Large-Scale Freezing of Biologics (Part III)

Understanding Frozen-State Protein and Solute Concentration Changes in Celsius Bags

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ABSTRACT

Protein bulk drug solutions (BDS) are typically frozen in various configurations ranging from plastic containers (carboys), stainless steel vessels and tanks, as well as plastic bags. Freezing protein BDS provides flexibility by enabling longer shelf-life for the drug substance and decoupling BDS manufacture from that of drug product. Despite the advantages offered by freezing of protein BDS, there are several technical challenges associated with freezing and thawing of protein drug substance. These include phenomena such as cryoconcentration and protein denaturation on ice-water interfaces. Celsius bags are used in the biotechnology industry for freezing BDS using a cryoprocessing unit. This study evaluated the extent of cryoconcentration in Celsius bags by creating a comprehensive map of the solute distribution in terms of osmolality and protein concentration for a formulated monoclonal antibody (mAb) solution in 8.3-L bags. The authors assessed the effect of freezing rate, solute (i.e., trehalose) concentration, and fill volume. A considerable degree of cryoconcentration for the solute was seen as a function of fill depth as well as the distance from the active cooling surface, with highest levels near the bottom and middle of the bag. Freezing rate affected the extent of cryoconcentration when the fill volume was low. Higher solute concentration resulted in a larger concentration gradient in the frozen state compared with low solute concentration.

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PEER-REVIEWED

Article submitted: May 29, 2012.
Article accepted: Jul. 10, 2012.

The freeze-thaw operation is a crucial step in protein therapeutics production, and is typically applied to protein bulk drug substance (BDS) to enhance its shelf-life and manage the logistics of drug substance campaigns independent of drug product demand. Freezing reduces the degradation rate of the drug substance, protects against microbial contamination or growth, and enables transport without risk of agitation and air-liquid interface-induced denaturation. However, despite these advantages and the seemingly simple technical operation, freezing and thawing of protein BDS presents significant fundamental physico-chemical challenges as well as processing challenges at large scale. Some of these challenges include cryoconcentration, pH and ionic strength changes, phase separation, phase changes, ice-water interface-induced denaturation, as well as reproducibility and uniformity of processing when performed at commercial scales. These aspects have been reviewed by the authors in earlier publications (1–3). The authors have also studied (in parts I and II of this series) the behavior of solutes and proteins in scaled-down systems designed to mimic 300-L cryovessels

to gain an understanding of the environment to which the protein is exposed under different processing conditions and in different parts of the cryovessel (4, 5).

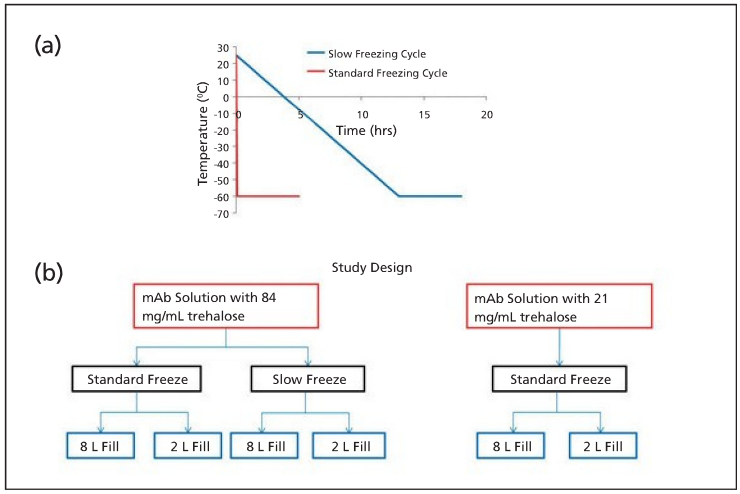
In this report, part III of this series, the authors extend their study to disposable bag systems designed for cryoprocessing. The Celsius Pak system (Sartorius Stedim) is designed to provide an active freeze/thaw processing and storage option for BDS at smaller scales than cryovessels. The use of multiple bags (up to 8.3 L / 16.6 L) allows a batch to be split up into smaller aliquots to provide flexibility in drug product manufacturing. The Celsius Pak bags are processed (i.e., frozen or thawed) by a dedicated cryoprocessing unit that allows predefined temperature profiles to be run in a reproducible manner, irrespective of the batch size (6).

