Production, concentration and purification strategies for Bluetongue virus

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Abstract

For the treatment and prevention of bluetongue in ruminants, it is indispensable to invest in upstream and downstream processes in order to obtain harvest volumes for viruses vaccines and high purification yields. The experimental work presented here describes methods used to address these current challenges, and develop and optimize new alternative technologies for the concentration and purification of Bluetongue virus. BTV purification and removal of contaminants was achieved by a combination of different unit operations used in downstream processing, such as precipitation, ultracentrifugation, ultrafiltration, hydrophobic chromatography (HIC) and monolithic chromatography (CIM DEAE).

Keywords: Bluetongue Virus (BTV), Aqueous two-phase systems (ATPS), Hydrophobic chromatography (HIC), Monolithic chromatography

Introduction

Bluetongue virus is a member of the *Orbivirus* genus within the family *Reoviridae* and transmitted by Culicoides biting midges, encompassing 26 known serotypes, is transmitted to ruminants (mainly in sheeps and less frequently in cattle, goats, buffalos, deers and dromedaries) and causes thrombo-hemorrhagic fevers. [1][2][3]

Bluetongue virus is a non-enveloped virus with 90 nm in diameter and has a triple-layered icosahedral symmetry and also a genome of approximately 19 200 base pairs composed of ten linear segments of double-stranded RNA responsible for encoding seven structural proteins (VP1 to VP7) and four non-structural proteins (NS1, NS2, NS3 and NS3A). [2][4][5]

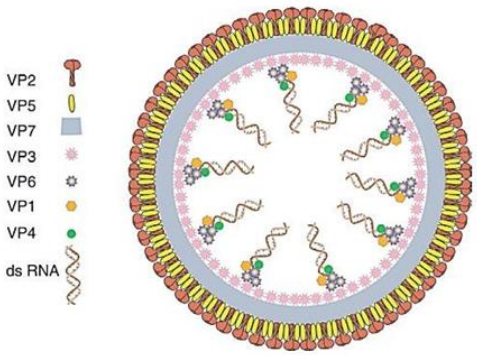


Fig. 1 - Representative scheme of BTV structural proteins and dsRNA.

The outer shell is composed of two structural proteins, VP2 that establishes the serotype and is responsible for receptor binding and eliciting host specific immunity, and the VP5 protein that interacts with the host's cell endosomal membrane. The intermediate layer is composed by a major immunodominant VP7 structural protein covering the subcore, which is the responsible for the serogroups specificity providing an epitope in ELISA tests for detection of antibodies against BTV. The subcore consists in VP3 protein, which houses the viral genome segments and three minor proteins involved in transcription and replication. Non-structural proteins probably participate in the control of BTV replication, maturation and export from the infected cell. [1][2][6]

Inactive vaccines are a good option for the prevention of BTV because they can induce reliable and protective immunity. However, they are expensive to produce and it requires large amounts of highly pure materials. So for the production of viruses there are two different methods: egg-based manufacture and cell-culture-based manufacture.[7] In this work the BTV virus was produced based in cell culture.

For the commercial of the vaccines is important the production of purified viruses with high quality and quantity, which requires efficient downstream methods for purification and concentration of viral particles. The three main steps of general downstream processing for virus particles are clarification, concentration and polishing. For clarification and concentration of BTV were tested three techniques: ultrafiltration-using 10KDa of molecular weight cut off Amicons, ultracentrifugation and PEG6000 precipitation.
For purification monolithic chromatography, hydrophobic chromatography and aqueous two-phase systems were performed.

1.1 Ultrafiltration

Ultrafiltration is commonly used for clarification in biotechnological production processes as an alternative to centrifugation methods. Ultrafiltration is a tangential flow filtration technique, which uses membranes with pore sizes in the range of 0.001 to 0.1 μm, and it is used to remove high molecular-weight substances, such as viruses, colloidal materials, and organic and inorganic polymeric molecules. [8][9]

1.2 Ultracentrifugation

Ultracentrifugation is an important tool in biochemical research, which, through rapid spinning, imposes high centrifugal forces on suspended particles, or even molecules in solution, and causes separations of such matter based on differences in weight. The use of ultracentrifugation has the potential to separate assembled virus from the empty capsids and it presents also sensibility in relation to viral strain differences.[10][11]

