İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

ADSORPTION KINETICS OF BOVINE SERUM ALBUMIN (BSA) AS AFFECTED BY CO-SOLVENTS

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BOVINE SERUM ALBUMİN (BSA)'İN ADSORPSİYON ÖZELLİKLERİNE KO-SOLVENTLERİN ETKİSİ

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LIST OF SYMBOLS

c	(mol/dm^3)	: Molar concentration
g	($9.81\ m/s^2$)	: Gravitational constant
k	(1.380658×10^{-23} J/K)	: Boltzman constant
s	(mm)	: Arc length of the profile measured from the drop apex
t	(s)	: Time
D	(m^2/s)	: Diffusion coefficient
K	(-)	: Equilibrium constant
M	(mol/dm^3)	: Molal concentration
P	(Pa)	: Pressure
R	(8.3143 J/mol K)	: Gas constant
R_0	(mm)	: Radius of curvature of a drop
S	(-)	: Dimensionless arc length of the profile of a drop
T	(K)	: Temperature
X	(-)	: Dimensionless co-ordinate
Y	(-)	: Dimensionless co-ordinate
γ	(10^{-3}N/m)	: Surface tension
μ	(kJ)	: Chemical potential
η	(Pas)	: Viscosity
π	(3.14)	: Pi number
ρ	(g/cm^3)	: Density
φ	(^{o})	: The angle made by R_0 and the *y* axis
β	(-)	: Dimensionless parameter
ΔG	(kJ)	: Free energy
ΔH	(J/mol)	: Change in enthalpy
Γ	(-)	: Preferential interaction coefficient
Φ	(-)	: Preferential interaction parameter
Π	(10^{-3}N/m)	: Surface pressure

Indices

c	: Co-solvent
eff	: Effective
int	: Differential interactions
s	: Solvent
steric	: Steric exclusion
tr	: Transfer
D	: Denatured
N	: Native
1	: Solvent
2	: Protein
3	: Co-solvent

PREFACE

The relationship between the molecular characteristics of proteins and their functional properties in food systems has been the focus of many studies. Since proteins are utilized as functional components in food systems in the presence of a variety of different co-solvents and their functionality is highly effected by their interactions with these co-solvents, it is important to study these interactions to develop a fundamental understanding of the influence of molecular environment and processing conditions on protein functionality. This study aimed to bring an in depth understanding to the effect of interactions of sugars with proteins to surface related protein functionality in food systems and question the reasons behind this effect .

The experimental part of the study was conducted in the Colloidal and Interfacial Food Science Laboratory in the Food Science and Technology Department at the University of Tennessee, Knoxville. It was supervised by Prof. Dr. Y.Onur Devres of Istanbul Technical University (ITU), Assistant Prof. Dr. Jochen Weiss of the University of Tennessee, Knoxville (UT), and Associate Prof. D.Julian McClements of the University of Massachusetts, Amherst (Umass).

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SUMMARY

Globular proteins are widely used as functional ingredients in the food industry, *e.g.*, emulsifiers and foam stabilizers because of their ability to adsorb at oil-water or air-water interfaces. In many applications proteins are dissolved in an aqueous solution that contains different types of co-solvents, *e.g.*, surfactants, alcohols and sugars. Co-solvents may alter the molecular and functional properties of globular proteins through a variety of different physiochemical mechanisms. Therefore, it is very important to establish the factors that determine the adsorption of globular proteins in presence of co-solvents and to characterize the interactions between protein-solvent and protein-co-solvent molecules.

In this study, the influence of co-solvents glucose, sucrose and fructose at concentrations ranging from 0 to 40 wt% on the adsorption kinetics of bovine serum albumin (BSA) at air-aqueous solution interfaces at 20^{o}C was measured. The change in surface tension with time was recorded using drop shape analysis tensiometer. Diffusion coefficients were calculated from the initial period of protein adsorption using the short term solution of the general adsorption model by Ward and Tordai for BSA dissolved in pure water were much greater than values obtained in the presence of sucrose, glucose and fructose. The relative decrease of the adsorption rate was significantly higher at sucrose concentrations less than 10 wt%. Results were attributed to an increase in solution viscosity reducing the molecular mobility of the protein molecules and preferential interactions of sugars with the protein surface, which contributes to a stabilization of the native non-absorbed state of BSA.

BOVINE SERUM ALBUMİN'İN ADSORPSİYON KİNETİĞİNE KO-SOLVENTLERİN ETKİSİ

ÖZET

Globüler proteinler emulsiyonları ve köpükleri stabilize edici özellikleri nedeniyle gıda endüstrisinde fonksiyonel bileşen olarak yaygın olarak kullanılırlar. Birçok uygulamada proteinler sıvı bir fazda surfaktanlar, alkoller ve şekerler gibi çeşitli ko-solventlerle bir arada bulunurlar. Ko-solventler globüler proteinlerin moleküler ve fonksiyonel özelliklerini farklı fizikokimyasal mekanizamalarla değiştirirler. Bu nedenle ko-solventlerin varlığında globüler proteinlerin adsorpsiyonunu belirleyen etmenlerin ve protein-solvent ile protein-ko-solvent arası etkileşimlerin tanımlanması çok önemlidir.

Bu çalışmada, ko-solventlerin (sukroz glukoz ve fruktoz) %0 ile 40 arasında değişen derişimlerde bovine serum albumin (BSA)'in hava-çözelti yüzeyinde 20^{o}C sıcaklıktaki adsorpsiyon kinetiğine etkisi incelenmiştir. Zamana bağlı yüzey gerilimindeki değişim damla şekli analizi tensiyoometresi kulanılanarak ölçülmüştür. Ward and Tordai'nin genel adsoprsiyon modelinin proteinin adsorpsiyonun başlangıç kısmına uygulanmasıyla elde edilen difüzyon katsayıları sukroz, glukoz ve fruktoz varlığında saf su çözeltisindeki değerinden az olarak bulunmuştur. %10'dan düşük sukroz derişimlerinde adsorpsiyon hızındaki bağıl azalış yüksek derişimlerdekine kıyasla belirgin derecede fazla olarak ortaya çıkmıştır. Sonuçlar çözelti vizkozitesindeki artış nedeniyle protein moleküllerinin hareketliliğinin azalmasına ve şeker molekülleri ile protein moleküllerinin seçici etkileşimine bağlı olarak tartışılmaktadır. Şeker moleküllerinin protein molekülerinin yüzeyinden seçici olarak uzaklaştırılmaları ile BSA moleküllerinin stabilizasyonunun sağlandığı ve bu nedenle BSA'nın hava-cözelti arayüzeyine difüzyonun azaldığı düşünülmektedir.

I. INTRODUCTION

Globular proteins are among major ingredients in food systems. These proteins may be naturally present in the raw ingredients or they may be added as functional components that provide specific physicochemical properties to the final product. Globular proteins have unique functional properties *e.g.,* their ability to catalyze biochemical reactions, bind and transport small molecules and adsorb to interfaces. These properties of proteins contribute to the physicochemical properties of food systems that they are found in (Creighton, 1993; Phillips *et al.,* 1994; Dickinson, 1992; Dalgleish, 1996; Damodaran, 1996).

It is very important to understand the relationship between the molecular characteristics of proteins and their functional properties in food systems (Kinsella, 1982; Damodaran and Kinsella, 1982). There have been many studies carried out on molecular or thermodynamic basis of protein functionality under controlled experimental conditions, *e.g.,* in distilled water at a particular pH, ionic strength and temperature (Mulvihill and Donovan, 1987; Mulvihill and Kinsella, 1987; Kinsella and Whitehead, 1989; Hunt and Dalgleish, 1995; Agboola and Dalgleish, 1996; Demedtriades *et al*., 1997a, b). In practice, proteins are utilized as functional components in materials that have a wide variety of different compositions, structures and processing requirements (Ganonkar, 1995). It is therefore important to develop a fundamental understanding of the influence of molecular environment and processing conditions on protein functionality.

The objective of this study is to understand the influence of sugars, which are weakly interacting neutral co-solvents, on the adsorption characteristics of bovine serum albumin (BSA). BSA was used as a model protein, for it is a very well documented globular protein. In most applications proteins are dissolved in a solution containing various cosolvents such as surfactants, alcohols and sugars. In this study fructose, glucose and sucrose were used to investigate the effect of sugars on protein adsorption kinetics, since these are the most common sugars found in food systems where proteins are utilized as functional ingredients.

The results of this study can help food scientists to understand the influence of protein-co-solvent interactions on the surface active functionality of food proteins and help them control properties of food systems where protein plays an important role with their adsorption dependent functionality.

2. GLOBULAR PROTEINS AS FUNCTIONAL INGREDIENTS

Globular proteins have compact structures that are roughly spherical in shape (Creighton, 1993). Most globular proteins used as functional ingredients in the food industry have a molecular weight of 10 to 100 kDa (Damodaran, 1996). Despite being highly compact, the structures of globular proteins are highly dynamic, with the polypeptide chain and side groups fluctuating between many different conformations (Onuchic *et al.*, 1997; Freire, 1998). The structures adopted by a globular protein under a particular set of environmental conditions depends on a delicate balance of physicochemical phenomena, including, hydrophobic interactions, electrostatic interactions, hydrogen bonding, van der Waals forces and configurational entropy (Dickinson and McClements, 1995; Damodaran, 1996). The main driving force behind the formation and stabilization of the compact structure of globular proteins is the hydrophobic effect, *i.e.*, the tendency for the system to reduce the contact area between non-polar groups and water (Tanford, 1991). Therefore, globular proteins tend to adopt conformations in which non-polar amino acids are located primarily in the interior away from water, whereas polar amino acids are located primarily on the exterior where they can form hydrogen bonds and electrostatic interactions with water. The compact structure of the protein is usually reinforced by the ability of amino acids to form relatively strong hydrogen bonds with neighboring amino acids within the structure's interior. Ultimately, it is the type, number and distribution of amino acids along the protein chain, as well as the environmental conditions that the protein chain experiences, which determine the structure of a protein (Creighton, 1993).

When the environmental conditions are changed the fraction of protein molecules within the different conformational states is altered. Generally speaking, proteins can be either in a "native" or a "denatured" state. The "native state" is the conformation adopted by a protein under the environmental conditions that it experiences in its natural environment. The "denatured state" is the conformation that the protein molecule adopted when it was completely unfolded, *i.e.*, a highly flexible random

coil. It has recently been realized that globular proteins can have structural intermediates between their native and denatured states, which are referred to as "molten globule" states (Holt, 2000). Any change in environmental conditions such as solution composition can change conformations adopted by proteins and different molecular conformations may have very different functional attributes.

To understand the molecular basis of protein functionality, it is necessary to have detailed information about the conformation and interactions protein molecules. The temperatures, pressures, mechanical stresses and solution compositions experienced by proteins in foods vary widely, and so the conformation of a protein in a food may be very different from that in its native state. The availability of more information about protein structure under different environmental conditions that are relevant to food systems would considerably advance our understanding of protein functionality.

Globular proteins are used as functional ingredients in foods for a variety of different reasons, *e.g.*, enzyme catalysis, flavor modulation, gelation, water holding, thickening, emulsification, and foaming (Phillips *et al*., 1994; Damodaran, 1996; Nakai and Modler, 1996). At the molecular level, these functional attributes are determined by the ability of the proteins to bind other molecules, to undergo conformational changes, to self-associate and to adsorb to interfaces. Different proteins have different molecular properties (*e.g.*, size, shape, flexibility, surface chemistry), thus they have different functional properties. The conformation, binding, self-association and adsorption of protein molecules under a given set of environmental conditions are normally a delicate balance between stabilizing and destabilizing forces (Dickinson and McClements, 1995). Many types of weakly interacting co-solvents present in foods are capable of altering protein functionality by altering protein conformation, binding, self-association and adsorption phenomena.

3. CO-SOLVENTS

Co-solvents in foods can be divided into four categories depending on their effect on the transition of proteins from native to denatured state.

3.1. Neutral Co-solvents

Neutral co-solvents neither promote nor oppose a protein transition, *i.e.*, the transfer free energy of the protein from solvent to co-solvent solution is the same for the native and denatured states. Practically, this may occur if the co-solvent and solvent molecules had approximately the same size (so that steric exclusion was small) and the same molecular interactions (so that differential interactions were small). Not many real co-solvents fall into this category (Timasheff, 1998).

3.2. Stabilizing Co-solvents

Stabilizing co-solvents oppose a protein transition, *i.e.*, the transfer free energy of the protein from solvent to co-solvent solution is greater for the native state than for the denatured state. At a molecular level a co-solvent may stabilize a protein through a variety of different mechanisms. For example, it may be more preferentially excluded from the denatured state than from the native state, it may be more preferentially accumulated by the native state than by the denatured state, or it may be preferentially accumulated by the native state and preferentially excluded from the denatured state. Co-solvents can act as stabilizers as long as the change in transfer free energy for the transition from the native to denatured state is positive.

Many simple sugars (*e.g.*, sucrose, glucose, raffinose, trehalose) and polyols (*e.g.*, glycerol) fall into the category of stabilizing co-solvents (Timasheff, 1993, 1998; Ebel *et al.*, 2000). It is widely believed that simple sugars stabilize proteins primarily through a steric exclusion mechanism.

