THE SEPARATION OF A GENE 3.5 DIRECTED ACTIVITY AND A LYTIC ACTIVITY ("LYSOZYME") IN T7 INFECTED E. COLI B CELLS.

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Summary. Bacteriophage T7 infected Escherichia coli B cells have been shown to contain two enzyme activities possessing the ability to liberate radioactive material from ^{3}H diaminopimelic acid labelled E. coli C cell walls. These two enzymes have been separated by chromatography on chitin. Only the one which binds to chitin possesses the ability to lyse chloroform-treated E. coli B cells.

Several reports concerning the study of T7 "lysozyme" have appeared in the literature (1-7). In all but one of these studies (1) the assay system used has been that described by Schweiger and Gold (8). This method measures the liberation of radioactive material from [^{3}H]diaminopimelic acid labelled E. coli cells precipitated on filter discs by trichloracetic acid, and is not strictly an assay for lytic activity. Lysis of bacteria seems to be a complicated process, and the sequence of events is not known in detail for any lytic system studied. The most relevant methods presently at hand for the determination of lytic activity are those which are based on the direct observation of the lysis of bacteria. Using such a method to determine T7 directed lytic activity (1), we found that the time course of appearence as well as the conditions for isolation and assay were so different from those described by many workers that the possibility of the existence of two enzymes was indicated. In the present paper we report the separation of two T7 induced enzyme activities, both possess the ability to liberate radioactive material from [^{3}H]diaminopimelic acid labelled E. coli C cells, only one, however, possesses the ability to cause lysis of sensitized bacteria. For convenience we refer to the latter as lytic activity, and the other as amidase activity since this is most likely the same enzyme as that described by Inouye et al (7).

Experimental.

Phages. T7 wild type was from the collection of Dr. F.W. Studier, Brookhaven National Laboratory, and T7am13, an amber mutant in gene 3.5 was obtained from Dr. H. Ponta, Max-Planck Institute für molekulare Genetik.

Preparation of T7 and T7am13 infected E. coli B extracts. 10 ml E. coli B cells were grown in tryptone medium at 37° with vigorous aeration and at a concentration of $5 \cdot 10^8$ cells/ml were infected with phage (m.o.i. 10). 11 minutes after the addition of wild type or 22 min. in the case of am13, sodium azide was added to a final concentration of 8 mM and the culture was immediately chilled in ice. The cells were harvested and resuspended in 1 ml 20 mM phosphate buffer pH 7.2. The suspension was frozen in dry ice/alcohol and then thawed. This procedure was performed twice. The cell extract was stored overnight at 4° before being centrifuged at 10.000 rpm for 10 min. and the supernatant was collected.

Isolation of lytic activity from the supernatant. 0.5 ml supernatant was applied to a 8x8mm deaminated chitin column (10) equilibrated with 0.05 M Tris buffer pH 7.4. The column was washed with the same buffer until the flow-through was free of A_{280}-absorbing material, fractions of 0,5 ml were collected. The lytic activity was eluted with 0,05 M phosphate buffer pH 7.2 containing 0.25 M KCl, 0,5 ml fractions were collected.

Determination of enzyme activities. a) Lytic activity: The assay for lytic activity was performed as previously described (1), except that the 3 mM phosphate buffer contained 0,5 mM $MgCl_2$. b) Amidase activity, i.e. liberation of radioactive material from [^{3}H]diaminopimelic acid labelled E. coli C cells: Assay was performed as described Schweiger and Gold (8).

Results and discussion.

a) Loss of T7 lytic activity in lysates during continued incubation. In the purification procedures of T2 (11) and T4 (9,12) lysozymes the enzyme has been purified from lysates. When T7 infected E. coli B cells were incubated

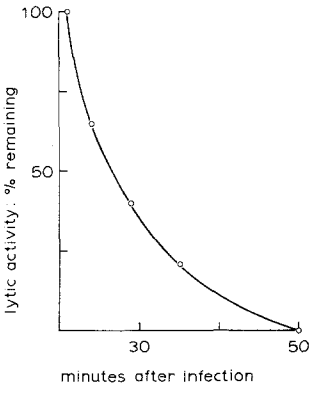

Figure 1. Loss of T7 lytic activity in lysates during continued incubation. 10 ml E.coli B were infected with T7 wild type as described in Experimental except that the temperature was 30°. Sodium azide was not added neither was the culture chilled, instead lysis was allowed to occur and aeration of the culture was continued. The onset of lysis was observed at 20 min. 100 µl aliquots of the lysate were removed at various times and assayed for lytic activity as described in Experimental. The activity measured at 21 min. after infection is referred to as 100 %.

with aeration until they cleared a drastic decrease in lytic activity in the lysate was observed (Fig 1). In order to obtain appreciable amounts of T7 lytic activity it was therefore necessary to prepare extracts of infected E. coli B cells as described in Experimental, where the cells were harvested prior to the onset of lysis. Inouye et al (7) have studied the specificity of a T7 induced enzyme activity which they found most likely to be the product of gene 3.5, the gene regarded to be the one responsible for T7 "lysozyme". However, under conditions where the onset of lysis is at about 20 minutes, they incubated their cultures until they cleared (usually 45 minutes for the wild type). At this time only traces of lytic activity remain (Fig 1) suggesting that the lytic enzyme was not responsible for the activity which they studied.

b) Separation of two T7 induced enzyme activities by affinity chromatography. The first step in the purification procedure for T7 lytic activity (to be published) is affinity chromatography on chitin. As can be seen from Table 1, when a supernatant prepared after infection with T7 wild type was applied to

Table 1. Separation of the two enzyme activities on chitin.

