Cryobiology 66 (2013) 339–342

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Brief Communication

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

C. Dini [a,b,*,1,2], P.J. de Urraza [a,b]

[a] Center for Research and Development in Food Cryotechnology (CIDCA-CONICET-UNLP) CCT La Plata, La Plata, Argentina
[b] Cátedra de Microbiología, Facultad de Ciencias Exactas, UNLP, Argentina

ARTICLE INFO

Article history:
Received 21 November 2012
Accepted 16 March 2013
Available online 26 March 2013

Keywords:
Phage
Lyophilization
Conservation
Storage
Lyoprotectant
Disaccharide

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

☆ This work was funded with ANPCyT project PICT-2006-00479 directed by Dr. G.L. De Antoni.

* Corresponding author. Address: Center for Research and Development in Food Cryotechnology (CIDCA-CONICET-UNLP) 47 y 116, La Plata 1900, Argentina. Fax: +54 (0221) 4254853.
E-mail address: cdini@biol.unlp.edu.ar (C. Dini).
[1] Fax: +54 (0221) 4890741.
[2] Fax: +54 (0221) 4249287.

0011-2240/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cryobiol.2013.03.007

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

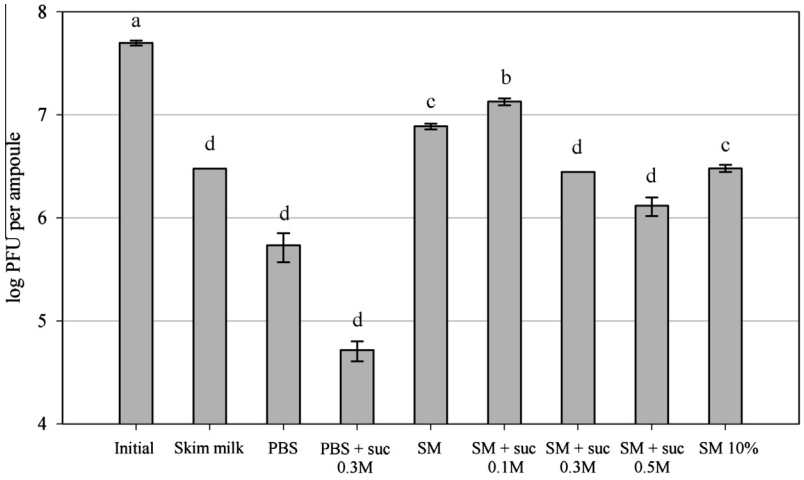

Fig. 1. Number of viable phage per ampoule before (initial) and after the freeze-drying process in skim milk, PBS, SM buffer (SM), PBS and SM buffer with sucrose (PBS + suc or SM + suc) and SM buffer diluted to 10% in mili-Q water (SM 10%). Different letters above the bars mean significantly different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

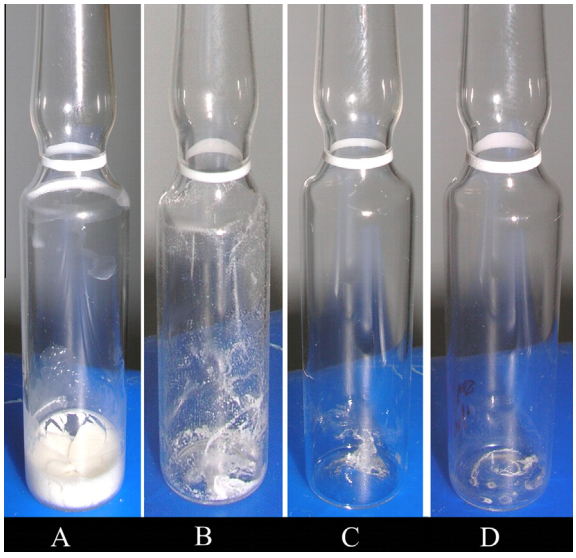

Fig. 2. Types of lyophilization powders obtained with A. – Skim milk, B. – PBS or SM buffer, C. – PBS or SM buffer with sucrose and D. – SM buffer diluted to 10%, as freeze-drying media.

Table 1
Number of viable phage per ampoule before (initial) and after the freezing and thawing process.

	Viable phage (log PFU)	Phage loss (log PFU)
Initial	7.70 ± 0.02^{a}	–
PBS	7.66 ± 0.09^{a}	ns
PBS + 0.3 mol l^{-1} sucrose	7.22 ± 0.09^{b}	0.48
SM + 0.1 mol l^{-1} sucrose	7.68 ± 0.01^{a}	ns
SM + 0.5 mol l^{-1} sucrose	7.44 ± 0.01^{b}	0.26

Different superscript letters mean significantly different values ($P < 0.05$). Phage loss: difference between viable phage before (initial) and after freeze-thaw. ns: not significant ($P > 0.05$).

