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A comparison between the protection of LDH during freeze-thawing by PEG 6000 and Brij 35 at low concentrations

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Abstract

The protection of lactate dehydrogenase (LDH) by low concentrations of the non-surface-active polyethylene glycol (PEG 6000) or the non-ionic surfactant PEG dodecyl ether (Brij 35) was investigated during freeze-thawing. The freeze-thawing process was performed with a controlled temperature history, and the protective mechanisms were elaborated. The systems were examined by differential scanning calorimetry (DSC), fluorescence spectroscopy and surface tension measurements. LDH activity assays were performed spectrophotometrically. Very low concentrations of PEG 6000 (8×10^{-5} mM) or Brij 35 (4×10^{-3} mM) protected LDH during freeze-thawing with a low cooling rate. With an increased freezing rate, higher concentrations of the additives were needed for full protection. No interaction was detected between LDH and Brij molecules. The strong interaction between LDH and PEG molecules disappeared with a small change in the protein structure, using a hybrid of LDH. The protein was nevertheless completely protected. The amount of Brij required for complete protection at high cooling rates correlated with the created ice surface area. The protection by PEG indicated a certain correlation with the ice crystal size and with the formation of a PEG hydrate. Brij or PEG hydrate molecules might compete with the protein for adsorption at the ice surface and thereby protect the protein during freeze-thawing. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: LDH; PEG 6000; Brij 35; Freeze-thawing; DSC; Fluorescence spectroscopy

1. Introduction

An increasing number of potential drugs are proteins or peptides, and they have limited stability in aqueous solutions. The most commonly used method for preparing protein pharmaceuticals is freeze-drying. Freezing is one important step in this process, but it induces several stresses capable of protein denaturation. Therefore, labile proteins require protection against both freezing and dehydration stresses. Protective additives are often added to recover sufficient activity. There are different types of stabilizing additives like sugars, amino acids, certain salts, polymers and non-ionic surfactants (Wang, 1999). Pharmaceutical formulations are thus quite complex, and it is important to understand the physical and chemical state of both protein and additives to predict the

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freeze-drying behaviour and the stability of the protein.

There are several possible mechanisms suggested in the literature for the protection of proteins. One theory is that the additive is preferentially excluded from the surface of the protein, and thereby the chemical potential of both protein and additive is increased. This thermodynamically unfavourable 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 favoured (Timasheff, 1982). In systems with high concentrations of polyethylene glycol (PEG), the polymer is preferentially excluded from the protein surface at room temperature (Arakawa and Timasheff, 1985). High concentrations of PEG stabilize proteins during freezing but not during freeze-drying (Carpenter et al., 1993), by the excluded volume mechanism. A second theory is that denaturation occurs at the ice–water interface and that the protective effect depends on the fact that the additive can compete with the protein for these interfaces (Chang et al., 1996; Hillgren et al., 2002b). Low concentrations of non-ionic surfactants provide a high degree of protection during freezing (Nema and Avis, 1993). The non-ionic surfactant Tween 80 protects lactate dehydrogenase (LDH) by this mechanism. The temperature history is crucial for the protection, since it affects the amount of ice–water interface that is formed during freezing (Hillgren et al., 2002b).

In this study, LDH from two different species was used as model protein, since LDH is inactivated during freeze-thawing in pure water (Carpenter et al., 1993). LDH from rabbit muscle is a commonly used enzyme in freeze-thawing and freeze-drying studies. LDH from *Bacillus stearothermophilus* (LDH-BS) was used to examine if the recovered activity during freeze-thawing is affected when the enzyme has a slightly modified structure. *B. stearothermophilus* is a thermophilic, heat-loving bacterium, and all proteins from this species have a high stability against denaturation at high temperatures (Kotik and Zuber, 1992). The non-surface-active PEG 6000 and non-ionic surfactant PEG dodecyl ether (Brij 35) were used as protecting additives. Brij is a non-ionic surfactant with some structural similarity with PEG.

The aim of the study was to investigate and compare the protection of the protein LDH by low concentrations of PEG 6000 and of Brij 35 during freeze-thawing. The freeze-thawing process was performed with a controlled temperature history. The protection mechanism during freeze-thawing was tentatively elaborated and discussed in the perspective of the different properties of the two additives.

2. Materials and methods

2.1. Materials

LDH was from rabbit muscle, crystalline suspension in 65% saturated $(NH_4)_2SO_4$ solution, pH 7.2 (ICN Biomedicals Inc.). LDH was from *B. stearothermophilus* (LDH-BS), a lyophilised powder of recombinant enzyme expressed in *Escherichia coli* (Sigma, St. Louis, MO). PEG with the formula $HO(C_2H_4{-}O)_nH$, PEG 6000, where $n = 140$, average M_w 5000–7000 was from Fluka Chemie AG (Switzerland). PEG dodecyl ether (Brij 35) was from Fluka Chemie AG. Nicotinamide adenine dinucleotide reduced form (NADH) in preweighed vial 0.2 mg and sodium pyruvate solution 22.7 mM pH 7.5 were from Sigma.

