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Ion-exchange chromatography method for the purification of genomic DNA fraction from *Mycobacterium bovis* Bacillus Calmette-Guérin

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Abstract

The goal of this study was to provide practical strategies for purifying genomic DNA fraction from *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG-DNA) by ion-exchange chromatography. A multistep process was developed to purify BCG-DNA. The process consisted of sonication, heating, trypsin digestion, ion-exchange chromatography, gel-filter chromatography, and lyophilization. After ion-exchange chromatography, BCG-DNA was highly purified and possessed potent biological effects. The methods described were efficient and had good reproducibility. Further, this was the first reported chromatography method to purify BCG-DNA.

Keywords: Purification; DNA

1. Introduction

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) has been used as a vaccine to prevent human tuberculosis for several decades [1,2]. In 1976, Morales reported for the first time that intracavitary administration of BCG had a therapeutic effect on superficial bladder tumor [3]. Recent studies have demonstrated that the antitumor function of BCG was related to its activation of B cell, NK cell, macrophage and inducement of T_{H1} cytokines [4–7]. However, severe side effects of BCG hamper its further use [8]. Therefore, many researchers are interested in separating active components with low toxicity from BCG, such as cell-wall skeleton from BCG (BCG-CWS), polysaccharide nucleic acid fraction from BCG (BCG-PSN), etc. [9]. In 1984, Tokunaga found MY-1, a fraction extracted from BCG which composed of 70%DNA and 28%RNA, could trigger immune defence mechanism by its unique structure. Several studies proved that it could induce T_{H1} immune response manifesting anti-tumor activity with low toxicity [10], it could augment the NK activity of peripheral blood lymphocytes from cancer patients, indicating its future application on humans [11,12].

Such regulation effect of MY-1 suggests potential role of DNA from BCG (BCG-DNA) in the modulation of the differentiation of T_H. To determine further the biological activity of BCG-DNA in animal models, the highly purified BCG-DNA are needed. Several techniques are available for the isolation of BCG-DNA; these include bacteriolycin digestion, phenol/chloroform extraction, cetyltrimethylammonium bromide (CTAB) precipitation, and phenol precipitation. A simple and fast technique has been validated for the isolation of BCG-DNA [10]. Tokunaga et al. used phenol/chloroform extraction + CTAB precipitation process to extract BCG-DNA (designated MY-1). But, the purity of MY-1 was low, composed of 70% DNA and 20% RNA. Another disadvantage of this method was the residue of toxic organic components in BCG-DNA. The aim of this study was to develop an improved method for highly purified BCG-DNA without contamination of organic solvent.

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2. Experimental

2.1. Chemicals

Sterile vials of heat-killed BCG (100 ml of a suspension containing 100 mg heat-killed BCG/ml) were provided by Vaccine and Serum Institute, Shanghai, China. Trypsin was obtained from Sino-American Biotechnology Co., China.

2.2. Apparatus

Ultrasonic Homogenizer was type 4710 (Cole–Parmar Instrument Co., Chicago, Illinois, USA). The BioCAD workstation (PerSpetive Biosystems Inc., Massachusetts, USA) was with a dual variable-wavelength UV–vis absorbance detector and an automatic autosampler. Chromatographic data were obtained and analyzed by BioCAD software.

2.3. Purification of BCG-DNA

2.3.1. Cell disruption

Heat-killed BCG were thawed and suspended in STE (0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0). We disrupted cells by sonication using an Ultrasonic Homogenizer (150 s; six output control; 60 duty cycle; totally, eight times). The disrupted cell suspension was centrifuged at 15,000 rpm for 15 min, the supernatant was collected.

2.3.2. Pretreatment procedures before ion-exchange chromatography

The supernatant was placed in a water bath at 100 °C for 10 min. Then the solution was left at room temperature for 20 min and centrifuged at 15,000 rpm for 15 min. Trypsin (10 mg ml^{-1}) was added to the supernatant to a final concentration of 1 mg ml^{-1}. The mixture was allowed to stand for 30 min at 37 °C and centrifuged at 15,000 rpm for 15 min. The supernatant was then precipitated with two volumes of 99.7% ethanol and kept for 10 min at −20 °C. The precipitate was collected, dissolved with PBS and were placed in a water bath again, the solution after centrifugation was collected.

