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International Journal of Pharmaceutics 237 (2002) 57–69

international journal of pharmaceutics

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# Protection mechanism of Tween 80 during freeze–thawing of a model protein, LDH

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Received 19 September 2001; received in revised form 11 January 2002; accepted 14 January 2002

**Abstract**

The purpose of the study was to investigate the protective mechanism of a non-ionic surfactant, Tween 80, at freeze–thawing with controlled temperature history of a model protein, lactate dehydrogenase (LDH). The system was examined by differential scanning calorimetry (DSC) and infrared spectroscopy (IR). LDH activity assays were performed spectrophotometrically. In all samples, independent of temperature history and addition of surfactant, all water was crystallized to polycrystalline ice at temperatures below −20 °C. The size and perfection of the ice crystals could be varied by a range of cooling rates giving different degrees of undercooling. At Tween concentrations below the cmc at crystallization, lower concentrations were required at low cooling rates compared to higher cooling rates to protect LDH. Concentrations above cmc of Tween reduced the protection at a cooling rate of 5 °C $min^{-1}$ and at quenching in $N_2$(l). The amount of Tween needed for complete protection correlated to the surface area of the ice crystals at a certain temperature history. Tween 80 protects LDH from denaturation at freeze–thawing by hindering its destructive interaction with the ice crystals. The protective effect might be obtained when Tween molecules compete with the protein for sites on the ice surface. The optimum concentration of Tween needed for complete protection is dependent on the temperature history. 

*Keywords:* Tween 80; LDH; Freeze–thawing; Temperature history; DSC; IR spectroscopy

## 1. Introduction

Protein instability in aqueous solution is a major difficulty in producing pharmaceutical formulations, therefore proteins are often freeze-dried to increase their stability. Freezing is an important step in this process, but it induces several stresses capable of protein denaturation. There are a wide variety of protective excipients added to the formulation, such as sugars, amino acids, polymers and nonionic surfactants. It has been found that low concentrations, below the critical micelle concentration (cmc) of nonionic surfactants, such as polyoxyethylene 20 sorbitan monooleate (Tween 80) provide a high degree of protection (Nema and Avis, 1993; Carpenter et al., 1997).

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0378-5173/02/$ - see front matter 
PII: S0378-5173(02)00021-2

There are several possible mechanisms suggested in the literature for protein protection by surfactants. One mechanism is specific binding/interaction with hydrophobic sites on the protein, where Tween can bind weakly to the protein surface (Bam et al., 1995; Katakam et al., 1995; Bam et al., 1998). The protective ability may then correlate with the amount of surfactant needed to saturate these hydrophobic patches, that is the molar ratio of surfactant to protein. The interaction between Tween and LDH in the system used here is very weak at room temperature, as was determined by steady-state and time-resolved fluorescence spectroscopy and diffusion NMR experiments (Hillgren et al., 2002).

Another possible mechanism of protection is a situation where the additive is preferentially excluded from the surface of the protein. This would create a thermodynamically unfavourable system, especially if the protein is denatured, because the surface area exposed to the solvent increases with protein unfolding. The most stable system, the native structure, is thus favoured (Timasheff, 1982; Carpenter et al., 1991). Polyethylene glycol (PEG) is supposed to protect proteins by this mechanism (Carpenter et al., 1991). Surfactants are, however, generally operative at concentrations where volume exclusion effects can be neglected.

A third theory is that denaturation occurs at the ice-water interface. Rapid cooling leads to formation of many small ice crystals, with larger surface area than the crystals produced by slow cooling. It has been shown in previous studies that fast cooling provides much more stress to proteins than a low cooling rate (Chang et al., 1996; Strambini and Gabellieri, 1996; Jiang and Nail, 1998; Kerwin et al., 1998; Kreilgaard et al., 1998). Surfactants may stabilize protein structure by competing with the protein for adsorption on the ice-water interface.

The stability of a protein is also dependent on its own concentration. A concentrated protein solution is more resistant to freeze-induced denaturation. This intrinsic stability may be due to damage and denaturation of the protein at the ice-water interface during freezing. If a finite number of protein molecules can be denatured at this interface, then increasing concentration will lead to a smaller percentage of damaged molecules (Carpenter et al., 1997). It is a well known fact that protein can be adsorbed to many surfaces and interfaces (Claesson et al., 1995) and several studies have also shown that different proteins are destabilized or have changed their secondary structure due to surface adsorption (Wang, 1999).

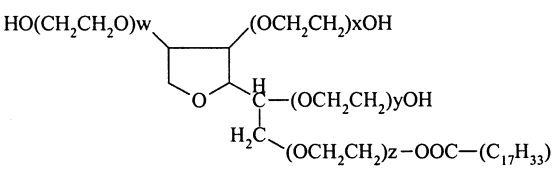
The purpose of this study was to further investigate the ice-water interface theory as a possible mechanism for protein protection by nonionic surfactant at low temperature. A controlled temperature history was used in the experiments by DSC and IR spectroscopy to investigate the ice aggregation state and the ice crystal size and energy. Lactate dehydrogenase (LDH) was used as a model protein in both low concentrations to avoid self-stabilization and in high concentrations. The effect of different concentrations of Tween 80 on the protein protection during the freeze-thawing process in relation to ice surface was also evaluated.

## 2. Experimental

### 2.1. Materials

Lactate dehydrogenase (LDH) from rabbit muscle, crystalline suspension in 65% saturated $(NH_4)_2SO_4$ solution, pH 7.2 (ICN Pharmaceuticals Inc., USA). Polyoxyethylene 20 sorbitan-monooleat (Tween 80) (Kebo, Sweden). Nicotinamide adenine dinucleotide reduced form (NADH) in preweighed vial, 0.2 mg and sodium pyruvate solution 22.7 mM, pH 7.5 (Sigma, USA). Sodium phosphate buffer 0.1 M, pH 7.5. Sodium citrate buffer 10 mM, pH 6.5.

Tween 80:




$w + x + y + z = 20$

## 2.2. Methods

### 2.2.1. Preparation of solutions

Prior to experiments, the LDH suspension was dialyzed against 10 mM sodium citrate buffer, pH 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., USA) and the concentration of the enzyme was determined spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Co., USA). The UV absorbance at 280 nm was linearly related to concentration in the range of 0.1–1.1 mg $ml^{-1}$.

