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# Effect of polyols on polyethylene glycol (PEG)-induced precipitation of proteins: Impact on solubility, stability and conformation

Vineet Kumar[a], Vikas K. Sharma[b], Devendra S. Kalonia[a,*]

[a] Department of Pharmaceutical Sciences, University of Connecticut, Storrs, CT 06269, United States
[b] Early Stage Pharmaceutical Development, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, United States

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ABSTRACT

Effect of polyols on the solubility of bovine serum albumin (BSA) in the presence of polyethylene glycols (PEGs) was investigated in order to strengthen the understanding of the observed effects of polyols and PEGs on protein properties in solution. Effect of polyols and/or PEGs on the thermodynamic (conformational) stability of BSA was measured using DSC and circular dichroism (CD). Glucose, sucrose, raffinose, glycerol and sorbitol, all reduced the extent of protein precipitation. Solubility of BSA in the presence of ethylene glycol increased in the case of PEG 1450 and PEG 8000, but was unaffected in the case of PEG 400. DSC studies indicated that smaller PEGs have destabilizing influence on protein structure. CD studies showed that smaller PEGs (ethylene glycol) induce subtle unfolding while stabilizing polyols induce subtle compaction. Results show that, effect of polyols on the apparent solubility of the protein correlates with their effect on the thermodynamic stability of the protein, smaller PEGs are not appropriate for estimating the activity of proteins in saturated solutions, and subtle changes in protein conformation can significantly affect protein precipitation. Though smaller PEGs have weak attractive interactions with protein molecules, perturbation of protein structure by PEGs can be balanced by utilizing appropriate stabilizing solutes.



## 1. Introduction

Polyethylene glycols (PEGs) are water-soluble synthetic polymers with a general formula $HO–(CH_2CH_2O)_n–H$ and have been utilized in various applications in the biotechnology industry (Winholz et al., 1976). PEGs are widely used as precipitants and crystallization agents for proteins, and as chemical agents for PEGylation of proteins (Francis et al., 1998; Gilliland and Davies, 1984; McPherson, 1985). Recently, stable formulations of dry protein powders have been developed by using PEG-induced precipitation and vacuum drying (Sharma and Kalonia, 2004).

Mechanism of precipitation of proteins by PEGs has been explained on the basis of the volume exclusion effects and is discussed in detail elsewhere (Atha and Ingham, 1981; Arakawa and Timasheff, 1985). According to this theory, protein molecules are sterically excluded from the regions of the solvent occupied by PEG molecules. As a result, protein gets concentrated and precipitates out as and when its solubility limit is exceeded. Thermodynamically, PEGs increase the chemical potential of the protein until it exceeds that of the pure solid state resulting in the precipitation of the protein molecules. Since steric exclusion of PEG also results in the preferential hydration of the protein, preferential exclusion should help maintain the protein structure. However, several authors have reported that PEGs could have tendency to induce subtle perturbation of the protein's native structure (Sharma and Kalonia, 2004; Atha and Ingham, 1981). Additionally, PEGs have also been shown to decrease the thermodynamic stability of proteins (Farrugia et al., 1999; Farrugia et al., 1997). It has been hypothesized that PEGs interact with protein molecules via hydrophobic interactions and that such interactions are responsible for the observed destabilization of protein structure (Arakawa and Timasheff, 1985). This slight perturbation of protein's native structure by PEGs is disadvantageous and can affect the long term stability of proteins in solution as well as in the solid state.

Sugars such as glucose, sucrose and trehalose and polyols such as glycerol and sorbitol are known to stabilize biological macromolecules in solution (Lee and Timasheff, 1981; Xie and Timasheff, 1997; Gekko and Timasheff, 1981a; McClements, 2002). Sugars and polyols prevent the loss of enzymatic activity, increase the thermal unfolding temperature of proteins and inhibit irreversible aggregation of macromolecules. Lee and Timasheff (1981) have shown that sugars and polyols affect protein properties in solution by a mechanism which is similar to the preferential exclusion

* Corresponding author at: U-3092, School of Pharmacy, University of Connecticut, Storrs, CT 06269, United States. Tel.: +1 860 486 3655; fax: +1 860 486 4998.
*E-mail address:* kalonia@uconn.edu (D.S. Kalonia).

