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Brief Communication

Effect of buffer systems and disaccharides concentration on *Podoviridae* coliphage stability during freeze drying and storage ☆

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ABSTRACT

The aims of this study were to determine the stability of *Podoviridae* coliphage CA933P during lyophilization and storage in different media, and to establish similarities between the results obtained and those expected through mechanisms described for proteins stabilization during freeze-drying.

PBS and SM buffer were assayed as lyophilization media. The effect of inorganic salts concentration as well as the addition of disaccharides on phage stability during freeze-drying and storage was also studied.

The addition of low sucrose concentration (0.1 mol l^{-1}) to SM buffer stabilized phage during freezing and drying steps of the lyophilization process, but higher sugar concentrations were detrimental to phage stability during freeze-drying. Sucrose stabilized phage during storage for at least 120 days. The lyoprotective effect of low concentrations of disaccharides during the drying step of the lyophilization of proteins as well as the stabilization of the freeze-dried product in time correlated with the results obtained for phage CA933P.

Main text

The development and commercialization of phage formulations for biocontrol of pathogens has had a strong growth in the last decades, which poses the need for the development of preservation techniques that allow long term storage of phage formulations.

In a previous work, *Podoviridae* phage CA933P proved to be a promising tool for the biocontrol of enterohemorrhagic *Escherichia coli* [6], but its application as a therapeutic agent requires the development of efficient conservation methods.

Lyophilization is a broadly process used for conservation and transport of biological products, but the available information about stabilizing agents used for the lyophilization of phage is scarce, being skim milk cited as one of the most common phage lyoprotectants [1].

The conservation of biological products is affected by so many factors that it is often necessary to empirically adjust the lyophilization conditions for each particular case [1]. Nevertheless, as phage external structures are assembled proteins (except for enveloped phage), some patterns in the results obtained for phage conservation in different media can be established by comparison with those expected for proteins through known stabilization mechanisms.

Disaccharides are commonly chosen as proteins cryo– and lyoprotectants because they can stabilize proteins by different mechanisms during freezing and drying steps of the lyophilization process. On the one hand, it has been described that high concentrations of disaccharides (above 0.3 mol l^{-1}) can protect proteins during the freezing step by a mechanism known as "preferential exclusion", where disaccharides are repelled by the protein's hydrophobic groups maintaining a high proportion of unfrozen water in the primary hydration sphere [4]. An approximation of the destabilization during the crystallization prior to dehydration in the freeze-drying process can be assessed by measuring the inactivation produced during freeze-thaw assays [5].

Saccharides have also been described as protein stabilizers during the drying process by interacting with them through hydrogen bonds, replacing water molecules on the protein surface and thus stabilizing their polar groups once the drying process is completed. This also reduces protein-to-protein interactions and the consequent aggregation and inactivation during storage of the lyophilized products [11]. Particularly, sucrose and trehalose have been reported to be good protein lyoprotectants [9,11].

Another stabilizing mechanism showed by disaccharides as lyoprotectants is the formation of amorphous systems. This structure is similar to that observed in the liquid state but their enormous

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viscosity values highly retard protein denaturation [11]. Additionally, amorphous systems dissolve more easily than crystalline solids [11].

It has also been postulated that the concentration of inorganic salts in the lyophilization media of proteins should be kept as low as possible; otherwise, parts of the mixture that crystallize more slowly during the freezing step may reach extremely high values of ionic strength which could distort the protein structure [9]. Moreover, reducing inorganic salts concentration produces amorphous stable systems in a wider range of operational temperatures [9].

In the present work, the stability of phage CA933P during lyophilization and storage in different media was determined. The protective effect of disaccharides in PBS and SM buffer was compared to that obtained with skim milk (reported as a common phage lyoprotectant) and was contrasted to that expected by stabilization mechanisms described for proteins.

Podoviridae coliphage CA933P [6] lysate was performed in LB medium composed of 1% (w/v) NaCl (Anedra, San Fernando, Argentina), 0.5% (w/v) yeast extract and 1% (w/v) tryptone (Biokar Diagnostics, Alonne, France) using enterohemorrhagic *E. coli* strain EDL933 (ATCC 700927) as host. Phage lysate was filtered through a 0.22 μm pore-size membrane giving a final concentration of 2.5×10^9 PFU ml^{-1} and kept at room temperature for further assays.

All assays were performed in triplicates. Sets of samples were prepared for each independent assay in sterile 1 ml glass ampoules by adding 20 μl of phage lysate (5×10^7 PFU) and 180 μl of each medium tested: Phosphate buffered saline (PBS; 137 mmol l^{-1} NaCl; 2.7 mmol l^{-1} KCl; 10 mmol l^{-1} Na_2HPO_4; 2 mmol l^{-1} KH_2PO_4; pH 7.2), SM buffer (100 mmol l^{-1} NaCl; 8 mmol l^{-1} $MgSO_4.7H_2O$; 50 mmol l^{-1} Tris–HCl; 0.01% (w/v) gelatin; pH 7.5), SM buffer diluted to 10% (v/v) with mili-Q water (SM 10%), PBS or SM buffer with sucrose (Anedra, San Fernando, Argentina) or trehalose (Mann Research Laboratories Division of Becton Dickinson and Co.) and UHT skim milk. Mixtures were frozen in a Nalgene® Mr. Frosty® Freezing Container (Thermo Scientific, US) at a chamber temperature of −80 °C (cooling rate of −1 °C/min) without a nucleation step, and kept at −80 °C for 24 h.

