Cryoprotection Mechanisms of Polyethylene Glycols on Lactate Dehydrogenase During Freeze-thawing

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ABSTRACT

The purpose of this study was to explore the cryoprotection mechanisms of high molecular weight polyethylene glycols (PEGs) (eg, PEG 4000 and PEG 8000) on lactate dehydrogenase (LDH). Ultraviolet activity assays, circular dichroism (CD) spectroscopy, gel filtration, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), ¹⁴C-PEG 4000 labeling and binding, and cryostage microscopic study were conducted. Different molecular weights and concentrations of PEGs in LDH formulations were treated by freeze-thawing. Higher molecular weights and concentrations of PEGs in LDH-PEG formulations obtained better activity and secondary structure recoveries of LDH after freeze-thawing. Insoluble aggregation of LDH was not observed in gel filtration studies. SDS-PAGE results suggested surface characteristic modifications of LDH by the larger molecular weight PEGs. The ¹⁴C-PEG 4000 labeling and binding study showed extensive non-specific interactions between the PEG 4000 and LDH molecules in a concentration-dependent manner. The bound LDH-PEG 4000/free PEG 4000 ratio increased when LDH or PEG 4000 concentrations increased. Cryostage microscopic study showed that PEG 8000 delayed the ice crystallization and eutectic transition of LDH formulation. It appeared that multiple mechanisms were at work during PEGs' cryoprotection of LDH. It was unclear whether the delayed eutectic characteristics of PEGs contributed to LDH cryoprotection. The favorable interaction, rather than preferential exclusion, between LDH and PEGs (eg, 4000) cryoprotected LDH.

KEYWORDS: lactate dehydrogenase (LDH), freeze-thaw, circular dichroism (CD), SDS-PAGE, ¹⁴C-PEG 4000 binding

INTRODUCTION

Previous studies have shown that polyethylene glycols (PEGs) protect lactate dehydrogenase (LDH) in a molecular-weight-dependent and concentration-dependent manner.¹ The focus of this study is to further elucidate the cryo-

protection mechanisms of high molecular weight PEGs on LDH.

Chilson et al² suggested that many factors caused the inactivation of proteins during freeze-thawing, such as the temperature changes, pH, and ions. They examined the effects of freezing and thawing under various conditions on catalytic activity, hybridization, and reactivity of sulfhydryl groups with p-hydroxymercuribenzoate. They explored different protection mechanisms for various enzymes, almost 30 years ago.² Since then, many researchers have studied cryoprotection, but no universal mechanism has been found to be applicable to all proteins.

Recently, a mechanism proposed by Timasheff et al³ has been widely accepted by other researchers. In Timasheff's mechanism, Timasheff explains protein stabilization phenomena in aqueous solutions by small molecular weight stabilizers, such as sodium lactate.³ A stabilizer favors the folded state of the protein when the folded and unfolded forms of the protein coexist in an equilibrium solution. In the presence of a stabilizer, less free energy for the folded native protein increases because there are less exposed surface areas than for the unfolded denatured protein; thus, the unfolded protein exists in the more thermodynamically unfavorable state.

During freezing, the water surrounding a protein would probably be drawn off into an ice crystal matrix and the presumed hydration shell would be disrupted, hence, the same hydration environment as in solution would no longer exist. However, many³ have argued that freezing does not necessarily invalidate the Timasheff mechanism, since a significant fraction of water remains unfrozen in protein formulations.

Our hypothesis was that PEGs could interact with proteins in multiple ways during cryoprotection. Higher molecular weight PEGs protected LDH better than lower molecular weight PEGs in solution and during freeze-thawing.¹ A likely explanation for this finding is increased affinity for binding in the higher molecular weight molecules. LDH in its native form is an oligomeric protein.⁴ An essential requirement for LDH cryoprotection by PEGs is that the stabilizers should favor the association of the tetramer.

Several investigators have proposed that PEGs function as chaperones,^{5,6,7} which are molecules that favor the folding of

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polypeptide chains into their native structures. This concept assumed that it was only possible to perform these tasks when protein molecules directly contacted the PEG molecules.

To confirm our hypothesis, a series of experiments was designed that included but was not limited to UV activity assay and circular dichroism (CD) methods. The changes from UV assay and CD secondary structures should be consistent and complementary to confirm the preservation of native proteins.

