5 mg cells) were added respectively, (a) 0.05 M Tris/HCl buffer pH 7.6, (b) 2×10^{-3} μg T4D lysozyme, (c) 30 μg inhibitor, and (d) 30 μg inhibitor and 2×10^{-3} μg T4D lysozyme. After 15 min at room temperature, during which time hexosamine liberation was linear, the reactions were terminated by boiling in a water bath for 1 min. Cell debris was centrifuged down at 14000 *g* for 20 min. 6 M HCl was added to the super-

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Table 1

Amount of inhibitor μg	% inhibition of lysozyme
0	0
1	50
10	77
25	89
25*	50

* After 12 min acid hydrolysis.

natant such that the final concentration of acid was 1M. Hydrolysis of the liberated acetylated sugars was performed at 100° for 1 hr in a water bath. The solutions were then neutralised with 6 M KOH and hexosamine determinations were carried out on duplicate 0.5 ml volumes [5].

3. Results and discussion

The effects of adding various amounts of inhibitor to 2×10^{-3} μg T4D lysozyme are shown in table 1.

In the presence of 25 μg of the inhibitor the activity was reduced by 80%. Inhibitor which had been subjected to acid hydrolysis and neutralised gave only 50% inhibition of the enzyme, indicating that the treatment had partially destroyed or inactivated the inhibitor. Acid treatment without boiling, followed by neutralisation, had no effect on the inhibitor.

Grossowicz and Ariel [6] showed that lysozyme-induced lysis of *Micrococcus lysodeikticus* cells did not occur in the presence of spermine. They were, however, able to demonstrate hexosamine production and thus showed that the polyamine was not an actual inhibitor of lysozyme itself but was merely acting as a stabilizer of the protoplasts formed by lysozyme. In one experiment, therefore, hexosamine determinations were performed in addition to the usual turbidimetric assay to investigate whether or not the inhibitor was actually an inhibitor of the enzyme itself. The results are shown in table 2.

The fact that both decrease in turbidity and hexosamine production were reduced in the presence of the inhibitor shows that it does not act in the same manner as spermine. Direct inhibition of the enzyme itself is indicated.

The inhibitor can be separated from lysozyme by adsorption of the enzyme to a chitin column, followed by elution. The inhibitor is not adsorbed and passes straight through. This method has allowed us to detect small amounts of lysozyme immediately after phage infection of bacterial cells [7].

Table 2

T4D lysozyme 2×10^{-3} μg	Inhibitor 25 μg	Hexosamine formed μg
–	–	8
+	–	95
–	+	8
+	+	18

The inhibitor has also been isolated from *E. coli K 12*, strain 41B, by the procedure described above and was found to have similar properties. It is, therefore, not specific to the B strain.

Cell-free extracts of *E. coli B* infected with phage T2 for 10 and 20 min have been prepared and the inhibitor capacity compared with that of uninfected extracts. Phage infection appears to have no effect upon the amount or efficiency of the inhibition.

A detailed analysis of the composition of the acid polysaccharide inhibitor will be the subject of another paper.

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