Effect of Temperature History on the Freeze-Thawing Process and Activity of LDH Formulations

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Purpose. The purpose of the study was to investigate the effect of freeze-thawing processes with different temperature histories on thermal transformations and on protein activity of lactate dehydrogenase (LDH) formulations. Polyethylene glycol (PEG 6000) and maltodextrin were used as cryoprotectants.

Methods. The thermal characterization was made by oscillating DSC (ODSC). LDH activity assays were performed spectrophotometrically.

Results. The crystallization of the solutions and the melting of the frozen samples occurred at fairly constant heat of crystallisation and heat of fusion values and temperatures. The main difference between the two investigated temperature cycles was an exothermic peak at −45°C, which might reflect the transition between the cubic and hexagonal ice structures. When PEG was added to the system an additional endothermic peak appeared at −15°C in the heating program. It was transformed into the shape of a glass transition at the same temperature when the heating rate was increased. The degree of crystallinity of the samples was evaluated as the quota between the c_p component of heat of transformation and the total heat of transformation values. Only minor differences between the two temperature histories and between the samples were observed. The c_p component of the melting endotherm revealed a complex melting process with two overlapping endothermic transformations. The good protein protecting ability of PEG obtained when cooling and heating rate was low, was greatly reduced with increasing rate. The addition of maltodextrin to PEG-containing solutions lowered the activity recovery.

Conclusions. The endothermic transformation of a PEG-ice structure at −15°C in the heating process is strongly correlated to the protective ability of PEG 6000 in the freeze-thawing process of LDH. To obtain the highest protein activity after the freeze-thawing process, the formulation shall be transformed by a low cooling and heating rate. The crystallinity of the system melting at about 2°C is independent of temperature history. The c_p component of the melting endotherm, however, shows a complex transformation, where two phases of different crystallinity and stability might be involved.

KEY WORDS: freeze-thawing; protein stability; oscillating DSC; lactate dehydrogenase; PEG; maltodextrin.

INTRODUCTION

Proteins are insufficiently stable for distribution and use in aqueous solutions, therefore they are freeze-dried to achieve long-term stability. The exact nature of the formulation may have a major impact on the freeze-drying process and the stability of the dried product.

There is a wide variety of additives that minimize protein denaturation, including sugars, polyols, aminoacids, methylamines and certain salts like sodium acetate or potassium phosphate. During the freeze-thawing process the stabilizing solutes are preferentially excluded from contact with the surface of the protein, thus increasing the chemical potential of both the protein and the solute. This thermodynamically unfavorable effect is greater for the denatured form because the surface area exposed to the solvent increases with protein unfolding and therefore the native structure is favored (1). Stabilization of proteins in the dried state is due to compounds that serve as "water replacers" by hydrogen bonds to the protein. For example, polyethylene glycol (PEG) stabilizes proteins during freezing but fails to stabilize dried proteins, while the sugars protect proteins during dehydration by hydrogen bonding to the dried protein (2).

The nucleation and growth process of ice in an aqueous solution greatly affects the cold denaturation of the protein during the freeze-thawing process. The rates of ice crystal nucleation and growth initially increase with increasing degree of supercooling to a certain degree and then decrease with decreasing temperature because of rapid decrease in molecular mobility. If cryodamage is a function of the amount of ice formation, the extent of the damage during warming may be greater than in cooling (3). The effect of, for example, PEG as a cryoprotectant is to reduce the ice nucleation temperature. A certain reduction is to be expected from the collegative properties of the solutions, but the magnitude of the effects cannot be accounted for by only these properties. The reduction in the nucleation rate is supposed to be due to its effect on the diffusional motions of water. PEG is suggested to stabilize the structures that might exist in undercooled water (4).

Differential scanning calorimetry (DSC) is an often used technique for studying glass transitions, melting behavior, degree of crystallinity and other aspects of freeze-dried formulations. In a development of the method, modulated or oscillating DSC (MDSC, ODSC), the usually linear heating or cooling program is modulated by an oscillating time-temperature wave. The technique allows a Fourier transform separation of the parent DSC signal into two components, the c_p (reversible) component and the kinetic (irreversible) component. Practical benefits of the ODSC technique include separation of reversible and irreversible thermal events and improved resolution of closely occurring and overlapping transitions such as recrystallisation and glass transition (5). At an appropriate degree of oscillation it is possible to determine the degree of crystallinity of the phases investigated (6).

