Design of a Scalable Strategy for the Initial Recovery and Capture of Bacteriophages: Nuclease Digestion and Phenyl Boronate Chromatography

Viúla, Nídia (86738)

Dissertation, july 2021, 2020/2021; Prof. Ana Azevedo

Integrated Master in Biological Engineering, Department of Bioengineering, Instituto Superior Técnico

Abstract

The evolution of antibiotic resistant bacteria has become a growing global health concern. Studies have been made in order to develop an answer to this problem. The use of phages, microorganisms that infect bacteria, preventing them from replicating but also decreasing their concentration by inducing the host cell lysis, may be the answer the scientists are looking for. In this study an attempt to remove protein and DNA from a phage suspension (T4 phages specific for *E. coli*) without decreasing the phage infectivity was done. The techniques studied were a Denarase® incubation digestion and a Phenyl Boronate Chromatography. The chromatography was tested for four different buffer conditions (differences in the salt concentration and in the pH), being the best results (49.49% yield, 96.61% protein and 47.60% DNA removal) obtained in the third condition studied. The digestion was tested for four different incubation times and two different stopping activity methods (heat and cold). It was also performed before and after the buffer exchanged samples. The activity stop method with best results was the cold one and of the 4 times studied the 30 minutes and 1 hour and 30 minutes were the ones with the best results. However, the digestion to the lysate was the process with better results, with a yield higher than 65%, a protein and DNA removals higher than 80% and 99%, respectively.

Introduction

The antibiotics discovery and development was an important step in order to combat bacteria, however the misuses of these drugs has resulted in antibiotic-resistant bacteria. With the increasing rates of antibiotic resistance and the decrease of antibiotic development, scientists are compelled to find new ways of controlling bacteria growth.[1]

Bacteriophages, informally known as phages, may be the answer for the problem stated. These microorganisms consist in a virus that infects bacteria and archaea, replicating within them. They can also confer phenotypes to their host, transforming a non-pathogenic strain into a pathogenic one, regulating the host population. The relationship between phage-bacteria can vary from a simple predator-prey model to a complex symbiotic one.[2]

As the predator-prey relationship occurs in the phage life cycle, it is important to understand the interactions between the organisms. Phages present two different life cycles: lytic and lysogenic cycle. The lytic cycle consists of the infection of virulent phages by attaching themselves to a specific receptor (lipopolysaccharides, teichoic acids, proteins or flagella[3]) present in the host membrane and injecting the genetic material into the cell. The host cell will, then, provide the molecular building blocks and enzymes enabling the phage replication, producing progeny phage. The enlysin and holin, phage encoded proteins, will activate the cell lyses from the inside. The lysogenic cycle, which produces temperate phages, does not include the host cell lyse. In this cycle phages integrate their genome in the host cell one by inserting it into the host chromosome at specific sites. However, the phage DNA may also exist inside the cell as plasmids. In lysogenic cycle it is established a stable relationship as the prophage, phage DNA inserted in the host genome, is replicated with the host genome.[4]

After the understanding of phage's actions and potential, these organisms started to be used in different areas, food industry, agriculture and aquaculture industry, wastewater treatments, biotechnology or even pollution remediation. Even so, the most studied application is regarding health problems, the fight against antibiotic resistant bacteria. The use of phages in phage therapy, as therapeutic agents continues to be studied.[5] Phage therapy has the advantages of presenting a fast isolation, continuous infective work, ineffectiveness of eukaryotic cells (reducing the risk of infection on human microbiota) and more important, the ability to adapt in order to keep up with their host evolution. However, these organisms can not be used in therapy against intracellular pathogens as they are unable to enter eukaryotic cells.

The research and therapy using phages requires a high purified phage suspension. Various purification techniques have been studied over the years and the defined one, used nowadays, is polyethylene glycol precipitation followed by CsCl gradient ultracentrifugation. This technique, however, has some disadvantages such as it's difficult to perform and expensive. It, still, presents another huge disadvantage which is the fact that it is unfit for the purification of large phage amounts. In order to overcome these problems, a phenyl boronate chromatography and a nuclease digestion (with denarase®) purification were tested.[6]

The phenyl boronate chromatography is an affinity chromatography used with success in clinical trials through the use of boronic acid or boronates as ligands. Most boronate derivatives, at pH 8, show the ability to form covalent bonds with compounds that have cis-diol groups. Sugars, as glucose, present these cis-diol groups allowing their removal by boronates.[7]

The nuclease digestion can be a solution method to decrease DNA amounts as this molecule is difficult to remove by chromatography. A nuclease is an enzyme with the ability to cleave the phosphodiester bonds between nucleotides of nucleic acids. These enzymes are natural and play a huge role in repairing the DNA within the cells. They are, also, extremely important to prevent the vulnerability of the DNA to modifications of the metabolic and environment stressors.[8]

This study focuses on purifying a phage suspension (T4 phages specific for *E. coli*) by removing the proteins and the DNA while maintaining the phage infectivity.

