Benefits of Celsius Controlled Freeze-Thaw Technology

on the Recovery of Protein Activity

By Gaël Péron

Sartorius Stedim Biotech is the world leader in controlled Freeze-Thaw technology

Executive Summary

The recovery of a model protein activity was investigated under the controlled freeze and thaw conditions of the Celsius technology and compared with conventional freeze and thaw systems. L-Lactate Dehydrogenase (LDH) was selected as a model protein, as it has been extensively studied in freeze-thaw applications and its activity can be measured by a simple UV method. Addition of stabilizers such as BSA or Tween 80 to the LDH formulation was found to reduce the denaturation caused by the freeze thaw process or the long-term storage at refrigerated temperature. BSA supplemented LDH samples were frozen and thawed using conventional freezing methods representative of current practices in the biopharmaceutical industry (500 mL bottles to 1 L bottles in -20°C and -80°C freezers) and controlled Celsius freeze-thaw technology (30 mL, 8.3 L and 16.6 L Celsius-Paks in Celsius S³ and FT100 systems).

The results indicated that, with appropriate cryoprotectant and under poorly controlled freezing and thawing conditions LDH activity recovery was low (80%). However, higher activity recovery was observed for LDH samples processed using the Celsius controlled freeze-thaw technology (90%). The result is attributed to the higher freezing rate and reduced protein stresses observed in the Celsius FT systems. In addition, the scalability between the different Celsius platforms was demonstrated since the LDH activity recovery is approximately constant (less than 3% variation) for an operating volume range spanning from 30 mL to 16.6 L.

Introduction

L-Lactate Dehydrogenase (LDH) has been extensively studied for freeze-thaw applications as a model for labile protein drugs. Under poorly controlled conditions and without appropriate additives (1, 2), significant reduction of the LDH activity has been identified following freeze and thaw steps. Hilgren et al (1) demonstrates that small addition of Tween 80 to the protein buffer reduces the LDH denaturation by hindering the interaction with the ice crystals. Tamiya et al (2) showed that the addition of 0.1% albumin reducess LDH enzyme inactivation when frozen in a –20°C freezer. The importance of the freezing and thawing rate in maintaining the LDH activity is demonstrated by Cao et al (3). The objective of this study was to demonstrate the benefits of Sartorius Stedim Biotech Celsius controlled freeze-thaw technology with LDH as a protein model. Uncontrolled freeze-thaw techniques, commonly used in the biopharmaceutical industry, were used as a comparison point.

Materials and Methods

Materials

LDH (type VIII, from chicken heart, as an aqueous ammonium sulfate solution suspension), NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt), Tween 80 and pyruvic acid sodium salt were purchased from Fluka Chemie Sàrl (Switzerland). Tri-sodium citrate dehydrate (for analyses, ACS, ISO), citric acid monohydrate (for analyses, ACS, ISO) and lyophilized BSA (bovine serum albumin fraction V, for biochemical use) were purchased from VWR International SAS (France).

Liquid storage

A pH of 7.5 was chosen for the buffer solution because the enzyme has maximum activity between 7.4 and 7.9 (4). A first series of experiments was conducted with the formulations of LDH shown in Table 1. Samples of each enzyme solution were kept at 4°C and their activities were measured over time.

Table 1: Solutions of LDH used during the study

Solution name	LDH, 1 µg/mL	50 mM sodium citrate, pH 7.51	BSA, 1 mg/mL	TWEEN 80, 0.01% (v/v)
LDH alone	•	•		
LDH + BSA	•	•	•	
LDH + Tween 80	•	•		•

Enzymatic assay

The enzymatic assay follows the reduction of a UV absorbing co-factor: NADH, at 340 nm (5). The starting solution and samples were tested in triplicate and the average activity and corresponding standard deviations were calculated as well as the activity recovery. Linear decrease of the UV absorbance was confirmed at the time of enzyme addition. A physically inactivated enzyme (by heat-shock treatment) was used as negative control. As expected, no enzymatic activity was detected. In order to further validate the enzymatic test, the volume of the enzyme sample was reduced two-fold. As expected, a two-fold reduction of the LDH Activity was measured.