### 2.2.2. Assay of enzyme activity

LDH activity was measured spectrophotometrically with a Spectronic Genesys spectrophotometer (Milton Roy Co.). The 1.44 ml reaction mixture contained 55 μM NADH in 0.1 M 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 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 S.D. based on three determinations.

### 2.2.3. Differential scanning calorimetry (DSC)

The solutions were examined using a DSC 220C oscillating differential scanning calorimeter (Seiko Instruments Inc., Japan) equipped with the Exstar6000 software, version 3.4A. The samples were kept in non-sealed aluminium pans 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 with a controlled cooling rate between 0.5 and 90 °C $min^{-1}$ and a heating rate of 5 °C $min^{-1}$. The concentrations were 25 μg $ml^{-1}$ LDH and 0.23 μg $ml^{-1}$ to 3 mg $ml^{-1}$ Tween 80 in 10 mM sodium citrate buffer, pH 6.5.

Thermograms were recorded at both cooling and heating. The results are presented as mean values with the S.D. based on three determinations.

### 2.2.4. Infrared spectroscopy

Infrared spectra were collected using a Fourier transform infrared (FTIR) spectrometer, BioRad FTS-45 (Digilab Division, USA). The protein solutions were placed in an IR cell with $CaF_2$ windows. Water and carbon dioxide vapour was removed by purging the spectrometer with nitrogen gas. The resolution was 2 $cm^{-1}$ and all spectra were collected by 16 scans and the average was calculated. The temperature was 20–60 °C, obtained in a variable temperature cell using $N_2$(l) as cooling agent. The concentrations were 20 mg $ml^{-1}$ LDH and 0.186–267 mg $ml^{-1}$ Tween 80 in 10 mM sodium citrate buffer, pH 6.5, in the absence or presence of 5% $D_2O$. Spectrum without $D_2O$ was subtracted from spectrum with $D_2O$ and the OD-stretching mode of isotopically isolated HDO molecules at $\approx 2500$ $cm^{-1}$ were studied.

To study the secondary structure, spectral features arising from water vapour and buffer components were subtracted and the second-derivative spectra were calculated. To determine the similarity between two second-derivative spectra, the correlation coefficient, $r$, was calculated.

$$r = \frac{\sum x_i y_i}{\sqrt{\left(\sum x_i^2 \sum y_i^2\right)}}$$

In the equation, $x_i$ and $y_i$ are the spectral absorbance values of the reference and sample spectra at the $i$th frequency position (Prestrelski et al., 1993). The correlation coefficient between two spectra of a given protein equals 1 when there is no conformational change in the protein. The larger the change in conformation, the greater the differences between the spectra and the smaller the value of $r$.

### 2.2.5. Surface tension measurements

The surface tension measurements were performed by a du Noüy tensiometer (Krüss Optisch-

Mechanische Werkstätten, Germany) against air at 25–3 °C. All measurements were performed at ≈10–15 min after formation of a new surface (Persson, 1999). Surface tension ($\gamma$) of pure water was also measured at different temperatures and used to correct the measurements of Tween solutions. The concentrations were 0.5–0.0001% w/v (0.76 μM–3.8 mM) of Tween 80 in Milli Q water. The critical micelle concentration (cmc) of Tween 80 at different temperatures was obtained from plots of surface tension versus the logarithm of the concentration.

The surface area occupied per Tween molecule was calculated from the surface excess concentration, $\Gamma$, through the Gibbs equation (Jönsson et al., 1998).

## 3. Results and discussions

### *3.1. Behaviour of the components in the system at low temperature*

#### *3.1.1. Crystallization of ice*

To examine the aggregation state of water and ice, the OD-stretching mode of HDO molecules in an IR spectrum was followed. This vibration occurs at ≈2500 $cm^{-1}$. The wavenumber and the size of the peak are dependent on the environment of the OD-bond. It moves to a lower wavenumber and becomes narrower as the crystallinity increases (Franks, 1982). In water, the environment is amorphous and the mobility of water molecules is large. The OD-stretching mode then corresponds to a broad peak at 2500 $cm^{-1}$ (Fig. 1). In polycrystalline ice, the vibration frequency decreases to ≈2430 $cm^{-1}$ and the band becomes narrower. The peak positions are also dependent on the temperature, both before and after the crystallization, but the full width at half maximum (FWHM) is independent of the temperature (Franks, 1982).

In our experiment, the peak position decreases as the temperature decreases with a sharp change when the ice crystallizes at about −15 °C (Fig. 2a). The addition of Tween 80 and LDH made no significant difference to this behaviour. The OD-stretching band for amorphous solid water is found at ≈2450 $cm^{-1}$ and for polycrystalline ice, ≈2430 $cm^{-1}$ (Franks, 1982). At temperatures below −20 °C there is no liquid water or amorphous solid water detected by IR, only polycrystalline ice. If the cooling rate increased from 1 to 10 °C $min^{-1}$, there was still no detectable liquid

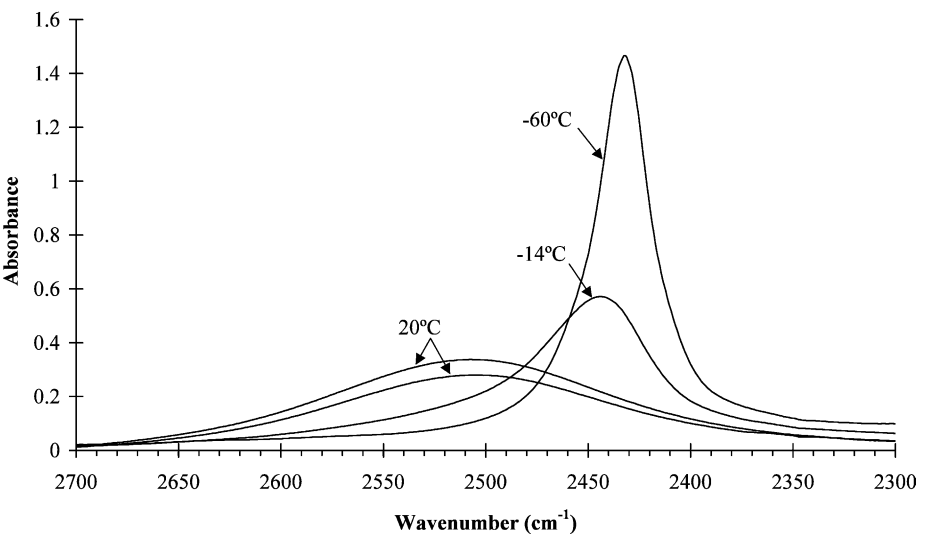



Fig. 1. The OD-stretching obtained by FTIR for sodium citrate buffer at different temperatures. There are two spectra at 20 °C, one before and one after freeze–thawing.