To achieve maximum accuracy when analyzing CD spectrum, a size exclusion method and a protein concentration confirmation should be conducted to exclude the possibility of CD spectrum changes caused by protein aggregation or concentration changes.⁸ In gel filtration studies, standard proteins can be separated according to their molecular weights. In this study, the larger molecular weight proteins were eluted earlier from a size exclusion column. The molecular weights of the proteins could be detected by comparing the elution time (commonly represented as the fraction numbers) with standard proteins of known molecular weights. In addition to the gel filtration method, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies can be used to identify insoluble aggregates from soluble aggregates, because different sizes of proteins carry different charges when coated by SDS molecules. SDS and a reducing agent, such as 2-mercaptoethanol, under an elevated temperature could dissolve the soluble aggregates. The SDS-PAGE studies complimented the gel filtration studies.

Arakawa and Timasheff first used the dialysis mechanism to separate the bound and unbound proteins in order to establish the preferential exclusion mechanism.⁹ Based on the same principle, an experiment was designed to detect the bound/unbound ratio of PEG 4000 by using radioactive labeled ¹⁴C-PEG 4000. Technically, it was more difficult and less accurate to use the dialysis method to measure the LDH concentrations inside and outside of the dialysis membrane since the molecular weight for LDH is 144 000 d. Commercially available ¹⁴C-PEG 4000 made it possible to probe the amount of free PEG 4000 and LDH-bound PEG 4000 by monitoring the radioactivity of ¹⁴C-PEG 4000. The total PEG 4000 binding could be determined by the radioactivity of ¹⁴C-PEG 4000 in the retained solution and in the filtered solution with a 30 000 molecular weight cutoff membrane positioned vertically between the upper tube and lower tube. Before centrifugation, free PEG 4000 and LDH-bound PEG 4000 existed in equilibrium in the upper tube aqueous system. Upon centrifugation, a greater radioactivity of hot ¹⁴C-PEG 4000 retained on the upper tube indicated that more cold-PEG 4000 molecules were bound to LDH.

None of the methods conducted in-process monitoring for freeze-thawing; however, detecting the amount of free PEG 4000 vs LDH-bound PEG 4000 suggests a binding status for LDH and PEG 4000 in the solution before freezing and after thawing.

A cryostage microscope provided a direct way to monitor the thermal events of protein formulations in real time. Because of the antifreeze characteristic of PEGs, many investigators¹⁰ considered that thermal properties of PEGs might contribute to the cryoprotection for LDH. Alden et al¹⁰ suggested that the endothermic transformation of PEG-ice structure (essentially a PEG-ice eutectic structure) at -15°C during the thawing process strongly correlated with the protective ability of PEG 6000 in the freeze-thawing of LDH. Therefore, a cryostage microscopic study was integrated to provide a direct way to monitor the freezing process. By monitoring the freezing events with and without PEG 8000, the cryoprotective mechanism by which different molecular weight PEGs cryoprotect LDH may be elucidated.

MATERIALS AND METHODS

Materials

LDH (type II, from rabbit muscle; as an aqueous ammonium sulfate suspension), NADH (β -nicotinamide adenine dinucleotide, reduced form, disodium salt), pyruvic acid sodium salt, PEG 400, PEG 4000, PEG 8000, and Tris (hydroxymethyl) aminomethane hydrochloride were purchased from Sigma Chemical Co (St Louis, MO). Lyophilized BSA (bovine serum albumin, Bio-Rad Protein assay standard II) was purchased from Bio-Rad Laboratory (Hercules, CA). Premixed running buffers, premixed sample buffer, and SDS-PAGE standards were purchased from Bio-Rad Laboratory (Hercules, CA). ¹⁴C-PEG 4000 was purchased from Amersham Pharmacia (Piscataway, NJ). Ultrafree-MC 30 000 NMWL filter units were purchased from Millipore Co (Bedford, MA). Scintillation liquid Ecolite (+) was purchased from ICN (Costa Mesa, CA).