Controlled vs. conventional freeze
In the first set of experiments, three variables were tested: the formulation, the container configuration and the freeze technology. The different formulations tested are reported in Table 1. Controlled freeze consisted of freezing Celsius-Paks (6), filled with 30 mL of the different enzyme solutions, in the Celsius S^3 system using the standard temperature set point profile reported in Table 2.

Table 2: Celsius S^3 Standard freezing profile

Elapsed time (in min)	Temperature set point (°C)
0	-15
40	-70
45	-15
120	-20
210	-30
250	-40

Conventional freeze was performed using 500mL and 1L bottles, filled with the enzyme solutions and stored in –20°C upright and –80°C chest freezers. All samples were thawed in a 37°C water-bath. They were removed from the water-bath as soon as the last piece of ice was melted.

Impact of freezing and thawing rate in controlled FT technology
A second set of experiments was conducted with the Celsius S^3 freeze-thaw module to study the impact of freeze and thaw rate on LDH activity recovery. The LDH+BSA solution was frozen in 30 mL Celsius-Paks placed in the Celsius S^3 freeze-thaw module, using temperature set point profiles reported in Tables 2 to 4.

Table 3: Celsius S^3 Fast freezing profile

Elapsed time (in min)	Temperature set point (°C)
0	-30

Table 4: Celsius S^3 Slow freezing profile

Elapsed time (in min)	Temperature set point (°C)
0	-15
30	-70
45	-10

The disposables were thawed using the methods shown in Table 5.

Table 5: Thawing conditions

Containers	37°C water-bath	4°C fridge	Celsius Standard Thaw
30 mL Celsius-Paks	•	•	•

Impact of scale-up in controlled freeze-thaw technology
The LDH+BSA solution was also frozen in 8.3 L and 16.6 L Celsius-Paks placed in the Celsius FT100 freeze-thaw module using a –60°C set point. The disposables were then thawed using a 30°C set point.

Results and Discussion

Liquid storage
Graph 1 shows enzymatic activity recoveries of the different formulations of LDH when stored at 4°C. The activity recovery was only 17.4% after 18 days of storage for the enzyme in sodium citrate buffer. The addition of 0.01% Tween 80 in the buffer solution had a positive impact since a 25% activity recovery was observed after 18 days of storage. However, that result was low compared to the one obtained with the addition of 0.1% BSA. An activity recovery of 72% was observed, representing a four-time increase compared to the unprotected enzyme. However this recovery is still low and would be unacceptable for a high value protein drug.

Controlled vs. conventional freeze
Graph 2 indicates that the activity recovery obtained with the Celsius S^3 system is approximately 100% for the formulations supplemented with either 0.1% BSA or 0.01% Tween 80. This represents a 50% gain in activity recovery compared to the unprotected enzyme formulation.

Graph 3 shows the activity recoveries obtained with the LDH + BSA formulation processed using conventional techniques. Higher recoveries are obtained for the bottles frozen at –80°C compared to -20°C. Under the investigated conditions, no impact of the processed volume was detected.

Comparison of controlled versus conventional freeze technology is shown in Graph 4.

A 20% gain in LDH activity recovery with the controlled freeze technology is observed for the BSA supplemented formulation.

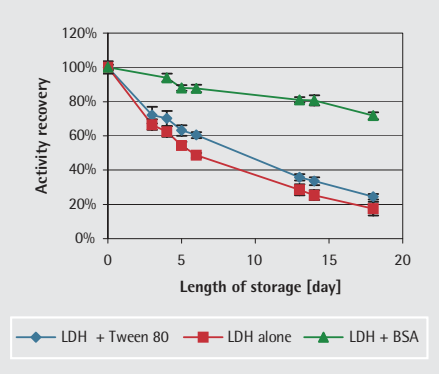
Impact of freezing and thawing rate in controlled freeze-thaw technology
The effect of the freeze and thaw rate on the LDH activity recovery was investigated with Celsius S³ freeze-thaw module.

Graph 5 shows that the impact of the freezing rate on the LDH activity recovery is low. For example, activity recoveries ranging from 89% and 90% are observed for all tested freeze rates and a constant 37°C thaw step. On the contrary, the impact of the thawing rate on the LDH activity is very significant. Regardless of the freezing rates, a higher activity recovery is observed for short thaw times. The optimal conditions for minimizing the activity loss are obtained with the standard temperature set point profile in the Celsius S³.