The purpose of this study was to investigate the effect of different temperature histories on the thermal events of the freeze-thawing process and on crystallinity of the protein formulations and to correlate the protein activity after the freeze-thawing process with the different temperature histories. The model protein chosen was lactate dehydrogenase and the additives of different character, polyethylene glycol, a protectant during the freeze-thawing process and maltodextrine, protecting during the drying stage of the freeze-drying process (7).

EXPERIMENTAL

Materials

a) L-lactate dehydrogenase (LDH) from rabbit muscle, crystalline suspension in 65% saturated ammonium sulfate pH 7.2 (ICN Pharmaceuticals, USA).

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b) Polyethylene glycol (PEG) with the formula HO $(C_2H_4–O)_n$, PEG 6000 where n = 140, average M_w 5600–7000 (Janssen, Belgium).

c) Maltodextrin (MD) with dextrose equivalent 16.5–19.5 (Aldrich, USA).

d) Nicotinamide adenine dinucleotide, reduced form (NADH) in preweighed vial 0.2 mg, Potassium phosphate buffer 0.1 mol/l pH 7.5 and Sodium pyruvate solution 22.7 mmol/l pH 7.5 (Sigma, USA).

Method

Preparation of Solutions

Different solutions were made by deionized and filtered water (Millex-GS 0.22 μm sterile filter) to concentrations of 25 μg/ml LDH, 1% w/v PEG 6000 and 6% w/v MD 16.5–19.5.

Oscillating Differential Scanning Calorimetry

Various solutions of LDH, PEG 6000 and MD 16.5–19.5 were examined using a DSC 220C oscillating differential scanning calorimeter (Seiko, Japan). The samples (22.90–34.92 mg) were kept in aluminum pans in an atmosphere of nitrogen. The calorimeter was temperature- and heat-calibrated with indium, tin, gallium and mercury as standards. Two different temperature histories were used, cycles 1 and 2, both with an amplitude of 2°C, a frequency of 0.02 Hz and a heating and cooling rate of 2.5°C/min. The degree of oscillation, defined by amplitude(K) · frequency(s^{-1}) / heating rate(Ks^{-1}), was 0.96 (6).

The PEG solutions were also examined with an amplitude of 4°C, a frequency of 0.02 Hz and a heating and cooling rate of 5 or 10°C/min, which gives a degree of oscillation of 0.96 or 0.48. The frequency 0.02 Hz was selected, since that frequency in combination with the other parameters gave the best reproducibility of the measurements.

The temperature history of cycle 1 included freezing of the sample to −40°C and keeping it at this temperature for 10 minutes before heating to room temperature. In cycle 2 the sample was first frozen to −60°C and then kept at −40°C for 10 minutes before heating to room temperature. Thermograms were recorded at both cooling and heating. The results are presented as mean values with the standard deviations based on three determinations.

LDH Activity Assay

LDH activity was measured spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy, USA). The preweighed vial of NADH was dissolved in 2 ml phosphate buffer. In a cuvette of 1-cm lightpath 400 μl NADH-solution, 10 μl LDH and 750 μl phosphate buffer were mixed and left at 25°C for 20 minutes. The enzymatic reaction was started by adding 20 μl sodium pyruvate and monitored by measuring the decrease in absorbance at 340 nm. The activity of frozen LDH samples was calculated as percentage of unfrozen sample and presented as mean values with the standard deviations based on three determinations.

RESULTS AND DISCUSSION

H_2O-LDH-MD Systems

Thermal Transformations

Pure water and aqueous solutions of MD with and without LDH were investigated. The heating thermograms of the systems in cycle 1 and cycle 2 and of the cooling in cycle 2 are presented in Figure 1.