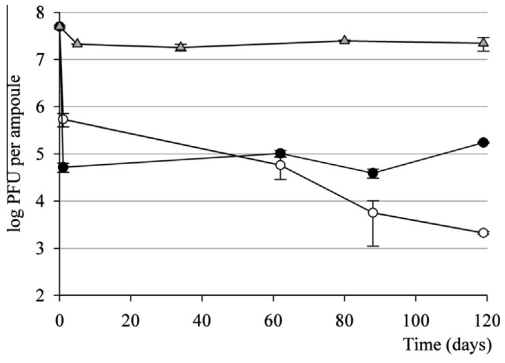

Fig. 3. Number of viable phage per ampoule during storage of the lyophilized products at 4 °C. Symbols: ○, PBS; ●, PBS with 0.3 mol l^{-1} sucrose and ▲, SM buffer with 0.1 mol l^{-1} sucrose.

storage at 4 °C, as observed in Fig. 3. Phage titer was kept for at least 120 days with respect to that obtained immediately after the freeze-drying process ($P > 0.05$) in agreement with that described for sugars on the shelf life of lyophilized proteins [11]. PBS without sucrose showed a progressive decrease in the number of viable phage during the 120 days of storage (Fig. 3), with a total loss of 2.41 log PFU with respect to the initial lyophilized product.

An important factor related to phage survival during the freeze-drying process and its stability during storage is the residual moisture content of the freeze dried products [10]. PBS lyophilized powders exhibited the lowest residual moisture content among the lyophilization media tested (Table 2), meanwhile the addition of sucrose significantly increased ($P < 0.05$) the residual water content of the freeze dried product resulting also higher ($P < 0.05$) than that obtained with skim milk (Table 2).

Table 2
Residual moisture content (g% on dry basis) of phage freeze-dried in different media.

Freeze drying medium	Residual moisture
Skim milk	0.036 ± 0.007^{c}
PBS	$<0.001^{a}$
PBS + suc 0.3 M	0.099 ± 0.021^{d}
SM	0.014 ± 0.004^{b}
SM + suc 0.1 M	0.032 ± 0.020^{c}
SM + suc 0.3 M	0.082 ± 0.008^{d}
SM + suc 0.5 M	0.155 ± 0.013^{e}
SM 10%	0.020 ± 0.005^{bc}

Different superscript letters mean significantly different values ($P < 0.05$).

The use of SM buffer as lyophilization medium showed a significant enhancement ($P < 0.05$) in phage stability during freeze-drying compared to that obtained with PBS (Fig. 1), increasing phage titer in 1.15 log after the freeze-drying process ($P < 0.05$) and allowing a phage survival percentage of 15 ± 1%. This effect was not related to inorganic salts concentration since dilution of SM buffer to 10% (v/v) in mili–Q H_2O (SM 10%) produced no significant change ($P > 0.05$) in the number of remaining viable phage after freeze drying with respect to the undiluted buffer (Fig. 1). The residual moisture content of the lyophilized products obtained with SM and SM 10% (Table 2), did not significantly differ from each other, but both were significantly higher ($P < 0.05$) than that obtained with PBS.

The addition of 0.1 mol l^{-1} of sucrose to the SM buffer significantly increased the number of remaining viable phage after freeze drying compared with that obtained with the buffer alone (Fig. 1). Particularly, magnesium (present in SM buffer) has been reported protect certain proteins during lyophilization in the presence of 0.1 mol l^{-1} sugars such as sucrose [2,11]. Raising sucrose concentration to 0.3 and 0.5 mol l^{-1} was detrimental to phage stability, resulting in lower phage titers than that obtained with the buffer without sucrose ($P < 0.05$).

The same effect was observed by Puapermpoonsiri et al. [10] on a *Myoviridae* phage of *Pseudomonas aeruginosa* and a *Siphoviridae* phage of *Staphylococcus aureus*. Authors reported an increased loss of infective particles with the addition of high concentrations of sucrose (0.5 mol l^{-1}) than that obtained with low concentrations (0.1 mol l^{-1}) after lyophilization, and the difference was attributed to the distinct residual moistures obtained for the lyophilized products on each condition. In the present work, a sucrose concentration dependency was also observed for the residual moisture content of the lyophilized products, resulting significantly increased ($P < 0.05$) when higher concentrations of sucrose were added (Table 2).