Solute and protein distribution have been studied for plastic bottles frozen in freezers by a passive freezing process (7–9). Concentration and osmolality mapping studies in plastic bottles showed that the (macro) cryoconcentration in plastic bottles is dependent on freezing temperature, protein concentration, and fill depth (7, 8). Lashmar et al. also evaluated freeze concentration for small-scale (30-mL) bags and found a twofold increase in protein concentration and osmolality (9). Large-scale bags have, to date, only been studied by Padala et al., who examined the Celsius Pak 8.3-L system using bovine serum albumin (BSA) as model protein and found an approximately 1.4-fold cryoconcentration in the lower middle part of the Celsius bag (10).

In the previous reports in this series, the authors have shown that (macro)-cryoconcentration in the cryowedge system (representing a cryovessel) is significantly affected by density-driven convection gradients, as demonstrated by protein concentration and osmolality changes during and after freezing (4, 5). A limitation of the cryowedge is that the depth of solution is limited to around 10 cm. The aspect ratio (i.e., liquid depth to diameter) is very small in the cryowedge compared with the real tank it is supposed to represent. Thus, convective effects may not be operational to the same extent as in the full-scale tank. In this third report of the series, the authors examine the solute distribution in the frozen state in Celsius bags as a function of fill depth, solute concentration, and processing (i.e., freezing) rate. The data suggest that convective effects become increasingly important in the distribution of solutes in practical systems, with fill depth being a key process parameter. Solute concentration determines the density gradients generated and therefore the extent of solute polarization.

In all of these studies, it must be remembered that using ice-core samples provides a measure of the macro-cryoconcentration only, that is, it provides a way to understand the solute movement in the system during freezing and the resultant solute polarization across the geometry. The true or micro-concentration is given by the phase or state diagram of the solute system, disregarding the nonequilibrium effects in a real-time process. The results reported from ice-coring analyses are dependent on the size of the core and the distribution of the core samples. Smaller cores and dense sampling will give a more realistic picture of the solute distribution (the macro-cryoconcentration) than otherwise.

Figure 1: (a) Freezing cycle profiles for freezing solution in Celsius bags and (b) study design of overall scheme.



MATERIAL AND METHODS

Materials

Solute distribution and solution property changes in frozen state in Celsius bags were monitored in 8.3-L Celsius bags as

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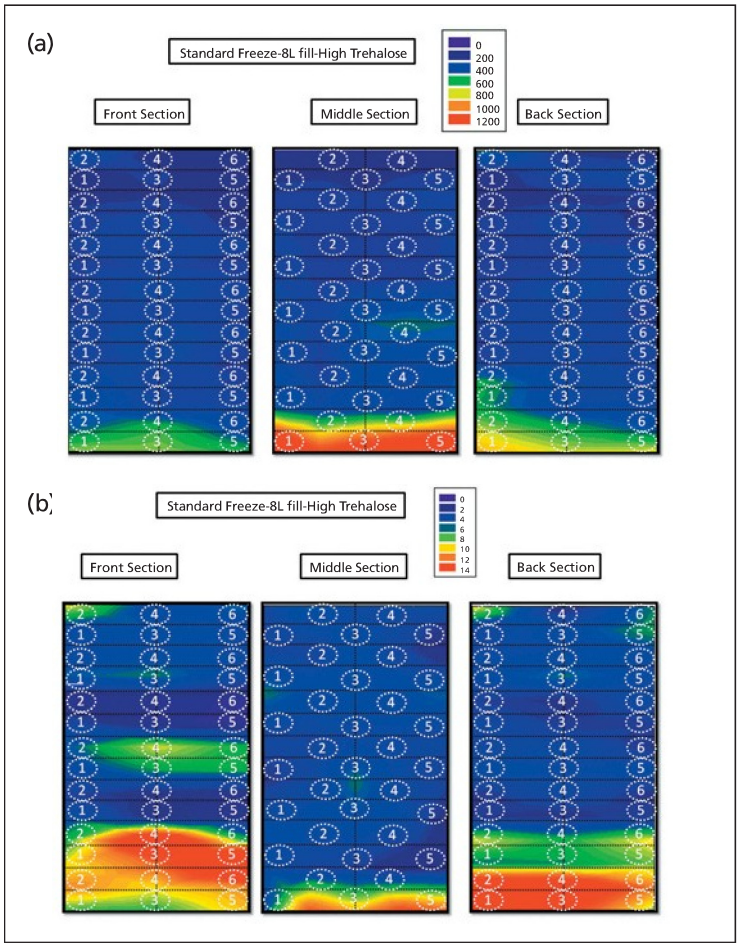
Figure 2: (a) Osmolality and (b) protein concentration map for standard freezing cycle for 8-L fill.



