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Purification and concentration of mycobacteriophage D29 using monolithic chromatographic columns

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A B S T R A C T

Bacteriophages are used widely in many fields, and phages with high purity and infectivity are required. Convective interaction media (CIM) methacrylate monoliths were used for the purification of mycobacteriophage D29. The lytic phages D29 from bacterial lysate were purified primarily by polyethylene glycol 8000 or ammonium sulphate, and then the resulting phages were passed through the CIM monolithic columns for further purification. After the whole purification process, more than 99% of the total proteins were removed irrespective which primary purification method was used. The total recovery rates of viable phages were around 10–30%. Comparable results were obtained when the purification method was scaled-up from a 0.34 mL CIM DEAE (diethylamine) monolithic disk to an 8 mL CIM DEAE monolithic column.

1. Introduction

Phages are viruses that infect bacteria. D'Herelle (1926) first used the phage to control outbreak of avian typhosis among farmed chickens. But the research was abandoned after antibiotics were discovered (Ho, 2001; Summers, 2001). There has been renewed interest in phage therapy because of the emergence of multidrug resistant bacterial infections (Chanishvili et al., 2001; Sharp, 2001). Phages are also used for other purposes, such as phage display (Benhar, 2001; Kassner et al., 1999; Larocca and Baird, 2001; Sergeeva et al., 2006), for DNA and protein vaccines delivery (Clark and March, 2004; Jepson and March, 2004; Ren et al., 2008; Wan et al., 2001), and phage bacteria typing (Balasubramanian et al., 2007; Bhowmick et al., 2007; Kumar et al., 2008).

Phages intended for use need to be purified to a high level preserving high infectivity. D29, a lytic phage, can form visible plaques after overnight incubation on a lawn of fast-growing *Mycobacterium smegmatis*. In the present work, D29 phages were purified by column chromatography. Currently available chromatography supports are mainly designed for protein purification with pore diameters adjusted to the protein size (Tyn and Gusek, 1990). However, chromatography columns for virus purification should have large pore diameters to enable access to a large binding surface area, which results in a high binding capacity (Kramberger et al., 2010). An appropriate chromatography support should be selected for phage purification. Recently, convective interaction media (CIM) methacrylate monoliths have been proven to be an efficient tool for purification and concentration of different viruses including phages (Boben et al., 2007; Gutiérrez-Aguirre et al., 2009; Kramberger et al., 2004, 2007, 2010; Smrekar et al., 2008; Whitfield et al., 2009). The aim of the study was to develop a purification method for mycobacteriophage D29 using CIM monolithic columns.

2. Material and methods

2.1. Bacteriophage preparation

The bacterial host *M.smegmatis* mc^{2}155 cells was propagated in 7H9 broth (Difco Middlebrook 7H9 broth; BD, USA) for 60 h. Approximately 2×10^4 plaque forming units (pfu) D29 phages were mixed with 1 mL bacteria stock and about 13 mL 7H9 top agar (containing 0.7% agar) (Michael et al., 1998). The mixture was plated on a 150 mm petri dish containing 7H10 agar (Difco Middlebrook 7H10 broth; BD, USA). Twenty such plates were prepared and incubated overnight at 37 °C. Both 7H9 and 7H10 broths contained 10% (vol/vol) oleic acid–albumin–dextrose–catalase (OADC; BBL

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Middlebrook OADC Enrichment; BD, USA) which was added when temperature of the medium was below 60 °C. After cultivation, the plates showed confluent lysis. Phage particles were collected by the addition of 10 mL phage buffer (containing 100 mM NaCl, 8.5 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris·Cl (pH 7.5), and 0.01% gelatin) to the surface of each plate. After six hours incubation at 4 °C, the phage-containing buffer was pipetted off the plates, and then filtered through a 0.45 μm pore size filter. The resulting phage solution was kept at 4 °C.

2.2. Viable phages particle enumeration

The titer of phage was determined by plaque assay (Adams, 1959). The result is expressed in plaque forming units. The phage suspension was diluted serially 10-fold with phage buffer, and each 100 μL phage solution was mixed with 300 μL *M. smegmatis* cells and 3 mL 7H9 top agar. The mixtures were plated on 90 mm petri dishes and incubated at 37 °C. After 16–20 h cultivation, PFU counting was done on plates containing between 30 and 300 plaques.

2.3. Determination of phage infectivity at different NaCl conditions

Different NaCl molarity of phage solution (0.1 M, 0.5 M and 1 M) was obtained by dissolving appropriate salt to phage buffer. Phage solutions were kept at 4 °C for 4 h, 12 h, 24 h and 7 d, respectively. Phage infectivity at each time point was determined with plaque assay technique.