1.3 PEG 6000 precipitation

Precipitation purification protocol is rarely used in downstream processes for purification of virus particles because this type of methods generally require a low pH that may compromise the virus viability as well as represent losses in biological activity. Besides this, PEG is a widespread polymer used as a fractional precipitating agent for the purification of proteins from a variety of sources due to its non-denaturing qualities, like being chemically inert, nontoxic, water-soluble and a synthetic polymer. Proteins precipitation occur in the presence of PEG due to the increase of the chemical potential, which means that when the solution exceeds the saturated level, the protein precipitates.[8][12][13][14]

1.4 Aqueous two-phase system

Aqueous two-phase systems (ATPS) are formed spontaneously upon mixing two aqueous solutions of different components, such as two polymers or one polymer and a salt prepared in an aqueous solution above a certain critical concentration.[15][16] The mixture will then separate into two immiscible phases and form an interphase between both phases, where the light phase (top phase) is rich in one polymer and the heavy phase (bottom phase) is rich in the second polymer or in salt. [17]
The partition for the proteins is dependent on their physico-chemical properties. The most soluble biomolecules usually partition

themselves to the lower phase, which is the more polar one, while the proteins prefer the top one which is less polar and is also more hydrophobic. [18][19]

1.4 Hydrophobic Chromatography

Hydrophobic interaction chromatography (HIC) is performed to separate and purify biological molecules based on their degree of hydrophobicity. In HIC to ensure the proper binding of the proteins to the column, high concentrations of salt are needed in order to expose, by salting out, the hydrophobic regions of proteins that would otherwise be turned to the inner parts. Once the biomolecule of interest is attached to the stationary phase, it is necessary to detach it in order to recover and separate it from the other molecules that might be attached to the ligands. Desorption is accomplished by reducing the ionic strength in the mobile phase, by building a decreasing gradient of salt concentration, which results in the weakening of the interaction between the biomolecule and the column. [20][21][22][23][24]

1.5 Monolithic Chromatography

Monolithic ion exchange chromatographic (IEC) purification has been proving to be a powerful method for the purification of virus particles with high yields and maintaining its infectivity. This technology is based on large pore sized monolithic anion exchangers, quaternary amine (QA) or diethyl aminoethyl (DEAE), which are applied to membranes containing icosahedral bacteriophage PRD1, which bind specifically to both matrices. Ionic interactions are the basis for purification of proteins by monolithic ion exchange chromatographic. The separation occurs due to competition between proteins with different surface charges for oppositely charged groups present in ion exchanger support. For IEC can be used two strategies: bind the target molecule to the column support with opposite charged and then wash away non-bound contaminants with same charged of the column or bind the impurities, and in that case, the protein of interest should be found in the flow through. [25][26][27][28]

2. Material and Methods

2.1 Culture expansion and virus inoculation

BHK-21 (Baby Hamster Kidney) cell lines were cultured in Glasgow (Gibco®, CA, USA) medium supplemented with 10% fetal calf serum and 1% of antibiotics (Gibco®). After two days in culture, the cells formed a monolayer and the culture was expanded for higher capacity flasks. After four days, there were enough cells for virus inoculation. Consequently, the growth medium was removed and 2 mL of BTV were inoculated for previous incubation for 30 min at 37°C in a 5% CO2 atmosphere. After 3 days of incubation, when extensive cytopathic effect was observable and the culture medium was removed and clarified by centrifugation 1500 G for 15 min. The supernatant was stored at 4˚C to ensure the complete inactivation of the virus until further experiments were carried out.

2.2 BTV concentration

2.2.1 Supernatants ultracentrifugation

The clarified supernatants were centrifuged in a SW-27 rotor (Beckman Coulter) for 90 min at 100 000 g at 4°C. The virus pellet was suspended in 1mL of one of the three buffers: PBS, 10mM phosphate or ammonium sulphate and then were stored at 4˚ C until being used in aqueous two-phase system and in chromatography experiments.