Purification of Enzymes

The LDH suspensions were diluted by addition of 10 mM potassium phosphate buffer at pH 7.5 and dialyzed overnight at 4°C. The LDH was centrifuged to remove any insoluble materials. A pH of 7.5 was chosen for the buffer solution because the enzyme has maximum activity between 7.4 and 7.8.⁹

Assay of Protein Concentration

Protein concentration was measured by the Bio-Rad method.¹¹ Lyophilized BSA was reconstituted to make a 1.4 mg/mL solution in high-performance liquid chromatography (HPLC) water, used as a relative standard. A standard curve was constructed by measuring the absorbance at 595 nm of BSA standard solutions at various concentrations at 25°C. Each LDH concentration was determined as the mean value obtained from 3 different samples.

Effect of PEG Molecular Weight and Concentration on LDH Activity Upon Freeze-thawing

Purified LDH was diluted to a concentration of 25 µg/mL and then added to different molecular weight PEGs to prepare PEG 10% (wt/vol) stock solutions. Lower concentration PEG samples were obtained by diluting the 10% PEG solution with LDH solution containing the 25 µg/mL LDH in buffer. One-hundred microliters of 25 µg/mL LDH solution was transferred to the 200-µL polypropylene Eppendorf microcentrifuge tubes (Eppendorf, Hamburg, Germany). Before freeze-thawing, 10 µL of LDH solution was used to measure the enzyme activity before freezing. The reaction was initiated by the addition of 10 microliters of a 25-µg/mL LDH solution with or without PEGs into the mixture at 25°C.¹ The remaining 90 microliters of LDH solution was frozen by immersing the closed Eppendorf microcentrifuge tubes in liquid nitrogen for 7 minutes. The samples were thawed slowly in the air at room temperature and assayed for residual enzyme activity. The results are expressed as the percentage of the pre-freeze activity recovered after freeze-thawing. Each activity percentage recovered value was calculated from the mean for 3 different samples. Initial experiments showed that PEG does not have absorbance at 340 nM. LDH activity was measured as a decrease in the absorbance at 340 nM using a UV-2401 PC UV-VIS Shimadzu spectrophotometer with 12 cell-positioners and a programmable temperature controller (Shimadzu, Kyoto, Japan).

Effect of PEG Molecular Weights on the Circular Dichroism Spectra of LDH

CD spectra were run on an Aviv CD spectrometer (model 62DS, Aviv, Lakewood, NJ) at 25°C, using a quartz cuvette with a path length of 0.5 cm for measurements in the far UV region. One milliliter, 25-µg/mL LDH samples with and without PEGs were prepared for CD spectra in the same manner as samples for the activity measurements. Every point interval was 0.5 second. The signals were averaged from 4 scans. The raw average signals were represented as molar ellipticity $\times 10^{-3}$.¹²

Effect of PEG Molecular Weights and Concentrations on Gel Filtration Spectrum of LDH

Two-hundred microliters of 200-µg/mL LDH injection solution with and without 1% PEG 8000 and PEG 400 were freeze-thawed as in CD studies. LDH samples before and after freeze-thawing were injected into a Superose 12 column (Amersham Biosciences Corp, Piscataway, NJ). Samples of 200 µL were chromatographed at flow rates of 0.50 mL/min in 20 mM Tris-HCl, pH 7.5, buffer containing 500 mM NaCl. The column eluent was monitored at 280 nm, UV 0.2, using

Pharmacia liquid chromatography controller LCC-500 (Pharmacia Corp, Peapack, New Jersey).

Effects of PEG Molecular Weights and Concentrations on the Migration of LDH in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGEs under reducing conditions were performed on 80 × 80 × 3 mm polyacrylamide gel with 10% stacking gel and 4% desolving gel. LDH samples were prepared the same way as for the activity studies. All samples were fully reduced and denatured at 100°C for 30 minutes. The electrophoresis was performed at a constant voltage of 250 V for 2 hours at room temperature. The gel was stained using Commassie brilliant blue R-250 (Pierce Biotechnology, Inc, Rockford, IL) and destained using a solution of 50% methanol and 10% acetic acid for 1 hour and 5% methanol, 7% acetic acid for 2 hours. The broad Bio-Rad protein standard solution containing several proteins of known molecular weights was also run with the LDH sample.