2.4. Preliminary purification of phage D29

2.4.1. Preliminary purification of the phage by polyethylene glycol (PEG) precipitation

The 100 mL of phage suspension was precipitated by PEG 8000 as described by Sambrook (1989). First, sodium chloride was added to the suspension to 1 mol/L. After 1 h incubation at 0 °C, phage particles were precipitated by the addition of PEG 8000 to 10%, followed by incubation at 0 °C for at least one hour. After the centrifugation at 11,000 × *g* for 15 min at 4 °C, the phage-containing pellet was resuspended by 10 mL phage buffer. The phage particles were separated out by rinsing the precipitate repeatedly. Precipitate was broken down by blowing and sucking the mixture gently using wide-bore pipette tips and was then stored at 4 °C for 24 h. Afterwards, the mixture was centrifuged at 6000 × *g* for 15 min at 4 °C. The phage-remaining in the supernatant was stored at 4 °C, and the precipitate was resuspended again by 10 mL phage buffer. The whole process was repeated for four times. Finally, all the incubated phage-containing suspensions were placed together, followed by filteration through a 0.45 μm filter, and then stored at 4 °C. The resulting phage solution was the starting material for purification by CIM column.

2.4.2. Preliminary purification of the phage by ammonium sulphate precipitation

The 100 mL of phage suspension was precipitated by the addition of ammonium sulphate to 60% saturation at 0 °C. After 2 h incubation at 0 °C, the suspension was centrifuged at 20,000 × *g* for 30 min at 4 °C. The precipitate was resuspended in 20 mL phage buffer. The suspension was shaken by hand, and then stored at 4 °C for 12 h to dissolve the precipitate completely. The phage-containing suspension was then filtered through 0.45 μm filter and stored at 4 °C. The resulting phage solution was the starting material for purification by the CIM column.

2.5. HPLC, stationary and mobile phases

All experiments were conducted using an AKTA™ prime plus system (GE, USA), consisting of two pumps, a UV detector, which operated at 280 nm, and an injection valve with 5 mL sample loop. All components were connected with polyether ether keton (PEEK) capillary tubes. The anion exchange methacrylate-based CIM DEAE (diethylamine) disk monolithic column (BIA Separations) was used for the experiments. The purification method for D29 phages was optimized on a 0.34 mL CIM DEAE monolithic disk (12 mm × 3 mm i.d., bed volume 0.34 mL) and then scaled-up to a larger 8 mL CIM DEAE monolithic column (Do: 15 mm, Di: 1.5 mm, L: 45 mm, bed volume: 8 mL). The columns were sanitized periodically by circulating 1 M NaOH through it. For chromatography experiments, the phage buffer (pH 7.5) mentioned in Section 2.1 was used as an equilibration buffer. The buffer has the function of phage preservation and dilution (Sambrook et al., 1989). Elution buffers were prepared by adding sodium chloride to equilibration buffers to final molarity of 2 M, followed by adjusting pH of the buffers to 7.5 using NaOH.

2.6. Total protein determination

Total protein was determined by the BCA (bicinchoninic acid) assay (Cwbio, Beijing, China), according to the manufacturer's instructions using bovine serum albumin (BSA) as a standard.

3. Results

3.1. Effect of NaCl concentration on phage D29 infectivity

An important limitation during purification of biological materials is stability under different conditions. When ion exchange chromatography is used, phage infectivity under different NaCl conditions has to be determined. In this experiment, phages D29 have been exposed to different NaCl concentrations for 4, 12, 24, and 7 d, respectively. The results showed that the phage was essentially stable in the NaCl concentration between 0.1 and 1 M when stored at 4 °C (data not shown). The phage D29 had infectivity retention of almost 100% up to 7 days in all NaCl concentrations.

3.2. Variability of plaque assay

The plaque assay was used for enumeration of viable phages. In order to determine variability of the plaque assay for phage D29, ten plates with the same sample having appropriate dilution were inoculated. This procedure was repeated twice and one result is shown in Fig. 1. Standard deviations were found to be 33% and 27%, respectively. The average value is 30%. The method was proved to be robust and reproducible, and can be used as an analytical method for evaluation during the development of purification methods.

3.3. Comparisons of methods for primary purification of the phage

The recovery rates of PEG 8000 and ammonium sulphate precipitation for phage D29 were 68.9% and 81.2%, respectively. Taking into account that variability of the plaque assay method is around 30%, it can be concluded that the recovery rates of the two methods are comparable. The protein removal capacity of the two methods is shown in Table 1.

3.4. Optimization of purification method using a CIM DEAE disk monolithic column

After primary purification, a portion of the resulting phage suspension (1 mL) was diluted four times with equilibration buffer