Journal of Chromatography B, 878 (2010) 1855–1859

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Purification of bacteriophage M13 by anion exchange chromatography

Razieh Monjezi[a], Beng Ti Tey[b,c], Chin Chin Sieo[a,c], Wen Siang Tan[a,c,*]

[a] Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia
[b] Department of Chemical and Environmental Engineering, Faculty of Engineering, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia
[c] Institute of Bioscience, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia

ARTICLE INFO

Article history:
Received 22 March 2010
Accepted 18 May 2010
Available online 24 May 2010

Keywords:
Filamentous bacteriophage
Virus purification
Anion exchange chromatography
Fast protein liquid chromatography
Cesium chloride centrifugation

ABSTRACT

M13 is a non-lytic filamentous bacteriophage (phage). It has been used widely in phage display technology for displaying foreign peptides, and also for studying macromolecule structures and interactions. Traditionally, this phage has been purified by cesium chloride (CsCl) density gradient ultracentrifugation which is highly laborious and time consuming. In the present study, a simple, rapid and efficient method for the purification of M13 based on anion exchange chromatography was established. A pre-packed SepFast™ Super Q column connected to a fast protein liquid chromatography (FPLC) system was employed to capture released phages in clarified *Escherichia coli* fermented broth. An average yield of 74% was obtained from a packed bed mode elution using citrate buffer (pH 4), containing 1.5 M NaCl at 1 ml/min flow rate. The purification process was shortened substantially to less than 2 h from 18 h in the conventional ultracentrifugation method. SDS-PAGE revealed that the purity of particles was comparable to that of CsCl gradient density ultracentrifugation method. Plaque forming assay showed that the purified phages were still infectious.

1. Introduction

M13 is a member of filamentous bacteriophage (phage) family, *Inoviridae*, that infects *Escherichia coli* (*E. coli*) via the F pilus and the viral progeny are produced without lysing the host cell [1]. The phage particle is approximately 1 μm in length and about 7 nm in diameter [2,3]. It consists of a single stranded DNA (ssDNA) genome with 6407 nucleotides containing 11 genes [4]. The viral genome is enclosed in a flexible cylinder shape protein coat [3]. The major coat protein, pVIII, makes up the larger fraction of the viral mass (98%); at one end of the phage there are five copies of minor proteins, pIII and pVI; the other tip of the phage is composed of minor coat proteins pVII and pIX [1]. In general, the protein shell can be divided into three parts: (1) the outer layer is occupied by negatively charged N-terminal region of pVIII which is highly rich in acidic amino acid residues that interact with the solvent and provide the phage a low isoelectric point; (2) the interior part of the shell contains 19 hydrophobic residues that are involved in intra-subunit hydrophobic interactions; (3) the inner surface has basic residues that are positively charged and interact with single stranded DNA core [3,5]. Currently, bacteriophage M13 has been used widely in phage display technology and many peptides and proteins have been displayed successfully on the surface of the particle [6]. The displayed peptides and proteins have been applied in various fields of study including protein–protein interactions [7–9], identification of mimotopes for the development of vaccines [10,11], investigation of immune response [12,13], evaluation of small molecules drug candidates [14,15], identifying of peptide ligands for a variety of receptor ligands [16], and development of diagnostic reagents [6,17–20]. Recently, M13 has been studied for its uses in nanostructures and nanotechnology for developing nanowires, nanosized energy storage unit and biocomposite fibers [21–23].

In spite of the broad applications of M13 in phage display technology and other biological fields, purification of the phage still remains a neglected area. Conventionally, purification of the phage to a high purity is done by cesium chloride (CsCl) density gradient ultracentrifugation which is time consuming, laborious and needs successive rounds of high speed centrifugation and ultracentrifugation.

Ion exchange chromatography is a popular technique for the purification of peptides, proteins, oligonucleotides, nucleic acids and charged molecules. The popularity of this chromatography is associated with its high resolving power, high capacity, simplicity and controllability. Although this method has been used to

Abbreviations: CsCl, cesium chloride; CV, column volume; CIP, cleaning-in-place; LB, Luria Bertani; IPTG, isopropyl-β-thio-galactoside; TBS, Tris-buffered saline; Q_B, dynamic binding capacity; pfu, plaque forming unit; PEG, polyethylene glycol; AEC, anion exchange chromatography.