3.3. Destabilizing Co-solvents

Destabilizing co-solvents favor a protein transition, *i.e.,* the transfer free energy of the protein from solvent to co-solvent solution is greater for the denatured state than for the native state. Destabilization may occur through a variety of different mechanisms. For example, the co-solvent may be more preferentially excluded from the native state than from the denatured state, it may be more preferentially accumulated by the denatured state than by the native state, or it may be preferentially accumulated by the denatured state and preferentially excluded from the native state.

The most common examples of destabilizing co-solvents are urea and guanidine hydrochloride, which are frequently used as protein denaturants at relatively high concentrations. These co-solvents are believed to preferentially bind to the surface of proteins, thus favoring the unfolded state over the folded state because the former has a larger surface area (Timasheff, 1998).

3.4. Combined Co-solvents

Some co-solvents may stabilize a particular state of a protein under some conditions but destabilize it under other conditions, *e.g.,* temperature, co-solvent concentration. An example of this type of co-solvent is sodium lactate, which has been proposed as an additive for improving the thermal stability and freeze-thaw stability of fish proteins. At relatively low concentrations sodium lactate acts as a stabilizer of protein structure, but at higher concentrations it acts as a destabilizer (MacDonald *et al*., 1996b).

4. THERMODYNAMICS OF PROTEIN-CO-SOLVENT-SOLVENT INTERACTIONS

Weak interactions between protein, co-solvent and solvent molecules are described in terms of three thermodynamic parameters: transfer free energy; preferential interaction parameter; and preferential interaction coefficient (Timasheff, 1993). The normal convention of referring to the solvent, protein and co-solvent molecules by subscripts *1*, *2* and *3* is used here.

4.1. Transfer Free Energy

The interaction of a protein molecule with a co-solvent solution compared to its interaction with pure solvent can be described in terms of the *transfer free energy*, ΔG_{tr} (Timasheff, 1993, 1998). The transfer free energy is the change in free energy of the system when a protein molecule is moved from pure solvent into a co-solvent solution of specified composition:

$$\Delta G_{tr} = \mu_2(\text{co-solvent}) - \mu_2(\text{solvent}). \qquad (4.1)$$

where μ_2 is the chemical potential of the protein molecule in the solution specified in brackets. A positive transfer free energy means that the transfer of the protein is thermodynamically unfavorable, *i.e.*, the protein molecule prefers to be surrounded by solvent molecules than by co-solvent molecules. A negative transfer free energy means that protein transfer is thermodynamically favorable, *i.e.*, the protein molecule prefers to be surrounded by co-solvent molecules than by solvent molecules.

4.2. Preferential Interaction Parameter

The effect of a co-solvent on the thermodynamic properties of a protein can also be described using the co-solvent solution in which the protein is immersed as the reference state, rather than pure solvent (as was the case for the transfer free energy).

The influence of the co-solvent is then described by the *preferential interaction parameter*:

$$\Phi_{3,2} = \lim_{m_2 \to 0} \left(\delta \mu_2 / \delta m_3 \right)_{T,P,m_2} \tag{4.2}$$

Here, m_3 and m_2 are the molal concentrations of co-solvent and protein, respectively, T is the absolute temperature, P is the pressure and μ_2 is the chemical potential of the protein. The preferential interaction parameter, $\Phi_{3,2}$, describes the change in the transfer free energy (chemical potential) of a protein when the co-solvent concentration is increased by a small amount. The definition of the preferential interaction parameter indicates that the pressure, temperature and protein molality should be kept constant, and that the protein concentration should be low enough for protein-protein interactions to be negligible. A positive preferential interaction parameter means that the protein transfer free energy increases (becomes more unfavorable) when co-solvent is added, whereas a negative preferential interaction parameter means that the protein transfer free energy decreases (becomes more favorable) when co-solvent is added. The transfer free energy of a protein from pure solvent to a co-solvent solution of specified composition (m_3) can be calculated from knowledge of the preferential interaction parameter as a function of co-solvent concentration:

$$\Delta G_{tr} = \int_0^{m_3} \left(\delta \mu_2 / \delta m_3 \right)_{T,P,m_2} \mathrm{d}\, m_3 \tag{4.3}$$

4.3. Preferential Interaction Coefficient

The interaction between weakly interacting co-solvents and protein molecules can be conveniently described in terms of a *preferential interaction coefficient*, $\Gamma_{3,2}$ (Timasheff, 1998; Record *et al*., 1998):

$$\Gamma_{3,2} = \lim_{m_2 \to 0} \left(\delta m_3 / \delta m_2 \right)_{T,P,\mu_3} \tag{4.4}$$

Here, m_3 and m_2 are the molal concentrations of co-solvent and protein, respectively, T is the absolute temperature, P is the pressure and μ_3 is the chemical potential of the co-solvent. The definition of the preferential interaction coefficient indicates that the

pressure, temperature and co-solvent chemical potential should be kept constant, and that the protein concentration should be low enough for protein-protein interactions to be negligible. The preferential interaction coefficient describes the amount of co-solvent that must be added or removed from the bulk solution when the protein concentration is increased by a small amount in order to keep the chemical potential of the co-solvent in the bulk solution constant. If a co-solvent is preferentially excluded then $\Gamma_{3,2}$ is negative, but if it is preferential accumulated then $\Gamma_{3,2}$ is positive (Record *et al*., 1998).

It is possible to conceptualize the solution surrounding the protein molecules as consisting of two regions separated by a semi-permeable membrane: a "local domain" and a "bulk solution". The local domain is the region of solution that immediately surrounds the protein molecules. The concentration of co-solvent (M_3) and solvent (M_1) molecules in the local domain is different from the concentration of co-solvent (m_3) and solvent (m_1) molecules in the bulk solution because of the presence of the protein molecules. If $M_3/M_1 > m_3/m_1$, the co-solvent is preferentially accumulated; if $M_3/M_1 < m_3/m_1$, the co-solvent is preferentially excluded; and if $M_3/M_1 = m_3/m_1$, the co-solvent is neither accumulated or excluded. As more protein molecules are added to the solution it is necessary to either increase or decrease the concentration of co-solvent molecules in the bulk solution to keep its chemical potential constant depending on whether the co-solvent is preferentially accumulated or excluded, respectively.

The preferential interaction coefficient is related to the preferential interaction parameters of the protein and co-solvent:

$$\left(\delta m_3 / \delta m_2\right)_{T,P,\mu_3} = -\frac{\left(\delta \mu_2 / \delta m_3\right)_{T,P,m_2}}{\left(\delta \mu_3 / \delta m_3\right)_{T,P,m_2}} \tag{4.5}$$

Thus by measuring the preferential interaction coefficient of a protein as a function of co-solvent concentration it is possible to calculate the preferential interaction parameter and transfer free energy (provided the relationship between μ_3 and m_3 is known for the co-solvent solution). The change in chemical potential of the co-solvent with co-solvent concentration can be determined from osmotic pressure or water activity measurements (Timasheff, 1993).

5. WEAK PROTEIN- CO-SOLVENT-SOLVENT INTERACTIONS

When a protein molecule is introduced into a solution it alters the spatial organization of the solvent and co-solvent molecules in the system. When the local concentration of co-solvent molecules in the immediate vicinity of the protein surface is greater than in the bulk solution, the co-solvent is “bound", "adsorbed" or "preferentially accumulated" (Timasheff, 1993, 1998). On the other hand, when the local concentration of co-solvent molecules in the immediate vicinity of the protein surface is less than in the bulk solution, the co-solvent is "preferentially excluded". "Preferential interaction" can be defined as any physiochemical phenomenon that causes a change in the concentration of co-solvent and solvent molecules around a protein relative to their concentration in the bulk phase. The physicochemical mechanisms responsible for changes in the spatial distribution of co-solvent and solvent molecules around proteins are two types: differential interactions and steric exclusion.

5.1. Steric Exclusion

The steric exclusion effect results in a preferential interaction that depends only on the number, size and shape of the molecules involved (Parsegian *et al.*, 1995). If solvent and co-solvent molecules had the same size there would be no steric exclusion effect. However, co-solvent molecules (*e.g.,* sugars) are usually larger than solvent molecules (*e.g.,* water) and so there is a region surrounding each protein molecule from which the co-solvent molecules are excluded but the solvent molecules can enter. This region extends a distance approximately equal to the radius of the co-solvent molecules (assumed that they are spheres) from the protein surface. As a result there is a concentration gradient between the co-solvent-rich bulk aqueous phase and the co-solvent-depleted local-domain surrounding the protein molecules (Record *et al.,* 1998; Timasheff, 1998). The steric exclusion contribution to the transfer of a protein molecule from pure solvent to a co-solvent solution is therefore thermodynamically unfavorable because of the free energy required to maintain the

concentration gradient between the local domain and the bulk solution (Timasheff, 1993).

The steric exclusion contribution to the change in free energy ($\Delta\Delta G_{steric}$) that occurs when a protein is moved from pure solvent to a co-solvent solution is as follows:

$$\Delta\Delta G_{steric} = \Delta G_{steric,C} - \Delta G_{steric,S} \quad (5.1)$$

where $\Delta G_{steric,C}$ and $\Delta G_{steric,S}$ are the change in free energy due to steric exclusion when a protein is introduced into a co-solvent solution and into a pure solvent, respectively. The steric exclusion contribution to preferential interactions is usually thermodynamically unfavorable because co-solvent molecules are normally larger than solvent molecules.

Co-solvents may also be excluded from surface crevices or interior cavities in a protein because of their relatively large size, which also leads to a steric exclusion effect (Parsegian *et al.*, 1995). Exclusion of the co-solvents from these crevices or cavities leads to a concentration gradient between the co-solvent-excluded region and the co-solvent-rich bulk aqueous phase. Consequently, the protein molecule is put under "osmotic stress" and there is a tendency for the protein to alter its conformation to close the crevice or cavity. This may affect functional properties of proteins that are related to their binding of molecules to a particular site, *e.g.*, flavor binding, lipid transport or enzyme activity.

5.2. Differential Interactions

Molecules interact with each other through a variety of fundamental electromagnetic forces, *e.g.*, electrostatic, van der Waals and steric overlap (Israelachvili, 1992). These interactions are opposed by the thermal energy of the system, which means that entropy effects also have to be taken into consideration. The expressions that we commonly use to refer to particular types of molecular interactions that occur in aqueous solutions, such as "electrostatic interactions", "hydrogen bonding" and "hydrophobic interactions", actually describe combinations of these more fundamental electromagnetic forces and entropy effects. Protein, solvent and co-solvent molecules generally have different numbers, types and orientations of chemical groups on their surface. Their ability to participate in electrostatic interactions, hydrogen bonding and

hydrophobic interactions therefore differs considerably. The overall magnitude of protein-co-solvent interactions is therefore normally different from that of protein-solvent interactions (Timasheff, 1993). Consequently, there will be a tendency for co-solvent molecules to be either preferentially accumulated or preferentially excluded from the various chemical groups on a protein surface. When the attractive interaction of a protein group with a co-solvent molecule is stronger than with a solvent molecule, the co-solvent will tend to be preferentially accumulated. On the other hand, when the interaction is weaker the co-solvent will tend to be preferentially excluded. We refer to this mechanism of alteration in the spatial distribution of co-solvent and solvent molecules around a protein as the differential interaction contribution, because it depends on differences in the strength of protein-co-solvent and protein-solvent interactions.

The differential interactions of a protein with a co-solvent molecule can be classified as either "weak" or "strong". An interaction is described as strong when the difference between the protein-co-solvent and protein-solvent interactions is considerably greater than the thermal energy of the system (kT) (Jones and Chapman, 1995). Strong interactions between protein and co-solvent molecules lead to accumulation of the co-solvent around the relevant protein group at relatively low co-solvent concentrations, *e.g.*, a few mM. An interaction is described as weak when the difference between the protein-co-solvent and protein-solvent interactions is approximately equal to the thermal energy of the system. A typical example of this type of interaction is the relatively weak hydrogen bonding that occurs between uncharged polar groups on proteins, co-solvent molecules (*e.g.*, sugars and polyols) and solvent molecules (*e.g.*, water).