Prior to the experiments the LDH suspension from rabbit muscle was dialyzed against 10 mM sodium citrate buffer, pH 6.4–6.5. Citrate buffer was selected because it has minimal pH change during freezing (Carpenter et al., 1997). The dialyzed LDH was concentrated during centrifugation using Microsep Centrifugal Concentrators (Pall Filtron Co.), and the concentration of the enzyme was determined spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Co.) at 280 nm. The UV absorbance at 280 nm was linearly related to concentration in the range of 0.1–1.1 mg ml^{-1}. LDH-BS was used as supplied.

2.2. Assay of enzyme activity

LDH activity was measured spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Co.). 1.44 ml of reaction mixture contained 55 μM NADH in 0.1 M potassium phosphate buffer, pH 7.5, and an aluminium pan with 20 μl LDH sample (25 μg ml^{-1}). The enzymatic reaction was started by adding sodium pyruvate (1.9 mM) and monitored by measuring the decrease in absorbance at 340 nm. The recovered activity of a frozen LDH sample was calculated as the percentage of the activity of an identical unfrozen sample that had been stored in an aluminium pan. The activities are presented as mean values with the standard deviations based on three determinations. The recovered activity has sometimes large standard deviations, which reflect the difficulty to analyse the small volume of sample from the differential scanning calorimetry (DSC) experiments. Adsorption of protein to the aluminium pan and evaporation of water during freeze-thawing are crucial for the reproducibility of the experiment.

2.3. DSC

The solutions were examined using a DSC 220C differential scanning calorimeter (Seiko Instruments Inc., Japan) equipped with the EXSTAR6000 SOFTWARE VERSION 3.4A. The 20 μl samples, corresponding to about 20 mg, were kept in covered aluminium pans, not hermetically sealed, in an atmosphere of nitrogen. The calorimeter was temperature- and heat-calibrated with indium, tin, gallium and mercury as standards. The temperature history included freezing from 30 to −60 °C and then heating to 30 °C. The cooling rate was 5–20 °C min^{-1} and the heating rate was 2–5 °C min^{-1}. The concentrations of the solutions were 25 μg ml^{-1} of LDH, 0.025 μg ml^{-1}–500 mg ml^{-1} of PEG 6000 and 0.5–505 mg ml^{-1} of Brij 35 in 10 mM sodium citrate buffer pH 6.4. Thermograms were recorded at both cooling and heating. The results are presented as mean values with the standard deviations based on three determinations. A very high cooling rate (10^2–10^3 °C min^{-1}) was achieved by freezing the samples instantly in liquid nitrogen. These samples were not thawed with a controlled heating rate.

2.4. Surface tension measurements

The surface tension measurements were performed by a du Noüy tensiometer (A. Krüss Optisch-Mechanische Werkstätten, Germany) against air at 25–1 °C. All measurements were performed at about 10–15 min after formation of a new surface (Persson, 1999). Surface tension, γ, of pure water was also measured at different temperatures and used to correct the measurements of the samples. The concentrations of PEG 6000 solutions were 0.01 μg ml^{-1}–100 mg ml^{-1} (1.7 nM–17 mM) and of Brij 35 solutions 5.3 μg ml^{-1}–0.53 mg ml^{-1} (4.4 μM–0.44 mM) in 10 mM sodium citrate buffer pH 6.4. The critical micelle concentration (cmc) of Brij 35 at different temperatures was obtained from plots of surface tension versus the logarithm of the concentration. The surface area occupied per Brij molecule was calculated from the surface excess concentration, Γ, through Gibbs' equation (Jönsson et al., 1998).

2.5. Fluorescence measurements

Steady-state fluorescence measurements were performed on a Fluorolog-2 spectrofluorometer (SPEX Industries Inc.) equipped with the DM3000F SOFTWARE. The tryptophan (Trp) fluorescence was determined by excitation of LDH at 285 nm, and emission spectra were recorded at 21 °C and at 4 °C in the range 300–700 nm. The fluorescent probe pyrene was added to the solutions and excitated at 334 nm, and emission spectra were recorded at 366–390 nm at 21 °C and at 4 °C. The concentrations were 0.25 mg ml^{-1} LDH in the Trp fluorescence measurements and 1 mg ml^{-1} LDH in the experiments with pyrene. The concentrations of PEG 6000 solutions were 0.01 μg ml^{-1}–100 mg ml^{-1} and of Brij 35 solutions 5 μg ml^{-1}–0.5 mg ml^{-1} in all fluorescence measurements.