SM buffer with and without sucrose produced lyophilized powders similar to those obtained with PBS (Fig. 2B–D), which were also immediately resuspended with the addition of SM buffer.

The analysis of phage loss during crystallization showed a significant reduction of 0.26 log PFU in the number of viable phage after freezing at −80 °C and thawing at 25 °C when sucrose was added at a concentration of 0.5 mol l^{-1} (Table 1). The complete freeze-drying process in the same medium produced a titer loss of 1.58 log (Fig. 1), implying that most of the phage loss occurs during the drying step, as observed with PBS.

When sucrose was added at low concentration (0.1 mol l^{-1}) to SM buffer, the loss of infective particles during the freeze-drying process was 0.56 log PFU (Fig. 1), and this reduction was attributed to the drying step, since the freeze-thaw assay showed no significant reduction ($P > 0.05$) in phage titer during the freezing stage (Table 1). Therefore, low sucrose concentrations, besides allowing a total retention of phage viability during the freezing step, also increase phage protection during the drying step compared to higher sucrose concentrations.

SM buffer with 0.1 mol l^{-1} sucrose stabilized lyophilized phage CA933P during refrigerated storage, showing no significant change ($P > 0.05$) in the number of viable phage after 120 days compared to that obtained immediately after the lyophilization process (Fig. 3).

The results obtained show that the destabilization of *Podoviridae* phage CA933P during lyophilization in the presence of high concentrations of sucrose (0.5 mol l^{-1}) occurs during both the freezing and the drying steps, being the freezing step less critical than that of dehydration. When sucrose is added at low concentration (0.1 mol l^{-1}) no loss of phage titer is observed during the freezing step and there is an improvement in phage stability during the drying step, in agreement with the protective effect described for low concentrations of disaccharides during dehydration of proteins. The addition of sucrose increased the residual water content of the lyophilized products, being higher when sucrose concentration was raised.

The analysis of refrigerated storage of the lyophilized products showed a stabilizing effect of sucrose in time for both, the PBS with 0.3 mol l^{-1} of sucrose and the SM buffer with 0.1 mol l^{-1} of sucrose, in agreement with that described for sugars on the shelf life of lyophilized proteins [11]. SM buffer with 0.1 mol l^{-1} sucrose significantly improved the resistance of phages to the lyophilization process with respect to skim milk, and produced a lyophilization product which is easily rehydrated and stable for at least 120 days of storage at 4 °C.

Acknowledgment

The financial support from ANPCyT (PICT-2006-00479) to Dr. G.L. De Antoni is gratefully acknowledged.

References

[1] K. Carlson, Working with bacteriophages: common techniques and methodological approaches, in: E. Kutter, A. Sulakvelidze (Eds.), Bacteriophages Biology and Applications, CRC Press, Boca Raton, Florida, USA, 2005.
[2] J.F. Carpenter, L.M. Crowe, J.H. Crowe, Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations, Biochim. Biophys. Acta 923 (1987) 109–115.
[3] W.A. Clark, Comparison of several methods for preserving bacteriophages, Appl. Microbiol. 10 (1962) 466–471.
[4] J.G. Day, G. Stacey, Cryopreservation and Freeze-Drying Protocols, Humana Press, Totowa, NJ, 2007.
[5] P.V. Date, A. Samad, P.V. Devarajan, Freeze thaw: a simple approach for prediction of optimal cryoprotectant for freeze drying, AAPS PharmSci. Tech. 11 (2010) 304–313.
[6] C. Dini, P.J. De Urraza, Isolation and selection of coliphages as potential biocontrol agents of enterohemorrhagic and Shiga toxin-producing *E. coli* (EHEC and STEC) in cattle, J. Appl. Microbiol. 109 (2010) 873–887.
[7] Z. Hubálek, Protectants used in the cryopreservation of microorganisms, Cryobiology 46 (2003) 205–229.
[8] A.M. Kropinski, A. Mazzocco, T.E. Waddell, E. Lingohr, R.P. Johnson, Enumeration of bacteriophages by double agar overlay plaque assay, Methods Mol. Biol. 501 (2009) 69–76.
[9] P. Matejtschuk, Lyophilization of proteins, Methods Mol. Biol. 368 (2007) 59–72.
[10] U. Puapermpoonsiri, S.J. Ford, C.F. van der Walle, Stabilization of bacteriophage during freeze drying, Int. J. Pharm. 389 (2010) 168–175.
[11] W. Wang, Lyophilization and development of solid protein pharmaceuticals, Int. J. Pharm. 203 (2000) 1–60.