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mechanism of protein precipitation by PEGs. Despite the similarity that both PEGs and polyols are excluded from protein domain, exclusion of sugars and polyols is not completely steric in nature. It is believed that sugars are excluded due to their effect on the interfacial tension (protein–water interface) while polyols are excluded due to solvophobic effects (Lee and Timasheff, 1981; Gekko and Timasheff, 1981b). However, unlike PEGs, sugars and polyols show minimal binding affinity for proteins. Hence these cosolvents are not known to perturb protein structure. Rather, the unfavorable interaction of protein with a sugar results in an increase in the free energy difference between the folded and the unfolded state leading to stabilization and presumably to further compaction of the native state. In fact, indirect evidence of protein compaction in the presence of sugars has been reported in literature. For example, Kendrick et al. (1997) have observed that the rate of hydrogen–deuterium exchange of the peptide backbone of proteins and reactivity of the cystine residue of interleukin slow down in the presence of sugars. We hypothesize that protein precipitation in the presence of sugars may be useful in maintaining the folded state of the protein molecules.

It can be argued from the above discussion that similar to PEGs, sugars should also result in a decrease in the solubility of proteins because of the preferential exclusion effect. However, upon extensive search of literature it was realized that data in this regard is very scarce. Only one report exists wherein authors have investigated the effect of polyols (glucose and sucrose) on the precipitation of proteins by PEGs (Paleg et al., 1984). In this work, it was observed that addition of sugars increased the apparent solubility of proteins. Authors of this article hypothesized that increased solvation of proteins in the presence of sugars results in the observed effects on protein solubility. In one other related work, it was observed that addition of glycine betaine decreased the enhanced rate of albumin coagulation induced by PEG 6000 (Winzor et al., 1992). In this case, stabilization of the folded state of the protein molecules (by glycine betaine) was hypothesized to be the cause of the observed effects.

In this paper, a detailed investigation of the effect of sugars and polyols on the precipitation of bovine serum albumin (BSA) by PEGs has been conducted. Additionally, the effect of polyols and PEGs on structural and thermal stability of proteins has also been studied. The studies were performed in order to get a better understanding of the mechanism by which PEGs and/or polyols affect protein solubility. Furthermore, understanding of such a quaternary system would be helpful during the utilization of PEGs for the precipitation of proteins in their native state. The results obtained are consistent with the steric exclusion mechanism of protein precipitation by PEGs and demonstrate that subtle changes of protein structure induced by PEGs and/or polyols can significantly affect protein properties. The results further show that some polyols and PEGs do have attractive interactions with protein molecules and that these attractive interactions result in the perturbation of protein's native structure. However, structural perturbation by PEGs can be balanced by using appropriate stabilizing solutes. Presented results also show that smaller molecular weight PEGs are not appropriate for estimating the true activity of proteins in saturated solutions.

## 2. Materials and methods

### 2.1. Materials

All buffer reagents and chemicals used were of highest purity grade available from commercial sources and were used without further purification. PEG 400, glucose, sucrose and raffinose were obtained from Fischer Scientific (Fair Lawn, NJ). PEG 1450 was obtained from Spectrum (Gardena, CA). PEG 8000 was obtained from Dow Chemical Co. (Danbury, CT). D-Sorbitol and bovine serum albumin (A0281-5G) were obtained from Sigma (St. Louis, MO). Glycerol and ethylene glycol were obtained from Acros (Geel, Belgium). Buffers were filtered before use.

### 2.2. Solubility studies

Solubility of BSA in the presence of various molecular weight PEGs was obtained as a function of PEG concentration both in the absence and presence of different concentrations of sugars and/or polyols. The solubility studies were conducted at a temperature of 25 °C at a solution pH of 4.76 and an ionic strength of 0.030 M. pH 4.76 was selected as this is close to the p*I* of the protein, and BSA has lowest solubility around its p*I*. 10 mM acetate buffer was used for all studies and ionic strength was adjusted using NaCl. Briefly, BSA was precipitated by mixing appropriate quantities of buffered stock solutions of the protein, PEG, sugar and the buffer to get the desired concentration of the components. The final volume was made up to 1 mL and the concentration of BSA was set at 1 mg/mL. However, for studies that were conducted to compare the effect of glucose, sucrose and raffinose, the concentration of the protein was kept at 2 mg/mL.