Materials and Methods

T4 Phage Sample Preparation

A pre-inoculum of *Escherichia coli* (DSM 613) was prepared and used to inoculate a TSB (Tryptic Soy Broth) medium. When the inoculum reaches an OD_{600} of 0.2-0.25, the suspension is infected with phage T4 (DSM 4505) to a MOI (multiplicity of infection) of 0.1. The suspension with the phages is incubated at 37 ºC and 250 rpm until the OD_{600} reaches 0.1. Then a centrifugation of 8000xg at 4 ºC for 20 minutes is performed. This is followed by a filtration in a 0.22 µm pore size bottle top filter. The sample is then stored at 4 ºC for further use.[9]

Phenyl Boronate Chromatography

Phenyl Boronate (PB) Chromatography was performed at room temperature in 746 µL of PB (Phenyl Boronate) resin columns, which were connected to an Äkta start (GE Healthcare, Uppsala, Sweden). The column presented 5 mm in diameter and was packed up to 3.8 cm. Several different equilibration, wash and elution buffers were tested in four different conditions. Condition 1: equilibration and adsorption buffer - Milli-Q water, elution buffer - 1.5 M Tris-HCl, pH 8.5; Condition 2: equilibration and adsorption buffer - 15 mM of Tris-HCl, pH 8.5, elution buffer - 1.5 M Tris-HCl, pH 8.5; Condition 3[10]: equilibration and adsorption buffer - 15 mM Tris-HCl, pH 7; elution buffer - 1.5 M Tris-HCl, pH 8.5; Condition 4: equilibration and adsorption buffer - 15 mM Tris-HCl, pH 7, elution buffer - 1.5 M Tris-HCl, pH 7.

The column was equilibrated with 5 column volumes of equilibration buffer. After equilibration, it was injected into the column 1 mL of sample. The unbound material was washed with 5 column volumes of adsorption buffer and the bound material was eluted with the elution buffer. All buffers were run at a constant flow rate of 1 mL/min. All trials were done with the lysate (before buffer changes) and the stock (after buffer changes) as samples. The stock samples were obtained after nuclease digestion.[11]

Nuclease Digestion

In order for the samples used to be incubated with Denarase®, a change to the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8) was made using 20 mL Amicons (NMWC, of 100 kDa) with the support of a centrifuge (10 minutes, 4000 rpm, 4 ºC). The Amicons were first washed with Milli-Q water and then a concentration of the lysate was performed. The volume removed was equivalent to the one inserted in the nuclease buffer. The procedure was repeated until a 4 diafiltration volume exchange was completed. The nuclease concentration in each sample was 1 U/mL. The samples were incubated at 37 ºC for different time periods (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours). To stop the nuclease digestion, two different methods were used, heat (10 minutes at 72 ºC and cold, ice storage). For the ice stopped digestion only the 30 minutes and 1 hour and 30 minutes were tested.The samples were, then, used in a phenyl boronate chromatography with condition 3.

As the salt concentration (2 mM $MgCl_2$) may interfere in the phenyl boronate chromatography, a change of buffer (to the adsorption buffer 15 mM Tris-HCl, pH 7) was accomplished, by using Amicon Ultra 0.5 mL (membrane module with a NMWC of 10 kDa), helped by a centrifuge (14 000g, 10 minutes, room temperature). A Phenyl Boronate Chromatography was then accomplished for each sample.

In an attempt to study the enzyme action without previously doing a buffer exchange, the lysate was incubated with Denarase® at 37 ºC for different time periods (30 minutes and 1 hour and 30 minutes).