The change in free energy ($\Delta\Delta G_{int}$) that occurs due to the differential interaction contribution when a protein is moved from pure solvent to a co-solvent solution is given by:

$$\Delta\Delta G_{int} = \Delta G_{int,C} - \Delta G_{int,S}. \qquad (5.2)$$

Here $\Delta G_{int,C}$ and $\Delta G_{int,S}$ are the free energy changes associated with alterations in the molecular interactions that occur when a protein molecule is moved from a vacuum into a co-solvent solution or from a vacuum into pure solvent, respectively. When a protein molecule is introduced into pure solvent it is necessary to break some solvent-

solvent bonds and to create some protein-solvents bonds, which would result in a change in free energy of $\Delta G_{int,S}$. When a protein molecule is introduced into a co-solvent solution it is necessary to break some solvent-solvent, co-solvent-co-solvent and solvent-co-solvent bonds and to create some protein-solvent and protein-co-solvent bonds, which would result in a change in free energy of $\Delta G_{int,C}$. Ideally, one would like to calculate the free energy contributions due to differential interactions *a priori* from information about the properties of the molecules involved. In practice, this is not possible due to the complexities associated with calculating molecular interactions between a huge number of different chemical groups (Onuchic *et al*., 1997). Nevertheless, it is possible to obtain an indirect estimation about the sign and magnitude of these interactions from surface tension measurements (Cioci *et al*., 1994; Kita *et al*., 1994; Cioci, 1996; Lin and Timasheff, 1996; Cioci and Lavecchia, 1997). It should be noted that surface tension measurements only provide information about the free energy change associated with cavity formation, *i.e.*, creation of a protein-sized hole in a co-solvent or solvent solution. They do not provide information about protein-solution interactions, and so they do not give an adequate description of systems where there are significant differences between protein-co-solvent and protein-solvent differential interactions.

The surface of a protein molecule has many different types of chemical group, each with a different shape, size and polarity. Each of these groups interacts differently with co-solvent and solvent molecules, depending on their molecular characteristics (Timasheff, 1993). In addition, the surface groups on the protein molecules need not act independently of one another. The organization of co-solvent and solvent molecules around one surface group (*e.g.*, an ionized group) may influence the organization of co-solvent and solvent molecules around a neighboring surface group (*e.g.*, a non-polar group) (Israelachvili, 1992). The differential interaction contribution that is experimentally measurable, therefore reflects contributions of many different types of interaction at different regions on the surface of a protein, some that favor the co-solvent and some that favor the solvent. Consequently, the overall magnitude of the differential interaction contribution depends on the specific surface chemistry of the protein, as well as the specific molecular characteristic of the co-solvent and solvent molecules. One would therefore expect proteins with different numbers of non-polar, polar and charged surface groups to behave differently with the same co-

solvent and that the same protein would behave differently with different co-solvents, which is supported by the available experimental data (Timasheff, 1993, 1998).

5.3. Overall Preferential Interactions

The overall interaction of co-solvent and solvent molecules with the protein molecule can be described in terms of both steric exclusion and differential interaction effects (Timasheff, 1993, 1998):

$$\Delta G_{tr} = \Delta\Delta G_{steric} + \Delta\Delta G_{int} \tag{5.3}$$

The steric exclusion and differential interaction contributions may each have the same sign and therefore reinforce each other, or they may each have different signs and therefore oppose each other. The sign and magnitude of the two contributions may change appreciably with solution composition or environmental conditions, thereby altering the overall effect of the co-solvent on the protein transfer free energy. A co-solvent may therefore act as a destabilizer under some conditions and a stabilizer under other conditions (Timasheff, 1998).

6. INFLUENCE OF CO-SOLVENTS ON PROTEIN EQUILIBRIA

The functional properties of food proteins often depend on the protein molecules undergoing a transition from one state to another. For example, the protein molecule may have to undergo a transition from a folded to an unfolded state or from a non-adsorbed to an adsorbed state. To a first approximation, these types of transitions can be represented as an equilibrium between two states (Timasheff, 1993):

$$\text{State}(1) \leftrightarrow \text{State}(2) \qquad (6.1)$$

This equilibrium is characterized by a free energy change (ΔG) and an equilibrium constant K: $\Delta G = - RT \ln(K)$. The definition of the equilibrium constant depends on the type and nature of the physicochemical process taking place, *e.g.*, unfolding, ligand binding, self-association or adsorption. In the following sections we examine the influence of co-solvents on transitions relevant to selected protein functionality in foods.

6.1. Denaturation

Thermal treatment is one of the most important processing operations used in the manufacture of foods containing globular proteins, *e.g.* pasteurization, sterilization, cooking, freezing, chilling (Loncin and Merson, 1979). Many globular proteins unfold when their temperature is either increased above a particular temperature (heat-denaturation) or decreased below a particular temperature (cold-denaturation). Various other processing operations, such as high pressure treatment, dehydration, whipping and homogenization , may also cause protein molecules to unfold (Iametti *et al* , 1998, 1999; Allison *et al.*, 1998, 1999; McClements *et al.*, 1993; Clarkson *et al.*, 2000). Denaturation causes pronounced changes in the molecular and functional characteristics of proteins (Kilara and Harwalkar, 1996). The conformation of a protein at a particular temperature depends on a delicate balance between the factors that favor the folded state and the factors that favor the unfolded state. These factors

include hydrophobic interactions, electrostatic interactions, hydrogen bonds, van der Waals forces, steric interactions and configurational entropy effects (Creighton, 1993; Dickinson and McClements, 1995; Damodaran, 1996). The protein will tend to exist in those kinetically accessible states that have the lowest free energies under the prevailing environmental conditions, e.g., temperature, pressure, and solution composition. The major factor stabilizing the native state of globular proteins is believed to be the hydrophobic effect. The molecule tends to adopt a compact arrangement that minimizes thermodynamically unfavorable hydrophobic interactions by having the non-polar amino acids located in the interior and polar amino acids located at the exterior (Tanford, 1991). The major factor favoring the denatured state of globular proteins is their configurational entropy, which increases with increasing temperature. Globular proteins therefore tend to unfold when they are heated above a certain temperature because the forces favoring the denatured state (*e.g.,* configurational entropy) increase above those favoring the native state (*e.g.,* hydrophobic interactions). The presence of co-solvents in the solution surrounding a protein may either promote or oppose protein unfolding, depending on their differing interactions with the native and denatured states (Timasheff, 1998).

The thermal denaturation of many globular proteins can be represented by the following equilibrium (Creighton, 1993):

$$N \leftrightarrow D \tag{6.2}$$

Here, N represents the native state and D represents the denatured state. This equilibrium is characterized by a free energy change ($\Delta G_{N\rightarrow D}$) and an equilibrium constant $K_{N\rightarrow D}$: $\Delta G_{N\rightarrow D} = -RT \ln(K_{N\rightarrow D})$, where $K_{N\rightarrow D} = [D]/[N]$. The equilibrium is also characterized by denaturation temperature T_m, which is the temperature where the concentrations of the native and denatured states of the protein are equal ($K_{N\rightarrow D} = 1$; $\Delta G_{N\rightarrow D} = 0$). Co-solvents either increase or decrease the thermal denaturation temperature of globular proteins depending on whether they are preferentially excluded or accumulated (Harwalker and Ma, 1989, 1996; Arntfield *et al.,* 1990; Timasheff 1993; Jou and Harper, 1996). Preferentially excluded co-solvents tend to favor the folded state over the unfolded state because there is a reduction in the surface area from which the co-solvent molecules are excluded. On the other hand, preferentially accumulated co-solvents tend to favor the unfolded state over the folded

state because there is an increase in the surface area to which the co-solvent molecules can bind. Hence, preferentially excluded co-solvents will tend to increase the heat-denaturation temperature and decrease the cold-denaturation temperature of a protein, whereas preferentially accumulated co-solvents will do the opposite. In practice, preferential interactions depend on the precise nature of the changes in the surface area and surface chemistry of a globular protein when it unfolds.

The influence of sucrose on the thermal stability of BSA was studied by Baier and McClements (2001). The thermal transition temperature of BSA dispersed in aqueous solutions containing between 0 and 40 wt% sucrose was measured using an ultrasensitive DSC (Table 6.1). The thermal transition temperature increased as the sucrose concentration increased, indicating that the change in the preferential interaction coefficient for the transition of the protein from the folded to unfolded states was negative ($\Delta\Gamma_{3,2}$), i.e., the transfer was unfavorable. Selected results from various studies of the impact of sugars on the thermal stability of some globular proteins including BSA are summarized in Table 6.2. Sugars increase the thermal stability of most globular proteins, however, the magnitude of the enhancement depends on co-solvent type, co-solvent concentration, protein type and solution conditions (*e.g.*, pH and ionic strength).

Table 6.1 Thermal properties of aqueous BSA solutions determined by analysis of dynamic shear rheology (2 wt% BSA, pH 7.0, 100 mM NaCl) or ultrasensitive DSC (0.5 wt% BSA, pH 7.0, 100 mM NaCl) (Baier and McClements, 2001).

Sucrose (wt%)	**T_{gel} (°C)**	**T_m (°C)**	**ΔT_m (°C)**	**ΔH (kJ mol^{-1})**	**$\Delta\Gamma_{32}$ (mol mol^{-1})**
0	78.1 ± 0.4	72.9 ± 0.1	0	102 ± 3	0
10	80.5 ± 0.2	73.8 ± 0.1	+0.9	103 ± 4	-0.8
20	83.3 ± 0.8	75.3 ± 0.1	+2.4	112 ± 5	-2.1
30	86.8 ± 0.4	76.9 ± 0.2	+4.0	117 ± 5	-3.3
40	89.4 ± 0.7	79.2 ± 0.2	+6.3	119 ± 5	-5.0

Table 6.2 Selected examples of the influence of weakly interacting co-solvents on thermal stability of globular proteins. Abbreviations: BSA = bovine serum albumin; β-Lg = β-lactoglobulin; RNase A= Ribonuclease A; WPI = whey protein isolate; WPC = whey protein concentrate.

Protein	**Sugar**	$(\delta\mu_2/\delta m_2)_{T,P,m3}$ [a]	**References**
Rnase A (pH 2.8)	0.2 M Trehalose	+4.02	Xie and Timasheff, 1997
	0.3 M	+4.71	
	0.4 M	+4.50	
	0.5 M	+4.97	
	0.7 M	+4.44	
	0.1 M Sucrose	+3.8	Lee and Timasheff, 19 91
	0.5 M	+4.3	
	1.0 M	+4.3	
RNase A (pH 8.8)	1 M Glucose	+1.7	Arakawa and Timasheff, 1982
	0.4 M Lactose	+1.8	
BSA (pH 6.0)	1 M Glucose	+10.4	Arakawa and Timasheff, 1982
	0.4 M Lactose	+12.8	
	30% Sucrose	+4.0	Baier and McClements, 20 01
Lysozyme (pH 3.0)	1 M Glucose	+3.0	Arakawa and Timasheff, 1982
	0.4 M Lactose	+3.9	
Lysozyme (pH. 4.0)	30% Sorbitol	+7.2	Cioci and Lavecchia, 1997
	30% Sucrose	+6.3	
	30% Glucose	+6.3	
	30% Sucrose	+6.3	
ChyTrp A (pH 2.0)	1 M Glucose	+5.1	Arakawa and Timasheff, 1982
	0.4 M Lactose	+7.5	
β-Lg (pH 4.65)	0.4 M Lactose	+6.0	Arakawa and Timasheff, 1982
β-Lg (pH 7.0)	30% Sucrose	+2.9	Harwalkar and Ma, 1989
WPI (pH 7.0)	30% Sucrose	+4.9,+5.9	Kulmyrzaev, 2000a,b
WPC (pH 6.0)	30% Sucrose	+5.1	Jou and Harper, 1996

6.2. Ligand binding

Many globular proteins have regions on their surface that are capable of binding particular types of molecules (ligands). The ability of proteins to bind ligands has important implications for their application as functional ingredients in foods, particularly when they are used as flavor binders or enzymes. The precise nature of the interactions between proteins and ligands depends on the unique characteristics of the molecules involved (Wyman and Gill, 1990; Friere, 1998). A protein may have single or multiple binding sites on its surface. If there is more than one binding site, then the binding of a ligand to one site may or may not influence the binding of a ligand to another site. The conformation of a protein molecule may change after one or more ligand molecules are bound, which may alter the binding characteristics of subsequent ligands. Binding or conformational changes may either be reversible or irreversible. The free energy of the binding process depends on the molecular

composition of the solution surrounding the protein and ligand molecules. The reaction normally takes place in water (or a dilute buffer solution), but the presence of a co-solvent may either favor or oppose the reaction depending on the nature of the preferential interactions. If a co-solvent molecule is preferentially excluded from the protein binding site, then it tends to favor the conformation with the lowest surface area, which is the protein-ligand complex. Thus preferentially excluded co-solvents tend to favor binding, whereas preferentially accumulated co-solvents tend to oppose binding. Nevertheless, it must be remembered that the influence of a co-solvent on ligand binding also depends on the preferential interactions of the co-solvent with the ligand, which may be different in sign and magnitude than the preferential interactions of the co-solvent with the protein binding site.

A number of experimental studies have examined the influence of weakly interacting co-solvents on ligand binding. The affinity of oxygen for hemoglobin is increased in the presence of preferentially excluded co-solvents (sucrose, stachyose and polyethylene glycols) because the co-solvents favor the protein-ligand conformation with the lowest surface area (Colombo *et al.*, 1992).

