CHAPTER III

PURIFICATION OF BACTERIOPHAGE Λ

BY GEL FILTRATION
Stocks of bacteriophage \( \lambda \) obtained by lytic growth in a sensitive host bacterium contain constituents of growth medium, the bacterial DNA, RNA, and other cellular components. For biochemical, genetic and radiobiological studies, it is necessary that the phage must be free from extraneous material including host DNA and RNA.

Purification of viruses usually involves a series of fractionation procedures, necessarily performed under mild conditions to prevent destruction of functional structure of viruses. The most frequently used method of phage purification employ either differential centrifugation or cesium chloride (CsCl) gradient centrifugation followed by removal of CsCl from the phage fraction (Kaiser and Hogness, 1960; Yamamoto et al., 1970; De Luy et al., 1972). Though the method is simple, it is very expensive and not suitable in countries like India in view of the cost and maintenance of ultracentrifuge, nitrocellulose tubes and CsCl etc. The other method using hydroxyapatite chromatography was described by Miyazawa and Thomas (1965). Although, it does not involve the CsCl gradient step, but it allows most of the impurities to elute with phage \( \lambda \). We, therefore, have developed a technique of phage purification by gel filtration which is efficient for bacteriophage \( \lambda \), by passes the CsCl gradient step, and is relatively inexpensive and rapid. The principle of \( \lambda \) purification by gel filtration is simply based on the "sieve theory" which implies that the molecular weight of one type of molecule (\( \lambda \)) is above the
exclusion range of the gel used, while the other type of molecules (all other constituents of stock other than \( \lambda \)) are capable of penetrating the gel.

**Materials and Methods**

**Phage strains.** \( \lambda \)IE, \( \lambda \)1577, \( \lambda \)22, \( \lambda \)LI, \( \lambda \)20, and \( \lambda \)p21 (described in Chapter 11) were purified by this technique.

(a) **Preparation of phage lysate:** The crude lysate was made either by confluent lysis of C600 strain on nutrient agar plates or by lysis of infected bacteria in shaken tryptone broth (Adams, 1959; Miller, 1972; Chapter 11). Heavy particles were removed by centrifugation at 5,000 r.p.m. at 4°C for 15 min. 2 ml of the crude lysate was applied on the Sephadex column to check the efficiency of column for crude stock. The remaining lysate was taken for nuclease treatment.

(b) **Nuclease treatment:** The crude lysate was first treated with bovine pancreatic DNase (10 \( \mu \)g/ml) for 30 min at 42°C with moderate shaking. The fibrous particles were centrifuged at 5,000 r.p.m. for 10 min and the supernatant was then treated with pancreatic DNase (10 \( \mu \)g/ml) in presence of 0.01M Mg\(^{2+}\) for 30 min at 37°C with constant shaking. The lysate was again centrifuged at 5,000 r.p.m. for 10 min to remove fibrous particles. At this step, again 2 ml fraction was loaded on the
Sephadex column for purification and the remaining lysate was further treated with polyethylene glycol (PEG).

(c) **PEG-treatment:** The volume of nuclease-treated phage lysate was measured and crystals of sodium chloride were added to give final concentration of 0.6M. Polyethylene glycol (10% w/v type 6000) was added to the lysate. The lysate containing NaCl and PEG was stirred at 4°C over a magnetic stirrer for at least 4 hr. PEG-treated phage lysate looked slightly opalescent. This was centrifuged in cold at 10,000 r.p.m. for 20 min. The supernatant was gently poured off and the pellet containing phage particles was suspended in 2 ml Tris-Hg buffer (0.01M, pH 8.0). Thus a 100 ml lysate had been reduced to 2 ml. The 2 ml buffer containing phage was centrifuged once at 2,000 r.p.m. for 5 min to remove heavy materials.

(d) **Final Purification of Phages:** PEG-treated phage was finally purified by gel filtration using Sephadex G-100 at 10 to 20°C. The column specifications are as follows:

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of the column</td>
<td>1.4 cm</td>
</tr>
<tr>
<td>Mass of dry gel used (approx)</td>
<td>2.4 g</td>
</tr>
<tr>
<td>Bed length</td>
<td>25.0 cm</td>
</tr>
<tr>
<td>Bed volume</td>
<td>38.3 ml</td>
</tr>
<tr>
<td>Void volume</td>
<td>13.0 ml</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.30 - 0.35 ml/min</td>
</tr>
</tbody>
</table>

**Preparation and Use of Sephadex Column**

(e) **Preparation of slurry:** 3 g dry Sephadex G-100 was taken in
100 ml conical flask to which 50 ml distilled water was added and mixed with a glass rod. The flask was kept for 4 hr in a boiling water bath and then for 2 hr at room temperature for swelling and removing the air bubbles from the slurry. Sephadex was sedimented and the supernatant was discarded, and 10 ml fresh distilled water was added.