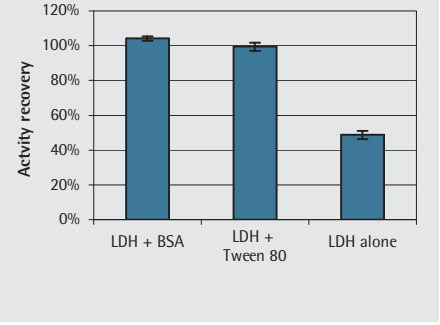
Impact of scale-up in controlled freeze-thaw technology
The LDH activity recovery obtained with the production-scale Celsius-system (FT100 freeze-thaw module) and large volume containers (8.3 L and 16.6 L Celsius-Paks) are presented in Graph 6 and compared to the one obtained at the lab-scale with the scale down system (S³ freeze-thaw module) and small volume container (30 mL Celsius-Pak).

The activity recovery at the large-scale varies from 88% to 92% and the result obtained with the Celsius S³ module is 90%. The scalability between the Celsius S³ and FT100 is good since similar level of LDH activity is recovered after one freeze-thaw cycle

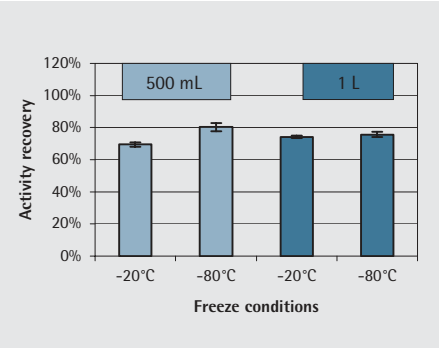
Conclusion
BSA supplemented LDH samples were frozen and thawed using conventional freeze and thaw methods and Celsius controlled freeze-thaw technology. The results showed at least a 10% increase in the LDH activity recovery with the controlled freeze-thaw technology. The results also demonstrate the scalability between the different Celsius platforms since similar level of enzyme activity (88-92%) was recovered for a 30 mL to 16.6 L volume range.



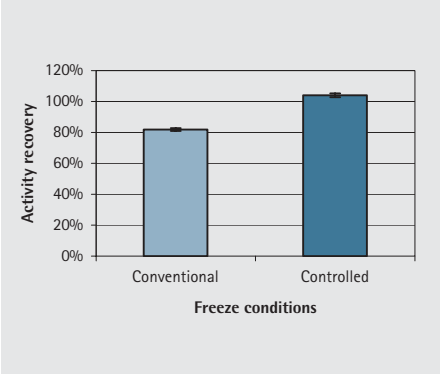
Graph 1: Activity recovery of different solutions of LDH stored at +4°C.



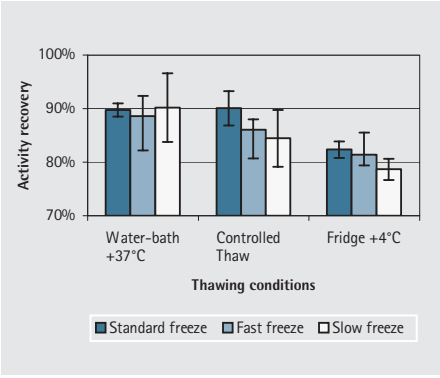
Graph 2: Activity recoveries for the LDH samples frozen with the controlled freeze technology and thawed in a 37°C water-bath.



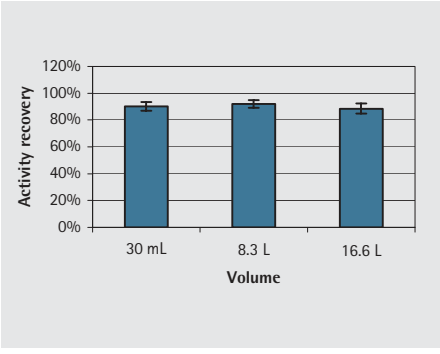
Graph 3: Activity recoveries for the bottles frozen with the conventional technique and thawed within the 37°C water-bath.



Graph 4: Conventional vs. Controlled freeze for LDH+BSA. Both containers were thawed in the 37°C water-bath.



Graph 5: Impact of freezing and thawing condition on LDH activity recovery



Graph 6: Comparison of the LDH activity recovery for increasing scale of operations

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