2.3.3. Ion-exchange chromatography

The solution was applied to a DEAE sepharose fast flow column (200 mm × 25 mm, Pharmacia Biotech, Sweden). Two methods were used for comparison: I, a linear gradient of 100% solvent A (10 mM Tris–HCl, 0.2 M NaCl, 1 mM EDTA, pH 5.0) + 0% solvent B (10 mM Tris–HCl, 2 M NaCl, 1 mM EDTA, pH 5.0) to 0% solvent A + 100% solvent B at 6 ml min^{-1} was used; II, a stepwise gradient of 100% solvent A + 0% solvent B, 80% solvent A + 20% solvent B, and 62% solvent A + 38% solvent B at 6 ml min^{-1} was used. The BioCAD workstation was used to carry out the experiments. The detector wavelength was set at 254/280 nm.

2.3.4. Gel-filter chromatography

The solution achieved after ion-exchange chromatography was applied to a Sephadex G-25 column (200 mm × 25 mm, Pharmacia Biotech, Sweden). The column was equilibrated and eluted with solvent A at a flow rate of 5 ml min^{-1}. The same BioCAD workstation was applied, the detector wavelength was set at 254/280 nm.

2.3.5. Lyophilization

The solution was lyophilized using a lyophilizer (type MAXI Dry Lyo, HETO-HOLTEN A/S, Denmark).

2.4. Quantification of DNA and RNA

DNA and RNA were quantified by the diphenylamine reaction with calf thymus DNA used as a standard and by the orcinol reaction with yeast RNA used as a standard, respectively. The size of DNA fragment was examined by 0.8% agarose electrophoresis.

2.5. Quantification of protein and polysaccharide

Protein was determined by a commercial kit (Micro BCA Protein Assay Reagent Kit, PIERCE) according to the manufacturer's instruction. Polysaccharide was determined by the anthrone–sulfuric acid reaction with glucose used as a standard.

2.6. Bioactivity assays

Bioactivity assays were performed using the following protocol. Spleens from 6- to 8-week-old naïve BALB/c mice were recovered under sterile conditions, and single-cell suspensions were prepared in RPMI 1640 supplemented with 10% FBS. The splenocytes were plated at 5×10^6/ml for the cytokine assays (1 ml/well) in triplicate in 24-well round-bottom polystyrene plates. BCG-DNA were suspended in RPMI 1640 and plated to final concentration of 0, 10, 50, and 100 μg/ml for IFN-γ evaluation. The splenocytes were incubated with 5% CO_2 at 37 °C for 72 h. The level of IFN-γ was determined by ELISA kits (Jingmei Biotech Co. Ltd., Shenzhen).

3. Results

Step 1: The sonication step disrupted cells quickly and completely, resulting in the release of DNA fraction.

Step 2: Most proteins in the supernatant were clarified by heating and trypsin digestion. A great deal of white floccule, denatured and precipitated proteins, could be observed in the solution. Moreover, BCG-DNA was made single stranded by boiling for 10 min [13].

Step 3: The solution was applied to the ion-exchange chromatography for separating BCG-DNA from proteins and polysaccharide. Fig. 1 showed the analytical linear gradient

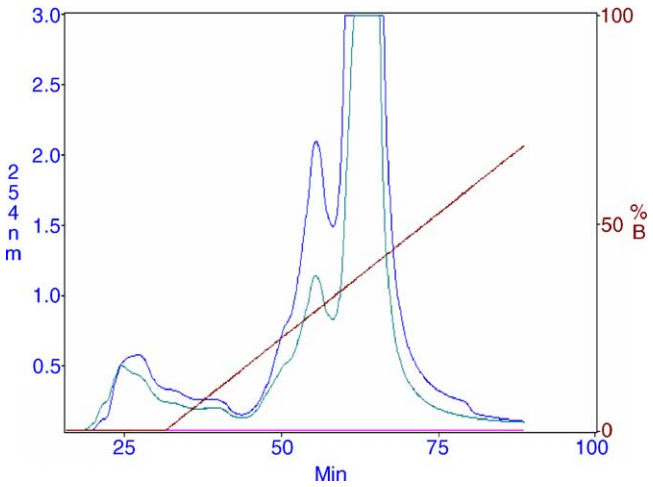

Fig. 1. Continuous gradient elution profile of ion-exchange chromatography. On the *x*-axis, 50 means 50 min. On the *y*-axis, 1.0 on the left side means OD_{254} = 1.0, 50 on the right side means B% = 50%; (■) 254 nm, (■) 280 nm, and (■) B%.