The final solutions were mixed well and equilibrated for 2 h. After equilibration, the solutions were centrifuged at 3000 × *g* for 30 min to separate the protein precipitate. Protein solubility was measured by measuring the absorbance of the supernatant at 280 nm and calculating the concentration based on $E^{1\%}$ of 6.67. Since the precipitate so obtained was amorphous in nature, the solubility obtained is referred to as apparent solubility rather than true solubility.

### 2.3. Differential scanning calorimetry studies

A VP-DSC micro-calorimeter from Microcal Inc. (Northampton, MA) was used. All samples were in acetate buffer (10 mM) at pH 4.76 with an ionic strength of 0.03 M. Scans were taken from 10 to 90 °C at a scan rate of 1 °C/min with a pre-scan thermostat of 10 min. A buffer scan was taken before the measurement of the actual sample. Buffer scan was subtracted from the sample scan. A protein concentration of 1.0 mg/mL was used. The analysis was performed using Microcal Origin software supplied by the manufacturer.

### 2.4. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were taken using a Jasco-710 CD spectropolarimeter (Easton, MD). The far UV–CD scans were performed in a 0.05 cm pathlength cell using a protein concentration of 0.25 mg/mL and a scan speed of 50 nm/min from 195 to 260 nm. A total of three spectra were accumulated and averaged. The CD data hence obtained was further analyzed using CD Pro software (http://lamar.colostate.edu/~sreeram/CDPro/main.html) and the SELCON3 program.

## 3. Results and discussion

Fig. 1 shows the effect of PEG 400, PEG 1450 and PEG 8000 on the solubility of BSA at pH 4.76 and an ionic strength of 0.03. Two significant observations can be made from this figure. First, an increase in the concentration of PEG results in an increase in the amount of the precipitated BSA. Second, the higher is the molecular weight of PEG, the greater is its effectiveness to precipitate the protein out of the solution. These results are consistent with the observations that have been reported in literature (Bhat and Timasheff, 1992). Such observations have been explained on the basis of the theory of steric exclusion. An increase in the size of PEG results in an increase in the

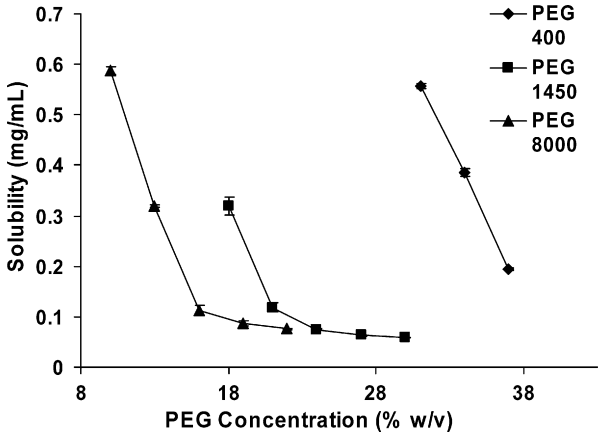


**Fig. 1.** Effect of different molecular weight PEGs on the apparent solubility of BSA at pH 4.76 and an ionic strength of 0.03 M (error bars are standard deviations).

volume of the solution from which proteins have to be excluded. A greater increase in the chemical potential of the protein in the presence of the higher molecular weight PEGs thus results in the observed phenomenon.

Fig. 2 shows the effect of sorbitol on the precipitation of BSA by PEG 1450. Addition of 15% sorbitol increased the apparent solubility of BSA. Such effects of sorbitol on the apparent solubility of BSA cannot be explained on the basis of the preferential exclusion theory. Since both PEGs and polyols are preferentially excluded (and hence increase the chemical potential of proteins), addition of sorbitol should have resulted in a decrease in the observed solubility. However, it should be noted that the present quaternary system is not a simple system. Such a system could have additional interactions such as those between PEG and polyol that may result in the observed effects on protein solubility.

