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Modulation of the thermodynamic stability of proteins by polyols: Significance of polyol hydrophobicity and impact on the chemical potential of water

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ABSTRACT

The exact mechanism of the modulation of chemical potential of proteins by polyols is not yet well understood. Present study investigates the role of hydrophobicity of polyols, and their impact on water activity and/or surface tension, in determining their stabilization/destabilization potential. Results with ribose and methyl-glucose show that the enhanced stability of proteins is not mediated via the effect on interfacial tension, a hypothesis that has so far been restricted to glycerol. An exemplary correlation between thermodynamic stabilization ($\Delta G_{f\text{-}uf}$), and polyol osmolality, confirms/generalizes the prominent role of water activity in the observed stabilization effects. Results show that even seemingly hydrophilic sugars such as deoxy-ribose can interact favorably with proteins, suggesting that properties other than the presence of hydroxyl groups also contribute to the net effect of polyols. We demonstrate that the hydrophobicity index of polyols and the net stabilization effect afforded to proteins have an excellent inverse correlation. These studies show that the weak hydrophobicity of polyols is critical for promoting their interactions with proteins, weakening of the hydrophobic forces within the protein interior and counteracting the polyol induced-solvent mediated stabilization effect.

1. Introduction

Sugars and polyols are widely used to impart thermodynamic stability to biological macromolecules in solution (Back et al., 1979; Lee and Timasheff, 1981; Uedaira and Udeaira, 1980). The general observations are that these additives prevent the loss of enzymatic activity (Bradbury and Jakoby, 1972), increase the thermal unfolding temperature and inhibit irreversible aggregation of proteins (Chi et al., 2003). To ensure the safety, efficacy and elegance of the aqueous formulations of proteins, it is important that the molecules be kept in a non aggregated state, and hence polyols are widely used as excipients in liquid formulations to enhance the shelf life of the active and non immunogenic species of a biological macromolecule.

The mechanism of stabilization of proteins by polyols has been primarily explained on the basis of the theory of preferential exclusion and is discussed in detail elsewhere (Gekko and Timasheff, 1981a,b; Lee and Timasheff, 1981; Xie and Timasheff, 1997a,b). However, the reason for the molecular origin of such preferential exclusion is still under debate. Sugars such as sucrose and trehalose increase the surface tension of water and are believed to be excluded from the protein domain because they increase the free energy at the protein water interface (Kaushik and Bhat, 1998; Lin and Timasheff, 1996). Glycerol on the other hand lowers the surface tension of water and has been hypothesized to preferentially hydrate proteins by enhancing the solvent ordering around the hydrophobic groups of the protein molecules (Gekko and Timasheff, 1981a; Kaushik and Bhat, 1998; Tiwari and Bhat, 2006). Any increase in the hydrophobic surface area of proteins on unfolding would thus be rendered even more unfavorable in the presence of glycerol. Liu et al. used molecular dynamic simulations and observed good correlation between protein stabilization effect at equivalent molarity of polyols and their molecular volumes (Liu et al., 2010). Additional work by these authors points to the role of indirect interactions such as ordering of water structure by polyols. Such ordering results in a decrease in the entropy of water present in the first hydration shell around the protein molecules. Greater entropic decrease on unfolding thus shifts the equilibrium towards the folded state. It is however unclear from the study as to why different polyols behave differently (in terms of stabilization effect) when used at equivalent weight percentages of the solution. For example, why is 20% (w/v) sorbitol a better stabilizer than 20% (w/v) glycerol.

The role of polyols on the surface free energy of water (surface tension/interfacial tension) in governing the extent of polyol exclusion, and hence determining protein stability, remains unclear.

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Recently, several authors have stressed on the importance of the effect of polyols on the chemical potential of water in determining their stabilization potential (Courtenay et al., 2000; Hammou et al., 1998; McClements, 2002; Miyawaki, 2007; Parsegian et al., 2000). Miyawaki (2007) investigated the difference in the hydration of the folded and unfolded states of the protein molecules and the relationship between this differential hydration and the observed effects of sugars on the thermal stability of proteins. Since, the number of bound water molecules was much higher for the unfolded state of the protein; it was hypothesized that sugars could produce the observed stabilization effect by merely affecting the chemical potential of water. However, the work was limited to a few cyclic sugars (no linear polyols were used) and one protein, and no explanation was given as to why different sugars provide differential stability at equivalent water chemical potential. Support for water activity hypothesis also comes from the work published by Parsegian et al. (2000) wherein, it was demonstrated that both osmotic stress (due to the affect of polyols on the chemical potential of water) and preferential hydration have the same thermodynamic origin (Preisler et al., 1995). It should thus be realized that an essential consequence of the effect of decrease in the chemical potential of water by polyols, and hence an increase in the chemical potential of proteins, is the exclusion of polyols from protein domain. The exact relationship between hydration water and preferential hydration hitherto remains under debate.

Timasheff and coworkers have argued that preferential exclusion effect can get compensated by preferential binding effect leading to a decrease in the stability parameters. Although, the origin of weak attractive interactions between molecules such as salts and proteins is not difficult to understand, the origin of the weak attractive interactions between highly polar trehalose, which has been observed to decrease the chemical potential of the protein under certain solution conditions (Xie and Timasheff, 1997a), and proteins remains unclear. Bolen et al. found that the transfer free energies of the side chains of some of the amino acids from water to sucrose solution are negative indicating favorable interactions between these amino acids and sucrose (Auton and Bolen, 2007; Bolen, 2004; Liu and Bolen, 1995; Qu et al., 1998).

Several polyols including sucrose have been observed to increase the solubility of proteins in solution (Antipova and Semenova, 1996; Conti et al., 1997; Farnum and Zukoski, 1999), an observation which theoretically counteracts exclusion. Since polyols increase the chemical potential of the protein molecules, it is anticipated that preferentially excluded co-solvents will favor the solid state over the dissolved state as this would minimize the area of the protein molecules that is exposed to the co-solvent environment. Further support to the observed effects of polyols on protein solubility (i.e. the increase in solubility by polyols) comes from the determinations of the second virial coefficients (B_{22} or A_2) of proteins in solution (Bajaj et al., 2004; Bonnette et al., 1999; Tessier et al., 2002). Experimental determination of B_{22} by different techniques such as light scattering and ultracentrifugation has shown that polyols in general decrease protein–protein attractive interactions in solution (Valente et al., 2005; Weatherly and Pielak, 2001).

A positive impact of a polyol on the stability of the protein may not necessarily translate into a positive impact on the solubility of the protein. In order to obtain maximum benefit out of the added excipient, careful optimization of the stability and solubility characteristics of the protein in the presence of the polyol is important. In the present market scenario, wherein monoclonal antibodies (mAbs) and mAb-like proteins constitute a major portion (>60%) of the total protein drugs currently in clinical and preclinical testing, the utility of sugars becomes even more important. Since, many diseases that are being targeted by these relatively low potency proteins are chronic and require frequent dosing, providing at home-outpatient administration option to patients by the subcutaneous or intra-muscular route is the desired way of delivery to increase patient compliance. The volume limitation (<1.5 ml) presented by these delivery routes, however, necessitates that the antibody and Ig-like therapeutics be formulated at high concentrations (>100 mg/ml). Increased aggregation propensity at high concentrations thus necessitate that excipients be used to minimize instabilities and hence increase the shelf life of the concerned biological macromolecule. Concerns however arise as development of high concentration aqueous formulations pose solubility issues for some of these molecules.