The thermograms are representative of both pure water and the solutions where MD is added. The main difference between the cycles is the small exothermic peak at −45°C. This peak seems to be connected to the ramp time of the heating process in cycle 2. It does not exist in the thermogram of the heating process in cycle 1, although this cycle started with the same ramp time at −40°C as in cycle 2. It is generally accepted that two polymorphs of ice can form when aqueous solutions are frozen at atmospheric pressure (3). Hexagonal ice is the stable polymorph and cubic ice, a metastable phase above

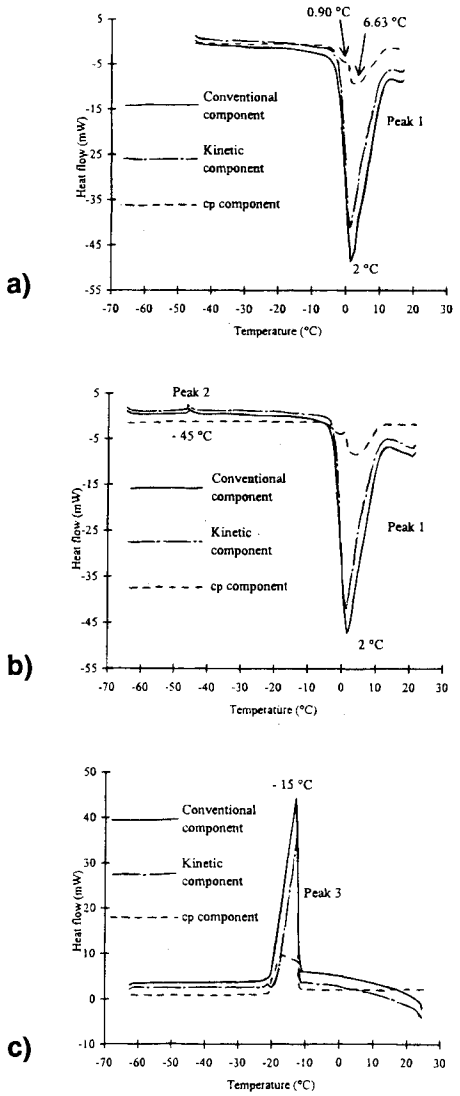

Fig. 1. Thermograms obtained by oscillating DSC of samples in the H_2O-LDH system in the interval −60°C − 30°C. HR = 2.5°C/min, A = 2°C and f = 0.02Hz. a) cycle 1, heating b) cycle 2, heating c) cycle 1 and 2, cooling.

−103°C. The transition from cubic ice to hexagonal ice occurs at −47°C (8). The obtained peak might reflect such a transition.

The melting endotherms are almost identical for cycle 1 and cycle 2. The separated reversible part, the c_p component, of the melting curve consists of a double melting peak, which shows the melting of two phases. The melting temperature of the c_p peaks were 0.90 and 6.63°C, respectively, showing different stability of the phases. The entalphies of the c_p peaks indicate different amount of reversing material in the two phases, in this study used as a measure of relative crystallinity. What the phases consist of can only be speculated about. Different sizes of crystals can be one explanation, since the temperature interval, where they were formed and were growing influences the size. At freezing of the systems, the crystallization process occurred at about −15°C. The broad exotherm of the c_p component might show overlapping peaks from different crystallized phases (Figure 1c).

The peak temperatures of the conventional component for both crystallization and melting at 2°C were rather constant for all samples. The standard deviations were large at crystallization, which reflects the sensitivity of the ice nucleation process for small differences in environmental conditions in very diluted solutions.

Heat of Transformations and Degree of Crystallinity

The heat of fusion and the heat of crystallization, ΔH values, of the two temperature cycles are fairly constant, for crystallization about −260 Jg^{-1} and for melting about 275 Jg^{-1}. A larger amount of ice is thus melted than the one that crystallizes in the integrated temperature interval at −15°C. The values show that the crystallization process is not terminated at −15°C but continues when the temperature is lowered to −40°C and −60°C, respectively. It is a well known fact that growth of nuclei formed at first crystallisation occurs during further cooling. Diffusion might however be too low at certain temperatures to allow growth of previously formed nuclei. But it can for example be large enough down to −40°C which would give the same result in cycle 1 and 2. The increased amount of ice melting may also be an annealing effect of the heating process since crystals grow with a velocity proportional to the mobility of water molecules. Because the mobility increases with temperature, the amount of ice forming during warming may be greater than that formed during cooling (3).