Plaque Assay

A pre-inoculum of *Escherichia coli* (DSM 613) was prepared and used to inoculate a TSB (Tryptic Soy Broth) medium. The bacteria were put in incubation until the OD_{600} was 0.2 at 37 ºC and 250 rpm. Successive dilution in SM buffer (0.1 M Tris-HCl pH 7.4, 0.1 M NaCl, 10 mM MgSO4.7H2O, 0.01% w/v gelatine) were made to the T4 phage samples. When bacteria OD_{600} is as expected, 50 µL of 1 M MgCl2 was added and 200 µL of the new solution was mixed with 100 µL of diluted samples. Top-Agar was melted and thermostated at 64 ºC. $MgCl_2$ with a concentration of 1 M was added to the top-agar, being 3 mL of this solution added to infected cells. The solution was mixed gently and poured onto TSA medium in petri dishes. The plates were incubated overnight at 37 ºC.

BCA Assay

It used a Pierce BCA (Bicinchoninic acid) Protein Assay Kit from Thermo Scientific. A standard curve of protein was done by diluting Albumin (BSA) Standard, where the diluent was Phosphate Buffered Saline (PBS), from Sigma. The samples were analysed in a microplate. To each well was added 25 µL of sample and 200 µL of reagent. The reagent was prepared by adding 1:50 µL of Reagent A and Reagent B, available in the kit. Absorbance at 562 nm was measured in a SpectraMax 340 pc, USA, equipment.

PicoGreen Assay

An invitrogen (Quant-iTTM PicoGreen TM dsDNA Assay Kit) from Thermo Fisher Scientific was used. First a standard curve was done by mixing λ DNA with 1x TE. A total of 100 µL of 1x TE and 100 µL of sample or standard is added per well. With the lights off, the PicoGreen 1:200 was prepared in 1x TE. 100 µL of the prepared PicoGreen was then added to each well. The plate is incubated for 5 minutes at room temperature, but kept from the light. The fluorescence is measured using Tecan. Note that for this assay, 2 pools (Pool 1 and 2) were done corresponding to the samples from each chromatographic peak, being the first peak (fractions T2, T3, T4, T5 and T6) corresponded to pool 1 and the second peak (fractions T17, T18 and 19) to pool 2.

SDS-Page

The samples were prepared by adding 25 µL of loading buffer 2x (312.5 mM Tris-HCl, 10% SDS, 50% glycerol, 0.05% bromophenol, pH 6.8) (Bio-Rad Laboratories, USA), 5 µL of 1 M DTT and 20 µL of protein sample. The sample is heated at 100 ºC for 10 minutes.

The resolving gel one is done by mixing 3 mL of Acrylamide/bis-acrylamide 40%, 2 mL of Resolving buffer 4x (1.5 M Tris-HCl, 0.4% SDS, pH 8.8), 4.445 mL of Milli-Q water, 0.005 mL of TEMED and 0.05 mL of ammonium persulfate, APS. The stacking gel is done by mixing 0.25 mL of acrylamide, 0.625 mL of stacking buffer, 0.5 M

Tris-HCl, 0.4% SDS, pH 6.8, 1.61 mL of Milli-Q water, 0.0025 mL of TEMED and 0.0125 mL of APS.

After solidification, the samples are put in the gel. The Precision Plus Protein™ Dual Color Standards ladder is also put in one or two wells of the gel. The gel is run at 100 mV until the samples reach the bottom of the gel. Afterwards, the gel is covered with Coomassie Blue to stain and destained with destaining solution (30% v/v ethanol, 10% v/v acetic acid) until the bands are visible.

Results and Discussion

Phenyl Boronate Chromatography

A phenyl boronate chromatography was done to the lysate using 4 different conditions, described in Materials and Methods. For every condition, a plaque assay, a BCA assay and a PicoGreen Assay were done in order to understand the phage purification in chromatography. The results obtained are available in Table 1.

Table 1. Protein Quantity (mg), DNA quantity (µg) and Infectivity (PFU) of samples present in the chromatographic peak corresponding to the higher phage concentration, for each condition studied and the lysate.

	Sample	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
Phenyl Boronate Chrom. (in the phage peak)	Lysate	4.87E-02	0.726	4.60E+09
	Cond. 1	3.33E-03	0.364	2.64E+09
	Cond. 2	3.12E-03	0.327	2.04E+09
	Cond. 3	2.47E-03	0.382	2.45E+09
	Cond. 4	2.82E-03	0.339	2.17E+09

All chromatograms obtained present a high intensity peak (2000 mAU) around the 2 mL of buffer (first peak), and a smaller peak after the 14 mL of buffer (second peak). From the plaque essay, it is possible to see that there are more infectable phages in the second peak than in the first peak and with the BCA assay, the protein quantity was obtained, showing that the quantity of protein is higher in the first peak. This shows that the chromatography allows a separation from the proteins and the infectable phages. Table 1 shows the infectivity, DNA and protein removals for the second chromatographic peak as this is the phage peak. In terms of protein quantity, condition 3 shows the lowest values (2.47E-03 mg). When analysing the DNA quantities, condition 2 presents the lowest quantities (0.327 µg). However, condition 1 shows the highest infectability (2.64E+09 PFU). In order to decide which condition should be used for further investigations the yield and percentage of protein and DNA removed (Table 2) were calculated and analysed.

