Development of a scalable process for the purification of bacteriophages

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Abstract: The recent development of antibiotic resistant bacterial strains has led to the development of new therapies for infections, such as phage therapy. Traditional phage purification methods are optimized for use in research and new methods for phage purification, suitable to large-scale production, are required, using, for example, chromatography. In this work, the purification of a phage solution from bacterial impurities was studied and a process was identified for further research. A phage stock was amplified and clarified though centrifugation, before concentration and diafiltration, leading to only small losses in phage titre, and removal of most bacterial proteins. Separation through anion-exchange chromatography (AEC) and multimodal chromatography (MMC) was then tested and optimized. and a combined AEC-MMC led to elimination of most proteins and DNA in AEC, followed by total phage recovery in MMC, with further protein removal, and possible elimination of smaller DNA fragments. This combined strategy is promising for phage separation, although further studies are required.

Keywords: Bacteriophage; Phage therapy; Downstream purification; Chromatography;

Introduction

Ever since World War II, the use of antibiotics has led to the decline of deaths resultant from bacterial infections, being considered a great advance in medical history. However, resistance to these compounds has become widespread among many pathogenic strains, leading to a challenging problem in medicine[1]. Due to these strains, there has been a focus in discovering and developing novel strategies for the treatment of bacterial infections. Among the many methods currently being investigated[2], bacteriophage therapy is a potential viable alternative to antibiotic treatment[3]. Discovered in 1915, bacteriophages or phages, are viruses that infect and destroy bacteria. Since their discovery, phage therapy has been proposed and attempted, but was mostly abandoned. However, with the need to find substitutes for antibiotic treatment there has been a surge in interest towards phage therapy[3].

Phage therapy presents many advantages when compared to traditional antibiotic treatment. The mode of lysis of phages is different from any antibiotic allowing them to bypass antibiotic resistance, the stricter spectrum of action prevents collateral damage on the gut microbiota, and phage therapy has shown better efficacy compared to antibiotic treatment[3]. However, phage production requires the use of bacterial strains[3] and, after fermentation, the culture broth contains several bacterial components and cellular debris that need to be removed[4]. Of note is endotoxin, which is found on the outer membrane of gram-negative bacteria, inducing adverse effects such as shock, tissue damage or even death, when administered to mammals. As such there is a maximum level of 5

endotoxins units (EU) per kg of body mass per hour allowed for pharmaceutical products[5]. Purification of phage preparations is thus a critical step in its production.

Several methods have been used in research for phage purification, including centrifugation in CsCl density gradients, ultracentrifugation, ultrafiltration and precipitation with polyethylene glycol. However, most of these methods are not suitable for industrial-scale production, and are not optimized for endotoxin removal[6]. As such, there is a growing need of developing novel strategies for phage purification. Among the methods currently in study, chromatography has been the most preferred, as it is an easily scalable process for use in industrial context.

Methacrylate monolithic media, also referred to as Convective Interaction Media (CIM), is composed by a single block, which features large pores and interconnected channels, allowing for convective mass transport and providing a larger binding surface, increasing the binding capacity. Monoliths have also been successfully scaled up for use in industrial processes[11]. Strong anionic CIM disks have been used to successfully purify phages [8–10] with successful scaling-up. Other chromatographic media and methods have also been tested, such as a packed-bed anionic exchange column[11], expanded bed anion-exchange chromatography[12], and methods such as size-exclusion[13] and affinity chromatography[14].

The aim of this work was to prepare a phage, stock, from a bacterial culture and develop a process for the purification of this preparation from bacterial components, such as proteins, DNA and endotoxins. To this end, a phage lysate was amplified, clarified with centrifugation, concentrated and diafiltrated. The resulting stock was then processed through anionic exchange (AEC) and multimodal chromatography (MMC), and the purified samples were analysed for their phage titre, as well as their content in bacterial proteins and DNA.

Experimental Process

Phage lysates were prepared by amplification in bacterial cultures. These lysates were then filtered, clarified through centrifugation or microfiltration. The clarified lysates were then processed for concentration and diafiltration. After stock preparation, different chromatographic methods were employed, including anion-exchange, multimodal, anion-exchange followed by multimodal and size exclusion chromatography. After each chromatographic trial, peaks were identified and recovered in order to analyse their phage titre through a plaque assay, bacterial protein content through SDS-PAGE and Bradford assay and bacterial DNA content though PCR and qPCR.

Discussion and Conclusion

The main objective of the work here presented was to study the purification of a preparation of a phage solution from bacterial contaminants and identify processes that might be interesting for further research. To this end, phage stocks were prepared, clarified and, for purification, several chromatographic approaches and combined strategies were attempted.

