THE APPEARANCE OF PHAGE ASSOCIATED LYSOZYME IN E. COLI B CELLS IMMEDIATELY AFTER INFECTION WITH PHAGE T2.

I.F. Pryme, P.E. Joner and H.B. Jensen

Department of Biochemistry, University of Bergen, Bergen, Norway.

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Jensen and Kleppe (1969) adopted the use of a chitin column as a step in the purification of T4D lysome. In the work reported here the chitin column has been used before lysozyme assay as a method of concentrating the enzyme and freeing it from its inhibitor(s) (Pryme, Joner and Jensen, 1969). We have thus been able to assay much smaller amounts of lysozyme than has been possible before. Using a standard method of lysozyme assay, Sekiguchi and Cohen (1964) found that the enzyme is first detected in bacterial cell-free extracts 8-10 minutes after infection with T-even phage in minimal medium. We have also found that lysozyme is just detectable at 8 minutes after infection using the same standard assay method, however, the chitin column technique has enables us to detect activity as early as 15 seconds after infection of E.coli B cells with phage T2. This activity very early in the infective period is not affected by the addition of chloramphenicol to the cells 5 minutes before the addition of phage, and, therefore, probably does not represent early synthesis of lysozyme. The activity has in fact been shown to originate from the infecting

phage. Lysozyme synthesis has been shown to begin about 6 minutes after infection. The data would suggest that infection is complete about 30 seconds after the addition of the phage to the bacteria.

Methods and Materials.

Lysozyme inhibitor was prepared from E.coli B cells as described before (Pryme, Joner and Jensen 1969). The lysozyme substrate was prepared according to the method of Sekiguchi and Cohen (1964). The cells were suspended in 3ml 0.05M Tris/HCl buffer pH 7.6, adjusted to an O.D. 450mμ of 0.7. Enzyme activity at 37° was followed in a Spectronic 600E recording spectrophotometer by measurement of the linear decrease in turbidity at 450mμ.

Cell-free extracts of E.coli B cells were prepared at different times after infection with phage T2. The cells were grown at 37° in 2 liters of M9 medium supplemented with 0.2% glucose, to a density of $5\text{-}6.10^8$ cells/ml and then infected with phage T2 (m.o.i. 5). A 200ml sample was taken before infection and poured into a centrifuge bottle containing 50g ice, 20mg chloramphenicol and 3 ml NaN_3. Similar samples were taken at 15 seconds, 35 seconds, 55 seconds and 2 minutes, and then at 1 minute intervals up to 8 minutes after infection. The cells were centrifuged down at 7500 rpm in the Sorvall RC-2 centrifuge and then resuspended in 3ml volumes of 0.02M Tris/HCl buffer pH7.6. The cells were broken in the French Press at 20001bs/sq.in. Cell-free extracts were obtained by a further centrifugation.

The procedure for the preparation and use of the chitin columns is described by Jensen and Kleppe (1969).

Osmotic shock of phage T2. 1.10^{12} phage (i.e. the same

amount used in the above infection) were sedimented in the Spinco Beckman Model L Ultracentrifuge. The pellet was resuspended in 10ml 0.1M $MgCl_2$ and shaken for 30 minutes at 4^{o}. Closs and Jensen (unpublished results) have shown that this treatment is sufficient to remove more than 90% of the lysozyme bound to the external surface of the phage. After the 30 minutes shaking the phage were again sedimented. The pellet was resuspended in 10ml 4M NaCl and then dumped into 100ml distilled water whilst stirring vigorously. Stirring was continued for 30 minutes at 4^{o}. The $MgCl_2$ 'wash' was diluted to 100ml with distilled water and poured on to a chitin column. The NaCl 'shockate' was also run on to a chitin column. The elution of lysozyme and subsequent assay were performed as above.

Results and Discussion.

(i) Preliminary experiments with the chitin column.

A mixture of T4D lysozyme and sufficient inhibitor to cause 100% inhibition was poured on to a chitin column. The effluent from the column was shown to inhibit added lysozyme whilst elution of the enzyme produced 100% recovery. It was thus possible to achieve complete separation of lysozyme from the inhibitor. The affinity of lysozyme for chitin is obviously greater than its affinity for the inhibitor.

When an extract of E.coli cells, prepared 8 minutes after infection with phage T2, was passed through a chitin column, three times the original activity of the extract was eluted from the column. It is thus evident that in infected bacterial extracts there exists significant inhibition of lysozyme.