Table 2. Protein Removal (%), DNA Removal (%) and Total Yields (%) of samples present in the chromatographic peak corresponding to the higher phage concentration, for each condition studied and the lysate.

	Sample	Protein Removal (%)	DNA Removal (%)	Yield (%)
Phenyl Boronate Chrom.	Cond. 1	96.26	49.09	55.99
	Cond. 2	96.73	53.69	33.41
	Cond. 3	96.61	47.60	49.49
	Cond. 4	96.85	46.67	53.45

From analysing Table 2 it is possible to see that the higher yield is achieved in condition 1 (55.99%), followed by condition 4 (53.45%), the lowest is observed in condition 2 (33.41%). As the yield is one of the major concerns, condition 2 is, for this reason, excluded from following studies. From the 3 remaining conditions the one with the higher DNA removal is the first condition (49.09%), followed by the third one (47.60%). Condition 4 is, then, excluded from the next steps. As condition 3 shows a higher protein removal (96.91%) than condition 1 (96.26%), condition 3 was chosen for the studies with the nuclease digestion.

Nuclease Digestion

- Heat Stopped Digestion

In order to decrease DNA quantity in the samples before a chromatography a nuclease digestion was done. To do this, a buffer exchange with the enzyme in use cofactor was performed. The samples, in the new buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8), were tested for amount of DNA, protein quantity and phage infectivity. The results are presented in Table 3. After a nuclease digestion with Denarase® is done for 4 different digestion times. The samples were also

tested for DNA and protein quantities and infectivity (Table 3). Posteriorly to the nuclease digestion, the samples could follow two paths, Directly to the phenyl boronate chromatography or another buffer exchange in order to understand the impact of salt concentration in the chromatography. For both paths the amount of DNA, proteins and infectivity were tested, being the results available in Table 3. After the second buffer exchange, into the adsorption buffer (15 mM of Tris-HCl, pH 7), a phenyl boronate chromatography was performed and, again, the samples were tested for DNA and protein quantities and infectivity (Table 3).

Table 3. Protein (mg), DNA (µg) quantities, Infectivity (PFU) of samples present in the nuclease buffer, after nuclease digestion, present in chromatographic peaks corresponding to the higher phage concentration and in the adsorption buffer, for each digestion time studied and the lysate.

Nuclease Buffer Exchange	Sample	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	Lysate	4.64E-02	1.96	7.20E+09
	Buffer	1.47E-02	1.39	2.34E+09
	Heat	1.41E-02	1.31	2.25E+08
Denarase® Digestion	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	1.29E-02	0.037	6.45E+07
	1 hour	1.43E-02	0.038	1.61E+08
	1 h 30 m	1.02E-02	0.167	1.73E+08
	2 hours	1.51E-02	0.059	2.31E+08
Phenyl Boronate Chrom. After Digestion	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	2.38E-03	3.75E-02	9.10E+06
	1 hour	2.29E-03	3.96E-3	5.96E+07
	1 h 30 m	1.00E-03	1.01E-03	6.42E+06
	2 hours	2.17E-03	5.58E-03	6.84E+06
Adsorption Buffer Exchange	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
Previous to Chrom.	30 min	1.81E-04	2.61E-03	6.90E+06
	1 hour	1.63E-05	2.33E-03	9.75E+05
	1 h 30 m	1.63E-05	3.48E-04	2.15E+06
	2 hours	4.37E-05	6.37E-03	2.36E+06
Phenyl Boronate Chrom. After Buffer Exchange	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	1.99E-03	2.82E-03	1.72E+06
	1 hour	2.19E-03	2.32E-03	4.61E+05
	1 h 30 m	2.60E-03	2.39E-03	8.13E+05
	2 hours	2.27E-03	3.10E-03	9.88E+05

It was also calculated the yield and the DNA and protein removals for each step of the process (Table 4).