Despite the presence of a vast amount of literature, the mechanism of protein stabilization/destabilization by polyols and the impact of polyols on protein solubility remain unclear. This lack of understanding prevents the best and the most productive utilization of an optimum polyol and/or sugar in the liquid formulation of a biological macromolecule. The present study was aimed towards answering the following fundamental questions: (1) what is the relationship between the effect of polyols on the surface tension of water (or on the activity of water) and their stabilization potential? (2) What is the nature and extent of the weak attractive interactions, if any, between polyols and proteins, and how may these interactions impact the stabilization effect and the solubility of the protein?

2. Materials and methods

2.1. Materials

All buffer reagents were of the highest purity grade available from commercial sources and were used without further purification. Ethylene glycol, glycerol, L-tyrosine, L-phenylalanine, L-tryptophan and biphenyl were obtained from Acros (Geel, Belgium). 2-Deoxy-D-ribose, D-ribose, alpha-methyl D-glucoside and sucrose were obtained from Fischer Scientific (Fair Lawn, NJ). Trehalose dihydrate, maltose monohydrate, lysozyme and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (St Louis, MO). IFNα2a was donated generously by Hoffmann-La Roche and was supplied as 1.6 mg/ml solution in 25 mM acetate buffer, containing 120 mM NaCl (total ionic strength = 142 mM). The protein was stored at −80 °C in vials and each vial was thawed before use.

2.2. DSC studies

A VP-DSC micro-calorimeter from Microcal Inc. (Northampton, MA) was used. Studies with lysozyme were conducted at pH 6.5 and 0.006 M ionic strength. The concentration of lysozyme was kept constant at 0.38 mM. Studies with BSA were conducted at pH 6.5 and 0.001 M ionic strength. Use of such low ionic strength was essential in order to ensure minimal interference from salt effects. Scans were taken from 10 °C to 90 °C at a scan rate of 1 °C/min with a pre-scan thermostat of 10 min. The instrument was allowed to run through the night with multiple scans of the buffer in both the sample and the reference compartments before the measurement of the actual sample. Buffer scan was subtracted from the sample scan and corrected for baseline. Buffers and samples were filtered through a 0.2 μm Millipore (Bradford, MA) syringe filters just before degassing. All samples and buffers were degassed for 5 min in Thermovac, the degassing accessory from Microcal Inc. (Northampton, MA), before their introduction into the sample holders of the DSC instrument. Thermodynamic parameters were obtained by fitting to a two state model. Good post transition baselines were obtained for both lysozyme and BSA. Lysozyme transitions showed good reversibility in the absence or presence of polyols (Fig. 1). The transition in the presence of deoxy-ribose was only partially reversible (40% enthalpy recovery in the rescan). The transitions under all

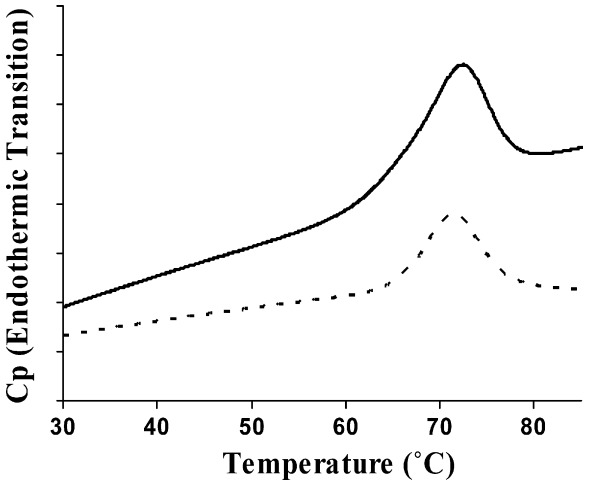


Fig. 1. The thermal transition of lysozyme in the absence of polyol at pH 6.5 and an ionic strength of 0.006 (raw data) at a concentration of 0.38 mM. Solid line represents the original scan and the dashed line shows reversibility following cool down and a prescan thermostat of 30 min.

solution conditions were hardly influenced by heating rates of slower than 60 °C/h allowing equilibrium analysis (Gekko, 1982; Sanchez-Ruiz et al., 1988). The effect of concentration of the protein on T_m was also insignificant.

2.3. Surface tension measurements

Surface tension of sugar solutions was measured on a KRUSS tensiometer (Hamburg, Germany) using a Du-Nouy ring. All measurements were made at a temperature of 25 ± 1 °C. The volume of the sample utilized was 30 ml. The method uses a platinum ring of known geometry. The principal of the instrument depends on the fact that the force necessary to detach the platinum ring immersed at the surface or the interface is proportional to the surface or interfacial tension. This force which is necessary to pull the ring is measured.

$$\gamma = \gamma^* \times F = \left(\frac{P}{l_b}\right) \times F \quad \text{where } l_b = 2\prod(R_i + R_a) \tag{1}$$

where γ = absolute surface tension value, γ^* = measured surface tension value, F = correction factor, P = maximum force at the ring, R_i = inner ring radius and R_a = outer ring radius.

2.4. Water activity and osmolality

Water activity is a useful parameter and can be used as a measure of potential reactivity of water molecules with solutes. In practice one can easily determine water activity of binary solutions. Any of the known colligative properties such as freezing point depression can be utilized for the calculation of the water activity in the presence of known concentration of the solute. Widespread data is present in literature that shows the effect of polyols on the activity of water, and hence we found no need to pursue the same (Comesana et al., 2001a,b; He, 2006; Ninni et al., 2000). The data present in literature was used for the calculations of the osmolality of the solute (Comesana et al., 2001a,b; Ninni et al., 2000). Relation between water activity and milliosmolality can be represented by the following equation (Cazier and Gekas, 2001; Courtenay et al., 2000).

$$\text{mOsm} = \frac{-10^6 \ln a_w}{M_w} = \left(\frac{10^6}{M_w}\right) \ln \frac{P^\circ}{P} \tag{2}$$

wherein a_w is water activity, M_w is the molecular weight of water and P° and P are the vapor pressure of pure water and solution, respectively.