¹⁴C-PEG 4000 Binding Experiment

The Millipore ultrafree 30 000 NMWL filter units include the upper tube and the lower tube, separated by a selective membrane with molecular weight cutoff of 30 000. The membranes were twice prewashed with 400 µL of 25-mM, pH 7.5, Tris buffer by centrifuging at 2000g for 5 minutes at room temperature. LDH concentrations varied from 25 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL to 200 µg/mL. PEG 4000 concentrations varied from 1% to 0.5% to 0.1%. One-hundred-fifty microliters of LDH solution, 10 µL ¹⁴C-PEG 4000, and 150 µL PEG 4000 solution were added onto the membrane of the upper tubes and mixed well, incubated at room temperature for 1 hour, and freeze-thawed as the activity studies. After incubation or freeze-thawing, the 310-µL solution was centrifuged at 2000g for 5 minutes at room temperature. The free ¹⁴C-PEG 4000 and LDH-bound ¹⁴C-PEG 4000 were separated using Millipore ultrafree 30 000 NMWL filter units. The filtrate including the repeated after-wash water, the retained materials, including the membrane, were transferred to the 4-mL scintillation liquid in the scintillation bottle, respectively, and auto-count of the radioactive disintegrations per minute (dpm) was performed in a Beckman LS 5000 TA series liquid scintillation counter (Beckman Coulter, Fullerton, CA). The bound and unbound ratio was represented by the ¹⁴C-PEG 4000 counts and corrected into the specific activity by the below equations and plotted:

$$\text{Specific activity} = \frac{\text{dpm } ^{14}\text{C-PEG 4000 added}}{\text{nmol PEG added}} \quad (1)$$

$$\text{Nanomole PEG bound} = \text{dpm bound} / \text{specific activity} \quad (2)$$

Ice Crystallization and Eutectic Transition of Protein Formulations by Cryostage Microscope

Four milligrams/milliliter LDH with and without 20% PEG 8000 were formed into a formulation with 2 mg/mL LDH and 10% PEG 8000. Two milligrams/milliliter LDH was obtained by dilution as control. Only a 3- μ L sample was dropped onto the freeze-dry stage of the chamber.

The freezing process was viewed under $\times 100$ and $\times 200$ magnification using a polarized microscope from McCrone Microscopes and Accessories (Westmont, IL). The thermal properties of protein formulations were measured both in images and in digital numbers. Linksys software recorded the dynamic microstructure formulations. The temperatures were recorded. Phase transition events were indicated by dynamic colors and brightness changes. Freezing started at room temperature to -50°C at $10^{\circ}\text{C}/\text{min}$ under atmospheric pressure.

RESULTS

Effect of Different Molecular Weight PEGs on Protection of LDH During Freeze-thawing

Figure 1A shows that incremental PEG concentrations and molecular weights (from 0.0001%, 0.01%, and 1%) in LDH-PEG formulations increased the LDH activity recovery. Low concentrations of PEG 400 failed to protect LDH activity. One percent PEG 400 recovered more than 80% LDH activity. In contrast, PEG 4000 and PEG 8000 at 0.01% showed significant protection for LDH. Figure 1B shows the relationship between PEG 4000 concentration and LDH activity recovery in a semi-log plot. When the concentrations of PEG 4000 increased from 0.0001%, 0.001%, 0.01%, 0.1% to 1%, the activity recovery of LDH increased linearly in the semi-log plot until 100% recovery. More than 1% PEG 4000 was needed for a complete LDH activity recovery. Our experimental results and the results of Lee et al¹³ also suggest that PEG 400 and PEG 8000 slightly activated some enzymes.

Effect of PEG Molecular Weights on LDH Secondary Structures Upon Freeze-thawing

Figure 2A shows that the secondary structure of LDH was partially preserved by 0.1% PEG 400 but completely preserved by 0.1% PEG 8000. Figure 2B shows similar results from 1% PEG 400 vs PEG 8000. The secondary structure of LDH was partially preserved by 1% PEG 400 but completely preserved by 1% PEG 8000. CD spectra were very similar in terms of ellipticity intensities in both formulations of 0.1% and 1% PEG 8000 with the same LDH concentrations. These data show that higher molecular weight PEGs preserved LDH secondary structure better than low molecular weight PEGs.