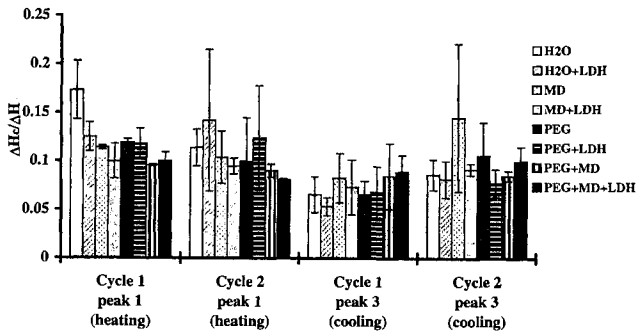

Fig. 2. Crystallinity, for different samples, expressed as ratio of the c_p component and conventional component of heat of transformation, $\Delta H_c/\Delta H$, for peak 1 and 3 with standard deviations.

A material with a high degree of crystallinity should melt and recrystallize in a narrow temperature interval. Such a reversibility should be observed by a greater reversible c_p component in the ODSC signals (6). The degree of crystallinity for the material can be expressed as the ratio $\Delta H_c/\Delta H$ where ΔH_c is the heat of fusion for the reversible process and ΔH is the heat of fusion for the total melting process. The range of values of the ratio varies greatly with the operational parameters used for the oscillation. When below the crystallinities of the samples are discussed, only differences between samples with the same selected variables are considered. In Figure 2, the crystallinity of the different samples are presented, as it is reflected both in the melting endotherm at 2°C and in the crystallization exotherm at −15°C. There are only minor differences between the two temperature histories for all samples. For pure water the results from the heating process indicate a slightly higher crystallinity than in the crystallization process. This fact might reflect the growth process of the primarily formed ice crystals during the heating process.

Activity of LDH

The activity of LDH after the freeze-thawing process is presented in Table I. It is measured as initial rate of degradation of the enzyme and as percentage of unfrozen sample. The values have large standard deviations, reflecting the problems that appear in activity determinations, when the very small solution volume from a freeze-thawing ODSC experiment is treated. Both evaporation of water during the experiment and adsorption of the protein to the aluminum pan might be crucial for the reproducibility of the experiment.

The activity of LDH in pure water and in solution with MD added was very similar about 20%. In other words 80% of the original activity has disappeared during the freeze-thawing process. In a procedure similar to cycle 2 in our investigation, but with much longer ramp times, Nema et al. determined the recovery of LDH in pure water to be 36% (9). Corveleyn et al reported an activity recovery of LDH of up to 90% after lyophilisation in the presence of maltodextrins with different dextrose equivalents (7). From our results it is evident that MD with 16.5–19 D.E. does not offer protection during freezing and thawing to 25°C. In the investigation by Corveleyn et al.,

Table I. Recovery of LDH Activity (25 μg/ml) After Freeze-Thawing with Different Temperature Histories, Heating Rate (HR),[a] and Additives, PEG (1% w/v) and MD (6% w/v)

Sample	Cycle	Activity[b] (% ± SD)
H_2O + LDH	1	19.3 ± 11.5
	2	21.8 ± 6.4
MD + LDH	1	4.6 ± 0.4
	2	15.7 ± 1.2
PEG + LDH	1	79.3 ± 13.3
	2	72.3 ± 23.3
	2	49.4 ± 16.4 HR = 5°Cmin^{-1}
	2	15.1 ± 11.2 HR = 10°Cmin^{-1}
PEG + MD + LDH	1	38.4 ± 4.7
	2	37.1 ± 0.6

[a] The heating rate is 2.5°Cmin^{-1} if otherwise is not noted.
[b] Unfrozen sample as reference.

the samples were freeze-dried with the primary drying temperature at −10°C. The difference between their and our results could be an effect of the fact that in the interval −10°C to 25°C we did not investigate the same system. Thus, temperatures above −10°C seem to be crucial for the destabilization of LDH in aqueous solution. If the water is removed, however, the protective ability is changed. MD seems to act as a water replacer after primary drying, but can not protect the protein when water is present.

The H_2O-LDH-PEG System

Thermal Transformations

A representative heating diagram of the systems where PEG is added is presented in Figure 3a. The thermograms show transformations similar to those of the H_2O-LDH-MD system at cooling and heating in cycles 1 and 2, with one exception.