2.5. Hydrophobic surface area

The surface areas of polyols were calculated using the computer program Hyperchem (Aurora, CO). The program utilizes atomic coordinates obtained from X-ray data. For polyhydroxy alcohols, the lowest energy conformations of the molecules were utilized for the surface area calculations. It was found that of all the possible conformations, the molecules that had the hydroxyl groups in *trans* positions had the lowest energies. For the purpose of calculations, only the water accessible surface area was taken into account (water accessible surface area is that area which is exposed to the environment and can come in contact with water molecules). Hydrophobic surface area shown in this paper is thus simply the area that is accessible to water and occupied by carbon and hydrogen atoms of the molecule. For sugars, the net hydrophobic or hydrophilic surface areas were calculated by averaging the contribution from each anomer. For example, in solution, glucose exists as a mixture of α-D-glucose (36%) and β-D-glucose (64%), and hence these percentages were used for the calculation of the average surface area. Anomer percentages have been reported previously (Miyajima et al., 1988).

2.6. Solubility studies

No effort was made to measure the effect of sugars on the extinction coefficients of the small molecules and/or the proteins (absolute quantization of the results was not intended).

2.6.1. Solubility of biphenyl

Solubility of biphenyl was measured at pH 6.5 (ionic strength 0.006 M) in the presence and absence of different sugars and/or polyols. For this purpose, excess of biphenyl (1 mg) was taken in 2 ml screw cap vials and 1.8 ml of the appropriate aqueous or polyol solution was added to the vial. The vials were sealed with Teflon and parafilm and kept on a shaker for 24 h at 25 °C. Extension of the shaking period to greater then 24 h did not affect the measured solubility.

Solubility was measured by reverse phase high performance liquid chromatography (RP-HPLC). The studies were performed by using a 30 nm, 250 × 2.1 mm inner diameter Vydac C_{18} column, 218TP54 (Grace Vydac, Hesperia, CA) attached to a UV detector with wavelength set at 250 nm. The mobile phase was 70% acetonitrile in water with 0.1% trifluoroacetic acid. An isocratic elution with flow rate 1 ml/min was used for quantification. A standard curve for biphenyl in acetonitrile was prepared by utilizing the above mentioned procedure. Biphenyl eluted out at 6 min. The column was equilibrated for 30 min prior to the measurements.

2.6.2. Solubility of IFNα2a

Solubility of a well characterized protein IFNα2a (Klaus et al., 1997; Sharma and Kalonia, 2003) was measured at pH 6.5 (ionic strength 0.006) in the presence and absence of different sugars and/or polyols. This pH was selected as IFNα2a is reported to have lowest solubility between pH 6.0 and 7.0 (which is also the pI of the protein) (Sharma and Kalonia, 2004). IFNα2a that was initially present at pH 5.0 was extensively dialyzed against the desired buffered solution by utilizing 5000 D cutoff Amicon ultracentrifuge tubes (Fischer Scientific, Fair Lawn, NJ). Repeated dialysis of the excess protein at the pI results in the precipitation of the protein (a clear indication that solubility limit has been reached). The suspension (solution with the precipitated protein) was taken out from the centrifuge tubes and 1 ml of the suspension was transferred to 2 ml

Eppendorf vial (Fischer Scientific, Fair Lawn, NJ). These Eppendorf vials were then shaken for 24 h at 25 °C. Extension of the shaking period to greater than 24 h did not affect the measured solubility. After 24 h, protein solutions were centrifuged at 5000 rpm for 30 min to separate the undissolved protein. Protein solubility was obtained by measuring absorbance of the supernatant at 280 nm (0.96 for a 1 mg/ml solution).

2.6.3. Solubility of amino acids

Solubility of tryptophan, tyrosine and phenylalanine was measured in the aqueous solutions of sugars and polyols. For this purpose, excess of amino acids (30 mg for tryptophan, 2.5 mg for tyrosine and 40 mg for phenylalanine) were taken in 2 ml screw cap vials and 1 ml of the appropriate aqueous or polyol solution (pH 6.5) was added to the vial. The vials were sealed with Teflon and parafilm and kept on a shaker for 24 h at 25 °C. Extension of the shaking period to greater then 24 h did not affect the measured solubility.

Solubility was determined by UV-absorbance at wavelengths of 280 nm, 274.75 nm and 257.50 nm for tryptophan, tyrosine and phenylalanine, respectively. The extinction coefficients for tryptophan, tyrosine and phenylalanine are reported in literature and are 5502 cm^{-1}/M, 1302 cm^{-1}/M and 195 cm^{-1}/M at the above mentioned wavelengths.

The transfer free energies of the amino acids from water to polyol solutions were calculated by utilizing the following equation (Liu and Bolen, 1995).

$$\Delta G_{tr} = RT\left(\frac{C_w}{C_p}\right) + RT\ \ln\frac{\gamma_w}{\gamma_p} \quad (3)$$

wherein C_w and C_p are the molar solubilities of the amino acid in water and polyol solution, respectively. Similarly, γ_w and γ_p are activity coefficients of the amino acid in water and polyol solution, respectively. However, since the determination of the activity coefficient term is extremely difficult, only the first term on the right hand side of the above mentioned equation was used for the calculations of the transfer free energies. It should be noted that although disregard of activity coefficient term may not be yield the best results, it is a widely followed procedure for such calculations (Liu and Bolen, 1995; Nozaki and Tanford, 1963). Our method for the calculations of the transfer free energies is similar to what has been used previously by (Liu and Bolen, 1995). The transfer free energy of the side chains from water to sugar solution was calculated by utilizing the method adopted by Liu et al. (solubility of glycine has been reported by these authors).

3. Results and discussion

Several reports in literature discuss the effect of polyols on the thermodynamic stability of proteins in solution. Thermal unfolding studies have been utilized for the estimation of the stabilization afforded by polyols where an increase in the midpoint of the unfolding temperature curve (ΔT_m) has been used as a stability parameter (Gekko and Morikawa, 1981). However, the shapes of ΔT_m vs. polyol concentration plots vary among studies even for closely related polyols and/or proteins. For example, work by Kaushik et al. showed that plots of ΔT_m vs. polyol concentration (molarity) were linear; while, Back et al. observed that stabilization effect (ΔT_m) increased nonlinearly with an increase in the concentration of polyol (w/w) (Back et al., 1979; Kaushik and Bhat, 2003). To get an understanding of the relationship between protein stability and polyol concentration, thermal unfolding studies were conducted on lysozyme in the presence of three polyols. Fig. 2A (% w/v or molarity) and 2B (molality) shows the effect of sucrose (a sugar), glycerol (linear polyol) and Methyl-Glucoside (2-methyl glucose) on the change in the thermal unfolding temperature of lysozyme at pH 6.5 and 0.006 M ionic strength. Protein stabilization increased with an increase in concentration of polyol, and all polyols (whether sugar or linear polyol) followed the nonlinear trend. However, the net stabilization afforded to the protein was different for different polyols. Although, the thermal denaturation of bovine serum albumin was found to be irreversible, similar increase in stabilization with increase in concentration of the polyols was also observed (data not shown). This trend of increased stabilization with increase in the concentration of polyol, is consistent with that reported earlier by Back et al. (1979).