a function of freezing rate, trehalose concentration, and processing volume (i.e., solution depth). Processing was carried out by using a FT-100 system that uses silicone oil (Silthermxx, Dow) as the heat-transfer fluid (HTF). An in-house IgG2 monoclonal antibody (mAb) solution at 5 mg/mL in a 20 mM histidine buffer, pH 5.5, with 0.2 mg/mL polysorbate 80 and either 84 mg/mL or 21 mg/mL trehalose dihydrate was used as a model protein for this study.

A bench-top band saw (Delta SM400 Shopmaster 3 Amp 9-Inch band saw) was used to cut the frozen Celsius bags into sections. As described previously, a Dewalt drill with a custom-made drill bit (diameter 1.5 cm) was used to take cores (approximately 2.5 cm long) from the frozen block (4, 5, 8).

Protein concentration in the cores was measured after thawing by UV280. Solute concentration was assessed by measuring osmolality of the thawed core solution using a freezing point osmometer (Advanced Instruments, Model 3250).

Celsius bag mapping in the frozen state

In-house mAb solution was frozen using either a slow or standard freezing cycle as shown in **Figure 1a**. In the standard freezing cycle, HTF is rapidly cooled to −60 °C and the freezing process is allowed to go to completion over 5 h. In the slow freezing cycle, the freezing was completed over 13 h. **Figure 1b** provides a road map of the study design. Celsius bags (8.3-L size) were filled with IgG2 mAb solution containing either 21 mg/mL trehalose or 84 mg/mL trehalose to volumes of either 2 L or 8 L, and frozen using the standard freezing cycle. In other experiments, an 8.3-L Celcius bag was filled with the mAb solution containing 84 mg/mL trehalose to a fill volume of 2 L or 8 L and frozen using the slow freezing cycle. Once the material in the bags was frozen, the bags were stored at −40 °C until further processing.

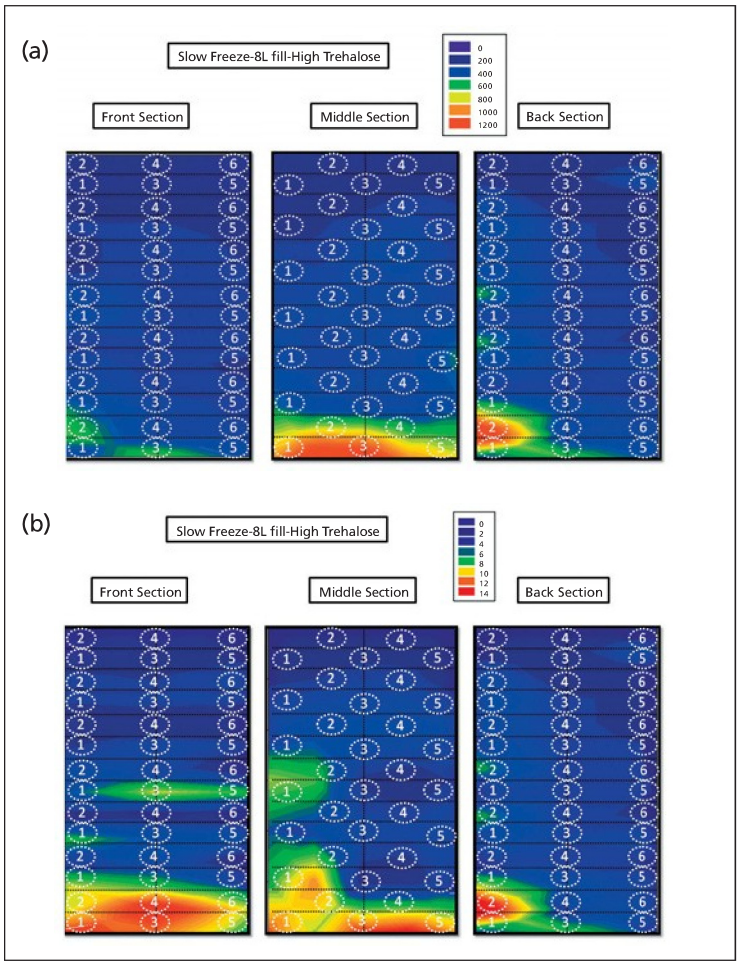
In the frozen state, the 2-L fill corresponds to an approximate depth of 20 cm and the 8-L fill to 80 cm.