2.2.2 Supernatants ultrafiltration

Due to the high volume of supernatant from cell culture, the supernatant was subjected to a ultrafiltration step utilizing an Amicon Ultra 15 mL (Millipore) centrifugal filters containing a 10 kDa molecular weight cut-off (MWCO) regenerated cellulose membrane. Approximately 12 mL of supernatant solution was placed in the filter unit and centrifuged at 14000g for 1 hour. After this first centrifugation the permeate solution was collected.

2.2.3 BTV concentration by polyethylene glycol-NaCl precipitation

The BTV was precipitated from the supernatant using 6% (w/v) polyethylene glycol 6000 (Sigma-Aldrich) and 0.4 M NaCl

(Sigma-Aldrich) with overnight stirring and centrifuged at 3000 × g for 30 min at 4 ˚C in a Sorvall GSA rotor. The resultant pellet was resuspended in 1 mL of phosphate buffered saline (PBS) (Sigma-Aldrich).

2.3 BTV concentration and purification by Aqueous two-phase

The ATPS were constructed on a % w/w basis using the quantities predetermined from stock solution of PEG 6000 (Sigma-Aldrich) and ammonium sulphate (Sigma-Aldrich). The virus was added in the end and consisted of a remaining percentage of the total ATPS weight. The phase components were thoroughly mixed on a vortex agitator and the formation of two-phase system was accomplished within 5 minutes. After the phase separation, the top and bottom phases were carefully separated with a syringe and taken for further electrophoresis and HPLC analysis.

2.4 Hydrophobic Chromatography

Three different chromatographic columns were used: HiTrap Octyl FF, Butyl HP and Phenyl HP (GE Healthcare Biosciences). All the chromatographic separations were performed on an Äkta Purifier system from GE Healthcare. All runs were performed at a flow rate of 1 ml/min. For HIC experiments were used four different concentrations of adsorption buffer: a solution of 1 M, 1,5M, 2M and 2,5M of ammonium sulphate in 10 mM of phosphate at pH7. During this operation the column pressure, UV absorbance at 280 nm and the conductivity were continuously monitored.

2.5 Monolithic Chromatography

Chromatography experiments were performed in an ÄKTA TM purifier HPLC system which works with the UNICORN TM control software. CIM® disk monolithic column (BIA Separations, Ljubljana, Slovenia) of diethyl amine (DEAE) was used during the experimental work. During the procedure, the absorbance was measured at 280 nm and the flow rate was 1 mL/min. Virus suspension volumes applied to the monolithic column were 1,5 mL.

2.6 Analytical Methods

2.6.1 SDS-PAGE electrophoresis

SDS–PAGE was performed to evaluate the purity of each phase collected of ATPS systems and each elution fraction from chromatography experiments. Samples were diluted in a loading buffer and denatured in reducing conditions with 0.1M of dithiothreitol (DTT) (Sigma-Aldrich) at 100˚C for 5 min. Samples were run at 200 mV using a running buffer containing 192 mM glycine, 25 mM Tris and 0.1% SDS, pH 8.3. Every gel was loaded with a Precision Plus Protein Dual-color standard from Bio-Rad. Gels were silver stained due to method's sensitivity according to the Bio Rad kit protocol.

3. Results and Discussion

Ultracentrifugation, PEG precipitation and ultrafiltration were used in order to find the best clarification and concentration method for BTV, while PEG/salt systems were developed to find the best system composition for the virus purification. Nevertheless, for purification of BTV, hydrophobic and anion exchange chromatography were tested.

3.1 Clarification Results

The three pre-purification methods tested aims to find the best feedstock resulting that will be used for the chromatography and aqueous two-phase systems experiments, in order to concentrate the BTV and decrease the load of impurities. Represented in figure 2 are the protein profile correspondent to the initial feedstock, which is the supernatant collected after the centrifugation of cell culture, and the three different feedstocks resultant from the three different pre-purification methods.