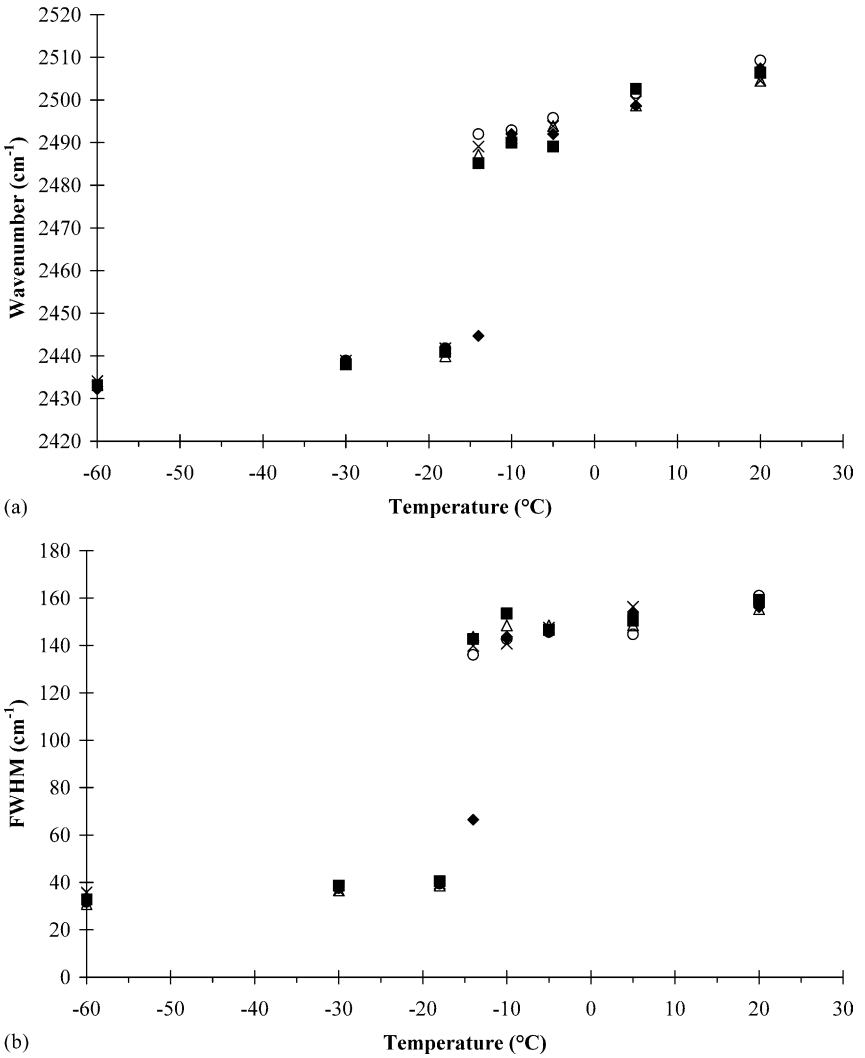



Fig. 2. The change of wavenumber (a) and the full width at half maximum FWHM (b) during cooling for the OD-stretching transition. (◆) Citrate buffer, (■) LDH 20 mg $ml^{-1}$, (□) LDH 20 mg $ml^{-1}$ and Tween 80 0.186 mg $ml^{-1}$, molar ratio 1:1, (×) LDH 20 mg $ml^{-1}$ and Tween 80 26 mg $ml^{-1}$, molar ratio 1:146 and (○) Tween 80 267 mg $ml^{-1}$.

water or amorphous solid water, only one single, symmetrical peak in the spectrum at 2434 $cm^{-1}$ (data not shown). The FWHM, for both water and ice, is independent of the temperature and the sharp decrease between −10 and −20 °C reflects the crystallization (Fig. 2b). The increased cooling rate, from 1 to 10 °C $min^{-1}$, did not change this behaviour (data not shown).

The experiments thus show that regardless of cooling rate and addition of Tween 80 and/or LDH, all water is crystallized to polycrystalline ice below −20 °C.

### 3.1.2. Denaturation of LDH

Denaturation of proteins can be monitored with different techniques. IR spectroscopy is one powerful method to study structural changes in any state of a protein, i.e. aqueous, frozen or dried. Nine characteristic vibrational bands or group frequencies that arise from the amide group