In order to obtain a better understating of the mechanism of the increase in the apparent solubility of the protein by sorbitol, we investigated the effect of equimolar quantities of glucose (a monosaccharide), sucrose (a disaccharide composed of glucose and galactose units) and raffinose (a trisaccharide composed of glucose, galactose and fructose units) on the solubility of BSA. Fig. 3 shows a plot of solubility of BSA versus the partial molar volume of the different sugars that were tested. Clearly, the solubility of BSA increased with an increase in the molecular weight of the polyol.

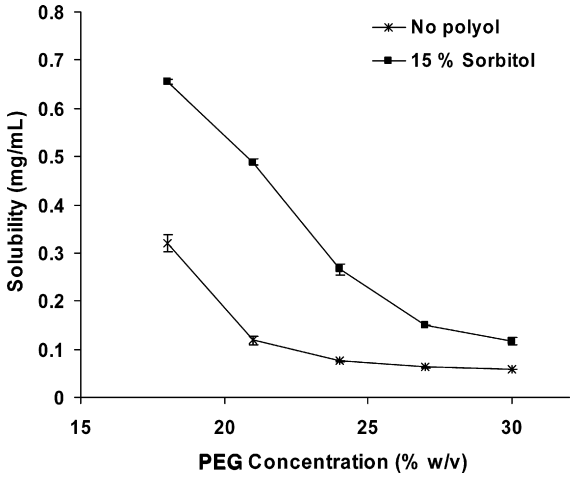


**Fig. 2.** Effect of sorbitol (15%, w/v) on the increase in the apparent solubility of BSA in the presence of PEG 1450 at pH 4.76 and an ionic strength of 0.03 M (error bars are standard deviations).

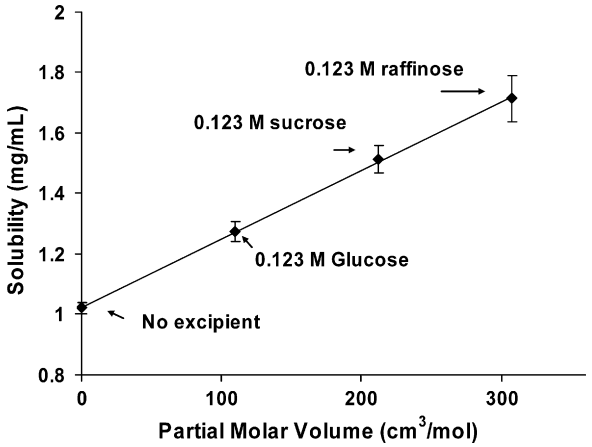


**Fig. 3.** Effect of glucose, sucrose or raffinose at a concentration of 0.123 M on the increase in the apparent solubility of BSA in the presence of 15% (w/v) PEG 1450 at pH 4.76 and an ionic strength of 0.03 M and the relationship of the relative increase in solubility with the partial molar volumes of the corresponding sugars.

However, it is also clear from this figure that the solubility increase would remain same if the polyols are used at equal weight percentage of the solution. Since all three polyols that are used here have been shown to provide nearly equivalent thermodynamic stability to proteins in solution (at equal wt.% of the solution) (Back et al., 1979), the increase in the solubility of proteins by stabilizing solutes seems to be related to the stabilization effect provided by polyols.

Additional studies were performed with polyols, sorbitol, glycerol and ethylene glycol that have been reported to provide differential thermodynamic stability to proteins (Back et al., 1979). Literature data show that of the three polyols, sorbitol provides the best thermodynamic stability to proteins in solution. Glycerol also acts as a stabilizer but is less effective than sorbitol in this regard (Back et al., 1979). Ethylene glycol on the other hand has been shown to decreases the thermal stability of proteins. Fig. 4(A–C) shows the effect 15% (w/v) concentration of these polyols on the apparent solubility of BSA at pH 4.76 in the presence of PEG 400, PEG 1450 and PEG 8000, respectively. Several observations can be made from Fig. 4. All polyols increased the apparent solubility of BSA. Sorbitol had the greatest effect on solubility. Glycerol and ethylene glycol followed in that order. These results clearly show that, in general, the effect polyols produce on the thermodynamic stability of proteins and on the apparent solubility of proteins, are correlated. However, an intriguing observation was the increase in protein solubility by ethylene glycol, which is otherwise known to destabilize proteins. This presumably is due to the attractive hydrophobic interactions that occur between smaller molecular weight PEGs (ethylene glycol can be considered as the lowest unit of PEG) and protein molecules.