Table 4. Protein and DNA Removal (%) and Total Yields (%) of samples present in the nuclease buffer, after nuclease digestion, present in chromatographic peaks corresponding to the higher phage concentration and in the adsorption buffer, for each digestion time studied and the lysate.

Nuclease Buffer Exchange	Sample	Protein Removal (%)	DNA Removal (%)	Yield (%)
	Buffer	68.32	29.08	32.50
	Heat	-	-	3.13
Denarase® Digestion	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	8.31	98.23	0.85
	1 hour	10.46	98.81	2.71
	1 h 30 m	27.19	89.57	2.08
	2 hours	18.71	93.71	3.01
Phenyl Boronate Chrom. After Digestion	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	94.92	98.14	0.14
	1 hour	95.03	99.64	1.31
	1 h 30 m	97.75	99.87	0.29
	2 hours	95.25	99.73	0.15

	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
Adsorption Buffer Exchange Previous to Chrom.	30 min	99.64	99.56	0.061
	1 hour	99.91	99.81	0.013
	1 h 30 m	99.95	99.38	0.188
	2 hours	99.94	99.41	0.026
Phenyl Boronate Chrom. After Buffer Exchange	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	99.93	99.79	0.029
	1 hour	99.93	99.81	0.011
	1 h 30 m	99.92	99.80	0.034
	2 hours	99.93	99.73	0.018

As for the nuclease buffer exchange 100 kDa ofNMWC Amicons were used, some proteins were removed (68.32%). There could also be some phage lost or some lose their infectivity, as shown by the 32.50% yield (Table 4). The digestion itself does not present a big effect on the phage's infectivity. In fact only the 30 minutes incubation shows a total yield lower than 2% being the 2 hours digestion the incubation with the higher yield, 3.01%. As expected, in the digestion, the DNA removal presents good values, with a highest removal for the 1 hour incubation (98.81%) and the lowest the 1 hour and 30 minutes digestion (89.57%). The chromatograms after digestion showed similar results in terms of peaks than the ones from the 4 conditions studied. The results from the PB chromatography show that the 1 hour digestion presents the higher yield (1.31%) and the 1 hour and 30 minutes the highest DNA and protein removal (99.87% and 97.75 respectively). After the digestion, a buffer exchange was done to understand the impact of salt concentration in the chromatography. This step shows a higher yield and protein removal in the 1 hour and 30 minutes digestion (0.188% and 99.95%, respectively). APB chromatography was performed to these samples, showing that the 1 hour and 30 minutes digestion maintains the higher yield (0.034%), however it has the lowest protein removal (99.92%). In terms of DNA, the 1 hour digestion shows the highest removal (99.81%). As the yield was low, meaning a huge part of phages was being lost, a cold stopped digestion was tested.

- Cold Stopped Digestion

The digestion process followed for the heat stopped digestion was repeated but with a cold stopped one. For this digestion were only studied 2 different digestion times (30 minutes and 1 hour and 30 minutes). As above, the samples were tested, in every step (nuclease buffer exchange, enzyme digestion, phenyl boronate chromatography after digestion, adsorption buffer exchange after digestion and phenyl boronate chromatography after adsorption buffer exchange), for the amount of DNA and protein and phage infectivity. The results obtained are available in Table 5.

Table 5. Protein (mg), DNA quantities (µg), Infectivity (PFU) of samples present in the nuclease buffer, after nuclease digestion, present in chromatographic peaks corresponding to the higher phage concentration and in the adsorption buffer, for each digestion time studied and the lysate.

	Sample	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
Nuclease Buffer Exchange	Lysate	5.94E-02	1.21	1.61E+10
	Buffer	1.99E-02	1.04	1.02E+10
	Cold	1.99E-02	1.00	9.30E+09
Denarase® Digestion	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	1.67E-02	0.336	8.55E+09
	1 h 30 m	1.93E-02	0.549	4.80E+09
Phenyl Boronate Chrom. After Digestion	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	2.44E-03	5.59E-02	7.04E+08
	1 h 30 m	1.85E-03	2.19E-02	6.71E+08
Adsorption Buffer Exchange Previous	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	5.30E-03	1.28E-02	2.19E+08

to Chrom.	1 h 30 m	5.26E-03	2.21E-02	1.58E+08
Phenyl Boronate Chrom. After Buffer Exchange	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	1.79E-03	6.69E-03	5.87E+07
	1 h 30 m	1.99E-03	1.34E-02	6.31E+07

With the results from Table 5, it was possible to calculate the phage yield and the DNA and protein removals, which are present in Table 6.