For clarification, both microfiltration and centrifugation were attempted. Although it was not possible to compare the phage losses between both methods, microfiltration was time-consuming. For protein removal, microfiltration appears to be more efficient, however,

acceptable removal was obtained for both methods.

For concentration and diafiltration, only small losses in the phage titre were detected. These steps appear to be important for phage stability, since the first stock, after centrifugation, remained stored for one month in the culture media, leading to phage precipitation and large losses in the titre. In both processes, loss of bacterial proteins to the permeate was also observed. In the first two stock, as the initial protein load was low, the removal of proteins by these processes led to a very low concentration, which prevented a correct appraisal of the protein removal efficiency of the chromatographic processes by Bradford assay and SDS-PAGE. These processes also led to removal of a portion of the DNA present in the clarified lysate.

For chromatography AEC, MMC and SEC were tested. In AEC, during initial optimization, phage particles seemed to elute preferably at a particular elution buffer concentration. Optimization of the injection load was also carried out, and larger injection volumes provided more intense peaks and concentrated fractions. However, when injection load optimization was being carried out, the elution profile of the particles seemed to change. While the reason for this change is unknown, it might be due to small-scale aggregation between phage particles, leading to less interaction between each particle and the media. During stock preparation, large-scale aggregation caused the loss of a significant portion of phages in the clarified lysate. While the phages are stable under the stock buffer, prolonged storage time might have led to some particle aggregation. In response, an optimized stepwise gradient mode was created, which allowed for satisfactory recovery of phage particles at different steps. This optimized strategy was thus employed in following AEC trials.

Regarding bacterial protein and DNA removal, it was seen that the filtration processes removed a large portion of the proteins in the lysate, leading to low levels in the stock. Although absolute values could not be obtained, it was possible to see that most of the remaining contaminants were removed from the phage fraction, although a residual portion still remained. Some small proteins were detected in different elution fractions, but it was not possible to confirm if these proteins were in association with the phage particles or were phage proteins. Additionally, the identity of some of the obtained peaks could not be confirmed, but could be caused by other, untested bacterial contaminants.

When MMC was attempted, very good phage recoveries were obtained. Due to the characteristics of the column, smaller impurities, like endotoxins and proteins will remain in the column, while phages are able to pass through, allowing purification. In the peaks obtained, only residual quantities of proteins, that were likely bound to the viral capsid, and bacterial DNA were detected. When a combined AEC-MMC strategy was attempted, although a low titre was obtained in AEC, which was not expected, full recovery was obtained in MMC, with elimination of most residual proteins, and bacterial DNA. While the endotoxin content was not detected, in theory the column used for MMC should result in removal. As such, out of the different approaches tested in this work, a combined AEC-MMC strategy seems to be the most promising for further studies.

A SEC strategy was also tested. However, due to time restrains, only two trials,

with different sample loads, were performed. Due to the differing results obtained in these trials, no conclusions could be drawn. Finally, the combined AEC-MMC approach was also tested with other type of bacteriophage. Although a significant amount of pfu was recovered from AEC, large losses were detected in MMC.

For the large-scale production of phages for therapeutic purposes, three conditions are required for the downstream process. It should be able to quickly process large volume of lysate, the phage recovery should be high enough for economic feasibility, and it should be able to remove most bacterial impurities such as endotoxins. While in this work identified a potential strategy for the purification of phage particles, several questions arose, that required additional research. Despite the importance of the removal of endotoxins from the phage preparation, it was not possible, unfortunately, to measure the efficiency of the methods here presented in their removal. As such, further tests, with this objective in mind, should be performed. Other tests should also be performed, such as determining the binding capacities of the columns to the phages.

Additionally, while AEC led to good recoveries and contaminant removal, phage elution was observed at two different conductivities. While phage aggregation could explain this change, it was not proved in this work. Electron microscopy could be used in order to clarify whether aggregation did actually occur, and if it interferes with chromatography. If the hypothesis is confirmed, the possible impact of phage aggregation on large-scale production should be studied. While it should be expected that downstream processing of the lysate would occur immediately following amplification, not allowing time for the agglomeration of the phage particles, the high titres necessary could induce this aggregation. Finally, the trials for SEC shown here were not conclusive.

In conclusion, in this work strategies for the downstream processing of bacteriophages has been studied and a combination consisting of centrifugation, filtration, and purification through AEC and MMC has been proposed. Further research is required in order to determine the endotoxin removal efficacy, and to test a possible integration of SEC into the process. Due to the growing need to develop new alternatives to antibiotics, work into this topic is sure to continue in the future.

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