3. Results and discussion

3.1. Protective ability of PEG 6000 and Brij 35

3.1.1. Recovered activity

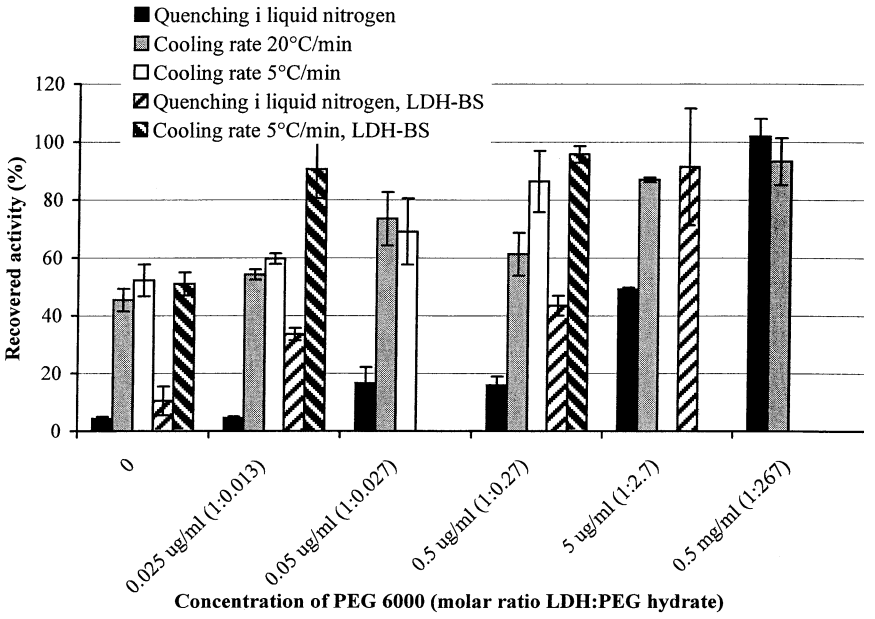
The recovered activity of LDH in pure buffer solution after freeze-thawing was $\approx 4\%$ when the sample was frozen instantly in liquid nitrogen. The activity increased to about 50% when the cooling rate decreased to 5–20 °C min^{-1} (Fig. 1). The addition of 0.5 μg ml^{-1} PEG 6000 (corresponding to 8×10^{-5} mM and molar ratio 1:0.27) offered a complete protection with the lowest cooling rate, 5 °C min^{-1}. With an increased cooling rate, quenching in liquid nitrogen, 0.5 mg ml^{-1} PEG (molar ratio 1:267), was needed for complete protection (Fig. 1a). The corresponding experiments with Brij 35 are shown in Fig. 1b. Complete protection of LDH with cooling rates of 5–20 °C min^{-1} was achieved with the addition of 5 μg ml^{-1} Brij (corresponding to 4×10^{-3} mM and molar ratio 1:24). When the sample was frozen in liquid nitrogen, 50 μg ml^{-1} Brij (corresponding to 4×10^{-2} mM and molar ratio 1:244) was needed. A solution of LDH-BS without protective additive had the same recovered activity as LDH from rabbit muscle (Fig. 1a). LDH-BS was completely protected by 0.025 μg ml^{-1} (4×10^{-6} mM) PEG 6000, when a cooling rate of 5 °C min^{-1} was used. A higher cooling rate needed a higher concentration of PEG for full protection similar to the behaviour of LDH from rabbit muscle. In this case, only 5 μg ml^{-1} PEG was needed to protect LDH-BS during freezing in liquid nitrogen. Thus, very low concentrations of PEG 6000 or Brij 35 were needed for complete protection of LDH during freeze-thawing. LDH-BS, with a slightly different structure than LDH from rabbit muscle, required even less PEG for full protection than LDH. With a high freezing rate, such as quenching in liquid nitrogen, a higher concentration of the additive was required for full protection. The behaviour is similar to the results obtained with Tween 80 as additive (Hillgren et al., 2002b), although even lower concentrations of Brij than of Tween protected the protein completely.

3.1.2. Protein denaturation

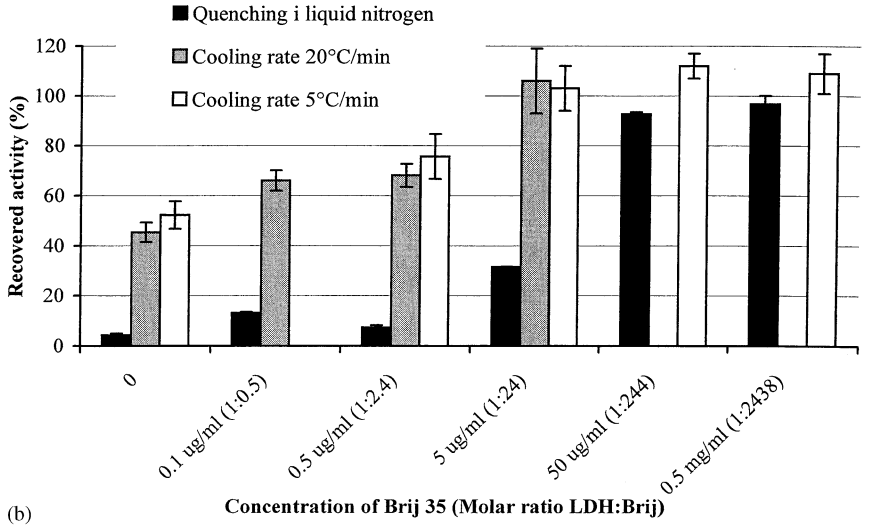
To investigate the occasional denaturation of the protein after freeze-thawing, fluorescence spectroscopy measurements were used. A valuable feature of Trp, which is the dominant intrinsic fluorophore in proteins, is its sensitivity to the local environment. Residues at the surface of a protein often show emission maxima at longer wavelength than residues in the core of the protein (Lakowicz, 1999). The wavelength of the emission maximum for Trp, at about 350 nm, can therefore indicate if the protein is denatured. The intensity of the emission maximum might also change as the protein denatures, due to increased molecular mobility and collisional quenching. The wavelength of the emission maxima of LDH and LDH-BS and their maximum intensities, when mixed with PEG 6000 or Brij 35, are shown in Fig. 2. The maximum intensity was constant over the investigated concentration range with both PEG 6000 (Fig. 2a) and Brij 35 (Fig. 2b). The wavelength of the emission maximum was in the range 344–350 nm with no significant change, as the concentrations of PEG or Brij were increased. If LDH is denatured, e.g. as in a solution with LDH and SDS, the emission wavelength passes through a minimum (Hillgren et al., 2002a). The results obtained here show that LDH is in its native state with any addition of PEG or Brij in the investigated concentration range. Furthermore, there was no change observed in the emission maximum wavelength or the intensity as the temperature decreased from 21 to 4 °C (Fig. 2), indicating a native protein also at lower temperatures.