Table 6. Protein and DNA Removal (%) and Total Yields (%) of samples present in the nuclease buffer, after nuclease digestion, present in chromatographic peaks corresponding to the higher phage concentration and in the adsorption buffer, for each digestion time studied and the lysate.

Nuclease Buffer Exchange	Sample	Protein Removal (%)	DNA Removal (%)	Yield (%)
	Buffer	66.50	14.05	63.55
	Cold	-	-	57.76
Denarase® Digestion	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	70.21	71.13	35.86
	1 h 30 m	67.32	52.59	30.47
Phenyl Boronate Chrom. After Digestion	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	96.26	95.20	4.36
	1 h 30 m	96.86	98.00	18.09
Adsorption Buffer Exchange Previous to Chrom.	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	91.24	96.55	1.39
	1 h 30 m	91.39	95.49	1.45
Phenyl Boronate Chrom. After Buffer Exchange	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	96.80	99.45	0.36
	1 h 30 m	97.31	98.79	0.34

In the cold stopped digestion it was also performed a nuclease buffer exchange, which shows a protein removal of 66.50% and a yield of 63.55% (much higher than the one obtained in the heat stopped digestion). The digestion shows a good DNA removal, being higher in the 30 minutes digestion (71.13%) and the yield is higher (between 30% and 35%) when compared with the heat stopped digestion. The phenyl boronate chromatography presented chromatograms with the same characteristics as the ones already described, and showed a higher yield, of 18.09%, for the 1 hour and 30 minutes digestion. The DNA removal is also higher in this digestion time (98.00%). The protein removal is between 96% and 97% for the times studied. When doing a buffer exchange into the adsorption buffer previously to the chromatography, it is possible to see that the yield decreases a lot, 1.45% is the highest (1 hour and 30 minutes). The DNA removal is higher for the 30 minutes digestion (96.55%) and protein removal for the 1 hour and 30 minutes (91.39%). The chromatography after the adsorption buffer exchange also showed chromatograms according to the ones already described and presents a higher yield for the 30 minutes digestion (0.36%). The DNA removal is higher than 98.50% for both times studied and the protein removal is higher than 96.50%. Once again, the yield is one of the biggest concerns as this study is only for the first steps of purification. With this in mind, and concluding that the buffer exchanges were losing infectable phages, it was decided to perform a nuclease digestion directly to the lysate.

- Digestion to the Lysate

As the amount of phages decreases when doing a buffer exchange, an attempt to decrease this loss and therefore increase the yield was done by removing these steps. A study of enzyme activity was then accomplished. The enzyme, diluted in equal parts in the nuclease buffer, was incubated directly to the lysate for 2 different incubation times (30 minutes and 1 hour and 30 minutes). After, a phenyl boronate chromatography was performed. The samples were tested in both steps (nuclease digestion and chromatography) for phage infectivity, DNA and protein quantities. The results are shown in Table 7.

Table 7. Protein Quantity (mg), DNA quantity (µg) and Infectivity (PFU) of samples after nuclease digestion and present in chromatographic peaks corresponding to the higher phage concentration, for each digestion time studied and the lysate.

	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
Denarase® Digestion	Lysate	5.53E-02	1.009	8.70E+08
	30 min	4.89E-02	0.008	6.75E+08
	1 h 30 m	4.84E-02	0.029	6.60E+08
Phenyl Boronate Chrom. (in the phage peak)	Time	Protein Quantity (mg)	DNA Quantity (µg)	Infectivity (PFU)
	30 min	9.55E-03	4.22E-04	5.96E+08
	1 h 30 m	2.35E-03	8.75E-03	6.41E+08

Using the results in table 7, it was calculated the phage yield and the DNA and protein removals, present in Table 8.

Table 8. Protein Removal (%), DNA Removal (%) and Total Yields (%) of samples after nuclease digestion and present in chromatographic peaks corresponding to the higher phage concentration, for each digestion time studied and the lysate.