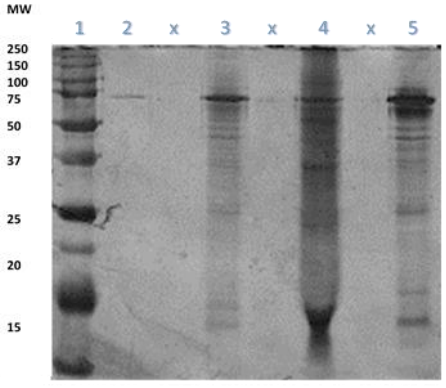


***Fig. 2**- Silver stained reducing SDS-PAGE analysis of the feedstocks. (A) Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 2: BTV clarified by ultrafiltration; Lane 3: BTV 100x concentrated by ultracentrifugation; Lane 4: BTV 100 x concentrated by PEG precipitation; Lane 5: Clarified BHK-21 cell supernatant (initial feedstock).*

By SDS-PAGE results, it is possible to verify the presence of several impurities from the culture medium supplemented with FBS where the cells were expanded, such as, bovine serum albumin (BSA, with 66 kDa), the major component of FBS.

The supernatant filtrated by ultrafiltration with a 10 KDa MWCO Amicons® Ultra centrifugal filter unit was only able to concentrated the BSA protein. This does not mean that other proteins are not present in this feedstock, but only that these are not being concentrated due to the small volume taken to the realization of the gel.

The pellet obtained by PEG precipitation was resuspended in TNE and analysed by SDS-PAGE, presents good results for concentration of proteins, however, is not a very selective method, because it involves not only the precipitation of viral proteins but also proteins that we wish to eliminate.

The ultracentrifugation of BTV shows a clearer protein profile, with well-defined bands that allow the identification of viral proteins as well as some impurities, but in less amount as compared with the initial supernatant. Moreover, is possible to observe two low molecular weight bands that may correspond to viral RNA, which also proves the presence of the virus.

The viral proteins of BTV have different molecular weights, which if there wasn't any impurities could be very well distinguished. The three minor components are VP1, VP4 and VP6 with molecular weight (MW) of 149 kDa, 76 kDa and 36 kDa, respectively. The core is composed of two major proteins VP3 with 100 kDa and VP7 with 38 kDa, and in turn is enclosed by the two outer virion proteins VP2 with 111 kDa and VP5 with 59 kDa, which forms the complete virus particle.

3.2 Aqueous two-phase system

The objective of using ATPS is to find new systems that can be used for BTV purification with high purification yields. In order to form two immiscible phases capable of separating the BTV it is necessary to select some parameters related to the desired system, such as pH, concentration of polymers and salts, molecular weight of the polymers to be used, since ATPS is strongly influenced by psychochemical properties. [55]

PEG-ammonium sulphate systems are the most used systems in ATPS due to high salting out capacity of ammonium sulphate; the fast formation of the two phases using relatively low concentrations and usually present high extraction yields. [55][69] In this work the partition of BTV was made by testing three different conditions where PEG molecular weight and the concentrations of PEG and ammonium sulphate compositions were varied (Table 1).

***Table 1** - ATPS conditions that were tested for BTV purification.*

PEG MW (Da)	PEG concentration (% w/w)	Ammonium Sulphate concentration (% w/w)	Name
6000	25%	15%	System 1
	15%	25%	System 2
	5%	30%	System 3
	Top phase	**Bottom phase**	

The molecular weight of PEG influences the partition since bigger polymers tend to form more aggregated structures making it harder for large proteins to partition to their phase. Based on this, the choice made was PEG 6000. The selection of the concentration was made based on phase diagrams, from where distinct points were chosen and all the systems were tested at pH 7.

To determine for which phases have the virus preferentially partitioned, samples from the top and bottom phase of each tested system were collected, and subsequently, analysed by SDS-PAGE followed by silver staining. The samples collected from all the conditions were running in a gel, which the results are represented below (Fig. 2).

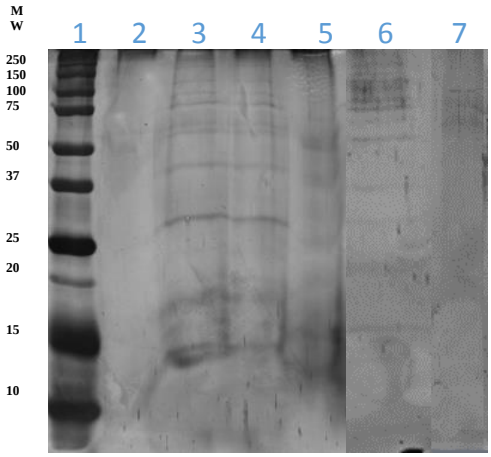


Fig. 3- Silver stained reducing SDS-PAGE analysis of the feedstocks. Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 2: Top Phase of system 2; Lane 3: Bottom Phase of system 2; Lane 4: Top Phase of system 3; Lane 5: Bottom Phase of system 3; Lane 6: Bottom Phase of system 1; Lane 7: Top Phase of system 1.