The frozen Celsius bags were cut and cores taken according to a predetermined template. This procedure ensured that the maximum amount of information would be obtained for concentration and osmolality distribution.

RESULTS

In previous studies, the authors demonstrated that the cryoconcentration and solute gradient in protein solutions progressively develops during the freezing step. If thawed without agitation, this gradient is maintained irrespective of container configuration (i.e., plastic bottles or cryowedge). The objective of this specific study was to map the solution properties in 8.3-L Celsius bags in the frozen state and study the effect of fill volume, freezing rate, and trehalose concentration and determine if there is any interdependence of these factors on observed cryoconcentration. Ice cores were taken from the frozen block and protein concentration and osmolality were measured. The

Figure 3: (a) Osmolality and (b) protein concentration map for slow freezing cycle for 8-L fill.



core sample from the frozen block represents an average concentration of solutes and protein at that position since the ice sampled in the core dilutes the sample once the core is thawed for measurement. The implications of this important but commonly ignored aspect of the measurement technique were discussed in a previous publication (5).

Celsius bags frozen state mapping: effect of freezing rate and fill depth

Standard and slow process cycles were employed for freezing the mAb solution containing 84 mg/mL of trehalose. The bags were filled with either 2 L or 8 L of solution. **Figure 2a** shows the osmolality map obtained for the standard freezing rate in an 8.3-L bag. The data are shown for front, middle, and back sections in the vertical plane. For the front and back sections of the bag, the osmolality ranged from 128 to 920 mOsm/Kg. The highest osmolality values were observed in slice 1 (the lowest horizontal slice) of the bag, with the middle section showing the greatest increase. The osmolality in the front and back sections corresponds to a more than three times greater cryoconcentration when compared with the initial osmolality of the solution (initial osmolality values was 280 mOsm/Kg). The extent of cryoconcentration increased for the middle section, where the highest osmolality of 1733 mOsm/Kg (approximately 6 times higher than the initial value) was measured. The solution is cooled at the walls, becomes denser, and flows down along the walls carrying the solute. The denser, concentrated solute is deposited at the bottom and the solvent convects back up through the middle section, leading to the concentration distribution observed.

The corresponding protein concentration map for the standard freeze thaw is shown in **Figure 2b**. The concentration in the front, back, and middle section ranged from 0.6 to 19.4 mg/mL. The highest concentration profile was observed for the bottom slices, 1 and 2. The observed cryoconcentration of protein at the bottom sections of the bag was approximately four times higher than the initial value. No significant differences in absolute maximum concentration levels between the middle and front/back sections was observed, although the distribution was not as compact as for the solutes in **Figure 2a**. This finding suggests that the solutes and protein are not migrating completely in tandem, with the protein lagging somewhat behind.

Osmolality and protein concentration maps for the slow freezing cycle are shown in **Figures 3a** and **3b**, respectively. The general trends are similar compared with the results for the standard freezing cycle. The osmolality ranged from 87 to 685 mOsm/kg for the front and back section of the Celsius bag. The osmolality for the middle section ranged from 87 to 1374 mOsm/kg. Protein concentration ranged from 0.6 mg/mL to 13.4 mg/mL for the front, back, and middle sections.

When the fill volume (i.e., fill height) is reduced, the extent of solute polarization is reduced as seen in **Figure 4** (standard cycle) and **Figure 5** (slow cycle). The slow cycle leads to a higher level of cryoconcentration in the middle section compared with the standard cycle (956 mOsm/Kg compared with 382 mOsm/Kg). Smaller protein concentration ranges are seen, with a maximum of 5.8 mg/mL in the middle for the standard cycle.

