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Purification and concentration of bacteriophage T4 using monolithic chromatographic supports☆

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Abstract

Phages are gaining importance due to their wide usage. In this work strong anion exchange monolithic chromatographic column was used for single step phage purification. Most of the proteins and DNA were removed and recovery of approximately 70% of infective virus was reproducibly achieved. 30 ml of phage sample was purified in around 10 min.

Keywords: Phages; Monoliths; CIM; Viruses

1. Introduction

Phages where discovered at the beginning of twentieth century [1] and are nowadays applied for different purposes such as antibacterial agents (solving the problem with growing antibiotic resistance) [2,3], for phage display (different proteins incorporated as coat proteins) [4,5], for development of phage-delivery vaccines (phage carrying vaccine antigens on the surface) [6,7], for targeted gene-delivery [8,9], and specific phage bacteria typing [10]. Regardless the field of use it is important that phages are purified to a high level preserving high infectivity [11].

When high purity product is required chromatography is a frequent method of choice. Although purification of viruses by column chromatography is thought by many to be a prerequisite for large-scale production, the technique is still problematic and poorly understood [12]. One of the major obstacles are currently available chromatographic supports being preliminary optimized for protein purification with pore diameters adjusted to the protein size [13]. Access of large molecules is therefore restricted by the pore diameter and as a consequence, the binding capacity of such media is low because large molecules are only adsorbed at the bead's outer surface [14].

With the introduction of new media like hydroxyapatite [15] and especially monolithic supports these problems seems to be overcome as there are few recent reports of fast virus purification preserving virus infectivity [16–18].

Our aim was to investigate whether Convective Interaction Media (CIM) monolithic columns can be implemented for purification and concentration of phages. Phage T4 (length, 220 nm; genome size, 174 kb) was used as a model system since it is one of the most investigated phages [19] and has already been used in preliminary phage therapy experiments [20,21].

2. Experimental

2.1. Sample preparation

Lauria–Bertani (Merck, Darmstadt, Germany) medium (LB medium) was inoculated with *Escherichia coli* (*E. coli*) (*DSM 613*) (DSMZ, Braunschweig, Germany) and grown at 37 °C under vigorous agitation. When the *E. coli* concentration reached 5×10^7 cell/ml, phage T4 (DSM No. 4505) (DSMZ, Braunschweig, Germany) stock culture (1×10^8 pfu/ml) was added to bacterial suspension. Incubation at 37 °C with agitation proceeded till the complete lysis of bacterial suspension, which was than centrifuged for 20 min at 6000 × *g* and 4 °C in a centrifuge (Sigma, Osterode am Harz, Germany). Afterwards, supernatant was filtered through 0.22 μm pore size filter (Millipore, Billerica, MA, USA). Phage solution was kept at 4 °C.

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2.2. Plaque assay technique

Titer of phage was determined with the plaque assay technique [22]. Result is expressed in plaque forming units (pfu). Liquid *E. coli* suspension in LB medium and phage suspension in buffer consisting of 60 mM Na_2HPO_4, 22 mM KH_2PO_4, 85 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$ were prepared. Autoclaved soft agar (0.75% agar) was thermostated on 50 °C to which equal volume (50 μl) of *E. coli* and phage solutions were added. After gentle mixing, suspension was poured across the agar plate. Plates with solidified soft agar were incubated overnight at 37 °C. By preparing different dilutions of phage solution before being added to soft agar different number of plaques on each agar plate were formed. On the plates where countable number of plaques was obtained their number was used for determination of phage concentration in original solution.

2.3. Determination of phage infectivity at different pH and NaCl conditions

Different pH values of phage solution were prepared by adjusting pH of phage solution using HCl or NaOH. pH values from 4 to 10 were prepared. Different NaCl molarity of phage solution was obtained by dissolving appropriate amount of salt. Concentration range from 0.1 to 2 M was prepared. Phage solution was kept for 4 h at room temperature. Afterwards, phage infectivity was determined with plaque assay described in Section 2.2.

2.4. HPLC equipment

All experiments were preformed on a gradient HPLC system consisting of two Pumps 64, an injection valve with 1 ml sample loop, UV–vis detector K-2501 with 10 μl volume-cell set to response time 0.1 and wavelength of 280 nm, all components connected with polyether ether keton (PEEK) capillary tubes. HPLC software and hardware were all from Knauer (Berlin, Germany). Flow rate was fixed at 2 ml/min.