	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
Denarase® Digestion	30 min	13.43	99.37	73.56
	1 h 30 m	13.16	97.87	72.41
Phenyl Boronate Chrom. (in the phage peak)	Time	Protein Removal (%)	DNA Removal (%)	Yield (%)
	30 min	82.36	99.96	69.33
	1 h 30 m	95.91	99.32	67.76

In this process (Table 8) it is observable that when incubating the Denarase® directly to the lysate, the DNA removal is really high (higher than 97%) for both digestion times in study. The 30 minutes digestion, however, showed a better DNA removal, 99.37%. In terms of yield, the digestion presents a higher yield when it occurs to the lysate (higher than 70%). Again the 30 minutes digestion presents the highest yield, 73.56%. After the digestion, a PB chromatography was performed on the samples. Once again, the chromatograms obtained show the same characteristics already discussed. When tested for phage infectability, DNA and protein quantities it was possible to calculate the yield (higher than 65% for both times studied), which is higher for the 30 minutes digestion (69.33%), the DNA removal (higher than 99% for both times), which is also higher for the 30 minutes digestion, and the protein removal. This removal was higher in the 1 hour and 30 minutes digestion (95.91%) than in the 30 minutes one (82.36%). When comparing the yield, DNA and protein removals of the processes described (heat/cold stopped digestion with buffer exchanges and digestion to the lysate), the DNA removal presents similar values and the protein removal is higher when the samples undergo buffer exchange. However, the phage infectivity is much higher in samples where the digestion was done directly to the lysate. Again, as this is the first purification step, it is important to maintain the highest infectivity possible. With this in mind, the digestion to the lysate should be the process considered for further studies. Due to this conclusion, in Figure 1 is presented the chromatogram corresponding to the 30 minutes digestion to the lysate.

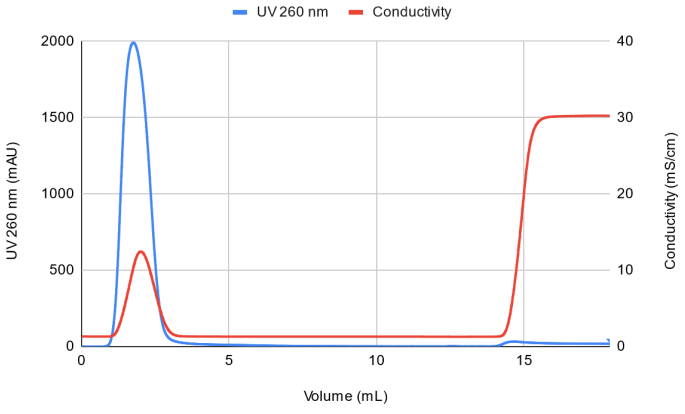

Figure 1. First triplicate of the 30 minutes digestion chromatogram, after the nuclease digestion to the lysate . It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per volume of buffer in mL.

SDS-Page

In order to confirm the presence of proteins and if it was possible to identify the phage's molecular weight an SDS-Page was performed to the samples from the nuclease digestion to the lysate. The two pools obtained for the Picogreen Assay were used. The results obtained are present in Figure 2.

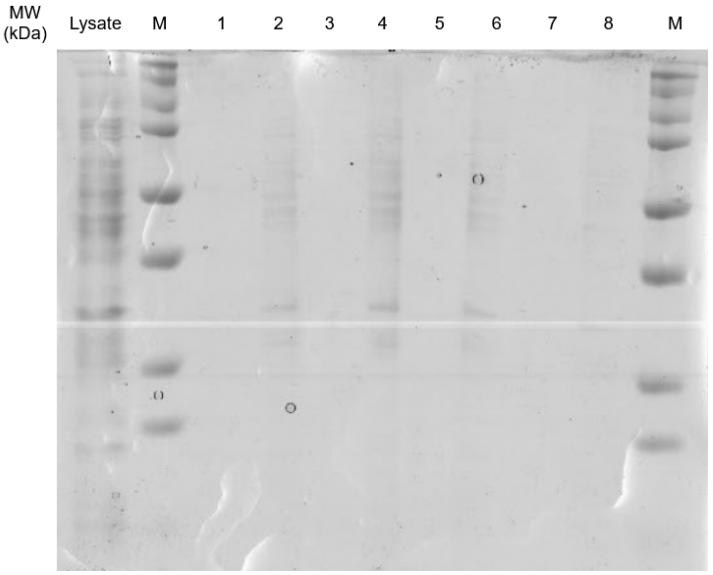

Figura 2. SDS-PAGE of the results of the nuclease digestion to the lysate followed by a Phenyl Boronate Chromatography for two different incubation times (30 minutes and 1 hour and 30 minutes) in duplicate. The gel was stained with Coomassie. The samples from the chromatography are organized in two pools according to the chromatographic peaks. The lysate well consists of a sample of lysate previously before the digestion and the chromatography. M wells correspond to the Precision Plus Protein™ Dual Color Standards ladder. Wells 1 and 3 present the second pool of the 1 hour and 30 minutes digestion to the lysate (duplicate). Wells 2 and 4 consist of the first pool of the 1 hour and 30 minutes digestion to the lysate (duplicate). In wells 5 and 7 it is possible to see the second pool of the 30 minutes digestion to the lysate (duplicate). In wells 6 and 8 it is observable the first pool of the 30 minutes digestion to the lysate (duplicate). In the first pool wells some bands can be seen.