For each independent freeze-thaw assay, two ampoules of each condition were thawed at 25 °C and phage titer was determined by the soft agar overlay method in LB agar medium [8]. For lyophilization assays, frozen samples were dehydrated in a Heto FD4 model tray type freeze-dryer (LabEquipment, Denmark) at a condenser temperature of −50 °C for 48 h (pressure <1 Pa). Residual moisture content of the freeze dried powders was determined by a gravimetric method using a Mettler-AE240 digital balance, accurate to 0.00001 g. For each independent assay, five lyophilized samples of each condition were dried at 70 °C in a vacuum of 5 mmHg (1 mmHg = 133.3 Pa) until constant weight was attained.

In phage stability analysis during freeze-drying and storage, ampoules were flame-sealed immediately after the freeze-drying process and stored at 4 °C for the time specified in each case. Phage counts was performed on each independent assay by resuspending two ampoules of each condition with 200 μl of SM buffer and blended for 10 min at 25 °C. Phage titer was determined in duplicates for each ampoule as described above.

Results were analyzed by the one way analysis of variance (ANOVA) with a significance level of 5% ($P < 0.05$) followed by Fisher's least significant difference test at a $P < 0.05$.

Phage titers obtained after lyophilization of phage in different media are shown in Fig. 1. Skim milk produced a phage titer loss of 1.2 log PFU after the freeze-drying process (Fig. 1), corresponding to a phage survival percentage of 6%. This is in agreement with the results obtained by Clark [3] who reported survival percentages of coliphages ATCC 8677-B and 11303-B1 to -B7 after freeze-drying in skim milk in the range of 25–<1%.

The use of PBS as lyophilization medium did not improve the stability of phage during freeze-drying (Fig. 1) with respect to skim milk ($P > 0.05$). However, PBS produced lyophilized powders (Fig. 2B) which were immediately resuspended with the addition of SM buffer, meanwhile lyophilization cakes obtained with skim milk (Fig. 2A) required approximately 10 min to be homogenized after rehydration.

The addition of sucrose to PBS in a final concentration of 0.3 mol l^{-1}, reported as the minimum amount required for proteins cryoprotection by preferential exclusion [4], did not improve phage stability during freeze-drying with respect to the PBS alone (Fig. 1). Neither did trehalose which exhibited a reduction in phage titer similar ($P > 0.05$) to that produced by sucrose (data not shown). Furthermore, the addition of 0.3 mol l^{-1} of sucrose to PBS reduced phage stability during the freezing stage (Table 1), contrary to what would be generally expected for proteins, for which increased concentrations of disaccharides have been reported to enhance protection during crystallization of the surrounding water [4]. This detrimental activity of sucrose during freezing was also reported for T4 phage [7]. However, the addition of 0.3 mol l^{-1} sucrose to PBS provided a protective effect to the lyophilized product during

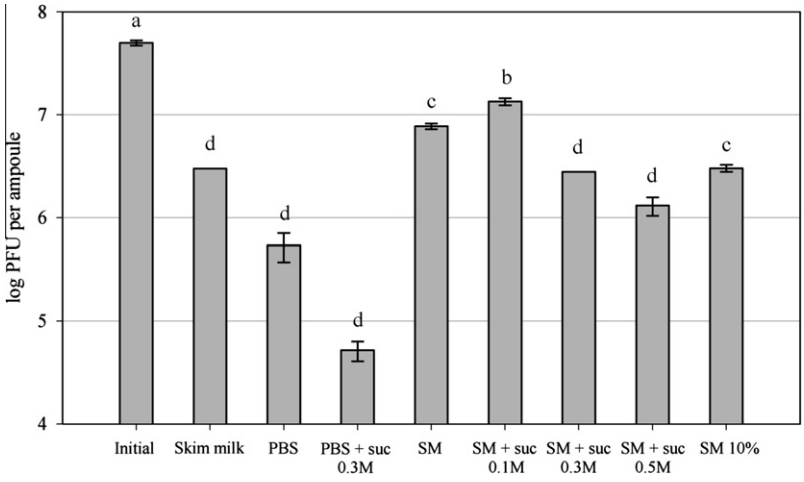

Fig. 1. Number of viable phage per ampoule before (initial) and after the freeze-drying process in skim milk, PBS, SM buffer (SM), PBS and SM buffer with sucrose (PBS + suc or SM + suc) and SM buffer diluted to 10% in mili-Q water (SM 10%). Different letters above the bars mean significantly different values ($P < 0.05$).

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