3.2. Characterization of PEG 6000 or Brij 35 in aqueous solutions

In the solid state, PEG crystallizes in a helical configuration (Maxfield and Shepherd, 1975). This helical structure is lost in an aqueous solution, and the configuration becomes a random coil (Yamauchi and Hasegawa, 1993). Although the helix is lost, the PEG chain is able to form a hydrogen-bonded complex, a hydrate of fixed composition (Antonsen and Hoffman, 1992). This complex includes two or three water molecules per repeat ethylene oxide unit (de Vringer et al., 1986). Brij 35



(a)



(b)

Fig. 1. The recovered activity of 25 μg ml^{-1} LDH or LDH-BS with different concentrations of PEG 6000 (a) or Brij 35 (b) after freezing to −60 °C and thawing to room temperature. The bars represent the standard deviation based on three determinations.

is a non-ionic surfactant, with the hydrophilic part of the molecule containing a chain with the same structure as PEG but with fewer monomers (Fig. 3).

3.2.1. Thermal transformations

Heating thermograms of solutions of PEG 6000 or Brij 35 at high concentrations are shown in Fig. 4. The endothermal peak between −3 and 5 °C

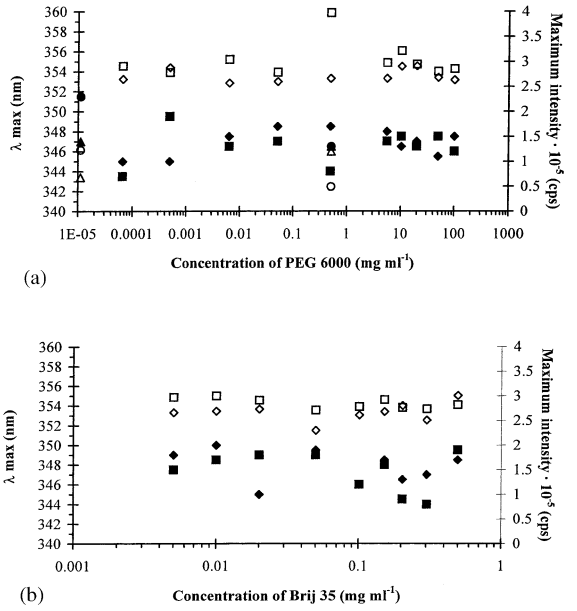


Fig. 2. The emission fluorescence of Trp in 0.25 mg ml^{-1} LDH in PEG 6000 (a) or Brij 35 (b) solutions with 10 mM sodium citrate buffer, pH 6.5. Filled symbols correspond to wavelength of the maximum and open symbols to the maximum intensity at 21 °C (◆, ◇) and at 4 °C (■, □). Samples with 0.25 mg ml^{-1} LDH-BS and PEG 6000 are also shown in the figure measured at 21 °C (▲, △) and at 4 °C (●, ○).

represents the melting of ice in all solutions. In solutions with PEG, two specific transformations appeared in the heating process. One is the endothermic peak between −10 and −15 °C, reflecting melting of a PEG–ice structure (Aldén and Magnusson, 1997), PEG hydrate mentioned above. The other specific transformation is the small exothermic peak at about −48 °C, which is associated with changes in the ice structure (Antonsen and Hoffman, 1992). The transition at −10 to −15 °C occurred in solutions with Brij 35 indicating the melting of a hydrate also here, probably a hydrate formed in the polyethylene

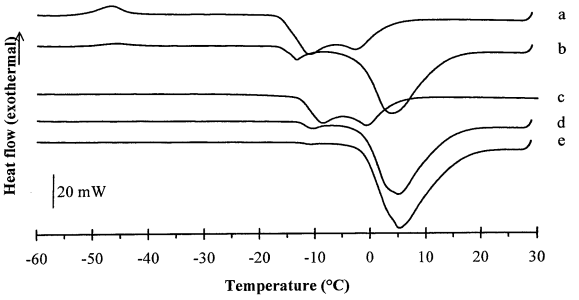


Fig. 4. Heating thermogram obtained by DSC of samples with different concentrations of PEG 6000 or Brij 35 in 10 mM sodium citrate buffer, pH 6.5. (a) PEG 6000 500 mg ml^{-1}. (b) PEG 6000 80 mg ml^{-1}. (c) Brij 35 505 mg ml^{-1}. (d) Brij 35 50.1 mg ml^{-1}. (e) Brij 35 10 mg ml^{-1}. Cooling rate = heating rate = 5 °C min^{-1}.

oxide part of the Brij molecule. The transformation at −48 °C did not appear with Brij.