By observing Fig. 3, it is possible to observe that the lanes containing top phases from system 1 and system 2 present a small number of bands, which are very smear. This gives the hypothesis of the partition of BTV particles in this aqueous two-phase systems is occurring to bottom phase, which is proved by the presence of several bands in lanes 3 and 6, which may be viral proteins in the presence of many impurities. However, this doesn't happen for system 3. In system 3, proteins are partitioning mostly to the top while just a few proteins are partition to the bottom phase, which is confirmed. Moreover, top phase from this system presents a protein pattern very similar to the bottom phase of system 2, which comes to confirm the inverse partition.

Since, the objective of ATPS is to use a system where the virus particles partition to salt phase, to then being collected and used for chromatography; results from system 3 were excluded for not showing any advantage for it.

The conditions tested in system 1 and 2 showed the presence of the major impurity protein present in the culture medium, which is bovine serum albumin (BSA, with 66 kDa), the major component of FBS. The presence of several impurities difficult the confirmation of the presence of the viral particles in bottom phases in both systems. Although, it is possible to identify some bands with molecular weight around, 100 kDa, 76 kDa, 60 kDa, 40 kDa that may belong to BTV proteins.

3.3 Hydrophobic chromatography

The systems from ATPS composed by 15% PEG and 25% of ammonium sulphate were chosen for the further HIC studies in three different concentrations of elution buffer (1M, 1,5M, 2M) using phenyl column.

Since there are no reported data describing the concentration and purification of BTV by HIC chromatography, in order to select the most suitable conditions for the virus downstream processing, initial studies were performed at neutral pH to find an appropriate salt concentration that would permit the binding of virus particles to the column without inducing the precipitation of the proteins. [38] For that, three different concentrations of ammonium sulphate were tested for the adsorption of the BTV particles presents in the bottom phases of systems 1 and 2. The column used for this experiments was phenyl HP and for the elution was used a 10mM phosphate buffer.

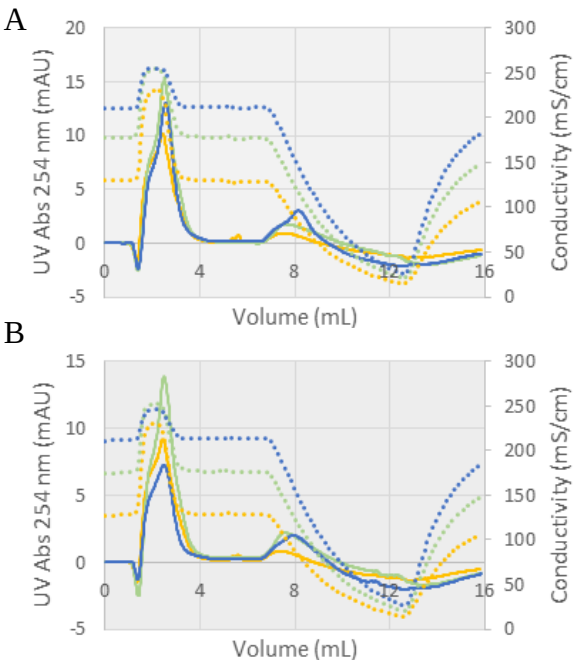


Fig. 4 – (A) Samples of bottom phase from system 1. (B) Samples of bottom phase from system 2. Absorbance at 254 nm (mAU) – yellow line and conductivity (mS/cm) - dashed yellow line (adsorption buffer 1 M ammonium sulphate at pH 7). Absorbance at 254 nm (mAU) – green line and conductivity (mS/cm) - dashed green line (adsorption buffer 1,5 M ammonium sulphate at pH 7). Absorbance at 254 nm (mAU) – blue line and conductivity (mS/cm) - dashed blue line (adsorption buffer 2 M ammonium sulphate at pH 7).