In order to further investigate the correlation between stability and solubility, thermal unfolding studies of BSA were carried out.

**Table 1**
Midpoint transition temperatures of the thermal unfolding of BSA as measured by DSC in the presence and absence of different excipients

| BSA (pH 4.76, IS 0.03) | $T_m$ (°C)[a] |
|---|---|
| No additive | 56.0 ± 0.7 |
| 10% (w/v) PEG 400 | 52.3 ± 0.5 |
| 10% (w/v) PEG 1450 | 54.2 ± 0.6 |
| 10% (w/v) PEG 8000 | 56.0 ± 0.4 |
| 10% (w/v) PEG 400 + 15% (w/v) sorbitol | 56.7 ± 0.6 |
| 10% (w/v) PEG 400 + 15% (w/v) glycerol | 53.8 ± 0.5 |
| 10% (w/v) PEG 400 + 15% (w/v) ethylene glycol | 51.0 ± 0.4 |

[a] Error bars are standard deviations ($n = 3$).

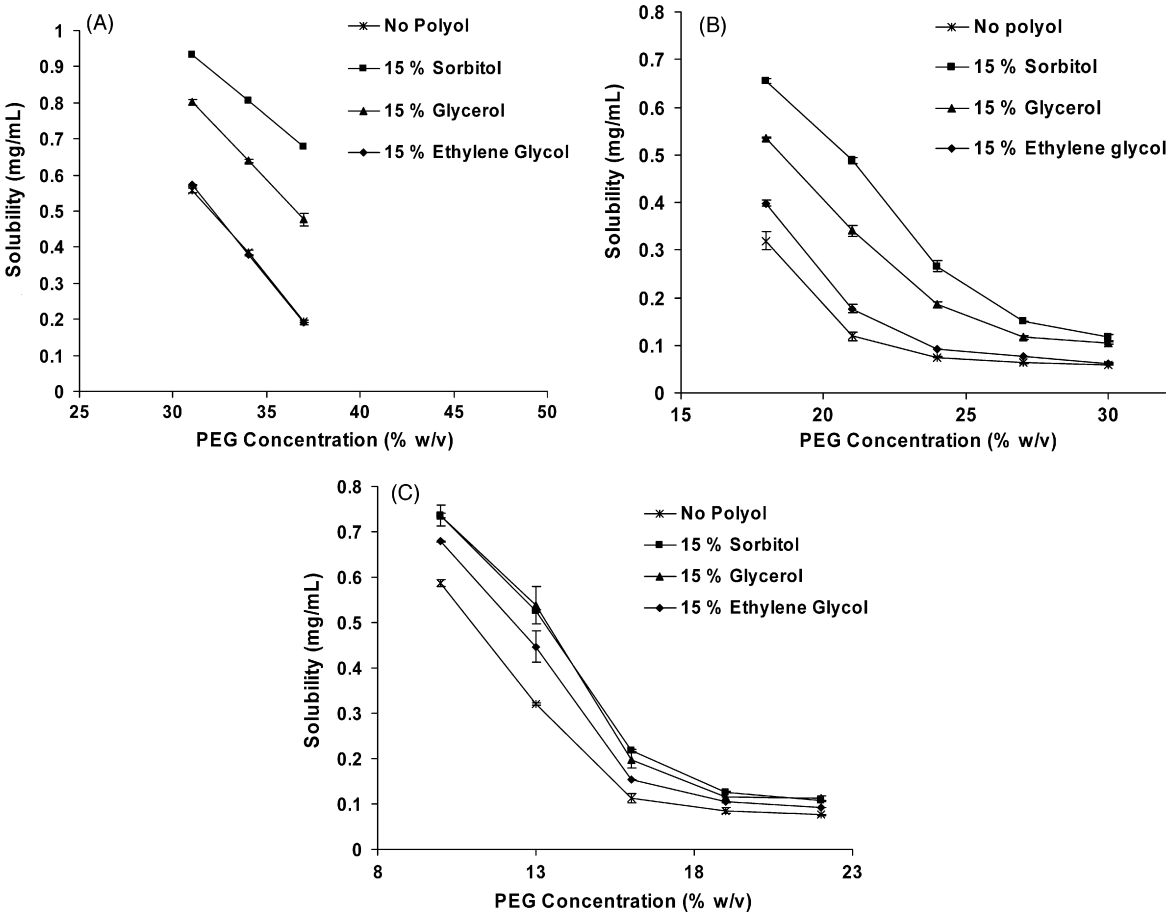


**Fig. 4.** Effect of 15% (w/v) sorbitol, glycerol or ethylene glycol on the increase in the apparent solubility of BSA at pH 4.76 and an ionic strength of 0.03 M in the presence of different PEGs (A) PEG 400, (B) PEG 1450 and (C) PEG 8000. Note the relative increase by different polyols.

Table 1 shows the transition midpoints (referred to as apparent $T_m$ since the transition was irreversible and resulted in protein aggregation) of BSA in the absence and presence of different excipients. $T_{onset}$ and $T_m$ showed good correlation (data not shown). $T_m$ of BSA in the absence of any excipient was found to be 56.0 °C. The $T_m$ was found to be 52.3, 54.2 and 56.0 in the presence of 10% (w/v) PEG 400, PEG 1450 and PEG 8000, respectively. It is clear that PEG 400 decreased the stability of BSA to the greatest extent followed by PEG 1450. 