* Corresponding author at: Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia.
Tel.: +60 3 89466715; fax: +60 3 89430913.
E-mail addresses: m.razieh22@gmail.com (R. Monjezi), btey@eng.upm.edu.my (B.T. Tey), ccsieo@biotech.upm.edu.my (C.C. Sieo), wensiangtan@yahoo.com, wstan@biotech.upm.edu.my (W.S. Tan).

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doi:10.1016/j.jchromb.2010.05.028

purify infectious pancreatic necrosis virus [24], alphaherpesviruses [25], and respiratory syncytial virus [26], but until now purification of M13 with ion exchange chromatography has not been reported. Since M13 has a net negative charge at neutral pH, an anion exchange adsorbent was selected and a purification process by using a fast protein liquid chromatography (FPLC) system was established. With the aid of anion exchange chromatography and the knowledge about the structure of phage M13, a rapid and simple method for the recovery of the virus from clarified bacterial culture was developed.

2. Materials and methods

2.1. Materials

SepFast™ Super Q column was purchased from BioToolmics (Durham, UK). It is a strong anion exchange and contains a strong anion Q, $N^+(CH_3)_3$ functional group. The base matrix is made of a composite of highly cross-linked polysaccharides. The column was connected to an ÄKTA FPLC chromatography system (Amersham Pharmacia Biotech, Stockholm, Sweden).

2.2. Preparation of feedstock

Bacteriophage M13 was propagated in *E. coli* strain ER2738 grown in Luria Bertani (LB) medium containing 20 μg/ml tetracycline. The culture was clarified at 4 °C by two successive 10 min centrifugation at 3840 × *g* and 9820 × *g* (JLA 16.250 rotor, Beckman, USA). The supernatant containing phage particles was used as feedstock for the subsequent purification process.

2.3. Anion exchange chromatography

Chromatographic process was performed at room temperature. A pre-packed SepFast™ Super Q column (1 ml) was connected to the ÄKTA FPLC system. The column was equilibrated with 10 column volumes (CV) of binding buffer (buffer A: 50 mM Tris–HCl pH 7.5). The feedstock was injected to the column through a 50 ml superloop. Then the column was washed with 10 CV of buffer A to remove unbound or weakly bound phage particles. The adsorbed phage particles were eluted with 10 CV of final elution buffer (buffer B) in isocratic mode. Flowthrough and eluate were collected with a fraction collector included in the system (Frac-950) at fixed volumes and as peaks during elution, respectively. Cleaning-in-place (CIP) for the column was conducted according to manufacturer's protocol. The required time in one purification cycle includes the time spent for equilibration, loading, washing, elution, and CIP.

2.4. Optimisation of elution condition

Prior to the elution, the pre-packed SepFast™ Super Q column was loaded with the feedstock, followed by washing step as described in Section 2.3. The optimal pH and ionic strength of elution buffer were determined.

2.4.1. Optimisation of elution buffer pH

The bound phage particles were eluted from the column in an isocratic mode using citrate buffer containing 1.5 M NaCl at various pH (3.5, 4, 5). Eluted phage fraction was collected and neutralized with Tris–HCl (1 M, pH 9). The amount of phage M13 in the eluate was determined by plaque forming assay as described in Section 2.6.

2.4.2. Effect of ionic strength

The effect of ionic strength on the elution of bound phages was investigated by varying the concentration of NaCl (1, 1.5, 2 M). The bound phage particles were eluted from the column with citrate buffer at optimal pH value obtained from pH optimisation experiment containing various concentrations of NaCl. The amount of total eluted phage particles collected throughout the operation was then determined by plaque forming assay as described in Section 2.6.

2.5. Dynamic binding capacity

The dynamic binding capacity of the pre-packed SepFast™ Super Q column (0.33 ml settled volume of adsorbent) for M13 particles was determined. Feedstock (1.4×10^{11} pfu/ml) was injected to the column at a flow rate of 1 ml/min. Collected fractions (10 ml) were then assayed for M13 concentrations. The dynamic binding capacity (Q_B) is equal to the amount of phage adsorbed per ml of adsorbent and can be determined by using the following formula:

$$Q_B = \frac{V_B C_O}{V_S} \quad (1)$$

where V_B is the volume at 10% breakthrough (ml), C_O is the initial concentration of the feedstock (pfu/ml) and V_s is the settled volume of the adsorbent (ml).