A solution containing lysozyme was diluted such that activity could no longer be detected by the standard assay

procedure, and it was then poured on to a chitin column. The enzyme was thereby concentrated and was eluted off and assayed in a concentrated form.

The use of a chitin column thus affords two advantages, first it enables lysozyme to be freed from its inhibitor (s), and secondly it provides a means of concentrating the enzyme.

(ii) Experiments with the cell free extracts.

Lysozyme activity in the infected cell free extracts was

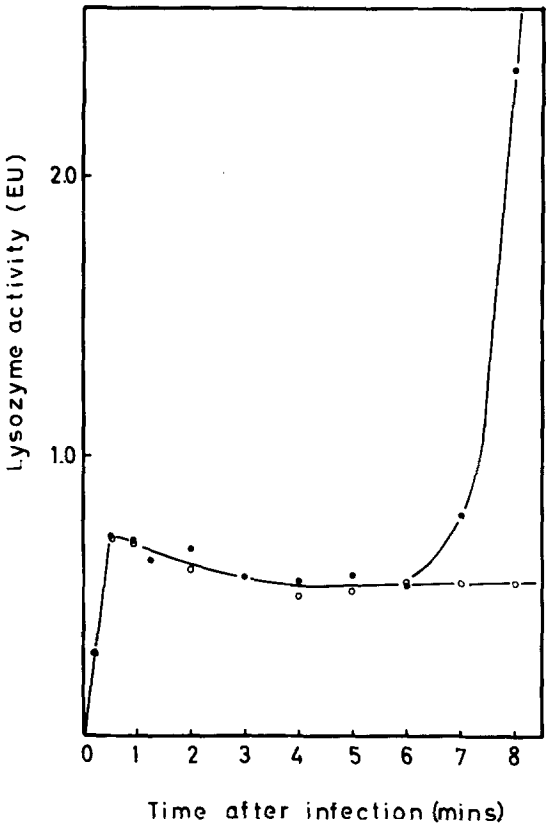

Figure 1. (●)-lysozyme activity appearing in E. coli B cells at various times after infection with phage T2.
(o)-as above, but chloramphenicol (100µg/ml) added to the cells 5 minutes before infection with phage.
One unit (EU) of lysozyme activity is expressed as that amount of enzyme which catalyses a decrease of optical density (450mµ) of 1.0/minute, i.e. $\Delta A_{450} = 1.0$.

determined after they had passed through chitin columns and the results are expressed in Figure 1. Appreciable activity was observed 15 seconds after infection, reaching a peak at 35 seconds. This level of activity remained reasonably constant until 6 minutes when a rapid increase occurred. When the experiment was repeated but chloramphenicol was added to the cells 5 minutes before infection, the appearance of lysozyme immediately after infection was not inhibited. The increase in activity after 6 minutes, however, was inhibited (Figure 1) and this obviously represented inhibition of the synthesis of new enzyme. Hosoda and Levinthal (1968) call proteins which appear before 7 minutes 'early' and those appearing after 7 minutes as 'late', hence according to this classification, lysozyme, on the basis of our results, could be called an 'early' enzyme. This supports the findings of Bolle et al (1968) who showed that lysozyme mRNA does not follow the hybridization pattern characteristic of 'late' messenger and they conclude, therefore, that lysozyme mRNA is not representative of 'late' mRNA species.

The fact that lysozyme appeared in the cells 15 seconds after infection in the presence of chloramphenicol, suggested that it must have originated from the infecting phage. The osmotic shock experiment confirmed this.

Lysozyme activity was detected both in the $MgCl_2$ 'wash' and in the NaCl 'shockate'. 23% of the total activity appeared in the former and 77% in the latter. This latter activity closely agreed with the maximal activity observed 35 seconds after infection. It was therefore obvious that this activity was entering the bacteria during the process of infection.

It seems likely that the lysozyme enters the bacterium

bound to the phage DNA, since taking into account the basic properties of lysozyme it would be expected to bind to DNA and hence the incorporation of lysozyme into newly formed phage particles is not difficult to visualise. We have in fact shown that DNA inhibits the lytic properties of T4D lysozyme, and that the addition of DNase restores the activity. The part of the curve which lies between zero and 35 seconds thus represents the rate of infection of the phage. As early as 15 seconds after the addition of the phage it appears that approximately 50% of the cells are infected, total infection occurring about 35 seconds after the addition.

In conclusion it may be stated that the use of the chitin column to concentrate the enzyme and free it from inhibitor(s) will provide a very useful tool for measuring extremely small quantities of the enzyme.

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