10% PEG 8000 on the other hand did not destabilize the protein. These results support the hypothesis that smaller PEGs have greater tendency to interact with the non-polar patches of the protein molecules. Thermal unfolding studies done by Farrugia et al. (1997) also show that lower molecular weight PEGs have greater destabilizing influence on protein structure. Table 1 further shows that addition of 15% (w/v) sorbitol effectively prevented the destabilization of BSA (in the presence of PEG 400). Glycerol also prevented the destabilization of BSA albeit to a lower extent then sorbitol. Addition of 15% ethylene glycol on the other hand had an opposite effect and resulted in a decrease of the $T_m$. Yet again, the effect of sorbitol, glycerol and ethylene glycol on the stability of BSA seems to follow the same order as that observed for the effect of these polyols on the apparent solubility of the protein.

Effect of polyols and PEGs on the secondary structure of BSA was studied by utilizing far UV–CD spectroscopy. Fig. 5 shows the effect of 10% (w/v) concentration of PEG 400 on the far UV–CD spectra of BSA in the absence and presence of 15% (w/v) concentrations of sorbitol, glycerol or ethylene glycol. For comparison, the spectrum of BSA in the absence of any excipients is also shown. Although, the observed differences are subtle, it is clear that addition of PEG resulted in a slight loss of the net negative molar ellipticity of BSA. Addition of sorbitol or glycerol on the other hand resulted in a somewhat increase of the observed negative molar ellipticity. A decrease

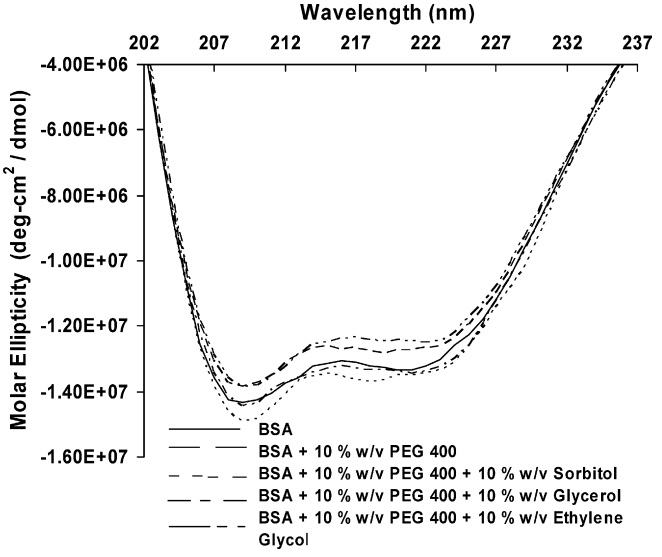


**Fig. 5.** Effect of different excipients on the perturbation or compaction of the native (secondary) structure of BSA at pH 4.76 and ionic strength of 0.03 M as measured by far UV–CD spectroscopy.