Is possible to observe the increase of the conductivity when the sample is injected which may represent a problem since if the concentration of ammonium sulphate is superior to the present in the sample, the hydrophobic regions will be exposed not only from viral particles but also from other proteins that we intended to eliminate. Other case that may happen is the precipitation of BTV and in this case, it will not bind to the column, and will appear in flow through (FT) peak. In order to confirm, in which fraction peak the BTV was eluted, a SDS-PAGE was performed (results not showed). From the SDS-PAGE performed was confirmed the presence of the virus in both fractions of flow through and elution peaks, The causes for presence of the virus in all fractions collected in FT and elution peaks may be the unspeciffically binding during the interaction to the column due to the support that is being used is not the most suitable for the purification or its conditions are not well function. Thus, in chosen support, we proceeded to further studies where two columns containing different ligands, butyl and octyl, were tested. The ammonium sulphate concentrations tested were 2M and 2.5M and this time, the injected samples did not undergo a pre-purification by ATPS, being only clarified by ultracentrifugation (Fig.5).

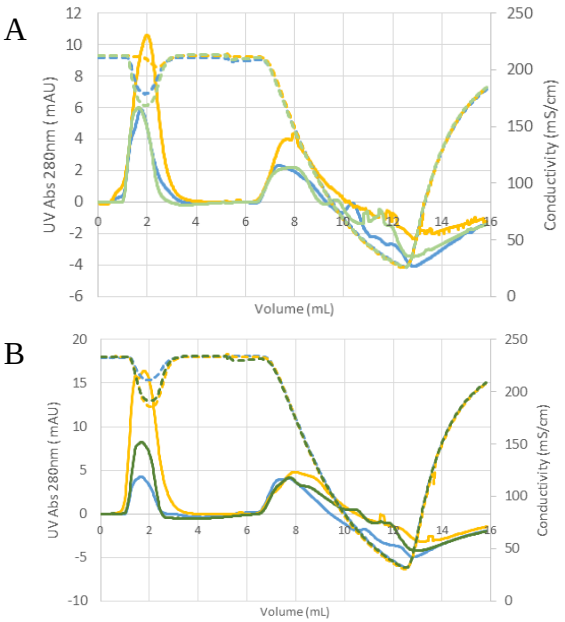


Fig. 5 – (A) HIC chromatography profile of BTV purification from ultracentrifugation samples, using as adsorption buffer 2M ammonium sulphate with 10 mM phosphate at pH 7 and elution buffer 10 mM phosphate at pH 7. (B) HIC chromatography profile of BTV purification from ultracentrifugation samples, using as adsorption buffer 2,5M ammonium sulphate with 10 mM phosphate at pH and elution buffer 10 mM phosphate at pH 7.Absorbance at 280 nm (mAU) – yellow line and conductivity (mS/cm) - dashed yellow line (Octyl ligand). Absorbance at 280 nm (mAU) – green line and conductivity (mS/cm) - dashed green line (Phenyl ligand). Absorbance at 280 nm (mAU) – blue line and conductivity (mS/cm) - dashed blue line (Butyl ligand).

To test the effect of three ligands in purification of BTV, a sample with 1,5mL containing a feedstock from ultracentrifugation suspended in adsorption buffer was prepared to be used in HIC. After injection of the sample, a decrease in conductivity was observed. SDS PAGE was performed in order to verify which species are being eluted and in each fraction collected.