	Lytic activity	Amidase activity
Flow through	0.000	11,294
Eluate	0.045	8,983

A cell-free supernatant was prepared after infection of E. coli B cells with T7 wild type and the lytic activity and the amidase activity were separated on a deaminated chitin column as described in Experimental. Each fraction was tested for enzyme activities as described in Experimental. The values presented are those found in fraction 2 of both the flow through and the eluate, which in each case contained the bulk of the enzyme activities. Lytic activity is expressed as the decrease in absorption at 450nm per minute per 25µl sample. The liberation of radioactive material from [^{3}H]diaminopimelic acid labelled E. coli C cells is expressed as cpm per 24 hours incubation at 37° per 25 µl sample. The values were corrected for the radioactivity released in the absence of enzyme.

a chitin column, the flow-through was found to be free of lytic activity. The lytic activity could be eluted by increasing the ionic strength of the buffer. Both the flow-through and the eluted lytic activity were shown to have the ability to liberate radioactive material from [^{3}H]diaminopimelic acid labelled cells.

c) Rechromatography of amidase on chitin. Since the material eluted from chitin with high ionic strength was shown to have the ability to release radioactive material from ^{3}H diaminopimelic acid labelled cells it was possible that this activity was due to amidase which had bound to the chitin and was simultaneously eluted with the lytic activity. Fraction 2 of the flow-through obtained after a T7 wild type supernatant had been applied to chitin was rechromatographed on a further column. The column was washed and eluted as for lytic activity as described in Experimental. Fraction 2 of the eluate was found to contain no A_{280nm}-absorbing material and possessed no amidase activity. It was thus apparent that the amidase does not bind to chitin and therefore the lytic activity was not contaminated with it.

d) The lytic enzyme loses both its lytic and amidase activities simultaneously.

One possible explanation for the existence of two activities was that the lytic enzyme, having lost its lytic activity, retained its amidase activity thus giving rise to the apparent presence of two separate enzymes. Fraction 2 eluted from chitin, containing T7 wild type lytic activity was stored at 4° for one week after which time neither lytic activity nor amidase activity was demonstrable. The amidase activity of the flow-through was not appreciably affected by storage at 4° for 3 - 4 weeks. These results further substantiate the existence of two well-defined enzymes.

e) Lytic activity and amidase activity in extracts of T7am13 infected E. coli B cells. When E. coli B cells were infected with T7am13, the first slight decrease in absorbance of the culture was observed at 22 min. In order to prepare extracts which could be compared with T7 wild type, the am13 infected cells were therefore harvested at 21 minutes. Enzyme assays were performed as for T7 wild type extracts. The release of radioactive material was followed for 30 min. incubation, during which time the release was linear. When compared with T7 wild type extracts prepared 11 minutes after infection it was found that the flow-through from deaminated chitin (specifically fraction 2) contained about 10% of the amidase activity and the eluate (fraction 2) from 6 - 28% of the lytic activity. Thus both activities were reduced, which could indicate that both enzymes are coded for by gene 3.5. However, amber mutants in gene 3.5 synthesizes DNA at a reduced rate, and T7 specific protein synthesis is at a low level (13,14). This may well explain the reduced lytic activity.

f) Other evidence for the existence of two enzymes. Studier (13) states that T7 "lysozyme" is synthesised between 5 and 15 minutes in common with other class II proteins. Pryme and Berentsen (1) using a turbidimetric assay observed a drastic increase in the amount of free lytic activity after 18 minutes, and according to the classification of Studier (13) the T7 lytic activity should therefore be classified as a class III protein. Hagen and Young (6) and Herrlich et al (3) using the filter technique have demonstrated

an activity which, appearing at 6 - 8 minutes, reaches a plateau at 15 minutes (class II). Hagen and Young (6) have observed reduced, but significant amount of "lysozyme" mRNA late in infection. The least complicated explanation for their results could be the overlapping of the activity of two different enzymes, one a class II and the other a class III protein. Schweiger et al (4) have studied the dependence of T7 directed synthesis of T7 "lysozyme" in vitro on Mg^{2+} concentration. They found that synthesis was maximal at approximately 11 and 13 mM Mg^{2+}, but with a distinct shoulder at 15 mM where non-early phage protein production is optimal. One of two possible explanations for these results was the induction of two "lysozymes" and in the light of our observations this would indeed seem to be the most reasonable suggestion.

g) The genes coding for the two activities. If gene 3.5 codes for the amidase, then which gene codes for the lytic activity? On the basis of their findings Pryme and Berentsen (1) have predicted that the lytic enzyme is incorporated into the T7 particles during their construction. Of the late genes without assigned function (13), the proteins specified by genes 7 and 13 are found in the phage particle but are not structural proteins (15). However, mutants from gene 7 - 19 have been shown to be similar to the wild type in both DNA synthesis and lysis (15), and it would thus appear that neither gene 7 nor gene 13 is responsible for the lytic activity. It is possible that the gene for lytic activity is one of those which still remains to be discovered (14).

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