6.3. Surface Activity

The ability of amphiphilic protein molecules to adsorb to boundaries separating two phases has a major impact on their effectiveness as emulsifiers and foaming agents. A protein dispersed in an aqueous solution that is in contact with another bulk phase (*e.g.*, air or oil) will partition between the bulk aqueous solution and the interfacial region according to its concentration and surface activity (Adamson, 1990). This partitioning can be described in terms of the change in surface or interfacial tension as a function of temperature, and the concentration of the protein in the bulk aqueous phase. As the protein concentration in the aqueous phase increases, there is a concomitant decrease in the surface tension due to an increase in the protein concentration at the surface (until the surface becomes saturated with protein). The thermodynamic driving force for protein adsorption is a balance between differential interaction and steric exclusion effects. Protein molecules are larger than water molecules and therefore there is an unfavorable steric exclusion effect that opposes protein adsorption. Nevertheless, this is more than compensated for by the differential interaction contribution that favors protein adsorption because of a reduction in the

contact area between the polar and non-polar components in the system. The presence of a co-solvent in the aqueous phase may either favor or oppose protein adsorption. There will be a region surrounding the protein where the co-solvent and solvent concentrations are different from those in the bulk solution. In addition, there will be a region immediately below the air-water surface where the co-solvent and solvent concentrations are also different from those in the bulk solution. If a protein moves from the bulk solution to the interface there is a reduction in the volume of these preferential zones. Thus, if a co-solvent molecule is preferentially excluded from both the protein surface and the interfacial region, then protein adsorption will be favored in the presence of the co-solvent. On the other hand, if a co-solvent molecule is preferentially accumulated around the protein surface and the interfacial region, then protein adsorption will be opposed in the presence of the co-solvent. In practice, the co-solvent may interact differently with the interfacial region and the protein surface, hence the impact of a co-solvent on protein adsorption may be more complex.

Many globular proteins undergo conformational changes after adsorption to an air-water or oil-water interface (Dickinson, 1992; Dalgleish, 1996). These structural alterations can promote interactions between neighboring proteins (*e.g.*, hydrophobic or disulfide bonds) leading to the formation of a viscoelastic interfacial region. Co-solvents influence these interfacial conformational changes because of their differing interactions with the folded and unfolded states of the adsorbed protein. It should be noted that protein adsorption may be irreversible in systems where extensive protein-protein interactions occur at the interface and so the thermodynamic analysis presented above is not strictly applicable. Even so, it does provide some useful insights into the influence of co-solvents on the tendency of proteins to adsorb to interfaces.

A number of studies have recently been carried out to examine the influence of co-solvents on the interfacial properties of proteins, *i.e.*, adsorption kinetics, surface activity, surface packing and surface rheology (Rodriguez-Nino *et al.*, 1997, 1998; Rodriguez-Nino and Rodriguez-Patino, 1998a, b; Wilde *et al.*, 1998; Rodriguez-Patino and Rodriguez-Nino, 1999). In the presence of convection effects, relatively low concentrations of sucrose were found to decrease the adsorption rate of BSA to an air-water interface, but relatively high concentrations were found to increase the adsorption rate (Rodriguez-Nino *et al.*, 1997). The decrease in adsorption rate at low

sucrose concentrations was attributed to its ability to increase solution viscosity, thereby slowing down the movement of the protein molecules to the interface and reducing the protein-interface encounter frequency. On the other hand, the increase in adsorption rate at high sucrose concentrations can be attributed to the ability of the sucrose to increase the adsorption efficiency *i.e.,* the fraction of protein-interface encounters that led to adsorption. Sucrose molecules are preferentially excluded from the immediate vicinity of protein molecules as well as from the region immediately below an air-water interface. Consequently, protein adsorption is thermodynamically favored in the presence of sucrose because the overall volume of the excluded regions is decreased after adsorption. In the absence of convection effects, sucrose was found to always increase the adsorption rate of BSA at an air-water interface, which suggests that the adsorption efficiency effect always dominated the encounter frequency effect (Rodriguez Nino *et al.,* 1997; Guzey *et al.*, 2001). The surface rheology of globular protein films adsorbed at an air-water interface has also been shown to be strongly dependent on the presence of co-solvents in the aqueous phase (Rodriguez-Nino *et al.*, 1997). Sucrose was found to decrease the surface dilational rheology of the adsorbed protein layer, which was attributed to its ability to stabilize the folded state of the protein. Consequently, there was less surface denaturation of the protein after adsorption, leading to a reduction in the number of reactive groups capable of forming protein-protein interactions.

7. INFLUENCE OF CO-SOLVENTS ON PROTEIN FUNCTIONALITY

7.1. Water solubility

The water solubility of a protein is determined by the relative magnitude of its interactions with other protein molecules compared to its interactions with other constituents in the solution, *i.e.,* solvent and co-solvent molecules (Damodaran, 1996). If protein-solution interactions are more favorable than the average of protein-protein and solution-solution interactions, then the protein molecules prefer to be surrounded by solution rather than by each other and so the protein tends to be soluble. On the other hand, if protein-solution interactions are less favorable than the average of protein-protein and solution-solution interactions, then protein molecules prefer to be surrounded by other protein molecules rather than by solution molecules and so the protein tends to precipitate out of solution. The magnitude of protein-protein, protein-solution and solution-solution interactions depends on the molecular characteristics of the protein, environmental conditions, and solution composition. Usually, the water-solubility of a protein decreases as its surface hydrophobicity increases and its net electrical charge decreases (Damodaran, 1996). The presence of weakly interacting co-solvents in the aqueous medium surrounding the proteins can alter their water solubility directly by altering the balance of the soluble - insoluble protein equilibrium or indirectly by altering the protein conformation. Theories for predicting the influence of co-solvents on the solubility of proteins are reviewed by Arakawa and Timasheff (1985).

The influence of co-solvents on the water solubility of proteins may be quite complex, depending on protein type, co-solvent type, co-solvent concentration, pH and temperature, which may account for the apparently contradictory affects of co-solvents on protein solubility reported in the literature. For example, glycols (glycerol, ethylene glycol and propylene glycol) and sugars (saccharose, sorbitol, sorbose and sucrose) were found to increase the water solubility of a variety of globular proteins near their isoelectric points (Antipova and Semenova, 1997a, b; Conti *et al.*, 1997),

whereas sucrose was found to decrease the water-solubility of a globular protein (ovalbumin) at pH values away from the isoelectric point (Antipova *et al.,* 1999). The influence of co-solvents on protein solubility has also been shown to be strongly temperature dependent, with the interactions going from net repulsive at low temperatures to net attractive at high temperatures (Antipova *et al.,* 1999).

7.2. Protein stabilization

The functional properties of globular proteins depend strongly on the molecular structure and dynamics of the proteins under the prevailing environmental conditions. A globular protein may undergo a transition from a "native" state to a "denatured" state in response to a change in its environment during extraction, isolation, utilization or storage, *e.g.,* temperature variations, dehydration, mechanical stresses or alterations of solvent composition (Harwalkar and Ma, 1989; Allison *et al.,* 1999; Kreilgaard *et al.,* 1999; Iametti *et al.,* 1998, 1999; Saunders *et al.,* 2000). A change in the molecular conformation of a globular protein often has an adverse impact on its functionality in food products. Consequently, a number of strategies have been developed to enhance the stability of globular proteins. Weakly interacting co-solvents are often added to aqueous solutions of globular proteins to stabilize them against unfolding or aggregation induced by temperature, mechanical stress or dehydration treatments.

7.2.1. Protection against freezing

The globular proteins in many foods are denatured by freezing/thawing in the absence of co-solvents, but are stable in the presence of co-solvents. Muscle proteins in fish and meat tissue are particularly sensitive to denaturation during freezing and/or frozen storage (MacDonald and Lanier, 1994; MacDonald *et al.,* 1996a; Kijowski and Richardson 1996; Chang and Regenstein, 1997; Wang and Xiong, 1998; Carvajal *et al.,* 1999; Nowsad *et al.,* 2000). Myofibillar proteins, such as myosin, partially unfold when the temperature falls below a particular level, which exposes non-polar groups normally located in their protein interior. The exposed non-polar groups can interact with similar groups on neighboring proteins leading to protein aggregation. This aggregation leads to adverse changes in the texture and water holding capacity of the muscle tissue, which is undesirable from a commercial standpoint. For this reason, low molecular weight co-solvents, known as cryoprotectants, are added to many

muscle food products to inhibit the loss of protein functionality during frozen storage. Sucrose and sorbitol are commonly added as cryoprotectants to fish muscle (Bakir *et al.*, 1994; MacDonald *et al.*, 1996a). The ability of sucrose and sorbitol to act as cryoprotectants is believed to be due at least in part to their preferential exclusion from the immediate vicinity of the protein surface. For simple carbohydrates and polyols, the most likely molecular mechanism contributing to preferential exclusion is the steric exclusion effect. In systems where steric exclusion dominates the transfer free energy is more unfavorable for the unfolded state (large surface area) than for the folded state (small surface area) of the protein. Consequently, co-solvents tend to enhance the stability of the folded state relative to the unfolded state, which would depress the cold denaturation temperature of the myofibrilar proteins. It should be stressed that sugars and polyols may also enhance the freeze stability of fish proteins through their ability to increase the glass transition temperature of the freeze concentrated solution (Carvajal *et al.*, 1999).

7.2.2. Protection against heating

If a globular protein is held at a sufficiently high temperature for a long time it will become partially or fully denatured, which may have an adverse effect on its functional properties, *e.g.*, surface activity, droplet stabilization, catalytic activity, or binding properties. For this reason, co-solvents are widely used to increase the stability of globular proteins during drying processes that involve heating, such as spray-drying and air-drying (Allison *et al.*, 1998, 1999; Murray and Liang, 1999, 2000). Air drying (78-88^{o}C) aqueous solutions of globular proteins (β-lactoglobulin and BSA) in the presence of relatively high concentrations of sugars has been found to improve their foaming capacity. One of the major factors that contribute to the retention of protein functionality after drying at high temperatures is the ability of the co-solvents to increase the thermal stability of the proteins (Murray and Liang, 1999, 2000). The folded state of the protein has better functionality for foam stabilization than the unfolded state. Sugars and polyols have also been shown to increase the thermal stability of many enzymes and other globular proteins (Gekko, 1982; Cioci and Lavecchia, 1994; Tzannis and Prestrelski, 1999a, b).

7.2.3. Protection against pressure treatment

The use of high-pressure processing is finding increasing use in the food industry for the production of food products (Thakur and Nelson, 1998; Tewari *et al.,* 1999). High pressure may induce the unfolding of globular proteins, which can lead to a loss of their functional properties (Lanier, 1998). Co-solvents have also been used to increase the stability of globular proteins to unfolding during high-pressure treatment (Dumay *et al.,* 1994). The unfolding and aggregation of protein in β-lactoglobulin solutions (pH 7.0) after pressure treatment (450 MPa, 25^{o}C for 15 min) has been studied in the presence and absence of sucrose. The presence of 5% sucrose in the aqueous phase of the solutions was found to reduce the extent of protein denaturation and aggregation during pressure-treatment. Similarly, sucrose has been shown to retard the denaturation and aggregation of ovalbumin (pH 7.0) treated at 400-800 MPa (Iametti *et al.*, 1998, 1999). Sugars and polyols have also been found to increase the stability of fish muscle proteins to high-pressure denaturation (50 to 200 MPa) (Ashie *et al.,* 1999). The most likely reason for the increased pressure stabilization of globular proteins by sugars and polyols is that they were preferentially excluded more from the unfolded state than from the folded state of the protein, although this has not been established experimentally.

7.2.4. Protection against dehydration

Many foods and ingredients that contain globular proteins are dehydrated because removal of water improves their physical, chemical and microbiological stability (Allison *et al.*, 1998, 1999, 2000). Nevertheless, many dehydration techniques can promote protein denaturation and loss of functionality.

In the absence of co-solvents, many globular proteins have been shown to lose their functionality during air-drying, spray-drying and freeze-drying processes, but in the presence of certain co-solvents protein functionality can be retained. At least three different physiochemical mechanisms have been proposed to account for the ability of co-solvents to enhance protein stability during dehydration processes (Allison *et al.*, 1998). First, co-solvents that are preferentially excluded from protein surfaces tend to favor folded over unfolded states of protein molecules, thereby retarding cold-, heat-, surface- and pressure-denaturation processes. Second, some co-solvents are capable of forming hydrogen bonds with the surface of dried proteins, thereby inhibiting

protein unfolding and aggregation by taking the place of water molecules (Allison *et al.*, 1999). Third, some co-solvents are capable of forming a highly viscous glass phase around the protein molecules that retards protein degradation by decreasing the molecular mobility of the system (Miller *et al.*, 1998; Allison *et al.*, 1999). It is likely that all of these mechanisms play some role in enhancing protein stability to dehydration, but the relative importance of each mechanism still needs to be established for particular systems.

Co-solvents that are used to increase protein stability during freezing, heating, high-pressure treatment and dehydration are known as cryo-protectants, thermo-protectants, baro-protectants and osmo-protectants, respectively. The physicochemical basis of the action of these co-solvent protectants is believed to be similar in many cases, *i.e.*, the co-solvents favor the folded state over the unfolded state. Thus a number of co-solvents that have been shown to increase the heat-stability of globular proteins, have also been shown to increase their stability to freezing (Carpenter and Crowe, 1988; MacDonald and Lanier, 1994; Dondero *et al.*, 1996) or high-pressures (Dumay *et al.*, 1994).