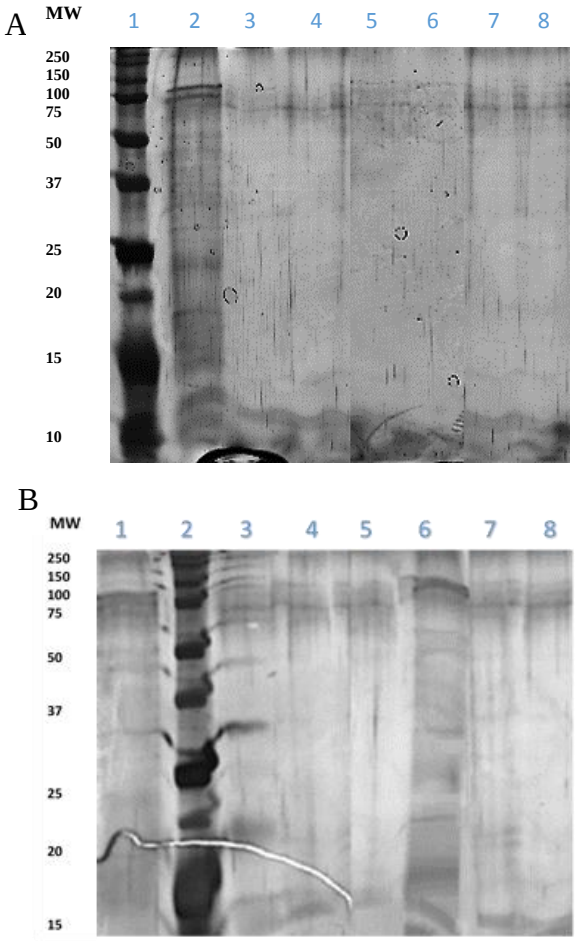
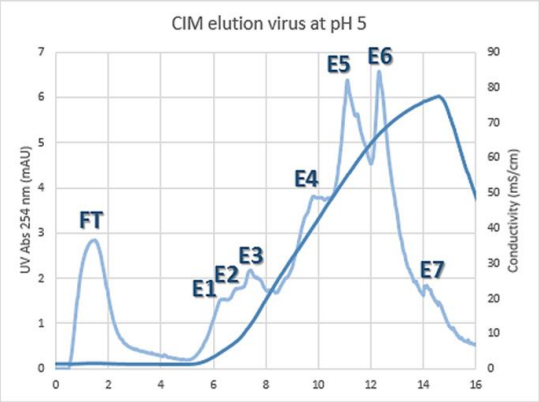


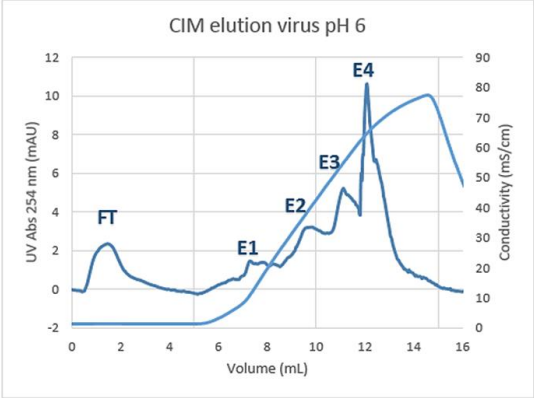
Fig. 6 - Silver stained reducing SDS-PAGE analysis of HIC chromatography samples (A) using as adsorption buffer 2M ammonium sulphate with 10 mM phosphate and (B) using as adsorption buffer 2,5M ammonium sulphate with 10 mM phosphate. Lane 1:Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa) at the left side; Lane 2: BTV virus after ultracentrifugation; Lane 3: Flow through of HIC experiments using octyl column; Lane 4: Elution peak of HIC experiments using octyl column; Lane 5: Flow through of HIC experiments using phenyl column; Lane 6: Elution peak of HIC experiments using phenyl column. Lane 7: Flow through of HIC experiments using butyl column; Lane 8: Elution peak of HIC experiments using butyl column.

The results presented in the gel A of SDS-PAGE do not allow draw many conclusions since the bands are not very clear, being only possible to identify a band with molecular weight around the 66 kDa very concentrated in all lanes, which might be the protein BSA, one of the many impurities. Although they are not very concentrated, other bands with molecular weight around 100kDa are present, which may be one of the viral proteins VP2, VP3 and VP6. In the case of being the viral proteins being present, the problem maintains: BTV is being eluted in both peaks, flow though and elution peak, independent of the ligand that is used. In the gel B, in lane 6, corresponds to the fraction collected from elution peak in chromatography experiment using phenyl column and seems to show similar bands with feedstock (lane 1). This result, is very promising, since the objective is to purify and collected the virus in the elution peak without seeing is presence in flow through samples, which means that virus particles have bound to the column and were eluted with the decreasing of the ionic strength.