The results show that solution depth as well as distance from the cooling surface determine the solute and protein distribution at the end of the freezing process. Solutes seem to cryoconcentrate in the middle section near the bottom while the protein is a slightly more widely distributed in the front and back sections too, but again near the bottom. The rate of cooling has a less significant role, probably because the immobilization of flow is completed in a similar time despite the different cycles.

Celsius bags frozen state mapping: Effect of trehalose concentration and fill depth

A second formulation containing trehalose at a lower concentration, 21 mg/mL instead of 84 mg/mL, was tested to understand if the trehalose concentration can contribute to the distribution through the density gradient that is generated. Both 8-L fill and 2-L fills were employed using the standard cycle. Results are shown in **Figures 6a** and **6b** (osmolality and concentration respectively) for the 8-L fill and in **Figure 7** for the 2-L fill. The overall distribution is similar to that of high trehlose solutions, with cryoconcentration effects seen near the bottom/middle sections. However, the maximum osmolality measured for the 21 mg/mL solution (8-L fill) was 280 mOsm/Kg, representing a 4-fold increase (from 70 mOsm/Kg) whereas it was 1733 mOSm/kg with 84 mg/mL trehalose (a 6-fold increase). The high trehalose concentration leads to a greater density gradient formation and thus a higher degree of solute polarization.

The differences were not as significant for the corresponding protein concentration. With the 8-L fill, the maximum protein concentration observed with 21 mg/mL trehalose was 15.9 mg/mL (see **Figure 6b**) compared with 19.4 mg/mL with 84 mg/mL trehalose (see **Figure 3b**). Similar results were observed for the 2-L fill. The highest protein concentration observed for both low and high trehalose concentrations was 5.8 mg/mL.

Figure 4: Osmolality and protein concentration map for standard freezing cycle (2-L fill).

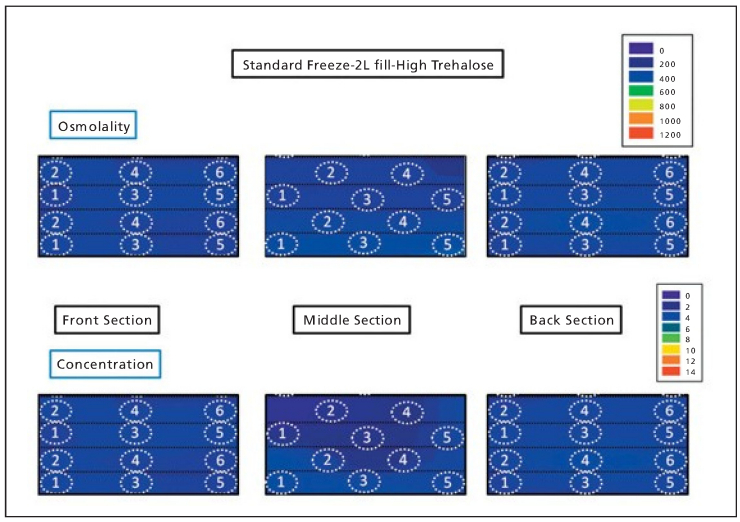
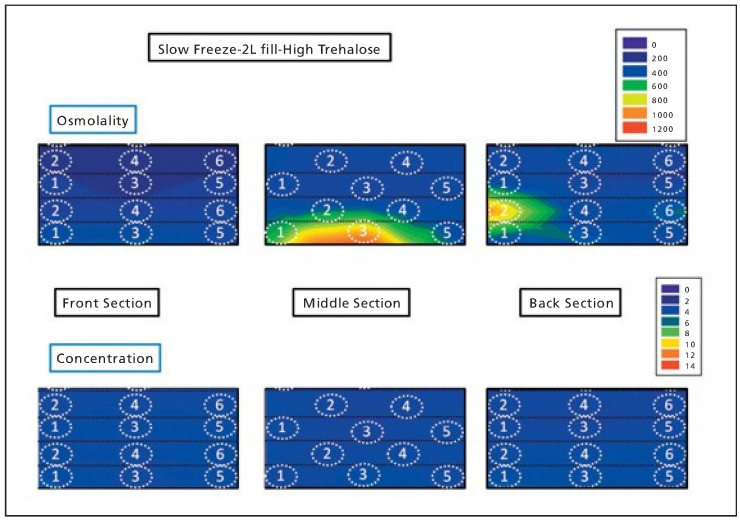