elution profile. The candidate peak and impurity peaks could not separate completely. According to the indication of the linear gradient elution, we applied a stepwise gradient elution as described above. As shown in Fig. 2, the two small peaks in Figs. 1 and 2 represented the impurities of proteins and polysaccharides in the product. The candidate peak and impurity peaks separated thoroughly.

Step 4: This step was applied for desalting. As shown in Fig. 3, there was only one candidate peak during the elution, representing the pure DNA product. After this step, the sample was ready to be lyophilized.

Step 5: This step was used to guard against the loss of the bioactivity of BCG-DNA, and was convenient for quantification. The lyophilization step produced a white flaky substance. The lyophilization is desirable for storage of BCG-DNA. The finished product was preserved at 4 °C.

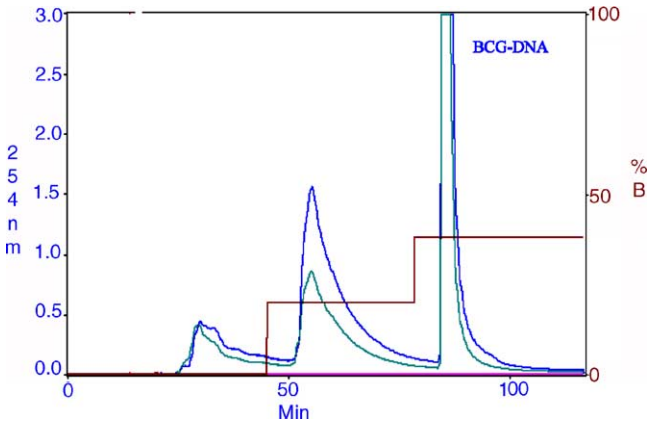

Fig. 2. Stepwise gradient elution profile of ion-exchange chromatography. On the *x*-axis, 50 means 50 min. On the *y*-axis, 1.0 on the left side means OD_{254} = 1.0, 50 on the right side means B% = 50%; (■) 254 nm, (■) 280 nm, and (■) B%.

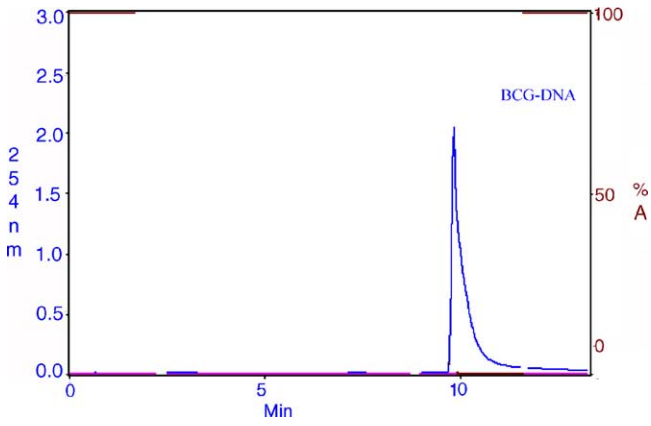

Fig. 3. Gel-filter chromatography profile. On the *x*-axis, 5 means 5 min. On the *y*-axis, 1.0 on the left side means OD_{254} = 1.0, 50 on the right side means A% = 50%; (■) 254 nm and (■) A%.