2.6. Plaque forming assay

The concentration of infectious phage particles was determined by plaque forming assay as plaque forming unit per ml (pfu/ml). Ten-fold serial dilutions of the phage suspension in Luria Bertani (LB) were prepared. Each dilution (10 μl) was dispensed into a sterile microcentrifuge tube containing 200 μl mid-log phase *E. coli* ER2738 culture. The culture was mixed with 3 ml of top agarose (1% Bacto-tryptone, 0.5% NaCl, 0.5% yeast extract, 0.1% $MgCl_2.6H_2O$, 0.7% agarose) which was equilibrated at 45 °C in a water bath. The mixture was poured evenly onto a pre-warmed LB agar plate containing 20 μg/ml tetracycline, 40 μg/ml X-gal and 0.2 mM IPTG. The plates were allowed to cool and incubated overnight at 37 °C. Plaques formed were counted and the concentration of phage was determined as pfu/ml. The amount of phage particles was calculated by multiplying the concentration of sample by respective volume. All assays were performed in triplicates.

2.7. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of eluted phage particles was analysed by SDS-PAGE under denaturing condition [27]. Electrophoresis was performed with a 15% resolving polyacrylamide gel on a mini-protein 3 apparatus (BIO-Rad, USA) at constant voltage. The gel was stained with Coomassie® Brilint Blue R-250 and destained with destaining solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

2.8. Calculations

Adsorption efficiency is defined based on the total amount of the target protein bound onto the adsorbent before elution, or:

$$\text{Adsorption efficiency}\,(\%) = \frac{I - (F + W)}{I} \times 100 \quad (2)$$

where I is the amount of total M13 in the feedstock, and F and W are the amount of total M13 loss in flowthrough and washing steps, respectively.

Yield of M13 after purification process can be expressed by the following equation:

$$\text{Yield}\,(\%) = \frac{E}{I} \times 100 \quad (3)$$

where E is the amount of M13 in elution.

Table 1
Binding efficiency at binding conditions; 50 mM Tris–HCl, pH 7.5.

Initial amount of feedstock ($\times 10^{12}$ pfu)	Amount of M13 lost in flowthrough ($\times 10^{4}$ pfu)	Amount of M13 lost in washing step ($\times 10^{2}$ pfu)	Binding efficiency (%)
2.8 ± 0.02	1.8 ± 0.4	2.5 ± 0.6	99%

3. Results and discussion

3.1. Adsorption condition

The adsorption of M13 onto the SepFast™ Super Q column was performed in 50 mM Tris–HCl at pH 7.5. The amount of unbound phage particles in flowthrough and the amount of particles lost during washing stage were determined by plaque forming assay. The percentage of adsorption efficiency was calculated based on Eq. (2). It was found that at this condition phage particles bound to the absorbent efficiently (about 99% of injected particles adsorbed) (Table 1). The isoelectric point (p*I*) and the stability of the target protein are two important factors that have to be considered for selecting a suitable binding condition. Since the surface of M13 has an acidic p*I* close to 4.2 [28], hence at pH 7.5 phages are negatively charged and bind strongly to the positively charged adsorbent. In addition, pH 7.5 is very close to pH 7.4 of Tris-buffered saline (TBS) which is the most commonly used buffer for phage storage and it has minimum effect on the stability of phage particles.

3.2. Dynamic binding capacity of the SepFast™ Super Q column for M13

The dynamic binding capacity (Q_B) of the pre-packed SepFast™ Super Q column for M13 was calculated from the breakthrough curve (Fig. 1). The Q_B at 10% breakthrough (33 ml) was 1.4×10^{13} pfu/ml adsorbent. Table 2 shows the results of the binding capacity with various volume of feedstock. The highest binding capacity was obtained with 20 ml feedstock volume, hence for the subsequent experiment 20 ml of feedstock was applied to the column to avoid any lost of phage particles during the adsorption stage.

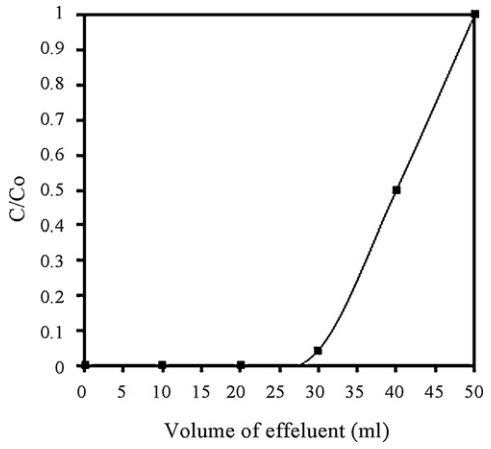

Fig. 1. Pre-packed SepFast™ Super Q column breakthrough curve. *C* is the M13 concentration in the effluent. C_O is the M13 concentration in the feedstock (1.4×10^{11} pfu/ml).