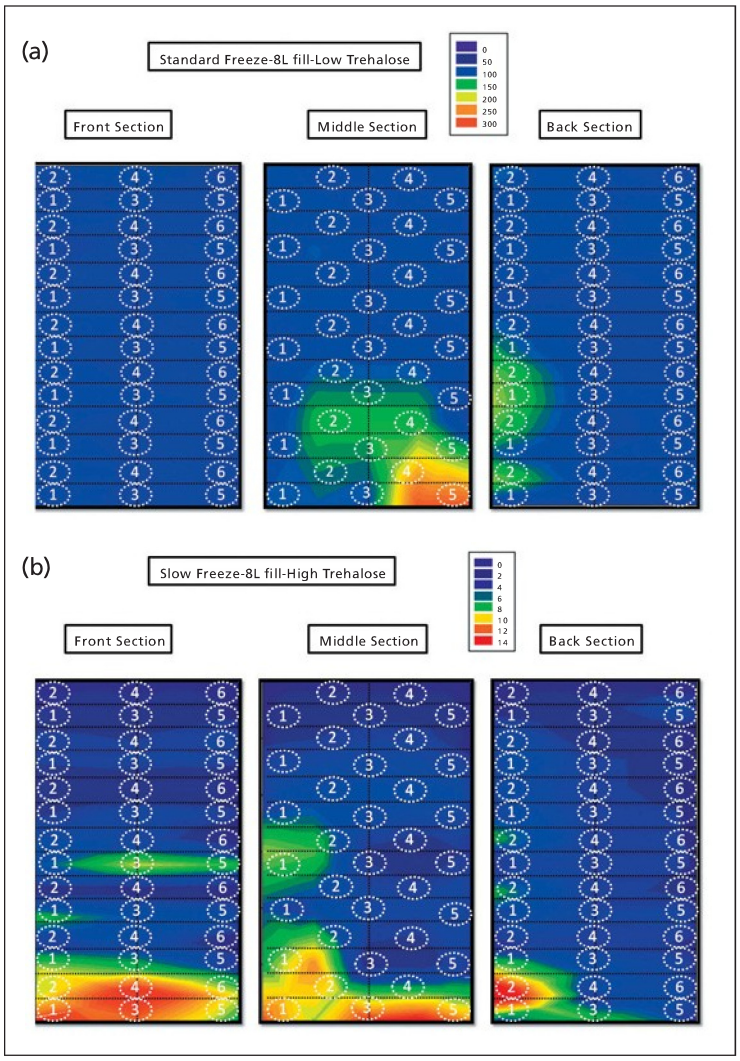
Figure 5: Osmolality and protein concentration map for slow freezing cycle (2-L fill).



Cryoconcentration and its effect

For all the conditions studied, an assessment of fraction of total protein mass affected by the cryoconcentration effect was performed. These findings are important since the highly concentrated protein

Figure 6: (a) Osmolality and (b) protein concentration map for standard freezing cycle for 8-L fill with low trehalose.



solution or reduced solute (cryoprotectant) concentration may affect aggregation rates. **Figure 8a** shows the data as percentage of affected protein mass for the various conditions studied. Most of the protein concentration fraction observed was for one-fold cryoconcentration or below (5 mg/mL or below). The slow freezing cycle with a 2-L fill containing high trehalose showed the highest fraction (92%) while the standard freezing cycle with an 8-L fill containing high trehalose showed the lowest fraction (60%) of protein in this concentration range. The highest fraction for two-fold and above was observed for the standard freezing cycle with an 8-L fill containing low trehalose followed by the standard freezing cycle with an 8-L fill containing high trehalose. The lowest fraction in this region was for the slow freezing cycle with a 2-L fill containing high trehalose. Similarly, the maximum fraction of cryoconcentration of three-fold and higher was observed for the standard freezing cycle with an 8-L fill containing high trehalose.

