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Short Communication

PREPARATION OF ENDOTOXIN-FREE BACTERIOPHAGES

JANUSZ BORATYŃSKI^{1*}, DANUTA SYPER², BEATA WEBER-DĄBROWSKA², MARZANNA ŁUSIAK-SZELACHOWSKA², GRYZELDA POŹNIAK³ and ANDRZEJ GÓRSKI^{2,4}

¹Laboratory of Biomedical Chemistry, Department of Experimental Oncology and ²Laboratory of Bacteriophages, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland, ³Institute of Organic and Polymer Technology, Wrocław University of Technology, ⁴Transplantation Institute, Medical University of Warsaw, Nowogrodzka 59, 02-006 Warszawa, Poland

Abstract: Bacteriophages (phages) are bacterial viruses that interact with bacterial walls and invade bacterial cells. Moreover, they disturb bacterial metabolism and lead to bacteria lysis. In the case of Gram-negative bacteria crude phage cultures, apart from the phages themselves, the bacterial debris, bacterial proteins and nucleic acids contain endotoxins. These endotoxins (lipopolysaccharides) possess a high degree of toxicity *in vitro* and *in vivo*, and their removal is essential for safety in antibacterial bacteriophage therapy. An effective, scaleable purification of bacteriophages from endotoxins was accomplished by sequential ultrafiltration through polysulfone membrane (30 nm) followed by chromatography on sepharose 4B and Matrex Cellulofine Sulfate. The phage fraction after gel filtration chromatography routinely contained endotoxins in the 150-2500 EU/mL range. The procedure yielded bacteriophages contaminated with as little as 0.4-7 EU/ml (Limulus assay). This value lies within the permitted level for intravenous applications (5 EU/kg/h by European Pharmacopoeia, 1997).

Key Words: Endotoxin, LPS, Phage, Bacteriophage Purification, Therapy

INTRODUCTION

Endotoxins (lipopolysaccharides, LPS) are an integral part of most of the Gram-negative bacteria. Approximately 70% of the bacterial surface consists of these molecules, which are responsible for the stabilization and organization of

* Corresponding author; e-mail: borat@iitd.pan.wroc.pl

bacterial walls. Bacteria shed LPS into the environment, thus making endotoxins ubiquitous.

The endotoxin molecule consists of three different parts: a non-polar lipid component (lipid A), a core oligosaccharide and heteropolysaccharides (surface O-antigen) [1, 2]. Lipid A is the most conservative part of LPS and is responsible for the toxicity. Lipid A or endotoxin have particular influence on monocytes and macrophages. Activated cells release mediators, such as TNF, interleukines, prostaglandins, colony stimulating factor (CSF), platelet activated factor (PDGF) and free radicals [3-5]. Exposure to endotoxins affects the structure and function of cells and organs, changes the metabolism, triggers a coagulation cascade, modifies haemodynamics, causes fever, and may result in shock [6, 7].

Therefore, removing endotoxins from materials designed for use on humans, or indeed any animals, is an important area of study in applied biotechnological science. Many reports on methods for endotoxin removal from biological fluids have been published. Their success was greatly dependent on the initial composition of the mixture. In the case of purifying water of endotoxins, filtration gives good results. Preparations obtained from Gram negative bacteria always include a mixture in which endotoxins are not only highly concentrated, but may also exhibit interactions with the desired products.

In the case of the UspA2 protein from the bacterium *Moraxella catarrhalis*, a potential vaccine candidate, the endotoxin formed a very tight complex, which could be dissociated with zwitterionic detergents. The pollution of protein by endotoxin was reduced approximately 20,000-fold by either ion-exchange or gel filtration chromatography [8]. Removing the tightly bound endotoxin from inclusion bodies requires solubilization with guanidine hydrochloride and ammonium sulfate in combination with hydrophobic interaction chromatography. An endotoxin reduction was achieved, from 10^6 EUmg⁻¹ in the solubilized material to 200 EUmg⁻¹ [9]. The synthetic peptide S3delta has a high affinity for endotoxin. A matrix based on this peptide was developed for the removal of LPS from water, buffers and protein solutions. At a starting LPS of approximately 100 EUml⁻¹, a post-purification level below 0.005 EUmg⁻¹ was achieved [10]. Cellulose membranes containing chitosan, metal chelator or a hydrophobic and positively charged membrane were used for removing endotoxin with an efficiency of over 80%. The recoveries of desirable substances were over 95% [11].

Deoxycholate and chitosan immobilized membrane cartridges were used for water and interferon purification. The endotoxin level of purified interferon was reduced ten-fold [12]. There are other reports on the employment of various other techniques for the removal of endotoxins. For example, adsorption onto immobilized, positively charged ligands such as histidine [13] polyethyleneimine [14], poly-lysine [15], or adsorption onto polymer particles [16], or extraction techniques [17].

Methods of endotoxin detoxification include whole-blood adsorption systems that are based on high-affinity interaction with immobilized albumin [18, 19], polymyxin B [20, 21] or adsorption onto dialysis membranes made from polyether polymer, polymethylmethacrylate or polysulfone [22]. Methods for removing endotoxins from protein solutions were recently reviewed [23, 24]. In this paper, chromatography on Matrex Cellulofine Sulfate preceded by ultrafiltration and gel filtration were employed for bacteriophage purification. The endotoxin level in such afforded bacteriophages would even allow their intravenous administration.