The amount of water molecules per PEG monomer in PEG hydrate was here calculated to be 2.25±0.83 (unpublished data). At the lowest concentration of PEG where the transition was detectable with DSC, the amount of water per PEG monomer was 11.6. The calculation is very sensitive to small errors in the measured enthalpy changes and to the concentration of polymer. In the Brij 35 samples, it was not meaningful to calculate the amount of water molecules per ethylene oxide unit, since the concentration of Brij, where this transformation is detectable by DSC, is far above cmc. Micelles are created, and the hydrophilic PEG part of the molecules might share water molecules or interact with each other.

The heat of transformation and the peak temperatures of the transformations monitored by DSC at the concentrations of PEG or Brij that were used in the freeze-thawing experiments are shown in Tables 1 and 2. The heat of transformation and the peak temperature during

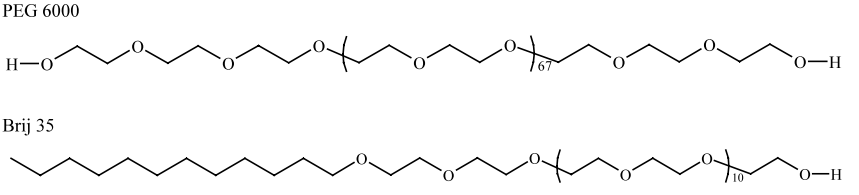


Fig. 3. The chemical structures of PEG 6000 and Brij 35.

Table 1
Peak temperature and heat of transformation for different samples of LDH (25 μg ml^{-1}) and PEG 6000

PEG 6000 (mg ml^{-1})	Heating rate (°C min^{-1})	Cooling rate 5 °C min^{-1}				Cooling rate 20 °C min^{-1}			
		T_{peak} (°C)	SD	ΔH (J g^{-1})	SD	T_{peak} (°C)	SD	ΔH (J g^{-1})	SD
Crystallization									
0.00005	5	−9.5	0.8	−267	7	−12.4	1.0	−197	6
0.005	5					−13.2	1.5	−195	13
0.05	2	−9.7	1.0	−266	11	−13.2	1.7	−200	16
0.5	2					−15.0	0.2	−180	4
5	2	−10.7	0.2	−265	3	−14.9	1.7	−192	9
Melting of ice									
0.00005	5	4.8	0.4	307	2	4.9	0.2	306	4
0.005	5					5.2	0.1	306	2
0.05	2	1.9	0.2	297	17	2.0	0.1	297	4
0.5	2					2.4	0.6	302	4
5	2	2.5	0.1	295	5	2.4	0.3	282	16
Melting of PEG hydrate									
0.00005	5	–		–		–		–	
0.005	5					–		–	
0.05	2	−15.2	0.2	0.029	0.011	−15.0	0.4	0.049	0.048
0.5	2					−14.7	0.2	0.080	0.003
5	2	−14.5	0.2	1.22	0.02	−14.5	0.1	1.13	0.14

crystallization and melting were not significantly changed with varying concentrations of additive. However, a larger heat of fusion value than heat of crystallization was obtained for all concentrations. A larger amount of ice is thus melted than the one that crystallizes in the temperature range of about −15 °C, assuming constant crystallinity. It is a well-known fact that growth of ice nuclei formed at first crystallization occurs during further cooling and heating if the diffusion is high enough. The temperature history did not affect the ΔH values of the melting. On the other hand, the heat of crystallization decreased as the cooling rate increased, from about −260 J g^{-1} with a cooling rate of 5 °C min^{-1} to −190 J g^{-1} at 20 °C min^{-1} (Tables 1 and 2). This indicates that less

Table 2
Peak temperature and heat of transformation for different samples of LDH (25 μg ml^{-1}) and Brij 35

Brij 35 (mg ml^{-1})	Cooling rate 5 °C min^{-1}				Cooling rate 20 °C min^{-1}			
	T_{peak} (°C)	SD	ΔH (J g^{-1})	SD	T_{peak} (°C)	SD	ΔH (J g^{-1})	SD
Crystallization								
0.0005	−10.3	0.7	−269	1	−14.6	0.7	−175	3
0.005	−10.8	0.0	−263	5	−13.6	2.7	−190	17
0.05	−10.4	0.6	−250	7	–	–	–	–
0.5	−10.0	0.6	−261	11	–	–	–	–
Melting of ice								
0.0005	5.3	0.2	304	2	5.2	0.1	304	2
0.005	5.3	0.4	302	2	5.3	0.5	299	5
0.05	5.1	0.1	279	35	–	–	–	–
0.5	5.0	0.2	286	25	–	–	–	–

Heating rate = 5 °C min^{-1}.

amount of ice is formed or that smaller and more imperfect ice crystals are created, giving a higher energy of the system at faster cooling (Hillgren et al., 2002b). The peak temperatures were changed with increased cooling or heating rate. For the crystallization process the peak appeared at a lower temperature and for the melting at higher temperature. A high cooling or heating rate creates a larger temperature gradient in the sample, and the peak temperature will increase in the heating process and decrease in the cooling part, since it corresponds to the point where the inner core of the sample is transforming and what is monitored by the instrument (Aldén et al., 1995).

3.2.2. Surface activity

Some of the additives used for protection of proteins are also surface-active, but there does not exist a correlation between surface tension and enzyme recovery (Nema and Avis, 1993). However, cmc of the solution might be important, since it changes the physico-chemical properties of the solution.