2.5. Stationary phase

Strong anion exchange (quaternary amine—QA) methacrylate-based CIM disk monolithic column (BIA Separations, Ljubljana, Slovenia) was used throughout the experiments. The column was periodically sanitized after processing every 300 column volumes of sample, by circulating 1 M NaOH for 2 h through it.

2.6. Mobile phase

125 mM NaH_2PO_4, pH 7.0 (loading buffer) and 125 mM NaH_2PO_4, 1.5 M NaCl, pH 7.0 (elution buffer) of different pH value were used. All chemicals were obtained from Merck (Darmstadt, Germany). Buffers were filtered through 0.45 μm pore size filter Chromafil (Macherey–Nagel, Duren, Germany) before use.

2.7. Protein determination

Protein concentration in the solution was determined by the BCA assay (Pierce, Rockford, US) according to the manufacturer's protocol using bovine serum albumin as a standard.

3. Results and discussion

In order to obtain infective virus during purification process we have to investigate chemical condition under which phage preserve high infectivity. This information is necessary to adjust chromatographic method accordingly to avoid undesired phage deactivation during processing. In parallel, we also investigated reproducibility of plaque assay method to be able to evaluate properly determined amount of infective virus after purification.

3.1. Effect of pH and NaCl concentration on phage infectivity

Important limitation during purification of biological material is its stability under different conditions [23,24] since this determines degree of flexibility during method development. When ion exchange chromatography is used, especially pH and ionic strength range of the mobile phase, within which phage infectivity remains intact, has to be determined. Despite phage T4 is known to be very stable in a wide range of NaCl [25] we verified this conclusions for our particular strain. Phage has been exposed to different NaCl concentrations and pH values for 4 h at room temperature. Results showed that phage was essentially stable in the pH range 4–10 and NaCl concentration between 0.1 and 1.5 M while 2 M concentration decreased infectivity. This values therefore represent constrains which should be taken into account during development of purification method.

3.2. Accuracy of plaque assay method

Precise determination of virus infectivity represents in many cases a serious challenge due to lack of high method accuracy [26]. In order to determine accuracy of plaque assay for phages (infectivity test) in our lab we inoculated set of 10 plates with the same phage sample having appropriate dilution. This procedure was repeated twice and data from one set are shown in Fig. 1. Calculated relative standard deviation was found to be 13 and 16% for two sets of plates giving an average value of 14.5%. The method seems to be robust and reproducible and can therefore be further used for evaluation during development of chromatographic method.

3.3. Development of chromatographic method

Recent work applying methacrylate monoliths for purification of tomato mosaic and cucumber mosaic viruses demonstrated that optimal recovery testing different buffers was achieved when strong anion exchange column was used [16]. Furthermore, since elution was achieved at 0.3–0.5 M NaCl, this

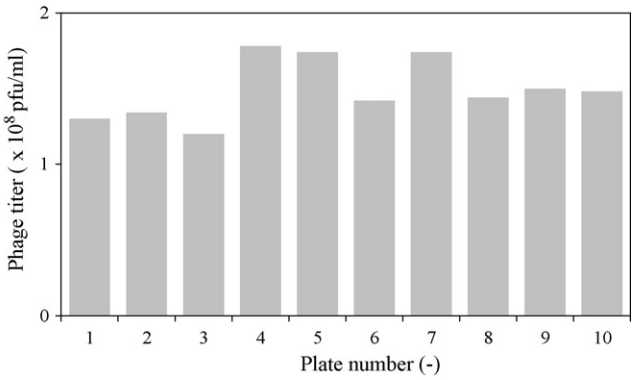

Fig. 1. Determination of plaque assay repeatability. 10 plates were inoculated with the same phage sample and phage titer was determined for each plate according to plaque assay method.

indicates possibility to remove DNA also in a single purification step which commonly elutes between 0.6 and 0.8 M NaCl concentration [27,28]. Based on these data we used CIM QA monolithic column and phosphate buffer as a mobile phase. Data of phage elution applying linear gradient of NaCl are shown in Fig. 2a. Collected fractions of 1.5 ml were analyzed via plaque assay to determine presence of infective phage and data are summarized in Table 1. While there is high UV absorbance response in first 2 min of purification run representing non-bound impurities, there was almost no phage detected with plaque assay in this fraction. This indicated that all phage binds to QA matrix under applied conditions. Majority, around 70% of loaded phage was eluted in fractions 2 and 3 having ionic strength between 0.3 and 0.5 M NaCl. All other fractions contained less then 3% of total loaded phage (Table 1).