Figure 3 shows the results from gel filtration studies. LDH

Figure 1. Effect of different molecular weights and concentrations of PEGs on LDH cryoprotection.

with PEG 400 at 1% had the same elution characteristic of the LDH control before freezing. However, the unprotected LDH solution showed a slight height erosion, which indicates a slight concentration decrease. PEG 8000 at 1% showed a slight curve shape modification, which suggests a possible LDH conformational modification. The only peak representing the LDH tetramer shows that unprotected LDH solution did not form into detectable insoluble aggregation or degradation. These results excluded the possibility that aggregation caused the ellipticity changes in CD spectrum.

Effects of PEG Concentrations on LDH Migration in SDS-PAGEs Under Reducing Conditions

Various concentrations of PEG 400 and low concentrations of both PEG 4000 and PEG 8000 had no effects on the migration rate of LDH and standard proteins (data not shown). However, in the presence of 1% PEG 4000 and PEG 8000, both LDH and standard proteins showed faster migration (data not shown). Figure 4 shows the effect of PEG 4000 concentrations on the mobility of freeze-thawed LDH. PEG 8000 within concentrations of 0.1%, 0.4%, 0.6%, 0.8%, and 1% and PEG 4000 within concentrations of 0.1%, 0.4%, 0.8%, 1%, and 2% caused faster LDH migrations in a con-

Figure 2. Effect of different concentrations of PEG 400 and PEG 8000 on LDH secondary structures upon freeze-thawing: (A) Effects of 0.1% PEG 400 and PEG 8000 on LDH secondary structures upon freeze-thawing. In A, from the top to the bottom are Tris control; LDH alone before freezing; LDH alone after freezing; 0.1% PEG 8000 after freezing; 0.1% PEG 400 after freezing. (B) Effects of 1.0% PEG 400 and PEG 8000 on LDH secondary structures upon freeze-thawing. In B, from the top to the bottom are Tris control; LDH alone after freezing; LDH-1% PEG 8000 after freezing; and LDH-1% PEG 400 after freezing.

centration-dependent manner (PEG 8000 data not shown).

Effect of Various Concentration LDH and PEG 4000 on PEG-LDH Binding Upon Freeze-thawing

Figure 5 shows the bound LDH-PEG 4000 as a function of increasing PEG 4000 and LDH concentrations after freeze-thawing. Apparently, at 3 PEG 4000 concentrations of 1%, 0.5%, and 0.1%, increasing LDH concentrations from 25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 150 $\mu\text{g/mL}$ to 200 $\mu\text{g/mL}$ caused a linear increase of the bound LDH-PEG 4000 amount. This result could not be explained if PEG 4000 molecules were largely excluded from LDH molecules and suggests an extensive nonspecific interaction between PEG 4000 and tetrameric LDH.

Effect of PEG 8000 on Ice Crystallization and Eutectic Transition of LDH Solution Under Cryostage Microscope

Figure 6 shows the different freezing process under the cryostage microscope for LDH formulation alone and for LDH-PEG 8000 formulation. PEG 8000 addition lowered the temperature for the ice crystallization event. In the absence of PEG 8000, the LDH solution showed the initiation of ice crystallization at -8.2°C , and the completion of the eutectic transition for the whole matrix at -38.1°C . In the presence of PEG 8000, LDH-PEG 8000 solution showed the initiation of ice crystallization at -19.1°C , and the completion of crystallization for the mixture of LDH-PEG at -50°C . This finding confirmed that PEGs at such a concentration caused

the delayed eutectic behavior for LDH formulations during freezing.

DISCUSSION

Although Timasheff's mechanism was predominant in explaining the protein stabilization phenomena in solution, it may not provide adequate explanation for some proteins in the frozen or solid state. The magnitude of protein-PEG interactions varied from protein to protein in a way that did not correlate with the simple exclude volume scenario.^{14,15} For example, Valine was not a protein stabilizer, although it was preferentially excluded from the lysozyme surface.⁹ Surfactants stabilized proteins by accumulating at the protein interfaces during freezing.¹⁶

Our activity and CD data for PEG 400-LDH formulations showed partial incremental activity and secondary structure protection, and complete protection of PEG 8000-LDH formulations. These data could not be explained if PEG molecules were preferentially excluded from LDH molecules. In Table 1, LDH concentrations held constant at 25 $\mu\text{g/mL}$. According to the exclude volume mechanism, the large molar ratio of PEG 400 to LDH at 36 000:1 suggested larger excluded volumes, more surface exclusion, and larger free energy differences between unfolded and folded states compared with PEG 8000 to LDH at molar ratio 18:1; however, the maximum activity recovery of PEG 400-LDH formulations only reached 85.9%. In contrast, PEG 8000 to LDH molar ratio at only 18:1 showed 90.6% activity recovery of LDH.