**Table 2**
Percent alpha helical content of BSA in the absence and presence of different excipients as determined by analyzing the CD data utilizing CD Pro analysis software and the SELCON3 program

| BSA (pH 4.76, IS 0.03) | Helical content (%) |
|---|---|
| No additive | 66 |
| PEG 400 | 71 |
| PEG 400 + sorbitol | 76 |
| PEG 400 + glycerol | 60 |
| PEG 400 + ethylene glycol | 61 |

of the negative molar ellipticity is an indicator of the loss of the corresponding secondary structural elements. An increase of the molar ellipticity on the other hand suggests a compaction of the folded structure of the molecule (Lee and Timasheff, 1981; Kendrick et al., 1997). In order to gain a better understanding of the effect of excipients on the helical content of BSA, the CD data were further analyzed using CD Pro software (SELCON3 program). Although, the results that were obtained (Table 2) do not show a clear trend (relative to that obtained by DSC experiments), it is evident that the addition of strong stabilizer i.e. sorbitol did result in an increase in the alpha helical content of the protein molecule. Addition of ethylene glycol (basic unit of polyethylene glycol) on the other hand resulted in a decrease in the helical content of the protein.

Though, it is anticipated that steric exclusion of PEG from protein domain should shift the equilibrium between the folded and the unfolded states of the protein towards the more compact folded state (few reports exist in literature that provide data in this regard), spectroscopic and thermal studies clearly indicate that attractive interactions between protein and PEG push the protein towards slightly expanded native state. It has also been shown in literature that the increase in the chemical potential of protein by PEG (and hence protein precipitation) is dependent on the area of protein molecule exposed to the solvent (excluded volume is dependent on the size of the protein molecules in question) (Atha and Ingham, 1981; Arakawa and Timasheff, 1985). Since the increase in the chemical potential of BSA by PEGs is much higher than a similar increase by comparable molecular weight polyols ((McClements, 2002; Bhat and Timasheff, 1992), a decrease in the volume of the protein molecules induced by sugars can result in the observed increase in the apparent solubility of the protein. Observed phenomenon can be better explained using a hypothetical thermodynamic box (numbers are also hypothetical) as shown in Fig. 6.

It is evident from the discussion and results presented above that the effect of polyols on the stabilization of proteins and the effect of polyols on the precipitation of proteins by PEGs are strongly correlated. However, the reason for the increase in apparent solubility of BSA by ethylene glycol still remains unclear. It is intriguing that PEG (a polymer of ethylene glycol) and ethylene glycol produce opposite effects on the apparent solubility of BSA. In order to get a deeper understating of the mechanism of the observed effect of ethylene glycol, some of the data from Fig. 4 was re-plotted as shown in Fig. 7. Effect of 15% concentrations of sorbitol, glycerol and ethylene glycol on the solubility of BSA in the presence of PEGs is shown in Fig. 7. It should be noted that the increase in the apparent solubility of BSA by polyols is plotted under the solution conditions when the solubility of BSA in the presence of either of the three PEGs, PEG 400, PEG 1450 or PEG 8000, was 0.32 mg/mL (in the absence of polyol, the chemical potential of the protein in the presence of either of the PEGs is proportional to the observed solubility and hence the potential is equivalent at equivalent solubilities). The observations as discussed under are thus independent of the choice of the base solubility i.e. 0.32 mg/mL. Several observations are apparent from this figure. The highest increase in solubility of BSA by sorbitol or glycerol is observed in the presence of PEG 400. The solubility increase due to sorbitol or glycerol decreases with an increase in the molecular weight of PEG. The highest increase in solubility due to ethylene glycol on the other hand is observed in the presence of PEG 8000. Careful observation of the data in Fig. 7 shows that the increase in the apparent solubility shows a gradual change in going from sorbitol to ethylene glycol. The observed effects of sorbitol, glycerol and ethylene glycol can be explained on the basis of DSC and CD studies discussed earlier. Sorbitol and glycerol produce the greatest effect in the presence of the most destabilizing PEG. This is because; sorbitol and glycerol being stabilizers shift the equilibrium towards the compact folded state. Ethylene glycol on the other hand produces the maximum effect when the protein species are most compact, i.e. in the presence of PEG 8000. Since lower molecular weight PEGs (such as PEG 400) have tendency to interact with proteins, it can be argued that ethylene glycol would also interact with protein molecules and that this interaction of ethylene glycol with the protein would be greatest (and hence more evident) in the presence of the higher molecular weight less interacting PEG 8000. This is a new finding and suggests that lower molecular weight PEGs and ethylene glycol can in fact act as solubilizers of hydrophobic molecules.