Table 2
Pre-packed SepFast™ Super Q column binding capacity.

Volume (ml)	Initial phage concentration (pfu/ml)	Phage in flowthrough (pfu/ml)	Phage bound (%)
10	1.4×10^{11}	1.37×10^{1}	100
20	1.4×10^{11}	4.43×10^{6}	99
30	1.4×10^{11}	5.50×10^{9}	96
40	1.4×10^{11}	7.30×10^{10}	48
50	1.4×10^{11}	1.45×10^{11}	0

3.3. Optimisation of elution condition

3.3.1. Effect of pH

The column loaded with 2.8×10^{12} pfu of M13 was eluted with isocratic elution using 10 CV of citrate buffer containing 1.5 M NaCl at different pH (3.5, 4, 5). The eluted phage particles were collected as a major chromatographic peak with a retention time of 1.61 min. Infectivity and recovery percentage of the eluted phage particles were determined by plaque forming assay. The highest recovery percentage was achieved when elution buffer at pH 4 was applied (Table 3). About 69% of bound particles were eluted at this pH.

M13 phage particles can be eluted by changing the condition favours for binding to a condition which is unfavourable for ionic bonding of the particles to the adsorbent. At pH lower than its p*I*, the phage has a net positive charge and can be eluted out from positively charged adsorbent. However, at pH lower than 3 the structure of the phage folds to a looser arrangement [29]. Hence, the lower recovery percentage of elution buffer at pH 3.5 is most probably due to the effect of low pH on the phage structure. On the other hand, at pH higher than the p*I* of M13 (pH 5) the particles have a net negative charge and bind onto the positively charged adsorbent. Consequently, lower recovery percentage at pH 5 was observed. Incubation of the phage particles at pH 4 showed that about 30% lost their infectivity (data not shown). As a result, the 26% loss of the recovery percentage is most probably due to the effect of elution condition on the phage particles.

3.3.2. Effect of ionic strength

The effects of ionic strength on elution efficiency of bound M13 particles are presented in Table 4. The results showed that the salt concentration of 1 M eluted the lowest amount of M13 compared to those containing 1.5 M and 2 M NaCl. The highest recovery percentage was achieved at elution buffer containing 1.5 M NaCl. Elution from ion exchanger is usually accomplished by increasing the ionic strength of the eluting buffer, as a result this reduces the forces between the adsorbent and the bound molecules. When salt

Table 3
Effect of pH on recovery percentage: citrate buffer containing 1.5 M NaCl.

pH	Amount of M13 particles in elution ($\times 10^{11}$ pfu)	Recovery (%)
3.5	5.40 ± 0.09	19.0
4	19.30 ± 0.09	68.9
5	5.00 ± 0.1	18.0

Table 4
Effect of concentration of NaCl in citrate buffer pH 4 on recovery percentage.

Concentration of NaCl (M)	Amount of M13 particles in elution ($\times 10^{12}$ pfu)	Recovery (%)
1.0	1.06 ± 0.01	37.0
1.5	2.20 ± 0.01	78.6
2.0	1.24 ± 0.01	44.3

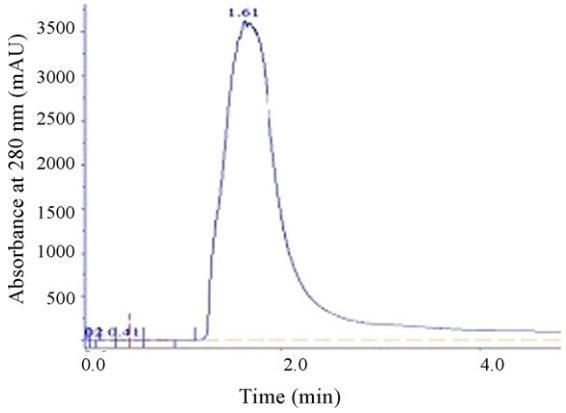

Fig. 2. Chromatogram of elution stage (for chromatography conditions, see text).

concentration increases from 1 M to 2 M, an increase in recovery percentage is anticipated, but the recovery was lower in 2 M NaCl than that of 1.5 M NaCl. This is most likely due to highly viscous elution buffer containing 2 M NaCl which may pose a problem in elution operation.