PEG is a polymer with poor surface activity. At low concentrations of PEG 6000, the surface tension between air and liquid was slightly reduced compared to pure water, from 72 to 65 mN m^{-1}. The surface tension was almost independent of polymer concentration (Fig. 5). At very high concentrations of PEG, 100 mg ml^{-1}, the surface tension decreased which might be due to adsorbed layers of PEG molecules at the liquid–air surface (Kim, 1997). The reduction in surface tension could also indicate an interaction between PEG chains creating a gel-like network, which is affecting the surface tension measurements. The absolute value of the surface tension increased as the temperature decreased to 1 °C, and was also at this temperature reduced by higher concentrations of PEG.

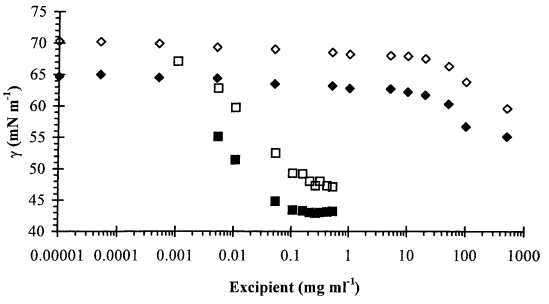


Fig. 5. The surface tension versus concentration at 25 °C (filled symbols) or at 1 °C (open symbols). Solutions with PEG 6000 (◆, ◇) or with Brij 35 (■, □) in 10 mM sodium citrate buffer, pH 6.5.

At room temperature cmc of Brij 35 in citrate buffer was determined to be 93 μg ml^{-1} (Fig. 5); in pure water cmc is about 108 μg ml^{-1} (Sivars and Tjerneld, 2000). When the temperature decreased to 1 °C, cmc of Brij 35 increased to 197 μg ml^{-1}, similar to the behaviour of Tween 80 (Hillgren et al., 2002b) and other non-ionic surfactants (Jönsson et al., 1998). The surface area occupied by one Brij molecule was calculated from the surface tension measurements and the surface excess concentration, Γ. The effective area of one Brij molecule was slightly increased, from 104 to 108 $Å^2$, as the temperature decreased from 25 to 1 °C.

3.3. Interactions between the additives and LDH

The interactions between LDH and PEG or Brij were studied with fluorescence spectroscopy. It has been found (Kalyanasundaram and Thomas, 1977) that the first to third peak intensity ratio, I_1/I_3, of the fine vibrational emission spectrum of the fluorescent probe pyrene is sensitive to the polarity of the surrounding solution, a high ratio indicating a hydrophilic environment. I_1/I_3 has been used to detect the interactions in polymer–surfactant and protein–surfactant systems (Goddard and Ananthapadmanabhan, 1993). Pyrene dissolved in water or the citrate buffer used here gives $I_1/I_3 \approx 1.9$, and a solution with 1 mg ml^{-1} LDH in citrate buffer gives $I_1/I_3 \approx 1.7$.

The micropolarity index, I_1/I_3, of PEG 6000 in the absence or presence of LDH is presented in Fig. 6a. The ratio in PEG solution was unchanged at lower concentrations of polymer and unchanged compared to pure citrate buffer. An aqueous solution of PEG in low concentrations thus creates a hydrophilic environment for pyrene. At PEG concentrations of about 100 mg ml^{-1}, I_1/I_3 decreased, as did the surface tension (Fig. 5). Thus, the surroundings of pyrene became more hydrophobic, indicating that the PEG chains start

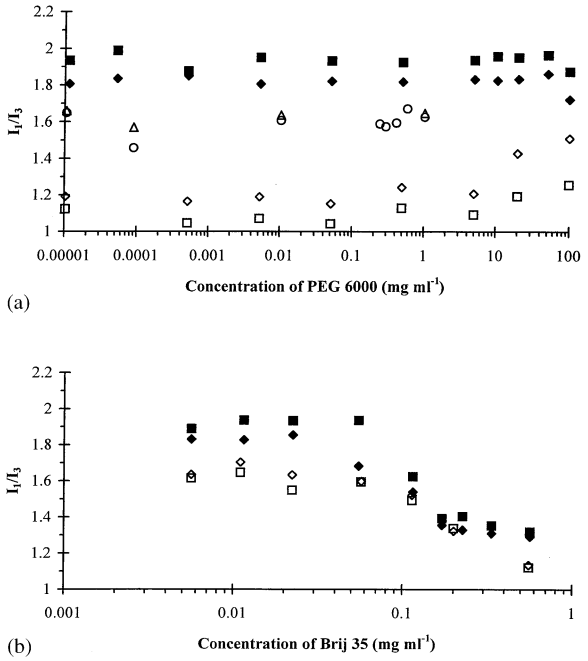


Fig. 6. The hydrophobic index, I_1/I_3, of pyrene in PEG 6000 (a) or in Brij 35 (b) solutions with 10 mM sodium citrate buffer, pH 6.5. Filled symbols correspond to a solution without protein and open symbols to a solution in presence of 1 mg ml^{-1} LDH at 21 °C (◆, ◇) and at 4 °C (■, □). Samples with 1 mg ml^{-1} LDH-BS and PEG 6000 are also shown in the figure measured at 21 °C (△) and at 4 °C (○).