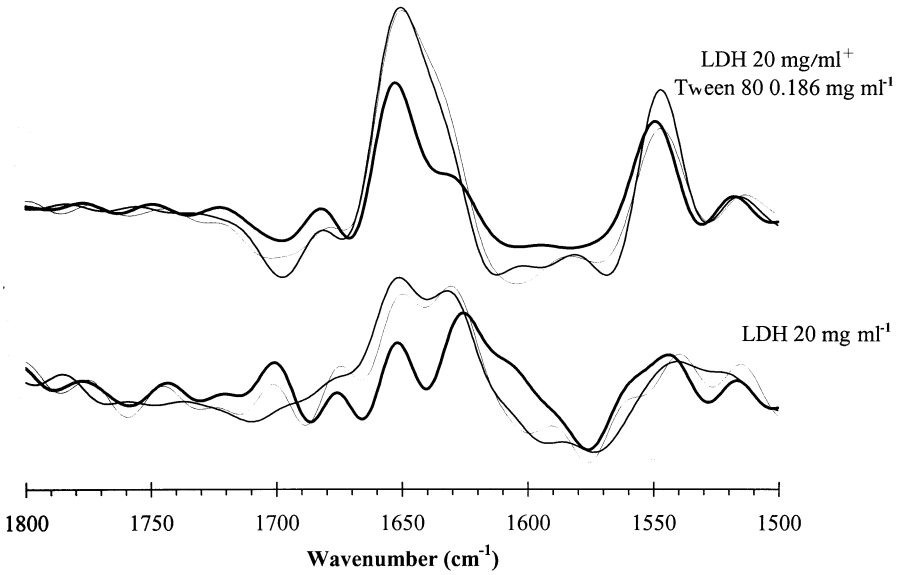



Fig. 3. Second derivative FTIR spectra plotted as the absolute values of 20 mg ml$^{-1}$ LDH with or without Tween 80 at different temperatures. ——, 20 °C; ——, −60 °C; ——, 20 °C after one freeze–thawing cycle.

of protein have been identified. The amide I band (1700–1620 cm$^{-1}$) is almost entirely due to the C = O-stretching vibration of the peptide linkages. This is the most studied band for determination of secondary structures, since it is sensitive to small variations in molecular geometry and hydrogen bonding patterns within the protein (Dong et al., 1995).

Fig. 3 shows the second-derivative IR spectra for LDH and LDH with the addition of Tween 80 at different temperatures. Each type of secondary structure gives a different C = O-stretching frequency in the spectrum. Strong bands near 1656 and 1636 cm$^{-1}$ arise from α-helical and β-sheet structures, respectively (Prestrelski et al., 1993). The correlation coefficient, $r$, defined in Section 2, is used to determine similarities between different spectra. If there is no change in the conformation of the protein, the correlation coefficient equals 1 and becomes smaller with greater changes in the protein structure.

The spectrum of 20 mg ml$^{-1}$ LDH at −60 °C is altered relative to the aqueous spectrum at 20 °C, with a correlation coefficient of 0.609 (Table 1). This indicates that freezing significantly changes the conformation of the protein. As the sample is thawing, the conformation seems to recover, with an $r$-value of 0.968 between LDH at 20 °C before and after freeze–thawing (Table 1). Relatively high concentrations of protein are required to obtain an IR spectrum of high quality and it is well known that increasing protein concentration leads to increased resistance to denaturation during freezing (Chang et al., 1996; Carpenter et al., 1997). We found that the recovered activity of pure LDH after freezing to −60 °C with identical temperature history varied with concentration and was 45% at 25 μg ml$^{-1}$,

Table 1
The correlation coefficient ($r$) between two second derivative IR spectra of 20 mg ml$^{-1}$ LDH and 0.186 mg ml$^{-1}$ Tween 80 at different temperatures

| Reference spectrum | Sample spectrum | $r$ |
|---|---|---|
| LDH at 20 °C | LDH at −60 °C | 0.609 |
| LDH at 20 °C | LDH at 20 °C | 0.968 |
| LDH at 20 °C | LDH+Tween 80 at 20 °C | 0.888 |
| LDH+Tween 80 at 20 °C | LDH+Tween 80 at −60 °C | 0.963 |
| LDH+Tween 80 at 20 °C | LDH+Tween 80 at 20 °C | 0.992 |

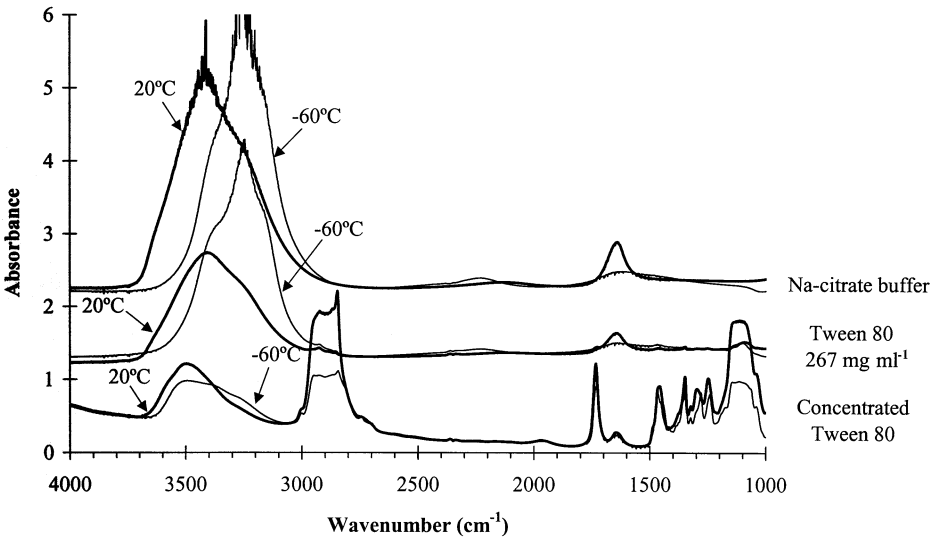



Fig. 4. FTIR spectra for different samples with Tween 80 and sodium citrate buffer at 20 and −60 °C, respectively.

63% at 0.1 mg $ml^{-1}$ and 103% at 10 mg $ml^{-1}$, respectively. Thus, concentrations of 20 mg $ml^{-1}$ protein, which were used in the IR experiment, do not denature if the complete freeze–thawing process is considered, although some conformational changes seem to occur at low temperatures.

Addition of Tween alters the conformation of LDH at room temperature (Table 1), indicating an interaction between LDH and Tween 80 at the high concentrations used here. The IR spectrum of LDH with the addition of Tween at −60 °C is only slightly changed compared to the spectrum of the same solution at room temperature. The conformation of LDH is also preserved during thawing with an *r*-value of 0.992 of this solution before and after freeze–thawing (Table 1). This shows that addition of Tween is hindering the denaturation of the protein during a freeze–thawing process.