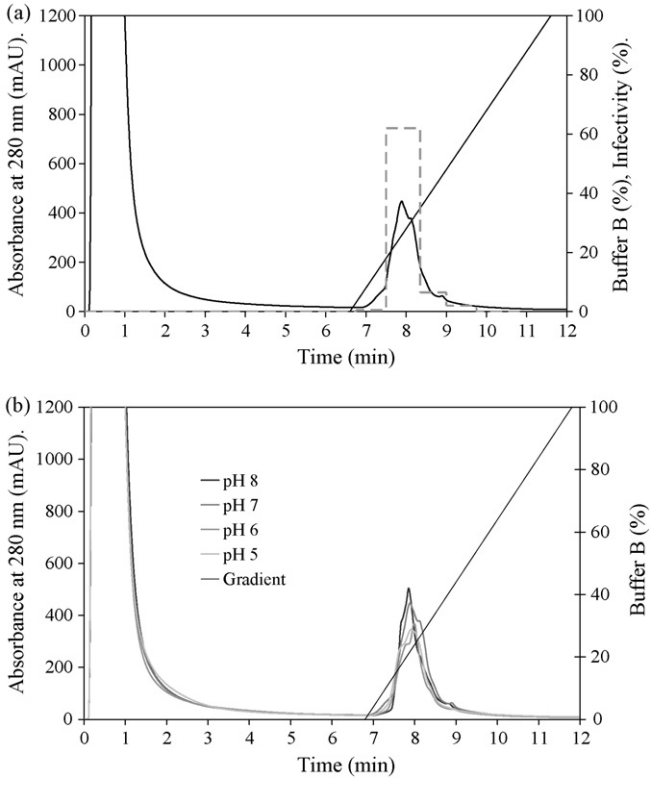

Fig. 2. Gradient elution of phage T4 on the monolithic column. Conditions: mobile phase: buffer A 125 M NaH_2PO_4; buffer B 125 M NaH_2PO_4 + 1.5 M NaCl; stationary phase: CIM QA disk monolithic column. Flow rate, 2 ml/min; gradient, 0–100% B (5 min). Sample, phage suspension; injection volume, 1 ml; detection, UV at 280 nm. Mobile phase pH: (a) 7; (b) different values.

Table 1
Titer of samples collected by phage purification which is shown in Fig. 2a

	Total pfu	Recovery (%)
Load	3.42×10^8	
Flow through: 0–1.5 min	2.4×10^4	0
Elution 1: 6.75–7.5 min	1.71×10^6	0.5
Elution 2: 7.5–8.25 min	2.12×10^8	62
Elution 3: 8.25–9.0 min	2.22×10^7	6.5
Elution 4: 9.0–9.75 min	6.84×10^6	2
Total elution	2.43×10^8	71

Further, we tested different pH values of mobile phase (Fig. 2b). Chromatograms are similar and elution salt concentration was the same, therefore no changes in binding strength occurred. In addition virus infectivity was also comparable. On this base we decided that further experiments are performed at pH value of 7 equalizing it to the value of phage growth medium.

When larger amount of highly concentrated phage is required, linear gradient can be changed into stepwise gradient where narrower peaks are achieved and fraction collection is easier. Based on data from a linear gradient, we introduced stepwise gradient consisted of four steps: 0 M–0.5 M–1.0 M–1.5 M NaCl. During elution with 0 M NaCl non-bound compounds were eluted and no phage is expected (see Table 1), during 0.5 M NaCl concentration step phage elution should take place, during 1 M NaCl step mainly DNA is supposed to eluted while at 1.5 M NaCl step other strongly bound impurities might elute. Typical chromatogram applying 1 ml of sample is shown in Fig. 3a. Indeed much higher and sharper phage peak was obtained while phage recovery (71%) was similar to the method of linear gradient. This method was further used for purification of 30 ml of sample containing phage titer 8×10^8 pfu/ml. Besides non-bound fraction there were two eluting peaks one during 0.5 M NaCl step (Fig. 3, peak 1) and other during 1 M NaCl step (Fig. 3, peak 2). In accordance to data from linear gradient, most of phage was eluted in peak 1, whereas peak 2 (1.0 M NaCl) probably contains majority of DNA. No elution peak in range of 1.5 M NaCl indicates that no other impurities have been eluted at even higher salt concentration. Eluted phage fraction of 3 ml volume contained 1.71×10^{10} pfu what was approximately 70% of loaded phage amount. Protein analysis determined with BCA showed concentration 0.4 mg/ml. This value consists of residual proteins and phage capside forming proteins. Taking into account initial protein concentration (4.5 mg/ml) in 30 ml of loaded sample, over 99% of proteins were removed in a single purification step. Entire procedure lasted approximately 40 min. When flow rate of 6 ml/min was applied, shortening the method down to 10 min, similar recovery was obtained. It can be expected that because of flow-unaffected monolith properties [29] faster purification can be achieved when even higher flow rate would be used.