Our evidence contradicted the prediction from the preferen-

Figure 3. Effect of PEG molecular weights and concentrations on gel filtration spectra of LDH upon freeze-thawing: (A) 200 μ g/mL LDH freeze-thawed with 1% PEG 8000; (B) 200 μ g/mL LDH freeze-thawed with 1% PEG 8000; (C) 200 μ g/mL LDH alone after freeze-thawing; (D) 200 μ g/mL LDH alone before freeze-thawing; (1) thyroglobulin (bovine, molecular weight 670 000); (2) gamma globulin (bovine, molecular weight 158 000); (3) ovalbumin (chicken, molecular weight 44 000); (4) myoglobin (horse, molecular weight 17 000); and (5) vitamin B-12 (molecular weight 1350). The elution time was represented by fraction numbers of the horizontal axis.

tial exclusion mechanism, which suggested that multiple mechanisms might be at work.

Protein concentration assays and gel filtration studies indicated that the ellipticity intensity changes were caused by secondary structural changes in LDH, and not by LDH concentration changes or aggregation.⁸ In addition, gel filtration and SDS-PAGE studies confirmed that no insoluble aggregates of LDH formed if PEGs protected the LDH during freeze-thawing. In the gel filtration result, a major single peak appeared between the peaks of gamma globulin (bovine, molecular weight 158 000) and ovalbumin (chicken, molecular weight 44 000). This indicated that upon freeze-thawing, LDH (molecular weight 144 000) did not aggregate or degrade. Since the molecular weights of standard proteins ranged from thyroglobulin (bovine, molecular weight 670 000) to vitamin B-12 (molecular weight 1350), any aggregated or degraded proteins with molecular weights within this

range would have shown peaks between thyroglobulin (peak 1) and vitamin B-12 (peak 5). PEG 8000 at 1% showed a slight curve shape modification in the elution curve, which suggested a possible LDH conformational modification, or a possible PEG 8000-LDH binding.

Further, PEG 8000 or PEG 4000 caused faster LDH running in SDS-PAGE, not because they were excluded from LDH molecules, but because they interacted with the LDH molecules.

SDS-PAGE results from Figure 4 show the faster migration of LDH in the presence of increasing concentrations of PEG 4000. Unpublished data from our lab also showed that LDH migration was faster with higher molecular weight PEGs (PEG 8000 > PEG 4000 > PEG 400). These observations were difficult to explain by preferential exclusion mechanism. According to the preferential exclusion hypothesis, PEG 400 or PEG 8000 should not affect the migration of LDH, since the LDH molecular weight and shape should not be affected by some excipients (eg, PEG 400 and PEG 8000) excluded from their surfaces, especially in a concentration-dependent manner.

Apparently, SDS-PAGE results indicated that the charges around LDH-PEG 8000 or LDH-PEG 4000 were higher than the charges around LDH. Possibly PEG-LDH interactions modified the molecular size and shape of LDH, favoring more SDS molecules to LDH surfaces. Thus, more negatively charged LDH-PEG complexes caused faster migration than the less negatively charged LDH. This was also illustrated in an earlier report: proteins with significant carbohydrate content ran as diffuse bands due to many factors, such as heterogeneity of molecular weight, charge, and SDS binding.¹⁷ In the current PEG-LDH samples, while attaching to LDH, PEGs might have similar properties to the carbohydrates causing a diffuse LDH band.