### *3.1.3. Phase transitions of Tween 80*

To be able to understand the cryoprotective effect of Tween 80 in protein solutions, it is of great importance to have some knowledge about the behaviour of the aqueous solution at low temperature. The cmc of Tween 80 in water at room temperature is, according to the literature, 10 μM (Sivars and Tjerneld, 2000) or 11 μM (Wan and Lee, 1974). In this study, the cmc in water was determined to be ≈13 μM. In a sodium citrate buffer solution at room temperature the cmc was 10–30 μM and the addition of LDH increased the cmc, as was determined in this laboratory by fluorescence spectroscopy and NMR (Hillgren et al., 2002). For nonionic surfactants, the cmc increases with decreasing temperature (Jönsson et al., 1998). If the temperature is decreasing to −3 °C, the cmc of Tween 80 in water increases to 130 μM, according to our experiments (data not shown). Measurements at lower temperatures were difficult to perform because of the freezing of the solution.

The IR spectra of different concentrations of Tween 80 in sodium citrate buffer and of pure buffer solution are shown in Fig. 4. Concentrated Tween, which may include up to 3% moisture, shows characteristic bands of OH-stretching (3600 $cm^{-1}$), CH2-stretching (2900 $cm^{-1}$) C=O-stretching (1750–1735 $cm^{-1}$) and HOH-bending (1600 $cm^{-1}$). When water is crystallized the OH-stretching vibration mode, reflecting both water and Tween OH-groups, changes from 3600 to ≈3300 $cm^{-1}$ (Franks, 1982). The intensity of the HOH-bending mode decreases rapidly as the hydrogen bond strength of the molecular environment increases, e.g. when water freezes to ice

(Devlin, 1990). As concentrated Tween is freezing, the OH-stretching mode is slightly shifted to a lower wavenumber, but the intensity of the HOH-bending mode is unchanged. In a sample with pure buffer or diluted Tween, both the wavenumber of the OH-stretching and the intensity of the HOH-bending are changed (Fig. 4). This difference indicates that in concentrated Tween 80, the water forms a structure different from water and ice when the temperature is decreasing. In a previous study, DSC measurement of a concentrated solution of Tween 80 showed a phase transformation at about −14 °C (Hillgren et al., 2002) that could indicate the presence of such a structure formed by Tween and ice/water at low temperature. Since Tween has a certain amount of side chains of polyethylene, the transition might reflect melting of a structure similar to the PEG-ice structure, proposed to be a hydrate (Antonsen and Hoffman, 1992), melting at about −15 °C (Aldén and Magnusson, 1997).

In the following experiments, however, the Tween concentrations are low enough to avoid the creation of such a structure and both OH-stretching and HOH-bending follow the pattern of pure water.

### 3.2. The ice-formation at the freeze–thawing process and recovered activity of LDH

#### 3.2.1. Influence of cooling rate on ice crystal formation

To investigate the protective ability of Tween 80 in the freeze–thawing process while the size of ice crystals and thus the amount of ice-water interface is varied, a series of DSC experiments were performed. A low concentration of LDH (25 μg $ml^{-1}$) was used to avoid intrinsic stability of the protein. At this concentration, the protein is more or less denatured during a freeze–thawing process (Aldén and Magnusson, 1997). The size of the ice crystals was varied by selecting a cooling rate between 0.5 and 90 °C $min^{-1}$ in the DSC experiments and a cooling rate of about $10^2$–$10^3$ °C $min^{-1}$ when the sample was frozen instantly in liquid nitrogen. The sample could not, however, immediately follow a cooling rate larger then 20 °C $min^{-1}$ in the DSC experiments, therefore the results presented in this study refer to strictly controlled cooling rates of 0.5–20 °C $min^{-1}$. The thermogram of 25 μg $ml^{-1}$ LDH in citrate buffer is shown in Fig. 5, where the cooling

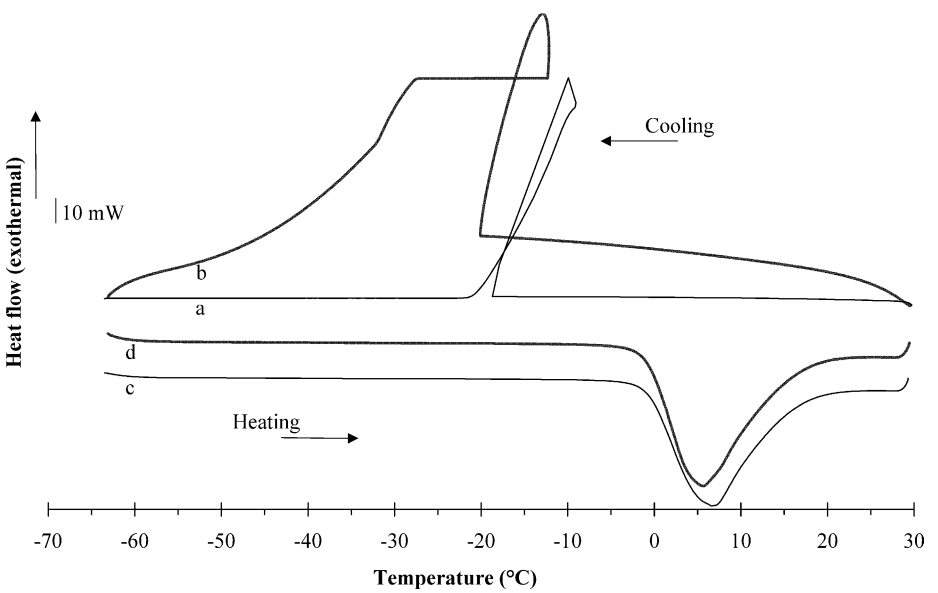



Fig. 5. Cooling and heating thermograms obtained by DSC for 25 μg $ml^{-1}$ LDH in sodium citrate buffer with a heating rate of 5 °C $min^{-1}$. (a) Cooling process with a cooling rate of 1 °C $min^{-1}$ and (c) the corresponding heating process. (b) Cooling process with a cooling rate of 20 °C $min^{-1}$ and (d) the corresponding heating process.