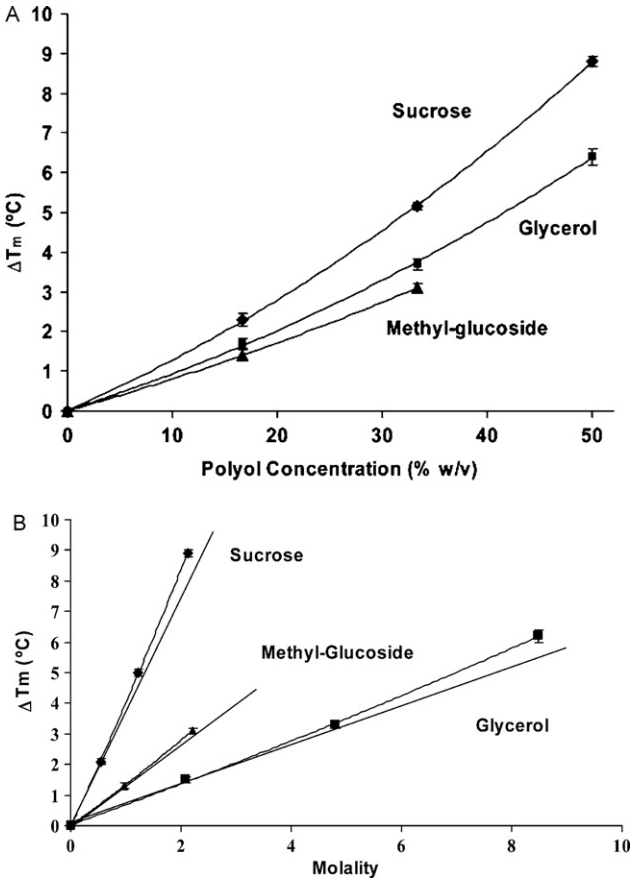


Fig. 2. Effect of sucrose, glycerol and methyl-glucoside on the change in denaturation temperature of lysozyme at pH 6.5 and an ionic strength of 0.006 (Error bars are standard deviations $n = 3$). (A) % w/v and (B) molality. Lines are drawn to guide the eyes.

The effect of other polyols is reported in Table 1, which shows the effect of 33.33% (w/v) concentration of polyols on the stability of lysozyme at pH 6.5 and 0.006 M ionic strength. Glucose increased the thermal unfolding temperature to the highest degree and hence was the most effective stabilizer. Deoxy-ribose (sugar) and ethy-

Table 1
Effect of polyols (33.3%, w/v) on the midpoint of the unfolding transition temperature of lysozyme at pH 6.5 and ionic strength of 0.006 as measured by DSC.

Polyol	(T_m (°C)[a]
Glucose	6.2 ± 0.1
Sucrose	5.0 ± 0.1
Glycerol	3.3 ± 0.1
Methyl-glucoside	3.1 ± 0.1
Ribose	1.3 ± 0.1
Ethylene glycol	−1.1 ± 0.1
Deoxy-ribose	−1.5 ± 0.1

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

lene glycol (linear polyol), on the other hand, decreased the thermal unfolding temperature of lysozyme, indicating that not all polyols stabilize proteins in solution. The destabilization of proteins by ethylene glycol has been noted earlier (Gekko and Morikawa, 1981) but the observed destabilization of lysozyme by Deoxy-ribose, a cyclic sugar, is a new finding. This is an important observation, especially, in view of earlier reports, wherein attempts have been made to relate the stabilization of proteins by polyols with the number of hydroxyl groups (especially equatorial in the case of cyclic sugars) present in the polyol molecule (Gekko and Morikawa, 1981; Uedaira and Uedaira, 1980). Uedaira and Uedaira had earlier observed that the denaturation temperature of lysozyme slightly increased in the presence of deoxy-ribose. The discrepancy in the results could be due to the use of a much higher concentration of lysozyme by Uedaira and Uedaira (60 mg/ml). It is important to emphasize that reversibility could have been an issue under such high concentration conditions. Since deoxy-ribose contains three hydroxyl groups and seems to be a fairly polar molecule, it can be argued that the presence of hydroxyl groups on the polyol molecule cannot be the only factor that determines the net stabilization effect afforded to proteins. Differential stability provided by near equal weight percentage of glycerol and ethylene glycol (at equal weight percentage both glycerol and ethylene glycol would provide equal number of hydroxyl groups in solution), also shows that presence of hydroxyl groups in the polyol molecule is not sufficient to impart stability to proteins in solution. Yet another observation can be made from Table 1. A comparison of the effect of glucose and methyl-glucoside shows that a small change in glucose molecule decreases the net stabilization effect by 50%. Considering the fact that protein folding is governed by hydrophobic interactions, a decrease in the net stabilization of proteins with an increase in the hydrophobicity of the co-solvent solution is not unreasonable (incorporation of the methyl group increases the hydrophobicity of the molecule). The net stabilization or destabilization effect imparted to proteins by a polyol thus has high dependence on both the presence of hydrophilic (hydroxyl groups) and hydrophobic groups in the polyol molecule and slight hydrophobicity can affect the stabilization potential afforded by such hydrophilic molecules as sugars/polyols.

Fig. 3(A) and (B) shows the effect of different polyols on the surface tension of water at 25 °C. Trehalose, sucrose, glucose and sorbitol increased the surface tension of water in a concentration-dependent manner. Ribose, methyl-glucoside and glycerol on the other hand, resulted in a much more pronounced decrease in the surface tension. Both sorbitol and glucose increased the surface tension of water to almost similar extent. Results from Table 1 and Fig. 3 show that not all polyols, which stabilize proteins in solution, increase the surface tension of water, and this is true not only for linear polyols but also for sugars. Therefore, the surface tension theory of preferential exclusion (Lin and Timasheff, 1996) is insufficient to explain the observed data and is presumably not the principal factor towards stabilization effect imparted by polyols. The present finding of a lacking correlation between surface tension and stabilization effect though not new adds significantly to the present database (the hypothesis has so far been based on restricted studies with glycerol).

From the reports in the literature, it is realized that steric exclusion cannot result in the observed effect of polyols on the stabilization of proteins in solution (at equal w/v %, smaller polyols should have greater excluded volume, e.g. ethylene glycol > glycerol) (McClements, 2002). This raises the fundamental question of what then is the cause of the observed stabilization of proteins by polyols. Recent articles by several groups have focused on the merit of the osmotic stress effect of polyols to describe their indirect effect on macromolecular conformations and reactions (Parsegian et al., 2000; Preisler et al., 1995). It has been recognized