3.1. Product purity analysis

Table 1 showed the percentage content of DNA, RNA, protein, and polysaccharide in the product achieved after the pretreatment step and the product achieved after ion-exchange chromatography which used stepwise gradient elution and gel-filter chromatography, respectively. To illuminate the effect of the ion-exchange chromatography, we also analyzed the percentage composition of the impurity peaks. There still existed much protein and polysaccharide after pretreatment, while the product after ion-exchange chromatography was relatively pure with only a small amount of impurity, suggesting an excellent effect of ion-exchange chromatography. The peak purity of the product was around 95.3%, which meets the preset technical specification of 95% purity. The analysis of the percentage composition of the impurity peaks showed that there were mainly protein and polysaccharide with trace of DNA in the two impurity peaks. Agarose (0.8%) electrophoresis showed that the size of BCG-DNA is 200–250 bp (Fig. 4).

3.2. Splenocyte activation

Our bioactivity assays showed that the purification process produced bioactive BCG-DNA. It acted as a Th1 immune

Table 1
Purity assay of BCG-DNA

Products	Percentage content of BCG-DNA(%)			
	DNA	RNA	Protein	Polysaccharide
Pretreatment	54	2.2	8	34
Impurity peaks	0.3	0.8	24.5	73.5
	0.2	0.5	5.2	92.6
Ion-exchange chromatography	94	1	0.6	4
Gel-exchange chromatography	95.3	0.5	0.1	3.5

Table 2
Effect of BCG-DNA on IFN-gamma level (pg/ml) released by mice splenocyte ($n = 3$, $\bar{x} \pm s$)

Group (μg/ml)	BCG-DNA	BCG-DNA digested with RNase	BCG-DNA digested with proteinase	BCG-DNA digested with DNase
0	52 ± 2.5	52.33 ± 2.2	52 ± 2.5	50 ± 2.0
10	$118 \pm 2.5^{**}$	$110 \pm 2.4^{**}$	$112 \pm 2.0^{**}$	$48 \pm 2.1^{***}$
50	$142 \pm 2.5^{**}$	$139 \pm 2.5^{**}$	$138 \pm 2.5^{**}$	$52 \pm 2.2^{***}$
100	$179 \pm 3.6^{**}$	$170 \pm 3.5^{**}$	$176 \pm 2.1^{**}$	$50 \pm 2.2^{***}$

** $P < 0.01$ vs. BCG-DNA, 0 μg/ml group.
*** $P < 0.01$ vs. the same concentration group of BCG-DNA.

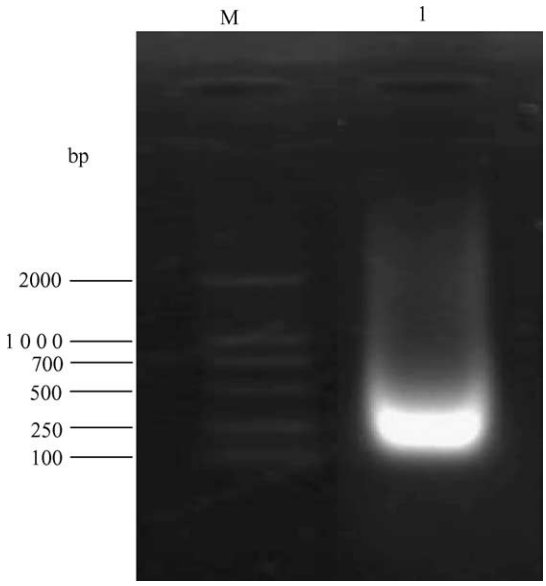

Fig. 4. Agarose (0.8%) electrophoresis of BCG-DNA; M, DL2000 marker and 1, BCG-DNA.

response immunostimulator, inducing significantly increased Th1-type cytokine (IFN-gamma) level (Table 2).

In addition to the experiment above, another assay was performed to test the bioactivity of BCG-DNA. In brief, the finished products were digested with DNase I (DNase I group), RNase (RNase group), and proteinase (proteinase group), respectively, and then were incubated with the splenocyte, no marked increase of IFN-gamma level was observed in DNase I group, while the similar increases of IFN-gamma were observed in BCG-DNA group, RNase group, and proteinase group (Table 2). Such results substantiated the bioactivity of BCG-DNA after purification by the process above.