7.3. Gelation

Globular proteins are often used in foods because of their ability to associate with each other and form three dimensional networks that entrap water and other water-soluble components (Zeigler and Foegeding, 1991; Clark, 1992; Doi, 1993; Damodaran, 1996). The appearance, rheology and water holding capacity of the gels depend on the spatial organisation and molecular interactions of the proteins within the gel network, which in turn depend on protein type, protein structure, aqueous phase composition, temperature and mechanical stresses. The creation of food gels with desirable quality attributes depends on an understanding of the relationship between the structure and interactions of protein molecules and the technologically important properties of the gel.

To act as an effective gelling agent a globular protein must be able to make intermolecular cross-links that lead to the formation of a three-dimensional network of aggregated proteins that extends throughout the volume of the system (Mulvihill and Kinsella, 1987). This network gives the system rigidity and traps water through capillary forces. The appearance of the gel depends on the interaction of the protein network with light. Globular proteins in their native state do not normally form gels

in aqueous solution because the intermolecular repulsive forces outweigh the attractive forces. To form a gel it is usually necessary to heat the solution to a temperature where the globular proteins unfold and expose non-polar and sulfhydryl amino-acid side groups that are normally embedded in the protein interior (Mulvihill and Donovan, 1987). Under appropriate conditions these reactive side groups interact with each other so that the proteins aggregate and form a gel. The presence of co-solvents could alter the gelation mechanism in a number of ways. First, they change the temperature at which the globular protein molecules unfold, which means that the system would have to be heated to a higher or lower temperature before gelation occurred. Secondly, co-solvents alter the magnitude of the attractive and repulsive forces between protein molecules, which can alter the structural organization of the protein molecules within a gel as well as the strength of the bonds between the proteins. Third, co-solvents increase the viscosity of aqueous solutions, which decreases the rate of protein-protein encounters. As a consequence co-solvents can alter both the formation and the final physicochemical properties (appearance, texture and water holding capacity) of protein gels in a complex manner.

In a recent study, Baier and McClements (2001) examined the influence of sucrose (0 to 40 wt%) on the thermal stability, and gelation of bovine serum albumin (BSA). Measurements of the effect of sucrose on the thermal stability of 0.5 wt% BSA solutions (pH 7) made using an ultrasensitive differential scanning calorimeter showed that sucrose increased the protein denaturation temperature and increased final gel rigidity. Sucrose molecules are preferentially excluded from the immediate vicinity of globular proteins (Timasheff, 1998), which generates an osmotic stress that favors a decrease in contact area between the protein surface and the surrounding solution. This osmotic stress influences both the thermal stability and the aggregation of protein molecules. The increase in osmotic stress with increasing sucrose concentration stabilized the native globular state of the proteins, which meant that the solutions had to be heated to higher temperatures before the protein molecules unfolded. The increase in osmotic stress also meant that once the protein molecules did unfold they had a greater tendency to aggregate, which accounted for the observed increase in gel rigidity.

7.4. Emulsification

Globular proteins are commonly used as emulsifiers in oil-in-water food emulsions because of their ability to adsorb to oil-water interfaces and protect oil droplets against aggregation (Dalgleish, 1996; Dickinson, 1994, 1997, 1999a, b). To be an effective emulsifier a protein must rapidly adsorb to the surfaces of oil droplets created during homogenisation. Protein adsorption lowers the interfacial tension, which facilitates droplet disruption and reduces the amount of energy required to generate small droplets. The creation of a protein membrane around the droplets also prevents the droplets from coalescing with each other during the homogenisation process. After homogenisation, the adsorbed protein film must be capable of imparting long-term stability to food emulsions against droplet coalescence and flocculation. The stability of an emulsion to droplet aggregation depends on the relative magnitudes of the attractive and repulsive interactions between the droplets, *e.g.*, van der Waals, steric, electrostatic, hydrophobic and depletion (McClements, 1999). To act as an effective emulsifier a protein must be capable of rapidly adsorbing to the surface of a newly created oil droplet during homogenization, reducing the interfacial tension (to facilitate further droplet disruption) and forming a protective membrane (to prevent droplets from aggregating).

The presence of weakly interacting co-solvents in the aqueous phase of an emulsion can influence protein adsorption, interfacial characteristics and droplet stability through a variety of different mechanisms. At a planar air-water interface co-solvents influence both the composition and physiochemical properties of the interface through their effect on the thermodynamics and kinetics of adsorption (Rodriguez-Nino *et al.*, 1997, 1998a, b; Taiwo *et al.*, 1997; Rodriguez-Nino and Rodriguez-Patino, 1998a, b; Wilde *et al.*, 1998; Rodriguez-Patino and Rodriguez-Nino, 1999). Co-solvents may also influence the physiochemical properties of emulsions by altering the interactions between protein stabilized emulsion droplets. Globular proteins often partially unfold after they adsorb to the surface of emulsion droplets because of the change in their thermodynamic environment (Dickinson and Matsumura, 1991; McClements *et al.*, 1993; Monahan *et al.*, 1993). This "surface denaturation" often promotes enhanced protein-protein interactions because unfolding leads to increased exposure of reactive amino acid side groups that favor hydrophobic interactions and disulfide bond formation. Enhanced protein-protein interactions between proteins adsorbed to the

same droplet cause an increase in the viscoelasticity of the interfacial membrane (Dickinson and Matsamura, 1991). On the other hand, enhanced protein-protein interactions between proteins adsorbed onto different droplets promotes droplet flocculation (McClements *et al*., 1993; Monahan *et al*., 1993). Preferentially excluded co-solvents would be expected to decrease the degree of surface denaturation of globular proteins in an emulsion, but to increase the strength of protein-protein interactions once the proteins had unfolded, whereas preferentially accumulated co-solvents would be expected to have the opposite effect. The influence of co-solvents on the viscoelasticity of droplet membranes and the degree of droplet flocculation would therefore be expected to depend strongly on co-solvent type, co-solvent concentration, protein type and environmental conditions.

7.5. Foaming

Foams consist of a condensed continuous phase (liquid as in whipped cream and ice cream or solid as in cake and bread) and a gaseous dispersed phase (Dickinson and Stainsby, 1982; Dickinson, 1992). Globular proteins are widely used in the food industry to facilitate the formation and stabilization of foams (Damodaran, 1996). The quality attributes of foams, such as appearance, texture and stability, are determined by the size and concentration of the gas bubbles distributed throughout the continuous phase (Damodaran, 1996). Protein molecules rapidly adsorb to the surfaces of freshly formed bubbles, reducing the interfacial tension and facilitating further bubble disruption. Once adsorbed to the surface of gas bubbles protein molecules protect them from merging with each other by generating repulsive forces between the bubbles, *e.g.*, electrostatic, steric and hydration repulsion. Many globular proteins undergo conformation changes after they have been adsorbed to the surface of a bubble, which promotes the formation of intermolecular protein-protein interactions, often through hydrophobic and disulfide bonds. As a result of these protein interactions the membrane surrounding the gas bubbles becomes highly viscoelastic and resistant to deformation.

Weakly interacting co-solvents may alter the formation and stability of foams in a variety of ways. Sugars have been shown to decrease the foam overrun after mechanical agitation, which has been attributed to their ability to stabilize the native state of the protein. In the presence of sugars, the denaturation of globular proteins at

the air-water interface is reduced. For example, trehalose and sucrose have been found to decrease the surface denaturation of a variety of globular proteins during foaming (Clarkson *et al.,* 2000). The increased stability of the proteins to surface denaturation means that fewer protein-protein interactions occur, and so the interfacial membrane is more prone to rupture. Consequently, the number of droplets that are stabilized by protein during the foaming process is reduced, leading to lower foam overrun. On the other hand, the presence of sugars has been found to increase foam stability, which has been attributed to their ability to increase the viscosity of the continuous phase and therefore reduce drainage of the liquid separating the bubbles. Sugars have been found to improve the foaming properties of spray-dried globular proteins, presumably by preventing thermal denaturation of the proteins during drying (Murray and Liang, 1999, 2000).

7.6. Enzyme Activity

Enzymes are globular proteins that are extremely efficient at accelerating the rate of specific biochemical reactions (Creighton, 1993). The protein molecule eventually returns to its original conformation once the conversion of the substrate to the product has taken place. Nevertheless, it must be stressed that enzymes normally undergo one or more temporary changes in their molecular conformation during the time that they bind the various ligand molecules involved in the reaction (Creighton, 1993).

The effectiveness of an enzyme at catalyzing a biochemical reaction may be altered by the presence of co-solvents in the surrounding medium through a variety of different physiochemical mechanisms (Cioci *et al.,* 1994; Gupta *et al.*, 1997; Jensen *et al.*, 1997; Lambert *et al.*, 1997). Firstly, co-solvents may alter the molecular conformation of enzymes in both their native and transition states (Luque *et al.,* 1998). Secondly, co-solvents may alter the thermal stability of an enzyme (Lozano *et al.*, 1994; Rajeshwara and Prakash, 1994; Cioci *et al.*, 1994; Mukorah *et al.*, 1998). Thirdly, co-solvents may alter the binding of substrates, products or cofactors to an enzyme (Cioci and Lavecchia, 1997; Parsegian *et al.*, 1995). Fourthly, co-solvents may retard the diffusion of molecules through the aqueous phase, such as reactants diffusing towards the enzyme or products diffusing away from the enzyme (Sierks *et al.*, 1997). These physiochemical mechanisms may act individually or together to change the overall kinetics and thermodynamics of the biochemical reaction. The

molecular characteristics of the protein and co-solvent molecules, as well as the prevailing environmental conditions, will determine whether a co-solvent promotes or retards a particular biochemical reaction. In some systems, co-solvents have also been shown to change the specificity of an enzyme for a particular substrate and even to change the type of reaction products produced (Mohapatra and Hsu, 1999).

A number of studies have shown that certain co-solvents are capable of increasing the catalytic activity of enzymes at relatively low concentrations, but decreasing the activity at relatively high concentrations. The increase in enzyme activity with co-solvent concentration at relatively low co-solvent concentrations has been attributed to the ability of the co-solvents to alter the conformation of the enzymes into a more active form. On the other hand, the decrease in enzyme activity that occurs at higher co-solvent concentrations has been attributed to retarded diffusion of molecules within the system (Lambert *et al.*, 1997; Sierks *et al.*, 1997). Even so, further studies need to be carried out to clarify the relative importance of the effects of co-solvents on the various mechanisms involved, *e.g.*, ligand binding, conformational changes and diffusion-limited reactions.

A number of studies have shown that co-solvents may either increase or decrease the physical and chemical stability of enzymes in aqueous solutions. One of the most detailed studies of the influence of weakly interacting co-solvents on the thermal stability of a food enzyme has been carried out on purified lipase from wheat germ (Rajeshwara and Prakash, 1994). The authors showed that glucose and glycerol increased the thermal stability of the native state of the enzyme. Measurements of the preferential interaction parameter of the protein in co-solvent solutions showed that the co-solvent was preferentially excluded by the protein. The stabilization of the enzyme by the co-solvents was therefore attributed to the preferential exclusion of the co-solvents from the enzyme's surface. Co-solvents have also been shown to influence the stability of enzymes to chemical degradation, *e.g.*, sucrose decreased the rate of methionine oxidation in substilisin (DePaz *et al.*, 2000). This phenomenon was attributed to the ability of sucrose to favor more compact native protein conformations, thereby limiting the accessibility of oxidizing substances to the protein site where oxidation occurs.

Weakly interacting co-solvents may also modulate enzyme activity by altering the capacity of enzymes to bind ligands (*e.g.*, reactants, products, cofactors). Polyols and

sugars decrease the heat-induced dissociation of the cofactor from the enzyme, probably through their ability to stabilize the more compact native protein conformations through a preferential exclusion mechanism (Cioci and Lavecchia, 1997).

In summary, co-solvents may alter the efficiency of enzyme catalyzed reactions through a number of different physicochemical processes, with the precise effect of the co-solvent on enzyme activity depending on environmental conditions, co-solvent type, co-solvent concentration and protein type.

8. EXPERIMENTAL TECHNIQUES TO STUDY ADSORPTION KINETICS

There are several experimental methods for studying adsorption kinetics: oscillating jet, drop volume, maximum bubble pressure, axisymmetrical drop shape analysis, growing drop tensiometry and some others. Table 8.1 gives an overview of some characteristics of dynamic surface and interfacial tension methods.

Table 8.1 Characteristics of some dynamic surface and interfacial methods (Duhkin *et al.*, 1995).

Method	**Time range**	**Temperature range (°C)**	**Suitability for liquid/liquid**	**Suitability for liquid/gas**
Drop volume	1 s-20 min	10-90	good	good
Elastic ring	10 s-24 h	20-25	bad	good
Growing drops and bubbles	0.01 s-600 s	10-90	good	good
Maximum bubble pressure	1 ms-100 s	10-90	possible	good
Oscillating jet	0.001 s-0.01 s	20-25	bad	good
Pendant drop	10 s-24 h	20-25	good	good
Plate tensiometer	10 s-24 h	20-25	possible	good
Pulsating bubbles	0.005 s-0.2 s	20-25	Bad	good
Ring tensiometer	30 s-24 h	20-25	bad	good

The drop methods, drop volume, drop pressure and drop shape are the most general ones. These methods are applicable to both liquid/liquid and liquid/gas interfaces and need only small amounts of solvent and solute. In addition, temperature control is easily arranged.