A similar analysis for osmolality fraction was performed. **Figure 8b** provides the analysis for various conditions studied. The highest fraction (83%) for 1X and below (280 mOsm/kg for high trehalose and 70 mOsm/kg for low trehalose) was observed for standard freezing cycle with 2-L fill containing high trehalose and the lowest fraction (6%) was observed for standard freezing cycle with 8-L fill containing low trehalose. The highest fraction (88%) for one-fold cryoconcentration and above was observed for the standard freezing cycle with an 8-L fill containing low trehalose and the lowest fraction (17%) was observed for the standard freezing cycle with a 2-L fill containing high trehalose. The highest fraction (4%) of three-fold and above cryoconcentration was observed for standard freezing cycle with 8-L fill containing high trehalose. Based on this analysis, it is clear that the actual fraction that is cryoconcentrated to greater than two-fold level is less than 20% of the complete system.

DISCUSSION

It is clear that macro-cryoconcentration is inevitable for all the practical-scale freezing systems. The actual distribution of ice and solute/protein may vary in the geometric space of the container depending on the process conditions and composition. Physically, the extent of this solute polarization will be proportional to the solution depth because of density gradient-driven convection during the processing. Furthermore, it is clear that freezing rates can have an effect on cryoconcentration, especially for low fill volumes in the Celsius bag system.

This analysis shows that less than 20% of the solutes are affected by the highest level of cryoconcentration (concentration

more than 2 times initial levels). The levels of cryoconcentration observed here compare well to those seen in previous studies, such as Webb et al. for plastic bottle, who reported that 91.6% of the total protein was at less than a two-fold level of cryoconcentration (11). Previous results using the cryowedge system suggested that more than 90% of the total protein was at less than two-fold level of cryoconcentration (5). Taken together, these results from plastic bottles, cryowedge, and Celsius bag system suggest that in most practical systems, the percentage of protein that is subject to more than two-fold cryoconcentration is in the vicinity of 10%.

The highest osmolality and protein concentration were observed near the bottom of the Celsius bag. The point of maximum osmolality observed is in the bottom/middle section, which is the farthest from the active cooling surfaces.

The authors have established that the freezing profiles have an effect on cryoconcentration, especially when the fill volume is low (8 L versus 2 L in this study). At the high fill volume, a maximum of six-fold cryoconcentration for standard freezing versus five-fold for slow freezing was observed. This observation can be explained by the fact that when the processing volumes are large, the freezing profile effects are not that pronounced and are largely governed by the processing volume. On the other hand, when the processing volume is small, the freezing profile has a greater effect on the outcome. In general, slow freezing allows more time for the ice to freeze and push solutes forward, thus leading to a greater degree of polarization.

The extent of cryoconcentration is higher when the trehalose concentration is higher. In our previous paper, we attributed this to the property of disaccharide solutions, which have significant temperature coefficients for density and viscosity compared with water or buffer solution alone (5). When a high-concentration disaccharide solution is subjected to cooling, smaller temperature differences will lead to larger density gradients (as compared with a low-concentration solution), resulting in greater convection effects, downwards at the cooling surface and in towards the center.

In their recent publication, Padala et al.

Figure 7: Osmolality and protein concentration map for standard freezing cycle for 2L fill with low trehalose.