4. Discussion

Although BCG-DNA is considered probably the essentially active component in BCG for its strong antitumor effect, limited data are available on the purification of BCG-DNA. The limiting factors in the purification of BCG-DNA have been the low recovery percentage and the contamination of toxic organic components. BCG-DNA, as an antitumor drug, is under clinical observation and hopefully intended as a drug for humans. So, it requires a high purity without residue of harmful organic compound. The extraction of DNA using chromatography has recently been reviewed [14] and although there are limited published methods, there is significant potential for ion-exchange chromatography to facilitate purification of BCG-DNA. The current method described has two major modifications from the traditional purification procedures [15]. The cells were disrupted by sonication quickly and thoroughly. The cells commonly were disrupted by bacteriolycin. But the cell wall of BCG is too hard to be disrupted by bacteriolycin. So, we experienced with sonication and found that BCG could be disrupted quickly and thoroughly under the given condition. Moreover, BCG-DNA could be broken down into small fractions of 200–250 bp. In the current method, heating, trypsin digestion, and chromatography were applied to clarify proteins without using organic compounds. In the conventional method, proteinase K and phenol/chloroform extraction are applied to clarify proteins [16], such processes are time-consuming and with high cost. The most key limitation of the conventional method is the contamination of toxic organic components in the product of DNA. Such contamination would hamper the further research on BCG-DNA in animals or human beings. Tokunaga et al. developed the phenol/chloroform + CTAB method to purify BCG-DNA (designated MY-1) which was composed of 70% DNA, and applied it in animals to investigate its antitumor activity. Such method was the same method as phenol/chloroform extraction but with a cetyltrimethylammonium bromide (CTAB) precipitation step included (at high NaCl, CTAB precipitates polysaccharides and proteins) to clear proteins. It was CTAB that may account for its relatively poor performance with pure DNA [10,17]. In the present research, ion-exchange chromatography was used to clear the rest of proteins. We found that too many impurities in the solution would reduce the efficiency of chromatography. So heating and trypsin digestion were applied to pretreat the solution to remove most of impurities of proteins. Heating and trypsin digestion were very simple and could cause proteins denatured and precipitated, thus clarify most of proteins. Then, ion-exchange chromatography was used. In the recent years, chromatography is applied widely, especially in the field of purification of proteins and nuclear acids. When loaded onto the column in a suitable buffer, the product of interest is retained on the column. After impurities such as proteins and polysaccharides are removed by washing, the BCG-DNA is eluted with the eluent with the increased ionic strength solvent. The chromatography technique provide an

inexpensive and efficient method for the purification of DNA. Anion exchange chromatography is often used to separate shorter DNA fractions. The separation is based on the total number of charges on each molecule. In the process of ion-exchange chromatography, we used the ionic strenghth and the pH value of the eluent as the elution strength to decrease the absorption equilibrium constant of the eluates [18]. We investigated the effect of initial pH of the eluent ranging from 5 to 8 on the retentivities of the eluates. The result showed that BCG-DNA had a strong retention on the DEAE sepharose fast flow column when the pH of the eluent ranged from 5 to 7. Considering that protein and polysaccharide had weak retention in low pH, we selected 5.0 as the optimal initial pH value of the eluent, so that the majority of BCG-DNA was retained on the column with little protein and polysaccharide retained on it. In the stepwise gradient elution, BCG-DNA could be effectively stripped from the column by the eluent with the increased ionic strenghth, after the weakly retained protein and polysaccharide were well separated. The resulting bands were sharp, which meant large peak capacity.

After the procedure of ion-exchange chromatography, the salt concentration of the product is high. But as we know, such product was going to be developed as an injection finally, and an injection cannot have such high salt concentration. So we applied gel filtration, whose aim is to decrease the salt concentration of the product.

After such series of procedures, highly purified BCG-DNA was extracted without contamination of toxic organic components. The recovery rate of BCG-DNA in the current method was 1.6‰, that was, the yield of BCG-DNA was about 16 mg from 10 g (wet weight) BCG, such yield was similar to that of the conventional method [19].

A novel method for purification of BCG-DNA has been developed. Extraction of BCG-DNA should provide a more complete understanding of the antitumor activity of highly purified BCG-DNA.

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