to interact with each other. A similar sharp decrease in I_1/I_3 was detected for the non-ionic cellulose ether, EHEC, at a concentration related to the critical overlap concentration of the polymer, c^* (Evertsson et al., 1996). In a solution with 1 mg ml^{-1} LDH, I_1/I_3 dropped from 1.7 to 1.1–1.2, as PEG was added. This indicates that hydrophobic zones are formed in the solution from the very first addition of PEG, pointing to a strong interaction between PEG and LDH. The protein was still in the native state, as was found by the wavelength maximum of Trp (Fig. 2a). A similar interaction pattern was detected between SDS and LDH by the same method, but in that case the protein was completely denatured (Hillgren et al., 2002a). PEG 6000 thus interacts strongly with LDH without denaturating the protein during freeze-thawing.

When the protein structure was somewhat changed, as here by using LDH-BS, a hybride LDH that has a different amino acid sequence at the surface, the ratio I_1/I_3 of the solutions with PEG did not indicate any interaction. The ratio was not significantly changed as PEG was added to the solution (Fig. 6a), and the protein was in its native state.

I_1/I_3 of Brij 35 in citrate buffer at concentrations below cmc is about 1.9, the same value as in pure citrate buffer. At cmc, I_1/I_3 steeply decreases, due to the formation of micelles. cmc of Brij 35 in citrate buffer obtained by this method is about 0.1 mg ml^{-1}, which is in accordance with the results obtained by the surface tension measurements. When LDH is present at 1 mg ml^{-1} in citrate buffer, I_1/I_3 is about 1.7 and pyrene may thus be distributed to more hydrophobic regions on the enzyme. The ratio was almost constant as Brij was added to the solution in low concentrations, indicating no interaction between Brij and LDH. At cmc of Brij, the ratio followed the curve of the pure surfactant solution. As more surfactant was added, micelles were created and pyrene was distributed to these hydrophobic aggregates and I_1/I_3 was largely reduced (Fig. 6b).

3.4. Protective mechanism

As the cooling rate increases, more nuclei of ice are created, forming crystals with a larger ice surface area than crystals formed during slow freezing. The possibility that the ice crystals will damage the protein increases with the ice surface area. If a non-ionic surfactant reduces the interaction between the protein and the ice surface, the protein is protected from denaturation. In an earlier study, it was found that the amount of Tween 80 needed for complete protection of LDH correlated with the area of the ice crystals that were formed (Hillgren et al., 2002b).

The minimum radius of ice crystals that can be formed and be completely covered with Brij monomers in a certain sample can be calculated. Those radii are presented in Table 3 for different concentrations of surfactant. The assumptions for the calculations were that the density of ice is 0.92 g cm^{-3}, that all ice crystals were perfect spheres

Table 3
Recovered activity of LDH (25 μg ml^{-1}) after freeze-thawing with different cooling rates and concentrations of Brij 35

Brij 35 (mg ml^{-1})	Molar ratio (LDH:Brij)	Minimum radius of ice crystals completely covered with Brij molecules	Cooling rate 5 °C min^{-1}		Frozen in N_2(l)	
			Recovered activity (%)	SD	Recovered activity (%)	SD
0.0005	1:2	12 mm	75.7	9.0	7.3	0.9
0.005	1:24	1.2 mm	103	9	31.5	0.1
0.05	1:244	120 μm	112	5	92.6	0.9
0.5[a]	1:2438	12 μm	109	8	96.7	3.4

[a] Concentration of Brij 35 over cmc (0.197 mg ml^{-1} at 1 °C).

and that Brij existed only as monomers at all concentrations (which of course is not true above cmc, 197 μg ml^{-1}). The area occupied by one Brij molecule at the temperature for ice crystallization was set to 108 $Å^2$, the area determined at 1 °C. At the concentration where Brij gave full protection with both low and high cooling rate, 50 μg ml^{-1}, the calculated minimum radius of ice was 120 μm which is a normal ice crystal size. A diameter of 100–200 μm has been determined for ice crystals by confocal laser scanning microscope (Evans et al., 1996). Full protection was, however, also reached with the lower cooling rate (5 °C min^{-1}) when the amount of Brij monomers could only cover an ice surface corresponding to much larger ice crystals, with a radius of about 1.2 mm. This indicates that the competition between Brij 35 and LDH for adsorption at the ice–water interface is part of a possible mechanism of protection for this surfactant but not the only explanation.

PEG is a very large molecule compared to the water molecule, and a mechanism that has been suggested for its protection of a protein is that PEG is excluded from the protein surface by steric exclusion. The stabilization by excluded volume is dependent on the PEG concentration and it becomes apparent only at relatively high concentrations, above 0.3 M which corresponds to about 2 g ml^{-1} PEG 6000 (Arakawa et al., 2001). The results in this study showed that very low concentrations of PEG, 0.5 mg ml^{-1} (8×10^{-5} mM), give full protection of LDH. Furthermore, the interaction between LDH and PEG 6000 was very strong, which shows that stabilization by excluded volume cannot be the only protective mechanism at the low concentrations of PEG used here.