(b) Preparation of Sephadex column. A washed glass column was closed by a pinch cock, alined vertically, and 10 ml distilled water was poured into the column. But piece of glass-wool was then cut out in the form of disc and put on the bottom of the column with the help of glass rod. Care was taken not to disturb the glass fibres, and to place it correctly. The glass rod was left pressing the glass fibre disc so that it do not move. All 10-15 ml slurry was gently poured down. After 10 min, the glass rod was gently taken out. The slurry was then poured up to the top with care that air bubble was not introduced. If air bubble appeared, it was removed by placing the tip of closed pipette near the bubble and then releasing the pressure of the pipette. Then all slurry was poured down, the column was kept closed for 2 to 3 hr for final setting. During the packing flow rate was kept identical to the working rate.

(e) Equilibration of column. For precision, accuracy and higher yield, it is necessary that pH and ionic strength of the Sephadex matrix (internal space of the Sephadex pores) must be identical to that of elution buffer. Therefore, elution buffer was passed through the column at the working flow rate till the pH of the buffer and effluent was the same. At this time the bed length of the column was also measured.
(d) Determination of void volume. Void volume was determined by loading 2 ml of blue-dextran (2.5 mg/ml) elution buffer on the column. The equilibrated column was taken and upper layer of elution buffer was removed by a pipette leaving behind approximately 2 ml buffer. The column was then opened and when just 2 ml buffer above the gel bed was about to be exhausted, it is closed and blue-dextran was very gently poured with a pipette. To have the uniform layering of blue-dextran, the tip of the pipette is uniformly moved in circular fashion. The column was then opened. The coloured layer moved down, when it reached the bed, 2 ml elution buffer was poured in circular fashion along the wall of column. The process was repeated twice and finally 10 ml buffer was poured. By this time, the coloured layer had moved down 5-10 cm below the top surface of the gel bed.

The volume of eluate was measured from the moment blue-dextran was applied till the coloured drop had come out from the column. This is the void volume. The column was re-equilibrated after determining the void volume.

(e) Purification of phage \( \lambda \) by Sephadex G-100 column. 2 ml phage stock was applied on the column and the elution was done by Tris-HCl buffer (0.01 M, pH 8.0). The procedure is identical as described for void volume. Fractions of 2 ml each were collected, but in the vicinity of phage peak, the size of the fraction was reduced to 1 ml. To compare the degree of purification, crude stock, nuclease treated and nuclease-PEG
treated stocks were loaded and eluted through the column.

**DNA, RNA and Protein Determinations of Eluted Fractions:**

Estimations of DNA, RNA and protein in each fraction were done according to the methods described by Burton (1956), Cesnotti (1955), and Loury et al. (1981) using calf thymus DNA, purified yeast RNA, and bovine serum albumin as standards respectively. Optical densities at 260 and 280 nm were also measured to follow the crude values of nucleic acid and protein in each fraction. Phage titre in each fraction was determined by plaque forming ability on C600 strain (Adams, 1959; Miller, 1972).

**RESULTS**

Typical elution profiles of DNA, RNA and protein for the purification of $\lambda\text{vir}$ are shown in Fig. 1. Elution patterns of the crude, nuclease-treated, and nuclease-PEG treated stocks loaded on the Sephadex column are respectively shown in (a), (b) and (c) compartments of Fig. 1. Fig. 1d shows the elution of phage $\lambda$ during gel filtration of the three stocks. Invariably 98% of the phage titre was recovered from the Sephadex column. Since the total phage particles were collected into 8 fractions of 1 ml each, the stock was eventually diluted 4 times. 92% of the total phage was recovered in 8 fractions of highest titre (at 12-16 ml of elution volume) with a dilution factor of only 2.8 (Fig. 1d).
Fig. 1. Gel chromatography of \( \lambda \) after various steps:
(a) Crude stock  (b) Nuclease treated stock  
(c) Nuclease-PEG treated stock  (d) Elution of  
phage particles.
It can be seen from Table 1 that crude lysate applied on the column contained 0.0, 0.60 and 0.45 mg of total protein, DNA, and RNA respectively. But the phage peak contained only 0.2 mg of protein, 0.34 mg DNA, and 0.24 mg RNA. Thus 97.4% of proteins, 43% DNA and 46% RNA were removed from the crude lysate as a result of gel filtration.

When the stock is treated with nucleases, a significant drop in protein, DNA and RNA was observed (Table 1), nevertheless, the column removes 95.8% of proteins, 65% DNA and 40% RNA from the phage fractions.