For dynamic adsorption studies several methods are suitable. The selection of a certain method depends on the experimental conditions, temperature, and time interval. These experimental conditions are governed by the surface activity of the surfactant or the polymer under study and its concentration.

The accuracy of all methods, over time intervals from several seconds to hours is usually ±0.1 mN/m. Special instruments enable measurements in the millisecond time scale, however such studies are performed with less accuracy (Duhkin *et al.*, 1995).

In this study, pendant drop technique was used to conduct surface tension measurements of samples.

8.1. Pendant Drop Technique

The pendant or sessile drop technique has been developed to determine interfacial tensions from the shape of drops without a direct contact of the interface. First experiments were performed by measuring characteristic drop diameters, and interpreting them on the basis of different tables. Later, the direct fitting of drop shape coordinates to the Gauss-Laplace equation was used to determine interfacial tension and contact angle data. The profile of an axyssimetric drop can be calculated in dimensionless co-ordinates from the following equation

$$\frac{d\phi}{dS} = 2 - \beta Y - \frac{\sin\phi}{X} \tag{8.1}$$

$$\frac{dX}{dS} = \cos\phi \tag{8.2}$$

$$\frac{dY}{dS} = \sin\phi \tag{8.3}$$

where *X*, *Y*, and *S* are made dimensionless by dividing *x*, *y*, and *s*, respectively, by R_o. *x* and *y* are the horizontal and vertical coordinate, s is the arc length of the profile measured from the drop apex and φ is the angle made by the radius of curvature and the *y* axis. $\gamma = \frac{\Delta\rho g R_0^2}{\beta}$ is a parameter which contains the density and surface tension of the liquid. The definition of all co-ordinates and characteristic parameters is given in Fig 8.1 (Duhkin *et al*., 1995).

Calculation of interfacial tension with fitting procedure is fast and precise. In order to fit the experimental coordinates of the drop shape, four parameters have to be adjusted: the localization of the drop apex X, Y, the radius of curvature R_0 and the parameter β. A software package called ADSA detects the drop edge coordinates and fits the Gauss-Laplace equation to these data. Different experimental set ups have been develop to determine the surface and interfacial tension from the shape of pendant drops. The experimental set-up for this study will be described later.

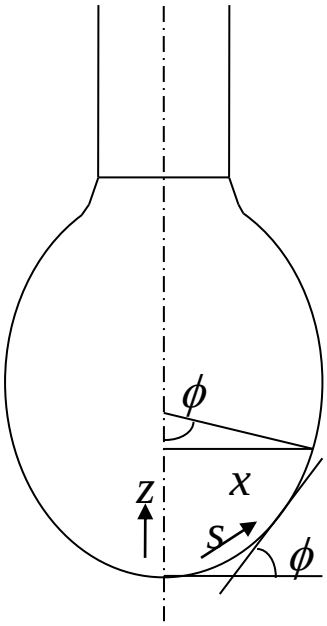

Figure 8.1 The geometry of a pendant drop (Duhkin *et al*., 1995)

9. THEORETICAL MODELS OF DIFFUSION-CONTROLLED ADSORPTION KINETICS

The adsorption kinetics of molecules at liquid interfaces can be described by qualitative and quantitative models. The first physically founded model for interfaces with time invariant area was derived by Ward and Tordai in 1946 (Duhkin *et al*., 1995). This model is based on the assumption that the time dependence of interfacial tension is caused by a transport of molecules to the interface and can be correlated to the interfacial tension (γ) of the adsorbing molecules. The so-called diffusion controlled adsorption kinetics model is as follows

$$\gamma(t) = 2\sqrt{\frac{D}{\pi}}\left(c_0\sqrt{t} - \int_0^{\sqrt{t}} c(0, t-\tau)d\sqrt{\tau} \right) \tag{9.1}$$

where D is the diffusion coefficient and c_o is the surfactant bulk concentration. The integral equation describes the change of surface tension with time t.

There are two general ideas to describe the dynamics of adsorption at liquid interfaces. The diffusion controlled model assumes the diffusional transport of interfacially active molecules from the bulk to the interface to be the rate-controlling process. If the diffusions assumed to be fast in comparison to the transfer of molecules between the subsurface and the interface the model is called kinetic-controlled (Duhkin *et al*., 1995).

Transport in the solution bulk is controlled by diffusion of adsorbing molecules if any liquid flow is absent. The transfer of molecules from the liquid layer adjacent to the interface, the so-called subsurface, to the interface itself is assumed to happen without transport. This process is determined by molecular movements.

The protein concentration distribution in the solution bulk at time t = 0 is assumed to be equal to c_0 for x<0 and zero for x>0. If a diffusion process starts at t>0, the concentration distribution is given by

$$c(x,t) = \frac{c_0}{2}\left[1 - erf\left(\frac{x}{2\sqrt{Dt}}\right)\right] \tag{9.3}$$

The adsorbed amount can be calculated for a model in which the change of surface concentration with time is assumed to be proportional to the concentration gradient at x=0, the location of the interface. This model is in accordance with the first diffusion law,

$$\frac{d\Gamma}{dt} = -D\frac{\partial c}{\partial x}, x = 0 \tag{9.4}$$

From equations (9.3) and (9.4) the first relation (9.5) is obtained which describes in a very simple way the change of adsorption with time,

$$\gamma(t) = 2c_0\sqrt{\frac{Dt}{\pi}} \tag{9.5}$$

This relation is very often used as a rough estimate and results from Equation (9.1) when the second term on the right hand side is neglected.

The quantitative description of adsorption kinetics process is much more complicated than the use of simplified methods mentioned above. The reader is referred elsewhere for a detailed review on the quantitative models of adsorption kinetics of surface-active molecules (Duhkin *et al*., 1995).

10. MATERIALS AND METHODS

10.1. Materials

Bovine serum albumin (BSA) (Lot: 10K1278) was obtained from Sigma Chemical Company (St. Louis, MO). The protein was of the highest native pure grade, prepared from Bovine milk using heat shock fractionation. BSA is a protein consisting of three specific domains and a molecular weight of 66,000 Daltons. It contains 582 Amino acid residues with 17 disulfide bonds and one free sulfhydryl group in its native state. *D*-fructose and α-*D*-glucose (purity 99+%) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sucrose was purchased from Fisher Chemicals (Fair Lawn, NJ). Double distilled and de-ionized water was used to prepare all solutions.

10.2. Methods

10.2.1. Solution preparation

All sugar and protein solutions were prepared by dissolving solutes in double distilled and de-ionized water and passed through a filter with 0.22 μm pore width (Millipore Corp. Bedford, MA) to remove additional impurities such as bacterial cells or undissolved material. A series of BSA solutions with different protein concentrations was prepared for surface tension measurements by diluting the protein stock solution to yield solutions that had protein contents between 3×10^{-4} and 6×10^{-11} M. Sugar solutions (fructose, glucose or sucrose) were prepared by dissolving sugars in distilled water to yield solutions with concentrations ranging from 0.5 to 40 w/w% (on a total weight basis). Solutions containing both BSA and sugars were prepared by dissolving BSA in the appropriate sugar solutions. All BSA solutions were prepared 2 hours in advance of the experiment and were stirred thoroughly to ensure proper hydration of proteins. The solutions were stored at 5°C in a refrigerator and used in one day. The pH of protein solutions was determined to be between 7.11 and 7.13.

10.2.2. Density measurements

Precise density data of solutions are required to ensure accurate determination of surface tension using drop shape analysis. The density of all solutions was initially measured using a specific gravity bottle obtained from Fisher Chemicals. The thermal expansion coefficient of the gravimetric bottle was determined over a temperature range between 15 and 25^{o}C (Julabo F 25 water bath) to determine the corrected volume of the bottle at the measurement temperature (20.0 ± 0.5^{o}C). Densities of the sugar solutions determined using this method were in good agreement with tabulated values reported in the literature (Linde, 2000). Measurements were later verified using a digital density meter (DMA 35N) obtained from Anton Paar GmbH (Graz, Austria). The accuracy of density measurements using the DMA 35N was ± 0.001 g/cm^{3}.

10.2.3. Surface tension measurements

A drop shape analysis tensiometer (Model DSA-G10 MK2, Kruss USA, Charlotte, NC) was used to determine surface tension. The tensiometer determines the shape of pendant drops or bubbles through numerical analysis of the entire drop shape. The relationship between the drop shape and the interfacial tension has been derived from the Young-Laplace equation of capillarity and a detailed description can be found elsewhere. The accuracy of surface tension measurements using drop shape analysis tensiometer is in the order of ± 0.2 x 10^{-3} N/m.

Surface tension measurements were carried out at controlled room temperature (20.0 ± 0.5^{o}C). Triplicate tests were performed for each measurement. An air bubble was formed at the inverted tip of a syringe that was submerged in a cuvette containing the protein and/or sugar solution. The syringe/cuvette system was positioned on an optical bank between the light source and a high-speed CCD camera. The CCD camera was connected to a video frame-grabber board for recording the image onto the hard-drive of a computer at a speed of 1 frame per second. The image was then analyzed using contour analysis of the drop profile and the interfacial tension was calculated by fitting the drop shape to the previously mentioned Young model. A schematic description of the drop shape analyzer is given in Figure 10.1.

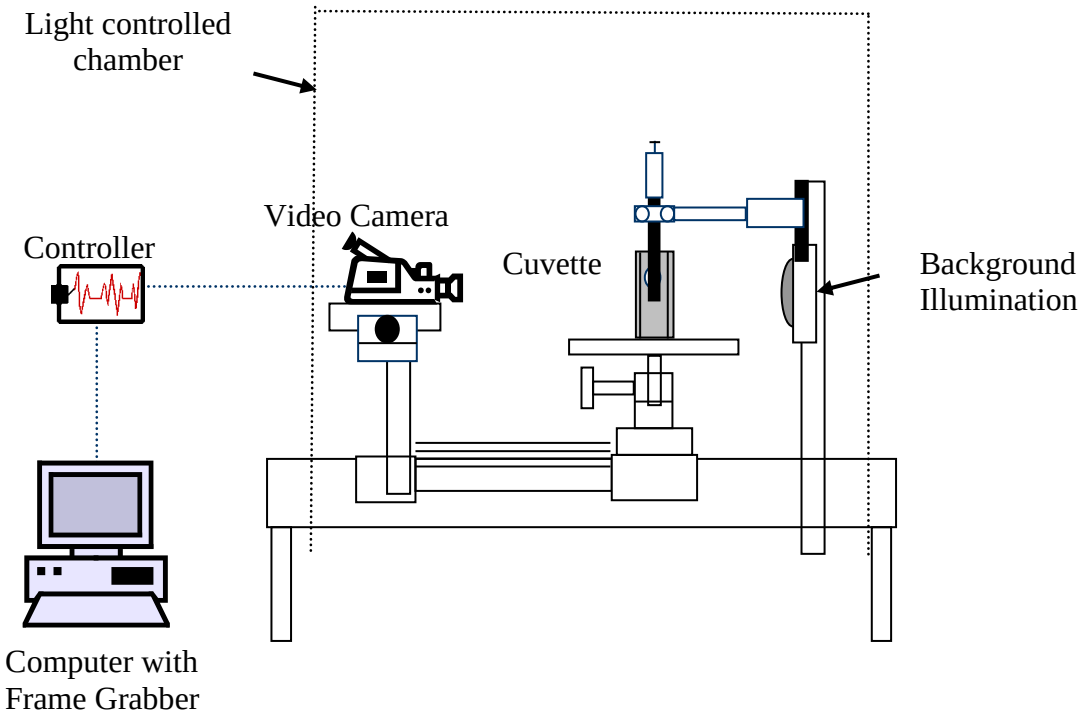

Figure 10.1 Drop shape analyzer DSA-G10 MK2 (Kruss USA, Charlotte, NC)

Data from surface tension measurements were plotted as surface pressure (Π). Surface pressure is the decrease in surface tension of a pure solvent caused by the addition of the protein. In other words, it is the difference between the surface tension of the protein solution and that of the solvent. Throughout this study surface pressure will be used to interpret adsorption kinetics data.

10.2.4. Effective diffusion coefficient calculations

A diffusion controlled adsorption model was applied to analyze the adsorption mechanism of bovine serum albumin solutions in presence of glucose, sucrose and fructose. The diffusion coefficients calculated were effective values (D_{eff}). This model (Equation 10.1) is an asymptotic solution to the model proposed by Ward and Tordai in 1946, which was given in the eighth chapter.

The difference between the surface tension of the solution and that of the solvent phase is defined as surface pressure. The slopes of surface pressure versus square root of time plots were used to determine the effective diffusion coefficient. At $t \rightarrow 0$ and for low protein concentration diffusion coefficient can be determined directly using the slopes of surface pressure (Π)– square root of time ($t^{1/2}$) plots from the equation

$$D_{eff} = \frac{\pi}{4}\left[\frac{1}{RTc}\left(\frac{d\Pi}{dt^{1/2}}\right)_{t\to 0}\right]^2 \qquad (10.1)$$

where R (8.3143 J mol^{-1} K^{-1}) is the universal gas constant, T (K) the temperature and c (mol/dm^3) the concentration in the bulk phase. Surface tension of water at 20°C is 72.8 x 10^{-3} N/m (Linde, 2000).