In systems where PEG is present, a small endothermic peak appears at about −15°C in the heating process. The phase transition occurs at about the same temperature as the crystallization during the cooling process. If it is assumed that a special "PEG-ice structure" is formed together with ice at −15°C during the cooling process, when PEG is present, and if this structure is stable at low temperatures, a transformation of this structure might occur during the heating process. The peak at −15°C (Peak 4), appearing only in solutions where PEG is added, is greatly affected by the heating rate. It is transformed to the shape of a glass transition when the heating rate is increased.

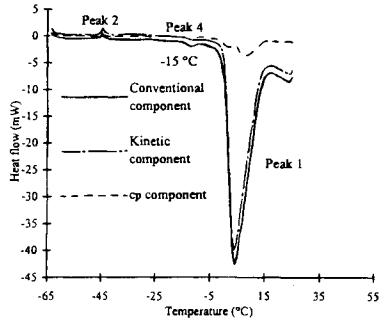

Fig. 3. Thermograms obtained by oscillating DSC of samples with 1% w/v PEG 6000, cycle 2, heating in the interval −60°C − 30°C. a) HR = 2.5°C/min, A = 2°C and f = 0.02Hz b) HR = 10°C/min, A = 4°C and f = 0.02Hz.

The glass transition temperature, T'_g, is in the interval −16°C to −15°C (Figure 3b). When below a *peak* is considered, the heating rate has been low enough to obtain an endothermic peak. If it represents a melting or expresses some other thermal event cannot be concluded from this experiment.

Heat of Transformations and Degree of Crystallinity

The heat of fusion and heat of crystallization values at 2°C and −15°C, respectively, for the freeze-thawing process in cycles 1 and 2 are fairly constant for the different samples, as was the case in the H_2O-LDH-MD system. Also in this system the absolute heat of fusion values of the heating process were higher than those for the crystallization process, 275 Jg^{-1} and −260 Jg^{-1}, respectively.

The ΔH values of the small exothermic peak (Peak 2) appearing at −45°C in cycle 2, and where the transformation of ice structure from cubic to hexagonal occurs, are presented in Table II. Both the heats of transformation and the transformation temperatures are constant. ΔH of the endothermic peak at −15°C (Peak 4) is significantly reduced when the protein is added to the PEG solution. When LDH is added the PEG-ice structure is less stable, which is reflected both in a smaller amount of the phase being transformed and in a lower transition temperature. The enzyme might interact with part of the PEG molecules and thereby the PEG-ice structure is partly destroyed. The ΔH value is also influenced by the lower freezing temperature in cycle 2. The crystallinity of the samples presented as the ratio $\Delta H_c/\Delta H$ shows only minor differences for all samples. The observed values of the crystallinity vary with heating rate, from 0.1 to 0.2, when the heating rate is increased from 2.5 to 10°Cmin^{-1} (Table III).

Activity of LDH

In Table I the recovered activities of LDH are shown. The recovery of LDH activity after the freeze-thawing process in PEG solution is 70–80% compared with unfrozen PEG solution. In a similar study, the recovery of LDH was found to be 85% with 0.2 M PEG 400 added to the solution (9). In a study by Carpenter *et al.*, solutions of 1–10% w/v PEG 8000, in buffer pH 7.5, fully protected LDH during freezing and thawing with a different temperature history (2). The activity of LDH is changed with rate of cooling and heating in cycle 2. It is decreased to 49 and 15%, respectively, when the heating rate is increased from 2.5 to 5 and 10°C/min respectively. It seems as if a slow thermal treatment in the −15°C range, where the structure of ice and PEG might transform, is crucial to retain the native structure of the protein.

The activity loss of the protein might well be an effect of both cooling and heating. It would be probable, that the cooling rate effects the properties of the protein, since the crystallisation and the growth of ice crystals are greatly influenced by the undercooling.