If the ice surface protection is part of the mechanism, there should be a correlation between the size of PEG molecules and the created ice surface area. The area that must be occupied by one PEG molecule to cover the surface of ice crystals with an assumed radius of 100 μm is calculated in Table 4. The same assumptions for the calculations have been made as in the case with Brij. A concentration of 0.5 μg ml^{-1} PEG indicated an almost complete protection of LDH (cooling rate 5 °C min^{-1}); the corresponding calculated area occupied by one PEG hydrate molecule was 1.2×10^5 $Å^2$. Assuming that the PEG coil is a perfect sphere, this area corresponds to a radius of 97 Å, which is somewhat larger than the radius of gyration for PEG 6000, estimated to be about 20 Å (Arakawa and Timasheff, 1985). As the cooling rate increases, the ice crystals that are formed are smaller than 100 μm and more PEG molecules are needed for complete protection. It might thus be possible that PEG by hindering the protein to reach the ice surface can protect it from denaturation at low concentrations.

PEG itself is not a surface-active molecule, and the tendency to bind to the ice surface is minimal. The formation of a PEG hydrate might, however, change the conditions for the ice surface interaction. The hydrated polymer chains can bind to the ice surface by hydrogen bonds between the ice and PEG hydrate (de Vringer et al., 1986) or by water molecules in the ice being part of PEG hydrate. The protein is thus hindered to reach the destructive ice surface.

It cannot be excluded that the interaction between LDH and PEG can stabilize the protein in the process. However, it was found that an even lower concentration of PEG was needed to protect LDH-BS than LDH from rabbit muscle, in spite of the fact that there was no interaction between PEG and LDH-BS. If all PEG molecules in this case are used to prevent the protein from interaction with the ice surface, a lower concentration should be expected for full protection.

It has been found that the formation of PEG hydrate is a slow process (de Vringer et al., 1986). A current study at this laboratory showed that the melting of PEG hydrate at −15 °C was not observed until the freezing temperature was lower than −30 °C, if a cooling rate of 1 °C min^{-1} was used (unpublished data). Therefore, with very high cooling rates, like cooling in liquid nitrogen, the hydrate probably does not form at all. If PEG hydrate is essential for the protection at the ice surface, it is reasonable that the protein protection decreases considerably at high cooling rates, especially as much larger ice surface is formed.

The PEG hydrate might also be active when Brij is used as an additive. Brij includes a PEG chain in the hydrophilic part of the molecule where PEG hydrate can form. The protection by Brij mole-

Table 4
Recovered activity of LDH (25 μg ml^{-1}) after freeze-thawing with different cooling rates and concentrations of PEG 6000

PEG 6000 (μg ml^{-1})	Molar ratio LDH:PEG hydrate	Area[a] ($Å^2$)	Radius[b] (Å)	Cooling rate 5 °C min^{-1}		Frozen in N_2(l)	
				Recovered activity (%)	SD	Recovered activity (%)	SD
0.025	1:0.013	2.4×10^6	435	59.7	1.8	4.5	0.6
0.05	1:0.027	1.2×10^6	308	69.1	11.4	16.5	5.6
0.5	1:0.27	1.2×10^5	97	86.4	10.6	15.9	3.0
5	1:2.7	1.2×10^4	31	–	–	47.0	3.3
500	1:267	120	3.1	–	–	102	6
1000	1:532	59	2.2	91.6	18.6	–	–
5000	1:2659	12	0.97	117	20	–	–

[a] Calculated area occupied by one PEG hydrate molecule, with two water molecules per repeat unit, covering ice crystals with a radius of 100 μm.
[b] Radius of a spherical PEG hydrate molecule corresponding to the calculated occupied area.

cules that are adsorbed to the ice surface might be reinforced by the presence of PEG hydrate. This can explain why extremely low concentrations of Brij 35 give full protection at low cooling rates.

4. Conclusions

Very low concentrations of PEG or Brij protected LDH from denaturation during freeze-thawing. The strong interaction between PEG 6000 and LDH can help stabilize the protein, but also if no interaction was at hand, like between PEG and LDH-BS, a complete protection was achieved. No interaction was detected between LDH and Brij 35 when the protection was complete.

The cooling rate was crucial for the protection by both PEG and Brij. Since different cooling rates form varying amount of ice surface, the denaturation at the ice–water interface seems to be important in the process. During freeze-thawing of PEG, a hydrate with approximately two water molecules per repeat unit appeared. PEG at the low concentrations used here might be able to interfere with the ice–water interface due to hydrogen bonds between PEG hydrate and the ice surface, or alternatively, the water molecules at the ice surface form part of PEG hydrate. PEG is thus able to compete with the protein for adsorption at the destructive ice–water interface. A certain correlation between the ice crystal size and the protection by PEG was found. The amount of the non-ionic surfactant Brij 35 needed for complete protection was correlated with the area of the ice crystals when the cooling rate was very high. At lower freezing rate, an additional effect was also involved. Brij molecules and PEG hydrate formed in the side chain of the molecule might compete with the protein for adsorption at the ice surface.

The protection of the protein during freeze-thawing thus seems to be complex. At very low concentrations of PEG or Brij the protection mechanism is probably a combination of effects. The amount of ice surface, however, seems to be crucial for the protection. Brij or PEG hydrate molecules might compete with the protein for adsorption at the ice surface and thereby protect the protein during freeze-thawing.

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