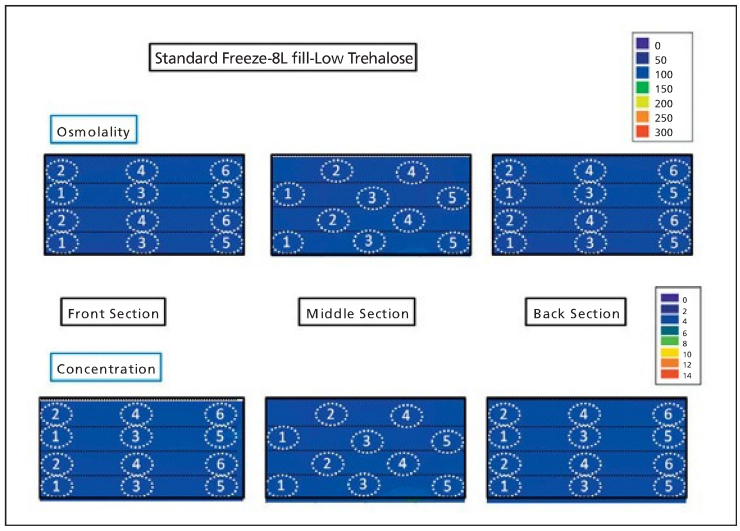
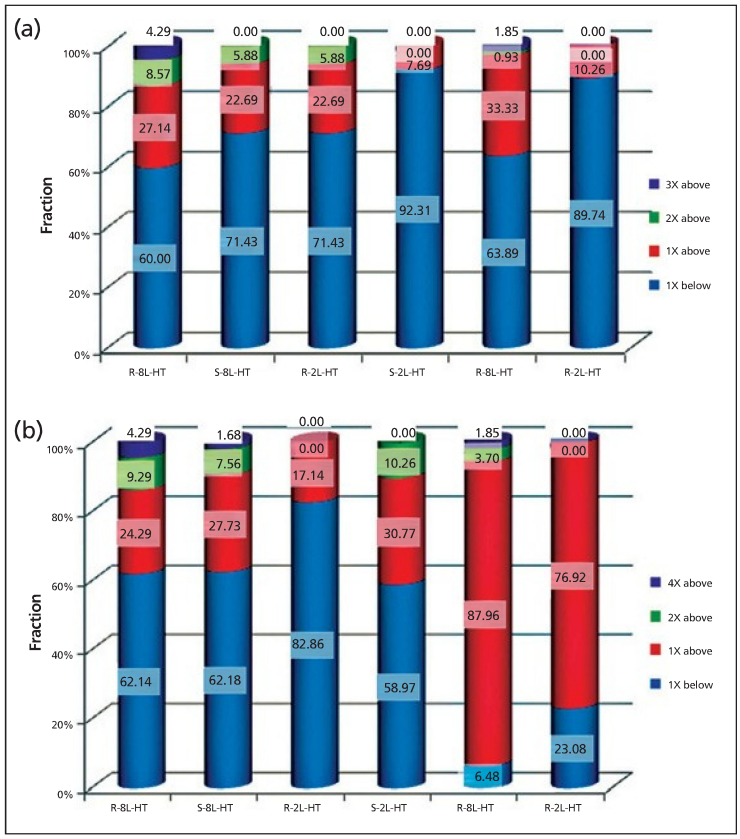


Figure 8: (a) Analysis of cryoconcentration effect on osmolality and (b) protein concentration effects for various conditions studied.



reported only a 1.4-fold cryoconcentration in the bottom center vertical of the Celsius bag, with no cryoconcentration effects for the front and back vertical sections of the bag (10). Although the general observation of cryoconcentration is similar to our study, the low extent of cryoconcentration reported is a function of the study design. The data presented in that specific study were based on 27 total cores as opposed to 140 total cores taken in our study. If a limited number of cores are taken or large cores are taken and the data are averaged over a large portion of the container, the reported cryoconcentration effect will be smaller than actual, as seen in Padala et al (10). This result can be understood by the simple thought experiment that if the whole bag is frozen and subsequently thawed as one sample and analyzed, no cryoconcentration would be reported, and would clearly be in error. This illustrates the importance of examining the design of the study when assessing results reported in the literature. Smaller but more numerous core samples taken from the frozen block at various positions provide a better picture of the solute polarization.

CONCLUSIONS

Cryoconcentration is inevitable in any system used to freeze bulk protein drug solutions of any practical size. The extent of solute and protein polarization will depend on freezing rate profile, the depth of solution, and the solute concentration, and is a consequence of density gradient-driven convective effects. The greatest changes will be observed near the center, farthest from the cooling surfaces and near the bottom in terms of geometry. Greater depth will allow for greater differences, as will high solute concentrations. As solution volume to be processed increases, the effect of the freezing rate profile decreases.

The so-called "controlled rate" freezing does not eliminate cryoconcentration. Controlled or more accurately, "active" freezing, however, enables a reproducible process that will in general be less subject to vagaries of freezer load, placement position, and other factors compared with a passive freezing process conducted in a freezer. For the same volume and geometry, an active freezing process will also generally be more rapid than passive freezing, reducing the time that the protein is exposed to the phase-transition environment where the greatest damage may occur (1, 12).

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