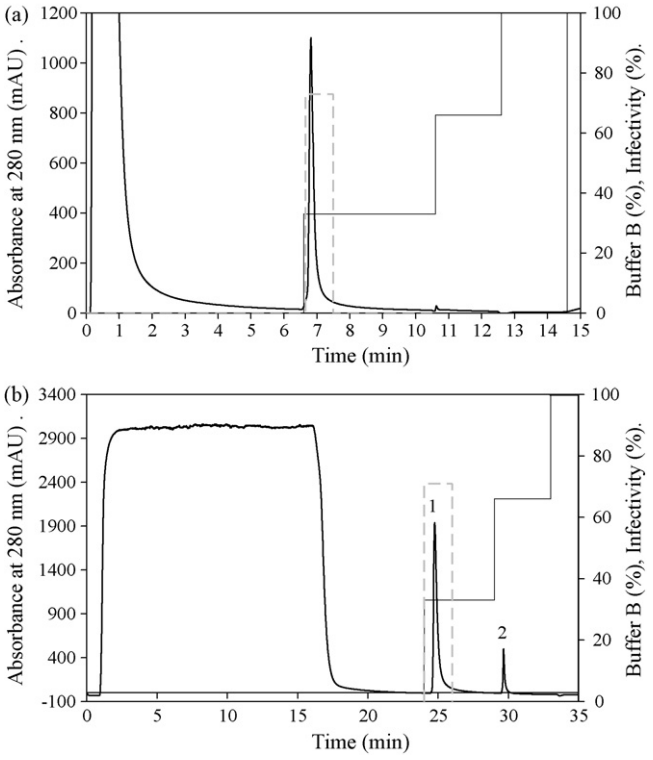

Fig. 3. Gradient elution of phage T4 on the monolithic column. Conditions: mobile phase: buffer A 125 M NaH_2PO_4, pH 7; buffer B 125 M NaH_2PO_4 buffer + 1.5 M NaCl, pH 7; stationary phase: CIM QA disk monolithic column. Flow rate, 2 ml/min; sample, phage suspension; detection, UV at 280 nm. (a) Gradient: stepwise consisting of four steps; 0% B (loading), 33% B (4 min), 66% B (2 min), 100% B (2 min); injection volume: 1 ml. (b) Gradient: stepwise consisting of four steps; 0% B (loading), 33% B (5 min), 66% B (4 min), 100% B (2 min); injection volume: 30 ml.

3.4. Method reproducibility

Method reproducibility is another important factor of particular concern in bioseparation [30]. To test it we repeated injection of 1 ml phage sample five times, determining phage recovery in terms of infectivity. As we can see from Fig. 4, phage recovery was always between 60 and 70% with a relative standard deviation of 9%. Taking into account that accuracy of plaque assay method is around 15% it can be concluded that chromatographic method for phage purification is robust and reproducible.

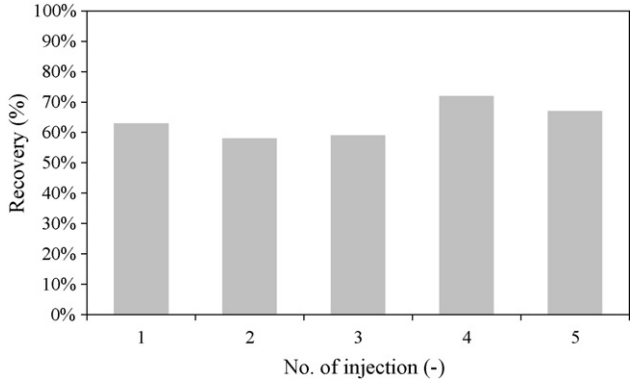

Fig. 4. Recovery of repeated purification procedure described under Fig. 2.

Data also indicate that performance of the column is preserved. Since phage is stable under applied chemical conditions, recovery around 65% is probably a consequence of phage mechanical damage during processing.

4. Conclusions

Experiments demonstrate that infective phages can be efficiently purified in a single chromatographic step using strong ion-exchange monolithic column. Since purification time is rather short this approach might be useful for fast purification of larger phage quantities when infective and pure virus is required.

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