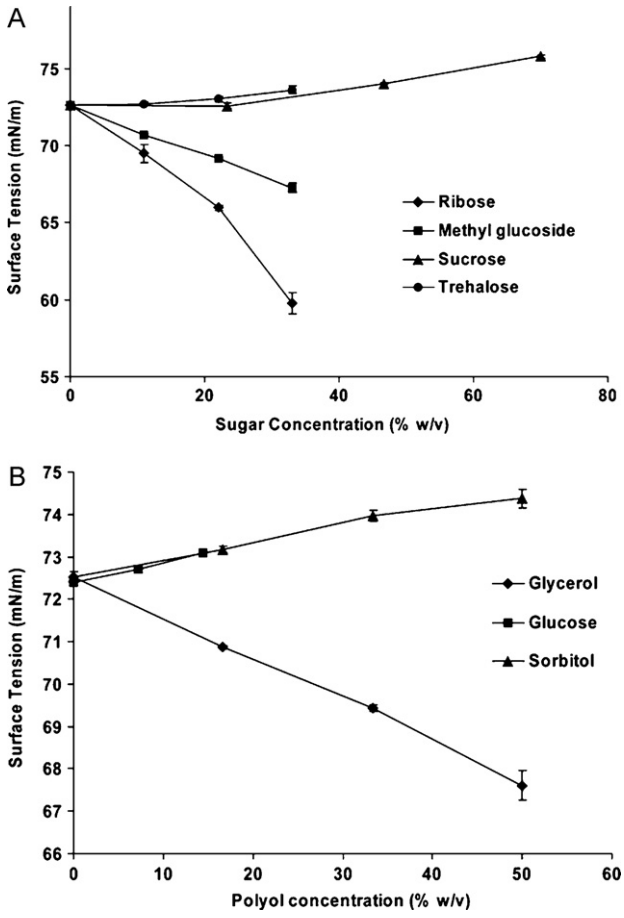


Fig. 3. Effect of polyols on the surface tension of water at 25 ± 1 °C as measured on Du-Nouy ring tensiometer for ribose, sucrose, trehalose and methyl-glucoside (A) and for glycerol, sorbitol and glucose (B) (lines are meant to guide the eyes).

that since proteins in the unfolded state require much greater number of water molecules for hydration, polyols and sugars can simply affect protein conformation by affecting the chemical potential of water (Miyawaki, 2007).

For a solution of a macromolecule containing N_w and N_s number of water and polyol molecules on one side of the semipermeable membrane in equilibrium with a reference solution containing n_w and n_s number of water and polyol molecules on the other side of the membrane (equilibrium dialysis is the traditional starting point for studying protein polyol interactions), Gibbs-Duhem constraint can be used to link the change in the chemical potential of the biological macromolecule to the changes in the chemical potentials of water and the polyol (Parsegian et al., 2000).

$$\partial G_M = -N_w \left(1 - \frac{(n_w/n_s)}{(N_w/N_s)}\right) \partial\mu_w = -N_{ew}\partial\mu_w \quad (4)$$

where N_{ew} is the excess (or deficit) number of water molecules in the vicinity of the macromolecule. The effect of solute on the chemical potential of water, and hence on the equilibrium between the folded and the unfolded states of the macromolecule can thus be described using Eq. (4) as,

$$\partial\Delta G_{FU} = -\Delta N_{ew}^{FU}\partial\mu_w \quad (5)$$

where $\Delta N_{ew}^{FU} = N_{ew}^{U} - N_{ew}^{F}$, $\partial\Delta G_{FU}$ is the difference in the free energy of unfolding of the protein in the absence and presence of solute, and $\partial\mu_w$ is the change in the chemical potential of water in the presence of the solute. Clearly, any difference in the number of water and/or solute molecules bound by the folded

Table 2
Thermodynamic parameters of lysozyme at pH 6.5 (IS 0.006) in the presence of sucrose and glycerol.

Co-solvent	ΔT_m (°C)	ΔH_m (kJ/mol)	$\Delta\Delta G^\circ$ (kJ/mol)[a]
Control (Buffer)	0 (T_m = 73.7)	508.5 ± 20.1	–
Sucrose (16.7%, w/v)	2.1 ± 0.1	547.5 ± 8.3	3.28 ± 0.26
Sucrose (33.3%, w/v)	5.0 ± 0.1	558.7 ± 4.8	8.06 ± 0.20
Sucrose (50%, w/v)	8.9 ± 0.1	572.6 ± 11.0	15.1 ± 0.41
Glycerol (16.7%, w/v)	1.5 ± 0.1	544.7 ± 6.3	2.40 ± 0.20
Glycerol (33.3%, w/v)	3.3 ± .1	575.4 ± 2.4	5.71 ± 0.38
Glycerol (50%, w/v)	6.2 ± 0.2	597.7 ± 4.1	10.80 ± 0.35

[a] At T_m in Buffer.

and the unfolded states of the macromolecule will impact the folded–unfolded equilibrium. Eq. (5) is equivalent to the Wyman-Tanford linkage function used for the thermodynamic analysis of stoichiometry of equilibiria, which is shown in Eq. (6) (Jenkins, 1998),

$$\frac{\partial \ln K}{\partial \ln a_w} = \Delta\nu_w - \Delta\nu_s \left(\frac{X_w}{X_s}\right) \quad (6)$$

where a_w is the activity of water, $\Delta\nu_s$ is the net change in the number of bound solute molecules, $\Delta\nu_w$ is the net change in the number of bound water molecules, X_w and X_s are molar fractions of water and solute, respectively, and K is the equilibrium constant.

Osmolality, which is an indicator of osmotic activity and incorporates the effect of solutes on the chemical potential of water, may thus be a good measure of the impact of polyols on the thermodynamic stability of proteins.

$$\Delta\mu_w = \mu_w^s - \mu^\circ = RT \ln a_w^s = -RT \left(\frac{m_s \cdot \phi^s}{55.5}\right) \quad (7)$$

where μ_w^s is the chemical potential of water in the presence of the polyol (solute), m_s is solute molality and ϕ^s is the osmotic coefficient. Eqs. (5) and (7) give,

$$\partial\Delta G_{FU} = \Delta N_{ew}^{FU} \cdot \left(RT \cdot \frac{m_s \cdot \phi^s}{55.5}\right) = \Delta N_{ew}^{FU} \cdot \left(RT \cdot \frac{Osm}{55.5}\right) \quad (8)$$

Modified Gibbs Helmholtz equation was used to determine the effect of polyols on the $\Delta\Delta G^\circ$ of lysozyme at the T_m of the protein.

$$\Delta G^{F-U} = \Delta H_m \left(\frac{T_m - T}{T_m}\right) - \Delta C_p \left[(T_m - T) + T \ln\left(\frac{T}{T_m}\right)\right] \quad (9)$$