Table 2
Heat of transformation and peak temperatures for 25 μg ml$^{-1}$ LDH with 0.23 μg ml$^{-1}$ Tween 80, molar ratio 1:1 and different cooling rates

| CR (°C min$^{-1}$) | Crystallization | | | | Melting | | | |
|---|---|---|---|---|---|---|---|---|
| | $T_{onset}$ (°C) | S.D. | $\Delta H$ (J g$^{-1}$) | S.D. | $T_{onset}$ (°C) | S.D. | $\Delta H$ (J g$^{-1}$) | S.D. |
| 0.5 | −17.2 | 0.2 | −257.7 | 4.1 | −0.9 | 0.1 | 286.9 | 5.4 |
| 1 | −15.4 | 1.3 | −267.2 | 2.2 | −0.8 | 0.2 | 301.5 | 3.3 |
| 5 | −17.1 | 1.6 | −268.7 | 2.8 | −0.7 | 0.1 | 306.2 | 3.0 |
| 20 | −21.5 | 1.3 | −174.8 | 11.9 | −0.8 | 0.1 | 307.6 | 9.3 |

rates were 1 and 20 °C min$^{-1}$, respectively and the heating rate in both cases was 5 °C min$^{-1}$. The thermograms show a crystallization peak at −10 °C to −20 °C and a melting peak at 5–6 °C. It also illustrates the complex temperature variation at the crystallization, since the sample temperature increases by several degrees as the crystallization process starts and before equilibrium is reached in the cooling process.

Table 2 shows the heat of transformation and peak temperatures for LDH and Tween 80 at molar ratio 1:1. There were no significant differences in the values between this composition of the sample and the other samples. The onset temperature for the crystallization decreased when the cooling rate increased to 20 °C min$^{-1}$, indicating a more undercooled solution. The heat of crystallization also decreased with increased cooling rate to 20 °C min$^{-1}$. This shows that the less sample transformed to solid state, the less amount of ice is formed or that the structure that is formed contains more energy. Since IR spectroscopy showed that regardless of cooling rate all water crystallizes to polycrystalline ice and no amorphous phase is formed, the decrease in $\Delta H$ for the crystallization must then depend on the size and perfection of the ice crystals. The crystallization temperature affects the size of ice crystals. As the cooling rate increases, the crystallization temperature decreases, the solution becomes more undercooled and the nucleation rate increases. This means that more nuclei of ice are created and thereby a larger amount of small crystals (Michelmore and Franks, 1982; Bronshteyn and Steponkus, 1995). A high cooling rate thus creates smaller and more imperfect ice crystals, giving a higher energy of the system than a low cooling rate. During slow freezing, the system is less undercooled, fewer nuclei of ice are formed and the crystals have time to grow and become more perfect. The same amount of ice in the shape of small crystals gives a much larger surface area than larger ones, which means more opportunity for the ice crystals to damage the protein.

The heat of melting and the onset temperature of melting were, however, not affected by the cooling rate. The same amount of ice with the same energy content melts regardless of cooling rate. This confirms that the crystallization and growth of crystals continues during the entire cooling process and also during heating at low temperatures (Bronshteyn and Steponkus, 1995).

### *3.2.2. The amount of Tween 80 needed for protection at different cooling rates*

The recovered activity of LDH in solution without the addition of Tween was ≈50% after freezing with a cooling rate of 1–5 °C min$^{-1}$. It decreased to some extent with a cooling rate of 20 °C min$^{-1}$ and was only 4% when the sample was frozen instantly, by direct insertion in liquid nitrogen (Table 3).

When Tween 80 was added in a certain concentration to the protein solution, the protective effect varied with the cooling rates that was used. The addition of 0.23 μg ml$^{-1}$ (0.17 μM) Tween 80, molar ratio 1:1, did not provide protection of LDH at high cooling rates (Table 3). Tween–LDH interactions are very weak at the molar ratio and temperatures used here, as found previously by fluorescence spectroscopy (Hillgren et al., 2002) and are obviously not sufficient for

protection. The recovered activity increased however compared to the formulation without Tween 80 when the cooling rate decreased to 1 °C $min^{-1}$. A cooling rate of 0.5 °C $min^{-1}$ with a larger undercooling (Table 2) decreased the activity again. The addition of 33 μg $ml^{-1}$ (25 μM) Tween 80, molar ratio 1:146, offered complete protection at cooling rates of 1–20 °C $min^{-1}$ (Table 3). When the sample was frozen in liquid nitrogen, the recovered activity increased from 4% in pure buffer to 25% when Tween 80, molar ratio 1:146 was added.

Table 4 illustrates the recovered activity of LDH in a low concentration with different concentrations of Tween 80 added at a certain temperature history. The sample was frozen with a cooling and a heating rate of 5 °C $min^{-1}$ or instantly frozen in liquid nitrogen and thawed at room temperature. The lowest concentration of Tween, 0.17 μM, did not protect LDH during freeze–thawing. At the lower cooling rate, 25 μM Tween 80 provided full protection and at higher concentrations, above cmc at crystallization, the protection was again reduced (Table 4). With a very high cooling rate, LDH was almost completely protected with the addition of 76 μM Tween 80, but the protection decreased at concentrations above cmc of Tween. The minimum amount of Tween needed for complete protection was thus dependent on the cooling rate and thereby the degree of undercooling and hence on the size of the ice crystals and the amount of ice surface.

### 3.2.3. Ice surface and amount of Tween 80

If Tween 80 is protecting LDH by reducing the interaction between protein and ice, there should be a correlation between the amount of Tween needed for protection at crystallization and the created ice surface during a specific freeze–thawing cycle. Nonionic surfactants have a strong temperature dependence of the adsorption on surfaces. The area occupied per Tween molecule, calculated from surface tension measurements (data not shown), increased as the temperature decreased. At room temperature, the occupied area of one Tween molecule is $\approx 60$ $Å^2$ and at temperatures below $-3$ °C, the area per molecule is larger than 80 $Å^2$. The minimum radius of ice crystals completely covered with Tween monomers at different Tween concentrations is calculated in Table 4. The assumptions made for the calculations are that all ice crystals are perfect spheres, that the area occupied by one Tween monomer at crystallization is 80 $Å^2$, that Tween exist as monomers at all concentrations (which of course is not true over cmc) and that the density of ice is 0.92 g $cm^{-3}$. At the concentration where Tween gives full protection at low cooling rates (25 μM), the molecules can cover the surface of crystals with a radius of minimum 268 μm. Some 76 μM Tween 80, which provides almost complete protection at both low and high cooling rates, can cover ice-crystals with a minimum radius of 89 μm. According to Evans et al. (1996), ice crystals have a diameter of $\approx 100$–$200$ μm if a freezing rate of up to 3 °C $min^{-1}$ is used.