H_2O-LDH-PEG-MD System

Thermal Transformations

When MD is added to a protein solution containing PEG the only difference in the thermograms concerns peak 4 (Figure

Table II. Heat of Transformation Values, ΔH, with Standard Deviations and Transformation Temperature, T_{peak}, with Standard Deviations for Peak 2 and 4 in the Heating Process

Sample	Cycle	ΔH peak 2[a] (J/g ± SD)	T_{peak} peak 2[a] (°C ± SD)	ΔH peak 4[a] (J/g ± SD)	T_{peak} peak 4[a] (°C ± SD)
H_2O	2	−31.5 ± 2.3	−45.39 ± 0.22	—	—
H_2O + LDH	2	−30.5 ± 1.9	−45.24 ± 0.13	—	—
MD	2	−30.6 ± 1.6	−45.23 ± 0	—	—
MD + LDH	2	−31.9 ± 1.0	−45.21 ± 0.03	—	—
PEG	1	—	—	2.1 ± 0.1	−14.02 ± 0.03
	2	−29.1 ± 2.6	−45.37 ± 0.12	2.0 ± 0.1	−13.47 ± 0.03
PEG + LDH	1	—	—	1.4 ± 0	−15.00 ± 0.13
	2	−30.2 ± 1.0	−45.37 ± 0	1.6 ± 0.1	−15.12 ± 0.02
PEG + MD	1	—	—	0.3 ± 0	−14.96 ± 0.05
	2	−32.9 ± 1.9	−45.39 ± 0.08	1.2 ± 0.1	−15.07 ± 0.10
PEG + MD + LDH	1	—	—	0.1 ± 0	−14.28 ± 1.11
	2	−31.5 ± 1.2	−45.37 ± 0.07	1.2 ± 0.2	−15.02 ± 0.05

[a] The denotation peak 2 and 4 derives from Figure 1 and 3.

Table III. Effect of Heating and Cooling Rate on Values of Heat of Transformation and Crystallinity, $\Delta H_c/\Delta H$, of LDH Solutions with 1% w/v PEG 6000 (Temperature Cycle 2)

Heating/cooling rate (°C/min)	Degree of oscillation	ΔH Peak 1[a] (J/g ± SD)	ΔH Peak 3[a] (J/g ± SD)	$\Delta H_c/\Delta H$ Peak 1[a]	$\Delta H_c/\Delta H$ Peak 3[a]
2.5	0.96	288 ± 9	−269 ± 9	0.12 ± 0.05	0.078 ± 0.014
5	0.96	300 ± 15	−239 ± 15	0.086 ± 0.015	0.11 ± 0.08
10	0.48	262 ± 6	−200 ± 14	0.20 ± 0.02	0.21 ± 0.01

[a] The denotation peak 1 and 3 derives from Figure 1 and 3.

3a). The peak appears at lower temperature and is smaller than in the solutions with only PEG added (Table II).

Heat of Transformations and Degree of Crystallinity

The heat of fusion and the heat of crystallization values of peaks 1 and 3 respectively, and the heat of transformation of ice (peak 2) are not changed compared with the H_2O-LDH-PEG system. The ΔH of peak 4 is, however, largely decreased, especially in cycle 1 (Table II). The freezing temperature seems to influence the stability of the PEG-ice structure transforming at about −15°C. When MD is present, freezing to −40°C hardly preserves the structure, while freezing to −60°C does so to a greater extent.

Activity of LDH

When MD is added to the protein-PEG solution, the activity of the protein after freeze-thawing is largely lowered, but not to the same level as when MD alone is used as protectant (Table I). MD affects the protective ability of PEG, probably by damaging the PEG-ice structure. This structure transformation at −15°C seems to be crucial for the recovery of protein activity.

CONCLUSIONS

The endothermic transformation of a PEG-ice structure at −15°C in the heating process is strongly correlated to the protective ability of PEG 6000 in the freeze-thawing process of LDH. An enhanced cooling and heating rate reduces the protection. The thermogram is transformed from an endothermic peak to a glass transition-shaped curve. The freezing temperature influences the amount and stability of the PEG-ice structure transforming at −15°C, and so do both the protein itself and the maltodextrin.

The crystallinity of the system melting at about 2°C and represented by $\Delta H_c/\Delta H$ remains constant independent of temperature history for a certain degree of oscillation. The c_p component of the melting process, however, shows a complex transformation, where two phases of different crystallinity and stability might be involved.

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