ΔC_p for lysozyme has been reported in the literature (≈6600 J/mol/K) (Cooper, 1992; Pfeil and Privalov, 1976). Fig. 4(A) shows the effect of the osmotic activity of the polyols on the $\Delta\Delta G^\circ$ of lysozyme at pH 6.5 and an ionic strength of 0.006 M (corresponding thermodynamic parameters are shown in Table 2). Clearly, as should be anticipated from Eq. (8), an excellent correlation is observed. Values of ΔN_{ew}^{FU} [or $\Delta\nu_w - \Delta\nu_s \cdot (X_w/X_s)$] can be determined from the slopes of the curves. For a completely excluded solute, $\Delta\nu_s \cdot (X_w/X_s)$ becomes zero. Greater affinity of the unfolded state of the macromolecule for the polyol molecules would however result in positive values for the $\Delta\nu_s \cdot (X_w/X_s)$ term. Differential slopes for sucrose and glycerol indicate differential affinity of the two species of solute molecules towards lysozyme. Glycerol seems to have higher binding affinity for the protein. Fig. 4B shows that ΔT_m and $\Delta\Delta G^\circ$ were also found to have a linear relation (at least in the temperature range studied here). Clearly, the effect of polyols on the activity of water seems to be the principal factor that governs the stabilization effect produced by these polyhydroxy molecules. The role of water activity was further investigated by studying the thermodynamic stabilization provided by three closely related sugar molecules sucrose, trehalose and maltose. Table 3 shows the properties of the three sugars (Banipal et al., 1997; Sato et al., 2004) including the effect of 50% (w/v) concentration (≈42%, w/w, for trehalose, 43%, w/w, for maltose and 44%, w/w, for sucrose) on

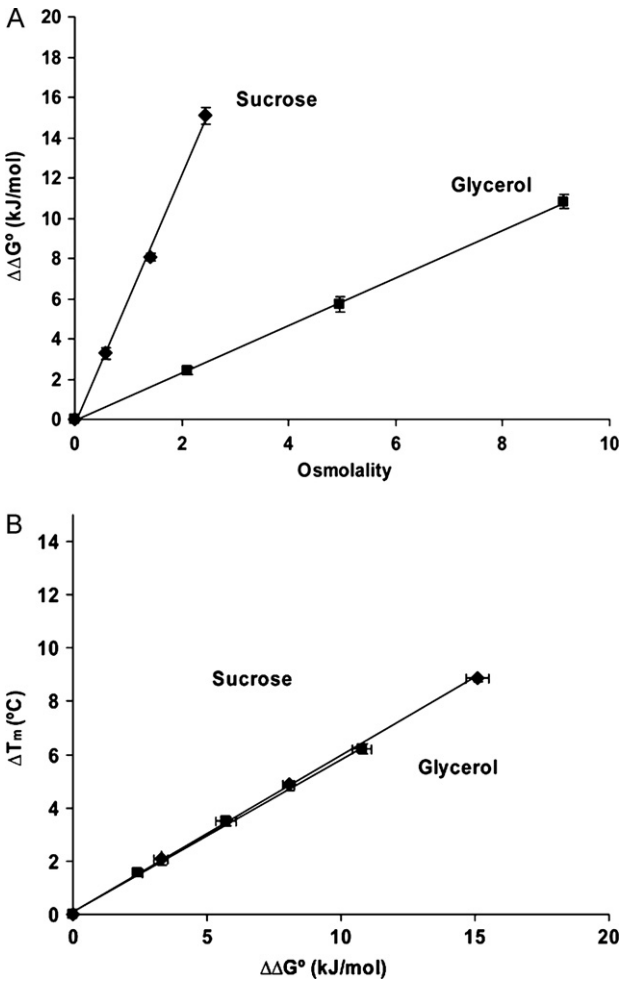
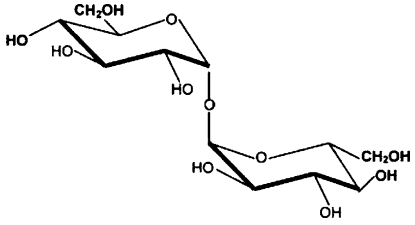
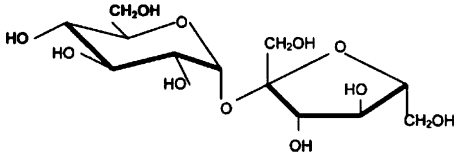
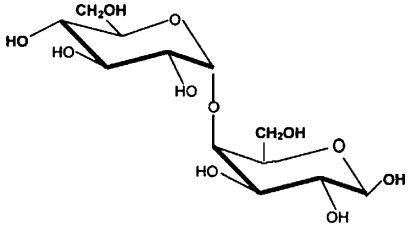


Fig. 4. Effect of polyols on the osmotic activity of water (water chemical potential) and on the unfolding free energy and ΔT_m of lysozyme (pH 6.5, IS 0.006) (A) Impact of polyol osmolality on the increase in the free energy of unfolding at the T_m of lysozyme in buffer. (B) Relationship between ΔT_m and $\Delta\Delta G^\circ$ as impacted by polyols.

the ΔT_m of lysozyme at pH 6.5 and an ionic strength of 0.006 M. Gaida et al. (2006) have shown that trehalose affects the chemical potential of water to the greatest extent followed by sucrose and maltose. Clearly, rank order stabilization effect does not correlate with the number of equatorial hydroxyl groups but correlates well with the effect of these polyols on the activity of water. Taken in conjunction with Miyawaki's work (Miyawaki, 2007), this exemplary correlation between thermodynamic stabilization ($\Delta G_{f\text{-}uf}$), and polyol osmolality thus confirms/generalizes the prominent role of water activity in the observed stabilization effects. The reason of the observed differences in the stabilization provided by different polyols at equivalent water chemical potential is further explored.

Our results as well as the data in the literature show that equal weight percentage of polyols that differ with respect to the number of hydroxyl groups impart differential stabilization to proteins (Fig. 5) (Gekko, 1982). In fact ethylene glycol (two hydroxyls) generally results in the destabilization of the protein structure. It was discussed earlier that introduction of a hydrophobic methyl group in glucose results in a considerable reduction of the stabilization power. Additionally, it is seen in the literature that some polyols increase the solubility of small hydrophobic compounds, a clear evidence of the weak hydrophobicity of these seemingly hydrophilic molecules (Bertau and Jorg, 2004; Janado and Yano, 1985; Liu et al., 2005; Nerurkar and Beach, 2005). In order to determine the relative hydrophobic nature of different polyols, solubility studies

Table 3
Properties of sugars and the effect of sugars (50%, w/v) on the midpoint of the unfolding transition temperature of lysozyme at pH 6.5 and ionic strength of 0.006 as measured on DSC.

Polyol	Mol. weight	Partial specific volume (cm^3/mol)[a]	Number of equatorial hydroxyl groups[b]	ΔT_m (50%, w/v polyol)[c]
 Trehalose	342.3	240.75	7.2	10.10 ± 0.1
 Sucrose	342.3	211.92	6.3	8.9 ± 0.1
 Maltose	342.3	228.14	7.2	7.80 ± 0.15

[a] Banipal et al. (1997).
[b] Sato et al. (2004).
[c] Error bars are standard deviations ($n = 3$).

were conducted on a strongly hydrophobic aromatic compound, biphenyl. Percent increase (or decrease) in solubility of biphenyl by polyols was taken as a measure of the relative hydrophobicities of different polyol molecules. Fig. 6 shows the effect of different polyols (33.33%, w/v) on the solubility of biphenyl and on the ΔT_m of lysozyme. All polyols tested increased the solubility of biphenyl. Deoxy-ribose and ethylene glycol were found to be the most effective solubilizers of biphenyl. It is clear from Fig. 6 also that an inverse relation exists between the hydrophobic nature of the polyol and its capacity to impart stabilization to proteins in solution.