11. RESULTS AND DISCUSSIONS

11.1. Influence of Protein Concentration on Adsorption Kinetics

The change in surface pressure with time was measured for aqueous BSA solutions with concentrations ranging from 3 x 10^{-4} to 6 x 10^{-9} M. Figure 11.1 shows the results of six selected concentrations within this concentration range.

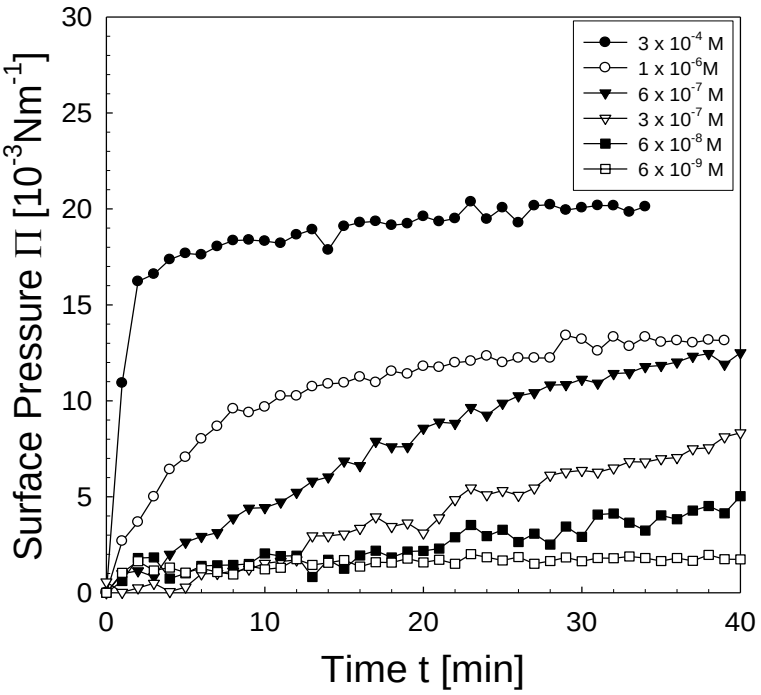

Figure 11.1 Adsorption of BSA at the air-water interface. BSA solutions had concentrations ranging from 3 x 10^{-4} to 6 x 10^{-9} M. As protein adsorbs at the interface, the surface pressure increases with increasing age of the interface.

At the lowest protein concentration (6 x 10^{-9} M), little change in surface pressure with time was observed, indicating that no appreciable adsorption of BSA to the air-water interface occurred. The surface pressure of BSA solution at this low concentration (6 x 10^{-9} M) remained nearly constant and was approximately equal to the value found for pure water at 20°C (72.8 x 10^{-3} N/m) (Linde, 2000). As the protein concentration increased, the surface pressure increased more rapidly with time indicating that BSA adsorbed more rapidly at the air-water interface. The equilibrium surface pressure

increased with increasing concentration, which correlates to more protein being adsorbed to the air-water interface. The results are in qualitative agreement with adsorption kinetic data reported earlier by other authors using globular proteins such as β-casein and β-lactoglobulin (Wüstneck *et al*., 1996). Previously in the literature it was given as a general rule that the adsorption rate of BSA increases as the concentration of BSA in the bulk phase increases (Rodriguez-Nino *et al.*, 1997).

In this study a protein concentration of 3×10^{-6} M as used as well as 3×10^{-4} M, since this concentration gave adsorption rates that could be used to accurately observe the initial period of adsorption where the surface pressure is $\leq 2 \times 10^{-3}$ N/m. The difference in the rate of adsorption between 3×10^{-4} M and 3×10^{-6} M BSA in pure water is given in Figure 11.2. The adsorption data of the initial period is required, because the Equation 10.1 is only applicable in the initial stages of adsorption, where the decrease in surface tension is less than 2×10^{-3} N/m. When the concentration of BSA is as high as 3×10^{-4} M the initial adsorption rate is so high that these data points cannot be recorded. Therefore, only the data from the measurements with 3×10^{-6} M BSA were used to analyze adsorption kinetics which will be given in the section 11.3.

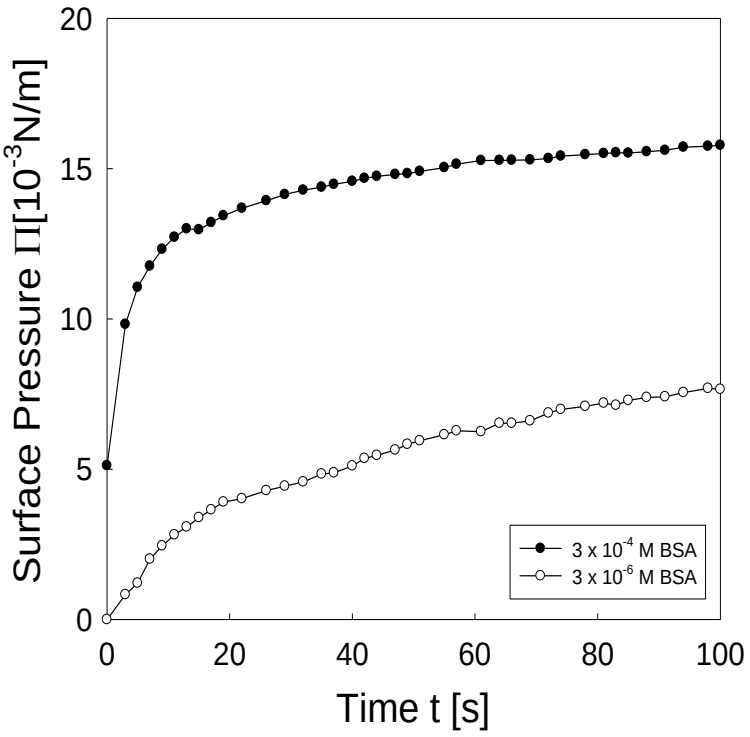

Figure 11.2 Adsorption rate of BSA at 3×10^{-4} M and 3×10^{-6} M concentrations

11.2. Influence of Sucrose Concentration on Protein Adsorption Kinetics

The variations in surface pressure with time for 0.02 wt% BSA solutions (3 x 10^{-6} M) with 0 to 40 wt% sucrose concentrations were also measured (Figure 11.3). At all sucrose concentrations, the surface pressure increased with time after the air bubble was introduced into the aqueous solution. The rate of increase in the surface pressure decreased as the sucrose concentration in the aqueous solutions increased. The largest change in adsorption kinetics and plateau surface pressure occurred when the sugar concentration was increased from 0 to 10 wt% sucrose.

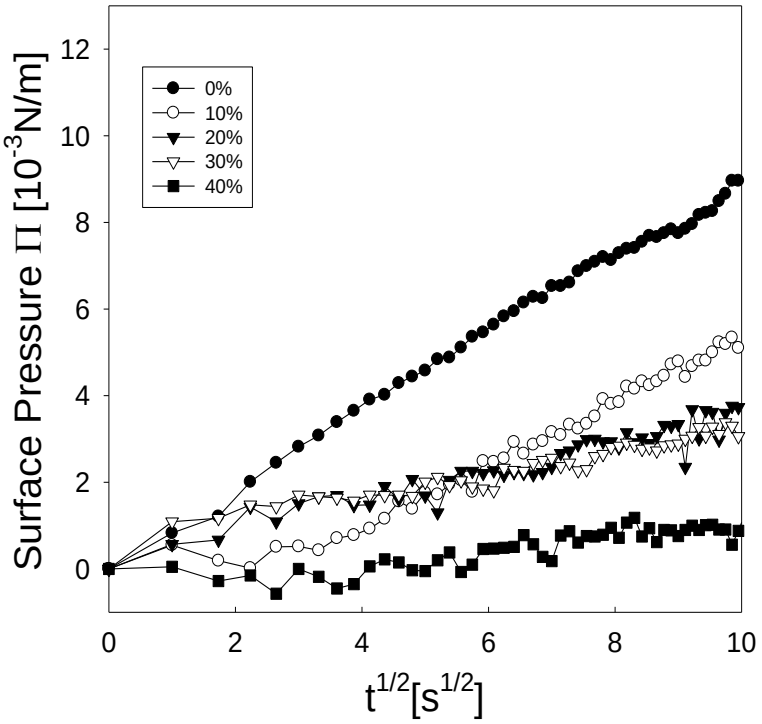

Figure 11.3 Adsorption kinetics of 3 x 10^{-6} M BSA at the air-sucrose-water co-solvent interface. Sucrose concentrations again ranged from 0 to 40 wt%. The change in surface pressure is plotted as a function of the square root of the interfacial age.

11.3. Analysis of Protein Adsorption Kinetics

The adsorption of a surface-active protein to an air-water interface can be divided into a number of different steps as demonstrated in Figure 11.4.

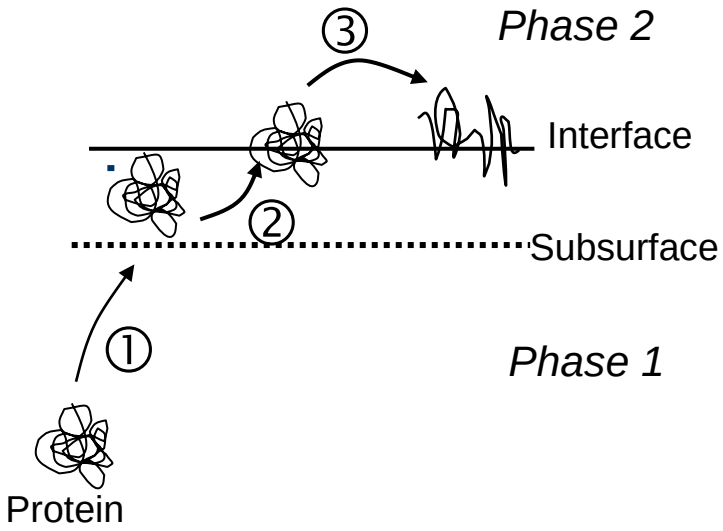

Figure 11.4. Steps of adsorption of a protein to an interface.

First, the protein must move from the bulk aqueous phase to the region immediately below the air-water surface (the "sub-surface"), which may be *via* diffusion or convection. Second, the protein must move from the sub-surface into the surface itself, which may depend on any existing local energy barriers. Third, the protein undergoes conformational rearrangements at the surface in response to the alteration in its molecular environment experienced when it moves from the bulk solution to the surface region. The surface tension measurements are primarily sensitive to the second of these processes, although the other processes could lead to some measurable change in surface tension (Miller *et al.*, 1998, 2000).

To determine the dependence of the diffusion coefficient of BSA on sugar concentration, short term solution of the diffusion model, that was given in chapter 10 (Equation 10.1), was used. For this purpose, the initial regions of the surface pressure versus square root of time profiles were analyzed (Figure 11.3). The assumptions underlying this equation are:

(i) the movement of a protein to the sub-surface is diffusion-controlled,

(ii) there is no energy barrier associated with the movement of a protein from the sub-surface to the surface and,

(iii) there are no conformational changes of the protein immediately after adsorption.

Diffusion coefficients calculated from dynamic surface tension measurements of 3 x 10^{-6} M BSA solutions containing 0 to 40% sucrose using the Equation 10.1 are shown in Table 11.1. The literature value for the diffusion coefficient of BSA in pure water is 5.90 x 10^{-11} m^2/s (Peters, 1985), and the value calculated in this study was 1.41 x 10^{-8} m^2/s, which is much greater than the literature value. On the other hand, Wüstneck *et al.* (1996) also reported similar results. The reason for the short term asymptotic solution of the diffusion model to overestimate the diffusion rate is that the adsorption process is more complicated than a phenomenon that can be described by classical diffusion. Nevertheless the results in Table 11.1 clearly show that the diffusion of BSA in presence of sucrose is slower and the diffusion coefficient decreases as the concentration of sucrose increases.

Table 11.1 Diffusion coefficients of 3 x 10^{-6} M BSA dissolved in sucrose solutions (0-40%) at 20°C.

Sucrose concentration (%)	**BSA D_{eff} (m^2/s)**
0.0	1.41 x 10^{-8}
0.5	1.25 x 10^{-8}
1.0	9.00 x 10^{-9}
3.0	4.25 x 10^{-9}
6.0	3.94 x 10^{-9}
10.0	2.33 x 10^{-9}
20.0	2.06 x 10^{-9}
30.0	3.87 x 10^{-10}
40.0	3.15 x 10^{-11}

The percent change in solution viscosity and effective diffusion coefficient of 3 x 10^{-6} M BSA relative to the viscosity of pure water and the diffusion coefficient of the BSA measured in pure water are shown in Figures 11.5.