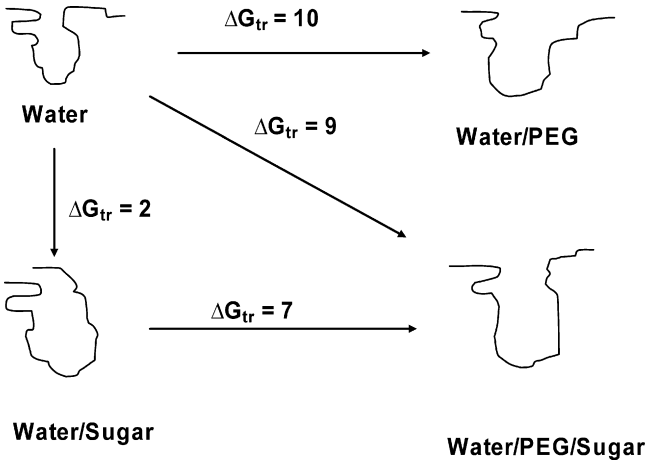


**Fig. 6.** Hypothetical thermodynamic box showing the transfer free energies of the protein from water to solutions containing different excipients demonstrating how the presence of polyols is anticipated to affect the chemical potential of protein (and hence its solubility). Note the observed changes in protein structure.

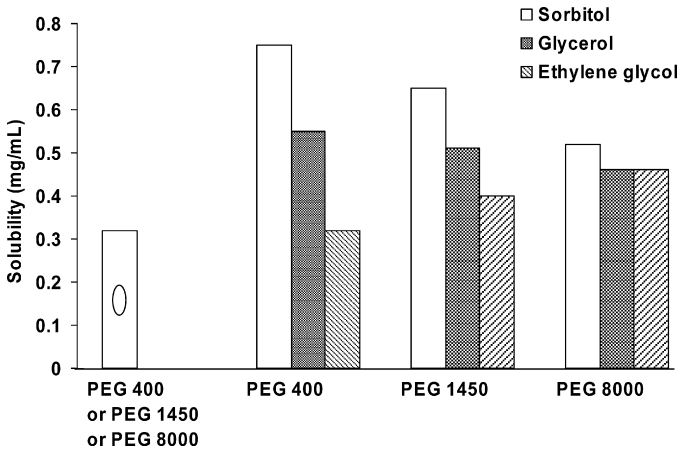


**Fig. 7.** Differential increase in the apparent solubility of BSA at pH 4.76 and an ionic strength of 0.03 M in the presence of different PEGs by various polyols (15% w/v). The solubility in the absence of polyols but in the presence of either of the PEGs (PEG 400, PEG 1450 or PEG 8000) as shown by the first column was 0.32 mg/mL.

## 4. Conclusions

The studies provided herein show that the effect of polyols on proteins' apparent solubility correlates with their effect on the thermodynamic stability of the protein. It is also shown that smaller molecular weight PEGs are not appropriate for estimating the true activity of proteins in saturated solutions and that subtle changes in protein conformation can significantly affect protein precipitation. Though, smaller PEGs and some polyols have weak attractive interactions with protein molecules, perturbation of protein structure by PEGs can be balanced by utilizing appropriate stabilizing solutes.

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