Role of hydrophobicity of polyols in determining the net stabilization effect was further investigated. Hydrophobic surface areas of different polyol molecules were calculated using Hyperchem program. These calculations show that, smaller mol. wt. polyols have larger values of hydrophobic/hydrophilic surface area ratio.

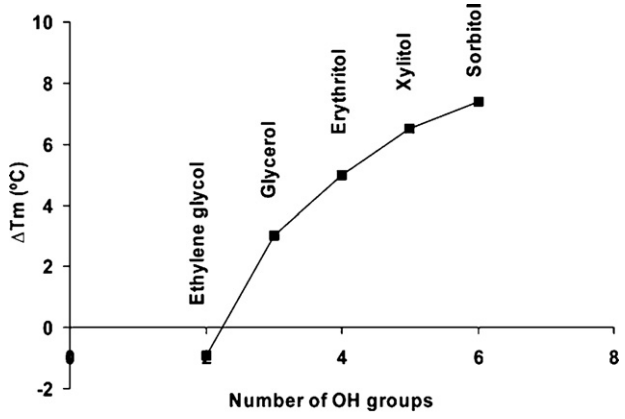


Fig. 5. Effect of 30% weight concentration of different polyols on the denaturation temperature of lysozyme as measured by DSC (Gekko, 1982).

In other words, the smaller the polyol, the larger is its relative hydrophobicity. Fig. 7A shows a plot of the ratio of the hydrophobic to hydrophilic surface area of polyols that were shown in Fig. 5 against the effect that these polyols produce on the T_m of lysozyme. A good linear correlation is observed ($R^2 = 0.97$). Fig. 7B shows a similar plot for sugars (T_m's are plotted at equal w/v concentrations of the sugars since data was not available for equivalent w/w concentrations. However, little difference is anticipated even if the data is plotted in terms of weight concentrations). A fair correlation is again observed (Fig. 7A and B have equal Y scales). These results show that the net stabilization of proteins imparted by polyols

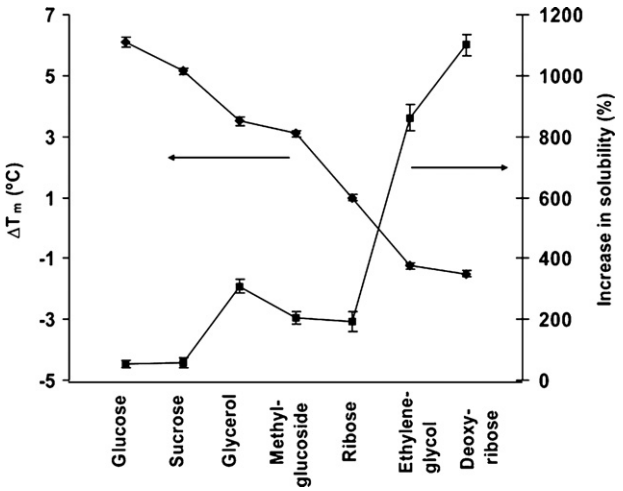


Fig. 6. Effect of 33.33% w/v concentration of different polyols on the denaturation temperature of lysozyme and on the % increase in solubility of biphenyl at pH 6.5 and an ionic strength of 0.006 M (for denaturation temperatures, error bars are standard deviations $n = 3$, and for solubility, error bars are deviations from average $n = 2$).

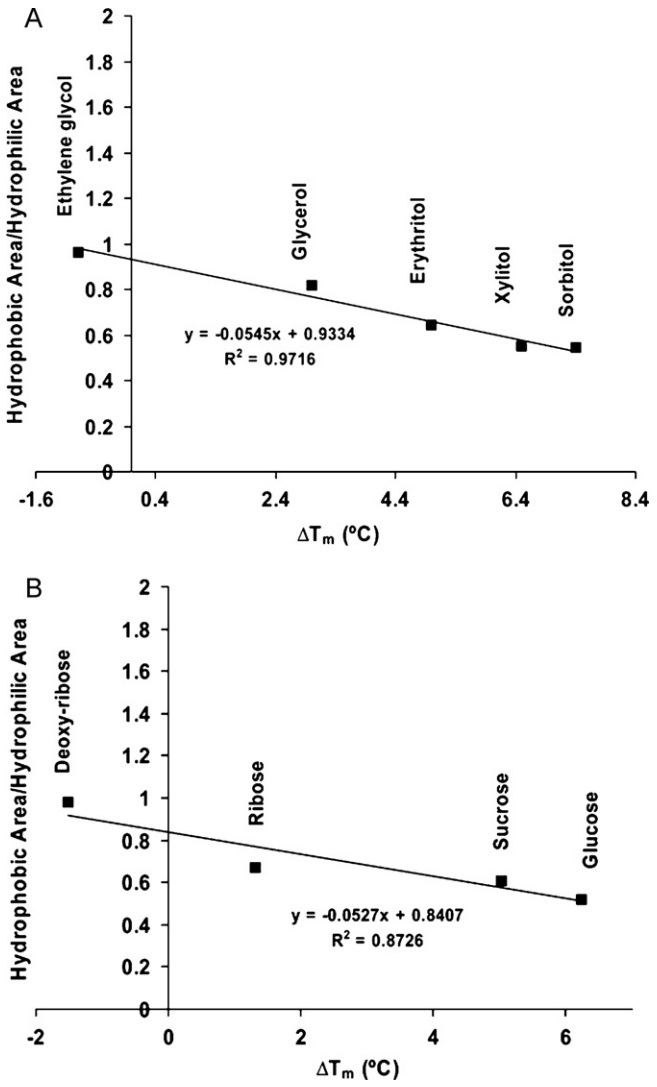


Fig. 7. Dependence of the denaturation temperature on the relative hydrophobicity of different polyols and sugars. (A) Effect of 30% (w/w) polyol on the denaturation temperature of lysozyme and (B) effect of 33.33% (w/v) sugar on the denaturation temperature of lysozyme.

(linear or otherwise) is dependent predominantly on two factors, osmotic activity and the hydrophobicity of polyols. Evidently, an increase in the hydrophobic nature of polyols pushes the protein towards an unfolded state because of the favorable interactions that occur between the non polar groups of protein and hydrophobic cosolvent molecules. It is important to note here that Liu et al. (2010) have previously tried to correlate the stabilizing power of the polyol (used at equivalent molarity) with the fractional polar surface area of the polyol hence suggesting that the stabilization is dependent on indirect interactions between the protein and the polyol. We take a different approach and correlate the destabilizing power and the hydrophobicity of polyols when the poyols are used at equivalent weight percentage of the solution. The destabilizing effect is a result of direct interactions between the hydrophobic surfaces of the two species of molecules. The stabilization on the other hand as pointed out by Liu et al. is an indirect effect (Liu et al., 2010). Indeed the impact of polyols on water activity, and/or water ordering and the impact of polyol hydration on the activity of water are all related and interdependent concepts (Gaida et al., 2006; Uedaira et al., 1989).