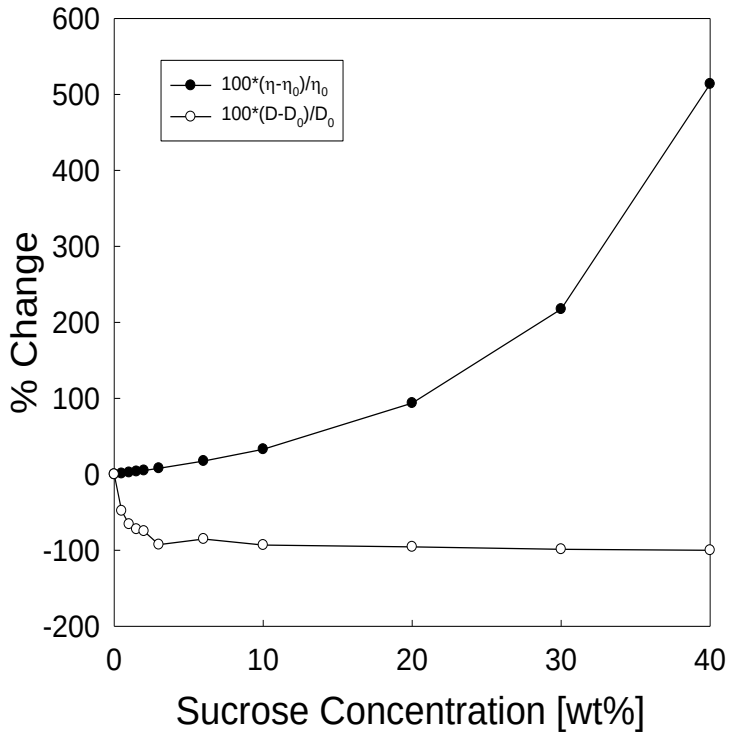

Figure 11.5. Influence of sugar concentration on the % change in solution viscosity and effective diffusion coefficient of 3 x 10^{-6} M BSA.

The decrease in diffusion coefficient of BSA in sucrose solutions is high at low sucrose concentrations whereas at high sucrose concentration the change in diffusion coefficient remains constant. This means that the decrease in the rate of adsorption at low sucrose concentrations is not only due to the decreased mobility of protein molecules in the aqueous phase and protein-sucrose interactions may play a role on the decrease in diffusion rate. This also explains the rapid adsorption rate of BSA at sucrose concentrations from 0-10% followed by a more gradual increase at sucrose concentrations higher than 10% (Figure 11.3).

11.4. Influence of Sugar Type on Protein Adsorption Kinetics

The effect of different types of sugars on the adsorption characteristics of BSA at air-water interface was also investigated using fructose and glucose as well as sucrose as co-solvents. The change in surface pressure of the protein solution in presence of 30% sucrose, glucose and fructose was given in Figure 11.6.

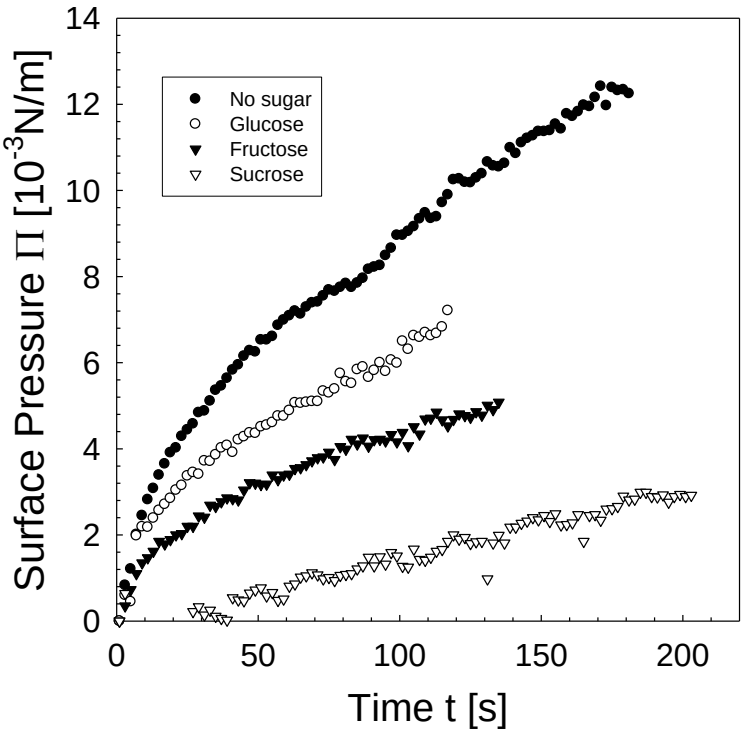

Figure 11.6. Comparison of adsorption kinetics of 3 x 10^{-6} M BSA dispersed in different sugar solvent at 30 wt%. The rate of surface pressure increased in the order of sucrose, fructose and glucose respectively.

As seen in Figure 11.6 the effect of sucrose is highest followed by fructose and glucose, respectively. In other words, at the same concentration in wt%, adsorption of the protein in presence of sucrose is lower than those in presence of fructose or glucose.

Figure 11.7 shows the difference in the increase in diffusion coefficient caused by sucrose, fructose and glucose. Calculated diffusion coefficients also indicate that BSA diffuses much slower in solutions containing sucrose than fructose or glucose.

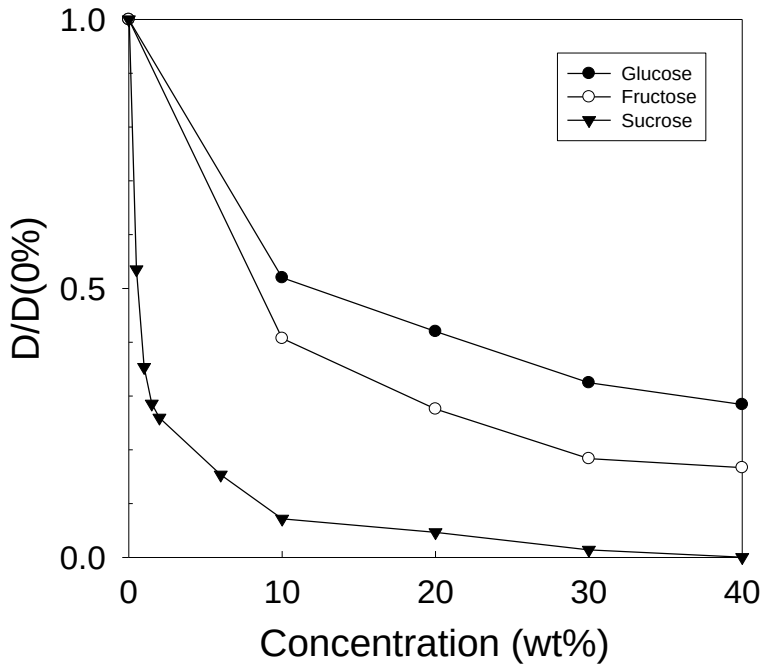

Figure 11.7. Diffusion coefficient of 3 x 10^{-6} M BSA dispersed in sugar solutions (sucrose, fructose, glucose) that ranged on concentration from 0 to 40 wt%.

Figure 11.8 shows the concentration dependent viscosity increase of solutions of sucrose, fructose and glucose. As seen from this figure, although the viscosity increase in water caused by sucrose is slightly higher than the viscosity increase caused by fructose and glucose, the difference is not high enough to explain the magnitude of the difference in adsorption kinetics of BSA in the presence of these sugars. The change in rate of adsorption of BSA in the presence of sucrose versus glucose or fructose might be explained by protein-sugar interactions that are dependent on molecular properties of these sugars.

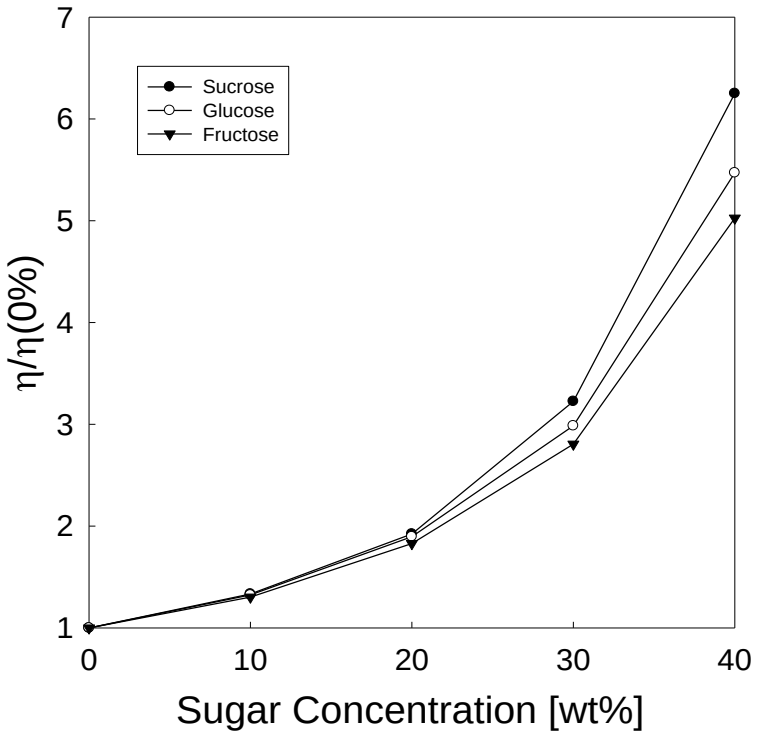

Figure 11.8 Viscosity of sugar solutions at concentrations ranging from 0-40% relative to the viscosity of water.

There are three mechanisms, which can explain the suppressed adsorption of BSA molecules in presence of sugars as follows:

(i) First, the addition of sugars increases the viscosity of the continuous phase. This increase in viscosity creates a friction between the continuous phase and the protein molecules moving to the interface, which slows down protein movement.

(ii) Another explanation for the decreased diffusion of protein molecules in sugar solutions is the preferential exclusion of sugar molecules from the surface of the protein molecules resulting in a more compact form of protein molecule. Protein molecule in this more compact form with less surface interacting with sugars is also less surface active for the same reason. Preferential interactions between proteins and sugars at high sugar concentrations were also reported by others (Record *et al*., 1998; Timasheff 1998). Considering the effect of sugar type on preferential exclusion, since sucrose has a larger molecular radius than glucose or fructose its exclusion from protein surface is higher than the others. This difference in steric exclusion might explain why the largest decrease in diffusion coefficient of BSA was observed in sucrose solutions.

(iii) The third mechanism is the increase in hydrophilicity. Sugars at low co-solvent concentrations bind to specific sites on the protein molecule (Antipova and Semenova 1997a, b). Thus, the bound layer of sugar around the protein molecule decreases its hydrophobicity, which would again lead to a decreased surface activity at the air-water interface.

12. CONCLUSIONS

This study has shown that sugars profoundly influence the adsorption kinetics of BSA to air-aqueous interfaces. There is a decrease in adsorption rate with increased sugar concentration. This effect is independent of sugar type examined in this study (sucrose, glucose and fructose). However, differences were found in the effects of these sugars on the adsorption kinetics of BSA, which could be explained by their molecular differences. The adsorption behavior of proteins in co-solvent systems can be attributed to a number of different effects:

(i) the ability of sugars to increase the aqueous phase viscosity,

(ii) the specific binding of sugars to patches of protein surfaces at low concentrations, and

(iii) the preferential interactions of sugars with protein surfaces at high concentrations.

In this study, the effect of sugars on protein adsorption kinetics was quantitatively established from surface tension measurement. Protein-co-solvent interactions, however, is very complex and more systematic research on a molecular basis is required. It is also the role of food scientists to integrate the concepts and methodologies developed in the fields of biophysics and biochemistry to understand protein-solvent-cosolvent interactions, and apply these to food systems to characterize protein functionality.

The impact of sugars on protein adsorption is of practical interest to food manufacturers to formulate certain food products, where proteins are used as functional ingredients to stabilize emulsions or foams in the presence of these co-solvents, i.e. bakery and confectionery products. A more in depth thermodynamic, molecular and functional analysis of protein-co-solvent interactions would ease to control protein functionality in very complex food systems.

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14. RESUME

Demet Güzey was born in Istanbul, Turkey in 1976. She completed her elementary and high school studies in the same city. She graduated from Üsküdar Anatolian Highschool in Istanbul in 1994, and started her studies in the field of Food Engineering at Istanbul Technical University (ITU). After receiving her B.S. degree in Food Engineering in 1999, she enrolled to the graduate school at ITU to pursue a M.S. degree in Food Engineering. Having been awarded with an assistantship she deferred her master track at ITU at the thesis stage and started a second graduate study in Food Science at the University of Tennessee (UT) in 2000. Currently she is a graduate research assistant in the Department of Food Science and Technology at the University of Tennessee, Knoxville, expecting to receive her M.S. in Food Science in August 2002. Meanwhile she has worked on a second project, supervised both by UT and ITU, towards the M.S degree in Food Engineering at ITU. During her graduate studies in 2002, Demet Güzey received Collins International Student Award given by The Department of Food Science and Technology at UT, the Provosts' Citation Award given by The University of Tennessee to graduate students for their academic achievements and the Honored Student Award given by The American Oil Chemists' Society.