MATERIALS AND METHODS

Crude bacteriophage suspensions (bacterial lysates) were prepared according Slopek *et al.* [25]. Polysulfone membranes with asymmetric pores of 30 nm in diameter were obtained from Institute of Organic and Polymer Technology, Wrocław University of Technology. Matrex Cellufine Sulfate was purchased from Millipore. The endotoxin assay was performed using the Limulus Amebocyte Lysate assay (LAL). Endotoxin tests were purchased from Charles River Laboratories International, Wilmington USA, and BioWhittaker, Walkersville, USA. The bacteriophage titer was assayed in an agar plaque test [25].

The bacterial lysate (2-4 l) was subjected to ultrafiltration (polysulfone membrane) under increased pressure (1 bar). The concentrated bacteriophage suspension (10-20 ml) was washed with physiological saline (3 x 150 ml). The resulting suspension (10-20 ml) was chromatographed on a sepharose 4B column (2.5 x 95 cm, eluent 0.063M phosphate buffer, pH 7.2, flow 0.3 ml min⁻¹) [26]. Bacteriophages were eluted in the highest-molecular weight fraction, and concentrated by filtration as described before. The partially purified phages were chromatographed on Matrex Cellufine Sulfate (2 x 10 cm, flow 0.2 ml min⁻¹). The first fraction eluted with 0.01 M phosphate buffer (pH 7.6) contained a mixture of bacteriophages and endotoxins. The second fraction, containing endotoxin-free bacteriophages, was eluted with 0.01 M phosphate buffer (pH 7.6) containing 1 M saline.

RESULTS AND DISCUSSION

Ultrafiltration and size-exclusion chromatography provide, in theory, a means of separating molecules differing in molecular mass. Their application to bacteriophage purification allowed a partial removal of most contaminants in phage suspension, and allowed their concentration. Unfortunately, endotoxins, despite their relatively low molecular weight (4-20 kDa for LPS monomer), are not effectively removed. Endotoxins aggregate forming structures similar to micelles and vesicles, with diameters up to 0.1 µm. The presence of detergents, chelators and proteins promotes the formation of structures like micelles (300-

1000 kDa) and monomers (4-20 kDa), while bivalent ions promote the formation of large structures like vesicles (>1000 kDa) [23].

Chromatography on the Matrex Cellufine Sulfate phase is a crucial stage in endotoxin removal. Matrex Cellufine Sulfate is a spherical cellulose matrix with a 3 kDa exclusion limit. On the one hand, this form grants resolution to the chromatography, but on the other hand, greatly lowers the load capacity of the solid phase and requires relatively pure material. The cellulose matrix contains a small amount of 6-mono-sulfate esterified glucose residues. Such a composition of the resin provides ion exchange and affinity interactions with a vast range of viruses and other macromolecules.

Tab. 1. Examples of the results of endotoxin removal from bacteriophages.

Bacteriophage	T4	T 4	F8
Target bacteria	Escherichia coli	Escherichia coli	Pseudomonas aeruginosa
Titer of bacteriophage in crude suspension	5×10^9	1×10^9	3×10^8
Volume of crude bacteriophage suspension [l]	4	3	2
Titer of bacteriophage after gel filtration on sepharose 4B	2×10^{11}	5×10^{10}	3×10^{10}
Volume of bacteriophage fraction after gel filtration [ml]	50	52	50
Endotoxin level after gel filtration in bacteriophage fraction [EU/ml]	2500	160	1 700
Volume of bacteriophage fraction after Matrex cellulofine sulfate chromatography [ml]	900	165	40
Titer of bacteriophage after Matrex cellulofine sulfate chromatography	5×10^8	2×10^7	4×10^8
Endotoxin level after Matrex cellulofine sulfate chromatography in bacteriophage suspension	7 EU/ml	0.4 EU/ml	1 EU/ml

At low ionic strength or in physiological buffers, virus particles or viral antigens bind to the chromatography media through affinity interaction, and are washed with high ionic strength solutions (i.e. 1 M saline) [27]. At the same initial conditions, nucleic acids, proteins and endotoxins pass through the column unbound. The literature reports that some viruses follow this principle (feline herpesvirus, human measles, human HSV 1, human parainfluenza type 3, feline

calicivirus), while others either bind to the matrix irreversibly (human RSV) or do not interact at all (human adenovirus type 8, murine leukemia virus, human polio virus type 1 and human echovirus type 8). These reported differences in interactions with the Matrex Cellufine Sulfate phase do not correlate with the virus dimension, the presence of the envelope, or the type of nucleic acid [28].

Our investigation proved that bacteriophages cultured from *Escherichia coli* and *Pseudomonas aeruginosa* bind to the chromatographic matrix, while endotoxins do not. The observation enabled highly purified bacteriophages to be obtained by scaleable affinity chromatography. The obtained bacteriophage preparations contained less than 10 EU/ml.

The presented procedure allows endotoxin-free bacteriophage preparations to be produced. The total yield of the purification procedure is 0.1-2.7%, and thus the ratio of bacteriophages to endotoxins increased 10- to 30-fold. It can be anticipated that optimizations of the processes will improve the yield. Purified bacteriophages contain $1.4\text{-}2 \times 10^{-8}$ EU/ *Escherichia coli* bacteriophage particle and 0.25×10^{-8} EU/ *Pseudomonas aeruginosa* bacteriophage particle. The endotoxin-free bacteriophages were tested for interactions with tumor and immunologically competent cells [29, 30].

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