3.4. Purification of M13 by FPLC system

The optimized elution conditions were used to purify M13 from a clarified *E. coli* fermented broth. 20 ml of M13 (2.8×10^{12} pfu) was applied to the equilibrated pre-packed SepFast^{TM} Super Q column through a 50 ml superloop, at a flow rate of 1 ml/min (the flow rate was kept constant throughout the entire purification process: equilibration, flowthrough, washing and elution stages).

Most of the M13 particles were eluted as a single sharp peak with a retention time at 1.61 min (Fig. 2). The SDS-PAGE of the fraction at this peak revealed a highly purified band of the major coat protein of M13 with purity comparable to that of CsCl gradient density ultracentrifugation purified M13 (Fig. 3).

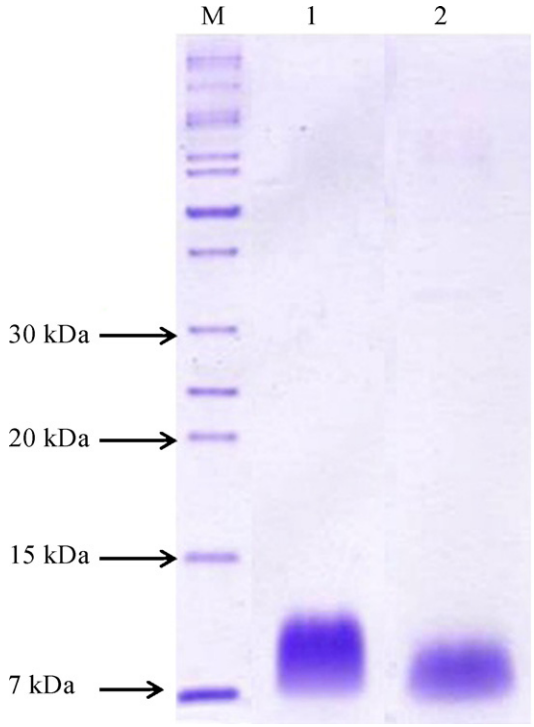

Fig. 3. SDS-PAGE of purified phage particles. Lane M: the protein markers in kDa; lane 1: M13 purified by CsCl density gradient ultracentrifugation; lane 2: M13 purified by AEC.

As a summary, the purification of M13 particles using the pre-packed SepFast^{TM} Super Q column connected to FPLC system has achieved a 74% recovery on average with a comparable purity to that of conventional CsCl gradient density ultracentrifugation method. Furthermore, in the purification of M13 by anion exchange chromatography method (AEC), clarified *E. coli* fermented broth containing M13 particles was applied directly to the column as feedstock. However, in the conventional method, phage particles in cell-freed culture supernatant have to be precipitated by PEG–NaCl overnight, and an additional 1 h of second PEG precipitation is needed. In addition, conventional purification method requires successive round of high speed centrifugation and ultracentrifugation [30]. Although these steps produce a very concentrated and highly purified bacteriophage sample, they also make the conventional method a time consuming and laborious method and also increase the cost of the purification process. In comparison, AEC is a single step, simple and fast method which can be accomplished within 2 h to produce a similar quality of bacteriophage.

4. Conclusion

In the current study, a simple, fast and efficient method for the purification of bacteriophage M13 was developed. 50 mM Tris–HCl buffer pH 7.5 and citrate buffer containing 1.5 M NaCl pH 4, were used as binding and elution buffers. The pre-packed SepFast^{TM} Super Q column was connected to an ÄKTA FPLC system. The purification process was performed at 1 ml/min flow rate and completed within 2 h. The infectivity and the recovery percentage of purified phage particles were determined by plaque forming assay. About 74% of adsorbed M13 particles were eluted successfully from the adsorbent. The 26% loss of the phage particles is most probably due to the effect of elution buffer condition on the phage structures during the elution stage. SDS-PAGE revealed highly purified M13 particles, with the purity comparable to that of conventional CsCl gradient density ultracentrifugation method.

Acknowledgements

This study was supported by the Research University Grant Scheme (RUGS) from Universiti Putra Malaysia and the Fundamental Research Grant Scheme (FRGS) from the Ministry of Higher Education of Malaysia.

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