Nuclease-PEG treated stock showed a sudden decrease in the protein, DNA and RNA as compared to nuclease treated stock (Table 1). The column removed 77% of the remaining proteins, 66% DNA and 56% RNA during the gel filtration.

The Table 1 also shows that minimum quantities of protein, DNA and RNA were present in the purified phage fraction with nuclease-PEG treated stock. Moreover, during the whole procedure these components reduced to 114, 20 and 13.5 foldes respectively, as compared to the initial crude stock.

**DISCUSSION**

It is obvious from Fig. 1d that purified phage particles were eluted in the void volume (14 ml approx), whereas most of the impurities which include bacterial DNA, RNA and
<table>
<thead>
<tr>
<th>Steps</th>
<th>Total 'applied' phage stock contained in mg</th>
<th>Total 'eluted' phage stock contained in mg</th>
<th>Phage titre of applied stock</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>DNA</td>
<td>RNA</td>
</tr>
<tr>
<td>Crude lysate</td>
<td>8.0</td>
<td>0.6</td>
<td>0.45</td>
</tr>
<tr>
<td>Nuclease treated</td>
<td>4.5</td>
<td>0.23</td>
<td>0.20</td>
</tr>
<tr>
<td>Nuclease-DIC treated</td>
<td>0.3</td>
<td>0.09</td>
<td>0.08</td>
</tr>
</tbody>
</table>
protein, and other molecules of low molecular weight were
eluted in the fractions quite different from those of the phage
(Fig. 1). The separation volume between phage and impurities
was approximately 20 ml for the column. It is also clear from
Fig. 1 and Table 1 that DNA, RNA and protein contents of the
stocks were decreased as we proceeded for the next steps of
purification, nonetheless Sephadex G-100 column was capable of
differentiating the phage and impurities at each step even from
nuclease-PEG treated stock.

The values of RNA in the finally purified phage fractions are not absolute and precise due to the vicinity of lowest
limit of colour detection. Furthermore, orcinol reagent imparts
a little colour also with DNA, protein and agarose (Sephadex)
which led to the overestimation of RNA content (Ceriotti, 1955).
Folin's reagent may also interfere to some extent with DNA, RNA
and agarose. It has also been reported that diphenylamino
reagent interferes with RNA, proteins and agarose (Burton, 1956).
As far as the presence of agarose in the phage fraction is
concerned, it is well established that a little amount of
Sephadex dissolves during elution. In view of this, the
quantity of DNA in the purified phage (2 x 2.8 x 10^{11} plaque
forming units/ml) comes out to be 0.026 mg\(^*\) which is comparable

\*Mass of 2x2.8x10^{11} phage particles was calculated according
to the following formula:

\text{Mass of } \lambda \text{ in grams} = \frac{\text{Molecular weight of } \lambda \times \text{no. of } \lambda \text{ particles}}{\text{Avagadro's number}}

Contd......
with the experimental values. Therefore, it may be concluded that phage purified from this technique did not contain any significant amount of extraneous DNA, RNA and protein.

Since the molecular weight of PEG, 6,000 does not exceed the exclusion limit of Sephadex G-100, PEG contamination in the nucleases-PEG treated stock is also ultimately removed during the gel filtration and thus the technique also by-passes the step of dialysis before loading on the column.

The technique is very handy, inexpensive, rapid, and efficient for the purification of bacteriophage λ. There was no significant loss of viable phage particles during the elution through Sephadex column. Owing to low molecular weight of nucleases, there is no possibility of their contamination in phage fraction. Furthermore, the technique can be employed for large scale purification of phage λ by recycling the PEG step. For that purpose, large amount (say 10 litres) of lysate can be concentrated 100 times by PEG treatment by dissolving the pellet in fresh medium. It will then be treated with nucleases and PEG followed by gel filtration. Care should be taken about 'holding capacity' of the Sephadex column. By holding capacity of the column we mean the maximum number of molecules which can freely interact to the Sephadex beads without any detectable hindrance. As one increases the amount of sample, the

\[ \frac{3 \times 10^7 \times 2 \times 2.0 \times 10^{11}}{6.0 \times 10^{23}} = \frac{18.0}{6.0} = 0.028 \text{ mg} \]
Effective interaction of the particle with the Sephadex will be
less so that more of the impurities will come with phage λ.
For our column dimensions, the maximum permissible amount of
phage particles to be loaded is 10 mg of λ. Hence, a phage
titre of $10^{14}$ plaque forming units/ml, obtained through
repeated PEG treatments can be applied on the Sephadex column
for their purification in a single gel filtration step.

In principle, the method is not only useful for the
purification of phage λ but it should be equally applicable
for other phages and simple viruses.