Table 3
The recovered activity of 25 μg $ml^{-1}$ LDH with addition of different molar ratio of Tween 80 during freeze–thawing at different cooling rates, CR and constant heating rate, 5 °C $min^{-1}$

| CR (°C $min^{-1}$) | LDH | | LDH + Tween 80 0.23 μg $ml^{-1}$ 1:1 | | LDH + Tween 80 33 μg $ml^{-1}$ 1:146 | |
|---|---|---|---|---|---|---|
| | Activity (%) | S.D. | Activity (%) | S.D. | Activity (%) | S.D. |
| 0.5 | | | 54.6 | 4.8 | | |
| 1 | 50.7 | 7.6 | 68.4 | 2.9 | 99.0 | 12.8 |
| 5 | 52.3 | 5.5 | 51.9 | 4.6 | 93.4 | 7.5 |
| 20 | 40.4 | 5.5 | 45.3 | 8.8 | 89.3 | 6.8 |
| $N_2$(l) | 4.3 | 0.7 | 4.4 | 1.1 | 25.5 | 0.9 |

Table 4
Recovered activity of 25 μg $ml^{-1}$ LDH after freeze–thawing with different cooling rates and concentrations of Tween 80

| Tween 80 | | Molar ratio LDH:Tween | CR = 5 °C $min^{-1}$ | | Frozen in $N_2$(l) | | Minimum radius of ice crystals completely covered with Tween molecules |
|---|---|---|---|---|---|---|---|
| | | | Activity (%) | S.D. | Activity (%) | S.D. | |
| 0 | 0 | – | 52.3 | 5.5 | 4.3 | 0.7 | |
| 0.17 μM | 0.23 μg $ml^{-1}$ | 1:1 | 51.9 | 4.6 | 4.4 | 1.1 | 3.8 cm |
| 25 μM | 33 μg $ml^{-1}$ | 1:146 | 93.4 | 7.5 | 25.5 | 0.9 | 268 μm |
| 76 μM | 0.1 mg $ml^{-1}$ | 1:447 | 80.0 | 4.9 | 89.9 | 12.6 | 89 μm |
| 0.23 mM | 0.3 mg $ml^{-1}$ | 1:1340 | 58.8 | 9.7 | 66.9 | 1.5 | 30 μm |
| 2.23 mM | 3 mg $ml^{-1}$ | 1:13398 | 78.0 | – | 58.4 | 2.3 | 3.0 μm |

The results obtained here indicate that the cooling rate of 5 °C $min^{-1}$ used in this study creates ice crystals of similar size.

### 3.3. The protection mechanism

A possible explanation for the concentration dependence of the protective ability of Tween 80 could be that denaturation of proteins occurs when the protein adsorbs to the ice crystal surface in the crystallization process. Tween can protect LDH by competing with the protein for sites on the ice surface, as is schematically illustrated in Fig. 6. At very low concentrations of Tween there are not enough Tween molecules to cover the ice surface and hence Tween cannot fully protect LDH from denaturation. If the concentration of Tween increases, but is still below the cmc at crystallization, the opportunity of the protein to interact with the ice crystals is reduced. When the amount of Tween molecules is large enough to cover the ice surface completely, LDH is fully protected from denaturation. If the Tween concentration increases further to about the cmc at crystallization temperature, there exist both micelles and monomers in equilibrium. Depending on the kinetics of micelle formation, the shape of the micelles and their affinity to different interfaces, there might be a distribution of micelles and monomers where part of the ice surface is uncovered and destructive interactions can occur again with the protein. As the concentration increases far above cmc at crystallization temperature, more micelles are formed and the ice surface

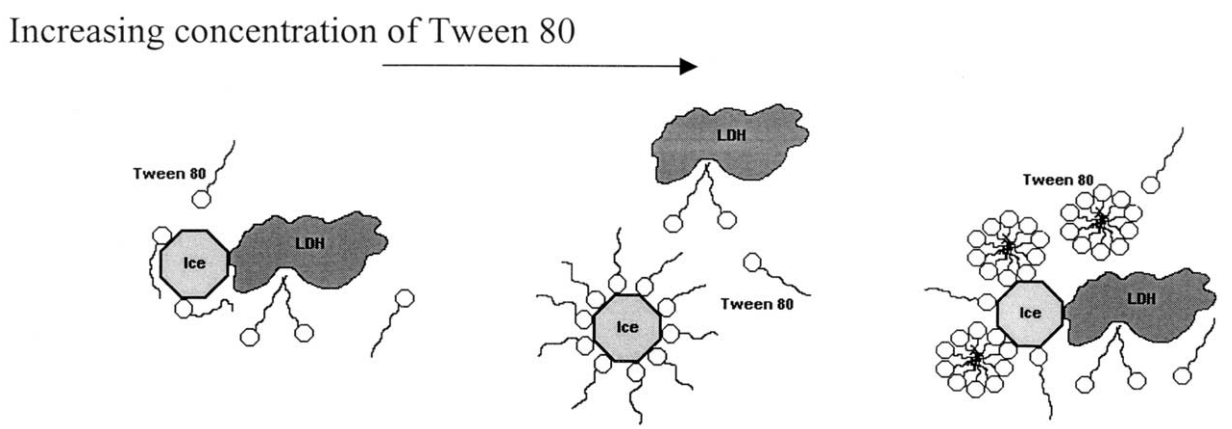



Fig. 6. Illustration of possible interactions between Tween 80, ice and LDH in the solid state at varying concentrations of Tween 80.

might be more or less covered. Since the ice surface area is correlated to the cooling rate and the undercooling of the system, a higher concentration of Tween 80 is needed for complete protection of the protein when the cooling rate is enhanced.