Support for our work comes from the work of Bolen et al. (Auton and Bolen, 2007; Liu and Bolen, 1995; Qu et al., 1998).

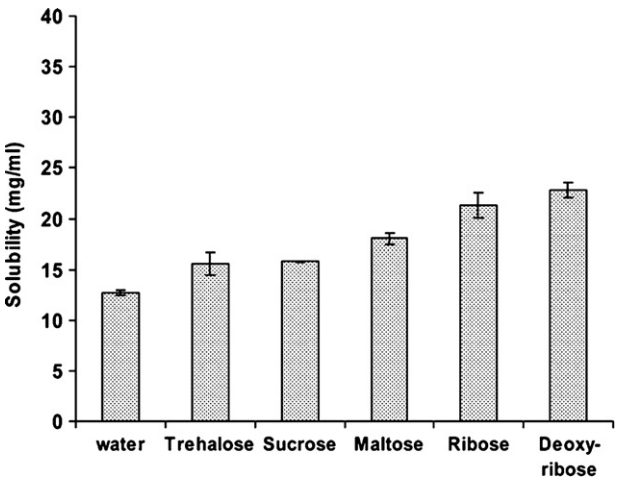


Fig. 8. Effect of different polyols (34.3%, w/v) on the solubility of tryptophan in water at 25 ± 1 °C.

These authors calculated the transfer free energies of amino acids from water to sugar solutions and concluded that such transfer of the non polar side chains of most amino acids is evidently favorable. We extended the work done by Bolen et al. and studied the effect of different polyols on the solubility of the most hydrophobic amino acid tryptophan (Fig. 8). The measured solubility in water closely matched the reported solubility (12 mg/ml) (Liu and Bolen, 1995). All of the polyols that were tested increased the solubility, albeit to different extents. An increase in the hydrophobicity of the polyol increased the solubility indicating that the solubility of tryptophan is significantly governed by its nonpolar side chain. It can also be observed that the trend of the increase in amino acid solubility (deoxy-ribose > ribose > maltose > sucrose > trehalose) is inversely related to the trend observed for protein stabilization (trehalose > sucrose > maltose > ribose > deoxy-ribose).

In order to investigate how polyols affect the solubility of amino acids that differ in the hydrophobicity of the side chain, effect of sucrose on the solubility of phenylalanine and tyrosine was studied. Fig. 9 shows the transfer free energies of the side chains of tryptophan, tyrosine and phenylalanine from water to 1 M sucrose solution. All the transfer free energies were found to be negative suggesting that the transfer of the hydrophobic side chains of all of these amino acids from water to 1 M sucrose solution is favorable. Additionally, Fig. 9 also shows that, of the three molecules, the

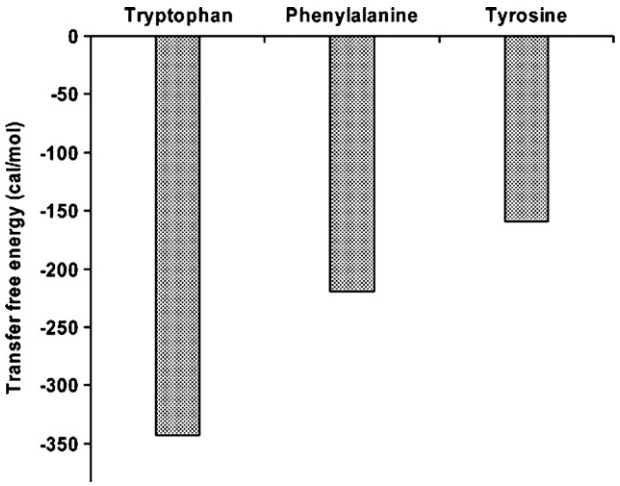


Fig. 9. Transfer free energies of the side chains of tryptophan, phenylalanine and tyrosine from water to 1 M sucrose solution at 25 ± 1 °C.

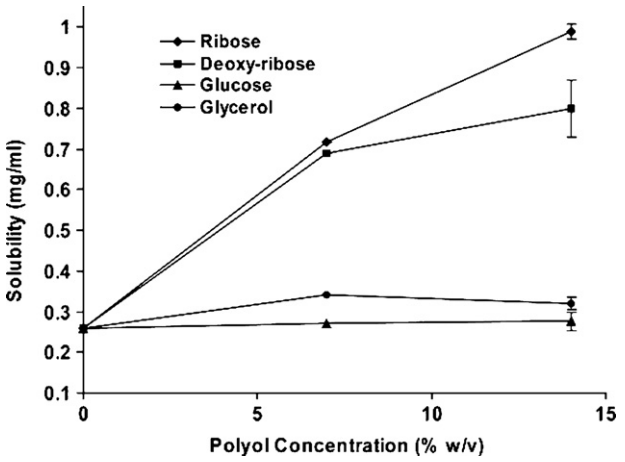


Fig. 10. Effect of polyols on the solubility of IFNα2a at pH 6.5 and an ionic strength of 0.006 at 25 ± 1 °C. Error bars are deviations from average of duplicate studies.

transfer of the side chain of tryptophan is most favorable while the transfer of the side chain of tyrosine is least favorable. It is known that the hydrophobicity of the three amino acids decreases in the order tryptophan > phenylalanine > tyrosine.

Finally, the effect of some of these polyols on the solubility of a relatively hydrophobic protein Interferon alpha-2a (IFNα2a) was studied at pH 6.5 and an ionic strength of 0.006 M. pH 6.5 is the isoelectric point and was selected as the protein is found to have lowest solubility around this pH. Ionic strength of the buffer was maintained low in order to further minimize the solubility of the protein. Fig. 10 shows that most of the polyols that were tested resulted in an increase in the solubility of the protein. Highest increase in solubility was observed in the case of ribose, while glucose was more or less ineffective in this regards. Though, it was anticipated that dexoy-ribose should result in the highest increase in IFNα2a solubility, the greater increase by ribose rather then deoxy-ribose may not be unreasonable. As shown in the case of lysozyme, deoxy-ribose can act as the destabilizer of IFNα2a structure. This unfolding of IFNα2a by dexoy-ribose will result in an increase in the propensity of the protein to aggregate and fall out of the solution.

4. Conclusions

Our studies provide support to the hypothesis that impact of polyols on the surface tension of water is not the primary factor that governs the exclusion of these cosolvents from the protein domain. A strong correlation between lysozyme stabilization and polyol osmolality points towards the prominent role of water activity (chemical potential of water) in mediating the polyol induced stabilization of proteins. An inverse relationship exists between the hydrophobicity of polyols and the net stabilization effect afforded to proteins. The hydrophobic character of polyols is critical for promoting their interactions with the protein molecules, weakening the hydrophobic forces within the protein interior and hence counteracting the polyol induced solvent-mediated stabilization effect.

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