From Figure 2 it is observable that the wells corresponding to the first pool, either of the 30 minutes digestion (Wells 6 and 8) or the 1 hour and 30 minutes digestion (Wells 2 and 4), present some bands. This confirms the presence of proteins in these samples. The wells regarding the second pools (Wells 5 and 7 and wells 1 and 2 for the 30 minutes digestion and the 1 hour and 30 minutes digestion, respectively) do not present a high amount of bands, confirming the less protein quantity in these samples. It is also possible to see that the lysate well presents a highest amount of bands, which confirms the protein removal.

Conclusions

As research and therapy using phages require a high purified phage suspension, a Denarase® and a Phenyl Boronate Chromatography were tested for removing the DNA and protein. The PB chromatography was studied for 4 different conditions, being the third one the one with the best results (49.49% yield, 96.61% protein and 47.60% DNA removal). From the various digestion processes studied (heat or cold stopped, digestion to the lysate), the cold stopped digestion presented better results than the heat stopped one. Also the results to the samples that did not undergo a adsorption buffer exchange were better in terms of yield. However, the digestion directly to lysate was the process with the best results (yield higher than 65%, protein and DNA removals higher than 80% and 99%, respectively), therefore this process should be the one considered for the next purification steps.

References

[1] Kaitlyn E.Kortright, Benjamin K.Chan, Jonathan L.Koff, Paul E.Turner; 2019; "Phage Therapy: A Renewed Approach to Combat Antibiotic-Resistant Bacteria"; Cell Host and Microbe, Volume 25, Pages 219-232.

[2] McGrath S, Van Sinderen D (editors), 2007, "Bacteriophage: Genetics and Molecular Biology", Caister Academic Press, 1st edition.

[3] Drulis-Kawa Z., Majkowska-Skrobek G., Maciejewska B.; 2015, "Bacteriophages and Phage-Derived Proteins – Application Approaches", Current Medicinal Chemistry, Volume 22, Pages 1757-1773.

[4] Doss J., Culbertson K., Hahn D., Camacho J., Barekzi N.; 2017; "A Review of Phage Therapy against Bacterial

Pathogens of Aquatic and Terrestrial Organisms"; Viruses, Volume 9, Page 50.

[5] João J., Lampreia J., Prazeres M., Azevedo A.; 2021 "Manufacturing of Bacteriophages for Therapeutic Applications", Biotechnology Advances, Volume 49, Page 107758.

[6] Adriaenssens E., Lehman S., Vandersteegen K., Vandenheuvel D., Philippe D., Cornelissen A., Clokie M., Garci A., Proft M., Maes M., Lavigne R., 2012, "CIMs monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles", Virology, Volume 434, Pages 265-270.

[7] Liu X., Scouten W., 2000, “Boronate Affinity Chromatography”, Humana Press, Affinity Chromatography, Volume 147, Pages 119-128.

[8] Nishino T, Morikawa K, 2002, "Structure and function of nucleases in DNA repair: shape, grip and blade of the DNA scissors", Oncogene, Volume 21, Pages 9022-9032.

[9] Smrekar F., Ciringer M., Peterka M., Podgornik A., Strancar A., 2008, "Purification and concentration of bacteriophage T4 using monolithic chromatographic supports"; Journal of Chromatography B, Volume 434, Pages 265-270.

[10] Gomes G., Azevedo A., Aires-Barros M., Prazeres D., 2012, "Validation and scale-up of plasmid DNA purification by phenyl-boronic acid chromatography", Journal of Separation Science, Volume 35, pages 3190–3196.

[11] Gomes G., Azevedo A., Aires-Barros M., Prazeres D., 2010, "Clearance of host cell impurities from plasmid-containing lysates by boronate adsorption", Journal of Chromatography A, Volume 1217, Pages 2262–2266.