## 4. Conclusions

During the freeze–thawing process with the same temperature history, very low concentrations of Tween 80 cannot protect LDH in low concentrations but higher concentrations of Tween, below cmc at crystallization, have full protective effect. With an increasing freezing rate, a higher concentration of Tween 80 is needed for full protection. These results suggest a protective mechanism of Tween that supports the ice-water theory, since only a weak interaction exists between Tween and LDH, not large enough for protection. At temperatures below $-20$ °C, only polycrystalline ice is present in the system. A low cooling rate at crystallization creates larger and more perfect ice crystals than higher rates. Since the ice surface seems to be a damaging environment for the LDH molecules, Tween 80 can protect LDH from denaturation by hindering its interaction with the ice. A varying amount of Tween molecules can cover the ice crystal surface totally or partly and thus reduce the possible protein–ice interaction. The amount of Tween that is needed for full protection at a certain temperature history correlates to the area of the ice crystals.

## Acknowledgements

The authors are very grateful to Dr Hans Evertsson for valuable discussions. Financial support from the Swedish Research Council for Engineering Sciences is gratefully acknowledged.

## References

Aldén, M., Magnusson, A., 1997. Effect of temperature history on the freeze-thawing process and activity of LDH formulations. Pharm. Res. 14, 426–430.

Antonsen, K.P., Hoffman, A.S., 1992. Water structure of PEG solutions by differential scanning calorimetry measurements. In: Harris, J.M. (Ed.), Poly(ethylene glycol) Chemistry Biotechnical and Biomedical Applications. Plenum Press, New York, pp. 15–28.

Bam, N.B., Randolph, T.W., Cleland, J.L., 1995. Stability of protein formulations: investigation of surfactant effects by novel EPR spectroscopic technique. Pharm. Res. 12, 2–11.

Bam, N.B., Cleland, J.L., Yang, J., Manning, M.C., Carpenter, J.F., Kelley, R.F., Randolph, T.W., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87, 1554–1559.

Bronshteyn, V.L., Steponkus, P.L., 1995. Nucleation and growth of ice crystals in concentrated solutions of ethylene glycol. Cryobiology 32, 1–22.

Carpenter, J.F., Arakawa, T., Crowe, J.H., 1991. Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. Dev. Biol. Stand. 74, 225–239.

Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W., 1997. Rational design of stable lyophilizes protein formulations: some practical advice. Pharm. Res. 14, 969–975.

Chang, B.S., Kendrick, B.S., Carpenter, J.F., 1996. Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci. 85, 1325–1330.

Claesson, P.M., Blomberg, E., Fröberg, J.C., Nylander, T., Arnebrant, T., 1995. Protein interactions at solid surfaces. Adv. Colloid Interface Sci. 57, 161–227.

Devlin, J.P., 1990. Vibrational modes of amorphous ice: bending mode frequencies for isotopically decoupled H2O and HOD at 90 K. J. Mol. Struct. 224, 33–43.

Dong, A., Prestrelski, S.J., Allison, S.D., Carpenter, J.F., 1995. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. J. Pharm. Sci. 84, 415–424.

Evans, J., Alder, J., Mitchell, J., Blanshard, J., Rodger, G., 1996. Use of confocal laser scanning microscope in conjunction with a conduction heat transfer stage in order to observe dynamically the freeze-thaw cycle in an autofluorescent substance and to measure ice crystal size in situ. Cryobiology 33, 27–33.

Franks, F., 1982. Water, a Comprehensive Treatise, Water and Aqueous Solutions at Subzero Temperatures, vol. 7. Plenum Press, New York.

Hillgren, A., Evertsson, H., Aldén, M., 2002. Interactions between lactate dehydrogenase and Tween 80 in aqueous solution. Pharm. Res. 19 (in press).

Jiang, S., Nail, S.L., 1998. Effect of process conditions on recovery of protein activity after freezing and freeze-drying. Eur. J. Pharm. Biopharm. 45, 249–257.

Jönsson, B., Lindman, B., Holmberg, K., Kronberg, B., 1998. Surfactants and Polymers in Aqueous Solutions. Wiley, Chichester.

Katakam, M., Bell, L.N., Banga, A.K., 1995. Effect of surfactants on physical stability of recombinant human growth hormone. J. Pharm. Sci. 84, 713–716.

Kerwin, B.A., Heller, M.C., Levin, S.H., Randolph, T.W., 1998. Effects of Tween 80 and sucrose on acute short-term stability and long-term storage at −20°C of a recombinant hemoglobin. J. Pharm. Sci. 87, 1062–1068.

Kreilgaard, L., Jones, L.S., Randolph, T.W., Frokjaer, S., Flink, J.M., Manning, M.C., Carpenter, J.F., 1998. Effect of Tween 20 on freeze-thawing- and agitation-induced aggregation of recombinant human factor XIII. J. Pharm. Sci. 87, 1597–1603.

Michelmore, R.W., Franks, F., 1982. Nucleation rates of ice in undercooled water and aqueous solutions of polyethylene glycol. Cryobiology 19, 163–171.

Nema, S., Avis, K.E., 1993. Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants. J. Parent Sci. Technol. 47, 76–83.

Persson, B., 1999. Drug–polymer interaction in relation to surface tension and polymer properties. Dissertation, Uppsala University, Uppsala.

Prestrelski, S.J., Arakawa, T., Carpenter, J.F., 1993. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization II. Structural studies using infrared spectroscopy. Arch. Biochem. Biophys. 303, 465–473.

Sivars, U., Tjerneld, F., 2000. Mechanisms of phase behavior and protein partitioning in detergent/polymer aqueous two-phase systems for purification of integral membrane proteins. Biochem. Biophys. Acta 1474, 133–146.

Strambini, G.B., Gabellieri, E., 1996. Proteins in frozen solutions: evidence of ice-induced partial unfolding. Biophys. J. 70, 971–976.

Timasheff, S.N., 1982. Preferential interactions in protein-water-cosolvent systems. In: Franks, F., Mathias, S.F. (Eds.), Biophysics of Water. Wiley, New York, pp. 70–72.

Wan, L.S.C., Lee, P.F.S., 1974. CMC of polysorbates. J. Pharm. Sci. 63, 136–137.

Wang, W., 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185, 129–188.