In addition, ¹⁴C-PEG 4000 binding experiments provided direct evidence of extensive binding (interactions) between PEG 4000 and LDH molecules. The binding between PEG 4000 and LDH increased in a concentration-dependent manner with LDH and PEG 4000. When LDH was mixed with PEG 4000 concentrations from 0.1% to 1%, bound/unbound ratios of PEG 4000 exceeded 1:1, which indicated that PEG 4000 protected LDH during freeze-thawing by extensive nonspecific binding. In Figure 5, PEG 4000 did not reach the saturation concentration for LDH binding because the PEG 4000 viscosity increase limited PEG 4000 molecules to pass through the 30 000-NMWL filter membrane.

Nonspecific PEG-protein binding provided a protective coating or shell composed of PEG amphiphilic complexes. This protective PEG coating or shell replaced the protective "hydration shell" in solution to stabilize and segregate LDH molecules during freeze-thawing.

Annunziata et al suggested a direct interaction of PEG with

Figure 4. Effect of PEG 4000 concentrations on freeze-thawed LDH mobility in SDS-PAGE under reducing conditions. The proteins ran from the top to the bottom of the gel: (1) 200 $\mu\text{g/mL}$ LDH before freeze-thawing (control 1); (2) 200 $\mu\text{g/mL}$ LDH after freeze-thawing (control 2); (3) 200 $\mu\text{g/mL}$ LDH with 2% PEG 4000 after freeze-thawing; (4) 200 $\mu\text{g/mL}$ LDH with 1% PEG 4000 after freeze-thawing; (5) 200 $\mu\text{g/mL}$ LDH with 0.8% PEG 4000 after freeze-thawing; (6) 200 $\mu\text{g/mL}$ LDH with 0.4% PEG 4000 after freeze-thawing; (7) 200 $\mu\text{g/mL}$ LDH with 0.1% PEG 4000 after freeze-thawing. LDH mobility was calculated as (migration distance of samples/migration distance of LDH control -1).

Figure 5. Effect of PEG and LDH concentrations on PEG-LDH binding upon freeze-thawing. The bound and unbound ratio was represented by the ^{14}C -PEG 4000 counts in the retained and in the filtrate. Bound PEG was corrected by total PEG amount and the specific activity; mean $\pm$ SD ($n = 3$).

protein surface residues.¹⁸ The weak attractive interactions between PEG and the protein bovine gamma D-crystallin, a protein in the eye lens (γ -D), induced a flattening of the polymer coils near the protein surface and a corresponding reduction of the depletion layer thickness. The liquid-liquid phase transition temperature was increased with PEG concentration

due to the attraction between the protein γ -D molecule and PEG.¹⁸

Heller et al claimed that PEG macromolecules made the water more structured up to 16 water molecules per monomer unit.¹⁹ This structuring of water by PEGs might reduce molecular mobility. Our unpublished data (October, 2000) also indicated that PEGs actually activated LDH activity in solution and under -20°C refrigerator storage. These data suggest that PEG could actively re-orient for the LDH and water molecules, either in solution, or during freezing. The nucleation of ice was effectively reduced. The structured water also decreased the anomalous cooperative hydrogen bonding between water and LDH molecules.^{19,20}

Apparently, crystallization or delayed eutectic behavior of PEGs did not provide cryoprotection of LDH. LDH alone would not form a eutectic. Also, it would not be reasonable to hypothesize that crystallization of PEG would contribute to protection of LDH since, in order to interact with the protein, the stabilizing agent must not be in the crystalline state. PEG 8000 at both concentrations of 0.1% and 1% reached almost 100% activity recoveries. In the absence of PEG molecules, the solute concentration in the non-ice phase could reach values as high as $\sim 75\%$ before a T_g or T_e was reached. Freeze-concentration induced phase separation, which could be detrimental for LDH activity

Figure 6. Ice crystallization and eutectic transition of LDH and LDH-PEG 8000 formulations under cryostage microscope. Left: LDH alone; Right: LDH with PEG 8000.