3.4 Chromatography using monolithic supports

In CIM DEAE chromatography, the working conditions are different from HIC, namely the type and concentration of the salts in the buffers, pH and working flow rate. The purification of BTV by anion-exchange chromatography using a monolithic bed was studied using a weak anion exchanger diethylaminoethyl (DEAE). The sample used in following experiments, was the feedstocking resulting from ultracentrifugation. The goal for this technique is to find the better conditions for BTV purification while preventing the binding of impurities to the monolith, by changing the work pH of the adsorption buffer, testing pH 5,6 and 7.





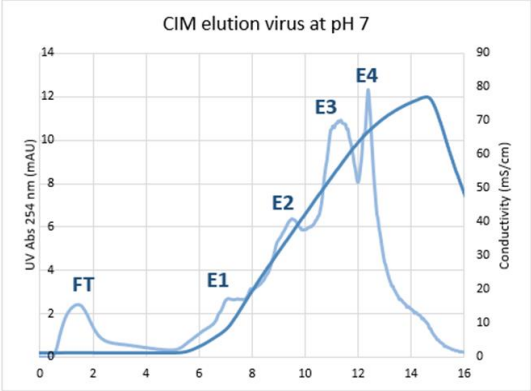


Fig.7 -CIM DEAE® chromatography profile of BTV purification from clarified BHK-21 cell supernatant, using as adsorption buffer 10 mM NaH_2PO_4 and elution buffer 20 mM NaH_2PO_4 with 1 M NaCl at three different pH.

In the chromatography using monolithic supports the flow through (FT) fractions collected present the unbound species that were washed out from the column, and the elution peaks fractions collected were the species that have bind to the column and that were removed by changing the buffer for elution conditions unfavourable for ionic binding of the molecules. In this way, it is expected as a good result that the flow through (FT) peak should only have impurities whereas one of the elution peaks would have the BTV. In order to evaluate the virus presence in the previous peaks an SDS gels were performed (results not showed).

From the three ion exchange experiments performed, the one that showed better results was the one performed at neutral pH for presenting a better resolution, which means the separation between the peaks eluted was better performed with less peaks that means less impurities and which come to be proved by SDS PAGE gel.

4. Final Considerations

The aim of this work is to find the most suitable method for clarification/concentration and purification of the BlueTongue Virus.

From the three clarification methods used, the feedstocks from PEG precipitation and ultracentrifugation were the ones that showed the best results, with more concentrated proteins however the results for ultracentrifugation showed fewer impurities present than PEG. Thus, the aqueous two-phase systems were performed using samples from these two feedstocks. The ultrafiltration using Amicons® proved to be a less efficient method than the others.

ATPS can be effectively used for the separation and purification of BTV, by using PEG/salts systems. In this work, three PEG/ammonium sulphate systems were studied and the results reported that BTV partitions to the bottom phase. ATPS tested with systems 1 and 2 show the appropriate conditions for the purification, even though, impurities presented in the culture medium partitioned to the same phase as the virus, which is a problem to be solved with the goal of preserving the virus infectivity and to obtain the pure virus. In future work, new protocols must be set in order to test new components and new concentrations so that the virus and its impurities partition in different phases.

Regarding chromatography, in HIC chromatography different ligands were tested with different concentrations of salt in the adsorption buffer. From the results testing phenyl, butyl and octyl ligands the best result was achieved with the phenyl ligand at 2,5 M of ammonium sulphate for the efficient adsorption of BTV to the column. Regarding the results obtained with the other columns and the other salt concentrations it was verified an unspecific binding of BTV to the columns, being possible to visualize in SDS-PAGE analyses the presence of the virus both in the flow through and elution fractions.

In ion exchange chromatography three-pH works were tested, being the neutral pH the one that presented the best results for BTV purification, since it was able to separate BTV from the other proteins contained in the initial feedstock.

CIM chromatography seemed to be more efficient for BTV purification since its affinity to it was higher when compared with HIC chromatography and the objective is to isolate the virus and purify it in only one elution peak with the less impurities possible in the fraction collected. In conclusion, in both chromatography experiments, neutral pH is suitable for the purification of the virus.

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