Table 1. Effect of Different Molecular Weight Polyethylene Glycols at Various Molar Ratios on Lactate Dehydrogenase Activity Recovery Upon Freeze-thawing*

PEG 400/LDH (M/M)	Activity Recovery (% $\pm$ SD)	PEG 4K/LDH (M/M)	Activity Recovery (% $\pm$ SD)	PEG 8K/LDH (M/M)	Activity Recovery (% $\pm$ SD)	PEG 20K/LDH (M/M)	Activity Recovery (% $\pm$ SD)
0	41.4 $\pm$ 0.2	0	41.4 $\pm$ 0.2	0	41.4 $\pm$ 0.2	0	41.4 $\pm$ 0.2
3.6:1	40.4 $\pm$ 0.7	0.36:1	41.6 $\pm$ 0.4	0.18:1	58.5 $\pm$ 0.3	0.0072:1	43.7 $\pm$ 0.4
36:1	36.2 $\pm$ 0.2	3.6:1	61.9 $\pm$ 0.3	1.8:1	75.8 $\pm$ 0.2	0.072:1	70.1 $\pm$ 0.4
360:1	42.7 $\pm$ 0.3	36:1	77.9 $\pm$ 0.4	18:1	90.8 $\pm$ 0.7	0.72:1	92.7 $\pm$ 0.4
3600:1	45.1 $\pm$ 0.7	360:1	80.9 $\pm$ 0.3	180:1	96.8 $\pm$ 0.2	7.2:1	95.8 $\pm$ 0.5
36 000:1	85.9 $\pm$ 0.3	3600:1	87.9 $\pm$ 0.2	1800:1	96.0 $\pm$ 0.3	72:1	98.3 $\pm$ 0.7

*PEG indicates polyethylene glycols; LDH, lactate dehydrogenase; and M/M, molar ratio between PEGs and LDH.

Figure 7. Cryoprotection modeling of oligomeric proteins (LDH) by PEGs: (A) PEG chain interacts with the amino acids of LDH monomers; (B) PEG chain bridges between LDH tetramers.

and secondary structures.²¹ In the presence of PEG molecules, “PEG-ice eutectic structure” was formed with an ice crystallization temperature of -15.0°C for PEG 6000.¹⁰ “PEG-ice eutectic structure” was formed with ice crystallization temperature around -19.1°C for PEG 8000, as shown by Figure 6. Compared with the ice crystallization temperature of -8.2°C in the absence of PEG 8000, PEG 8000 delayed ice crystallization and the eutectic transition of LDH-PEG 8000 formulation. However, when liquid nitrogen was used to flash-freeze the formulations to -192°C , ice crystallization and PEG crystallization occurred rapidly, along with the freeze-concentration and the induced phase separation between ice and the crystallized solutes.

Antifreeze characteristics of PEGs could not explain the LDH cryoprotection. Pulsed nuclear magnetic resonance (NMR) experiments showed a devitrification at -55°C in PEG solutions, which occurred at temperatures above T_g and a rapid drop in proton mobility at -62°C . Upon thawing, PEG crystallization occurred around -50°C , and melting of the crystallization occurred around -20°C to -15°C .²⁰ Regardless of the molecular weights of PEGs, the crystallization temperatures of PEGs were still colder than the temperature of ice crystallization during liquid nitrogen freezing at -192°C . Other excipients, such as dextrans, could depress the freezing points of LDH formulations but did not provide similar cryoprotection to that of PEGs. Mannitol crystallized during freezing but could not provide equivalent cryoprotection for proteins. Therefore, the thermal properties or crystallization

of PEGs could not explain LDH cryoprotection.

Presented here Figure 7 shows the proposed cryoprotection model of oligomeric proteins by PEGs. PEG 8000 of lower concentration provided better protection than PEG 400 of higher concentration, because PEG 8000 had longer chain lengths to interact with LDH molecules, thus PEGs prevented LDH from dissociation or secondary structural changes, as illustrated in Figure 7.

Short-distance preferential binding favors protein unfolding because the binding between proteins and small molecules, such as urea, disrupts hydrophobic interactions in the interior of the proteins. Timasheff’s mechanism to explain small molecular weight stabilizing excipients in solution was not applicable for surfactants or high molecular weight PEGs during freeze-thawing.

CONCLUSION

PEGs protected LDH activity by preserving the secondary structures of LDH. Higher molecular weight PEGs provided more potent cryoprotection to LDH than lower molecular weight PEGs. The higher molecular weight PEGs appear to be more cryoprotective because they have a larger interfacial area with LDH during freeze-thawing. Cryoprotection of PEG 8000 did not correlate with the delayed eutectic characteristic of PEG 8000. The results showed that extensive non-specific interactions between LDH and PEG molecules were protective for LDH activity and secondary structures.

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