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THE OSMOTIC SECOND VIRIAL COEFFICIENT AS A PREDICTOR OF PROTEIN STABILITY

By

Kusum S. Verma

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THE OSMOTIC SECOND VIRIAL COEFFICIENT AS A PREDICTOR OF PROTEIN STABILITY

By

Kusum S. Verma

Approved:

Wilbur William Wilson
Professor of Physical Chemistry
Department of Chemistry
(Major Professor)

Stephen Foster
Associate Professor of Physical
Chemistry, Department of Chemistry
(Committee Member)

Steven Gwaltney
Assistant Professor of Physical
Chemistry, Department of Chemistry
(Committee Member)

Stephen Foster
Associate Professor of Physical
Chemistry, Department of Chemistry
(Graduate co-ordinator)

Philip Oldham
Dean of the College of
Arts and Science

Name: Kusum S. Verma

Date of Degree: Dec 8, 2006

Institution: Mississippi State University

Major Field: Chemistry

Major Professor: Dr. Wilbur William Wilson

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Candidate for Degree of Master of Science

The number of protein containing therapeutic drugs is growing day by day. Lack of proper storage conditions can cause protein degradation or aggregation.

The osmotic second virial coefficient, B_{22}, is a thermodynamic parameter, which can predict protein interaction with other proteins and solvent molecules. B_{22} has been successfully used as predictor of crystallization conditions for a protein in the solution, and in this study an attempt has been made to relate B_{22} as a predictor of stability of the protein.

Static light scattering was used to measure B_{22} in our studies. B_{22} and the solubility of three proteins were measured in several excipient solutions. George et al. in 1997 related the osmotic second virial coefficient with the solubility of protein in a solution [9]. In this study we have attempted to relate solubility with B_{22} and stability of lysozyme, human serum albumin, and ovalbumin in buffer solutions containing various excipients.

DEDICATION

I would like to dedicate this research to my elders. Special dedication to (father) Mr. Santosh Kumar Verma, (mom) Mrs. Sukanya Devi Verma, (brother) Kamaljeet Verma and loving husband Mr. Binay Kumar Chaudhary.

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I would like to express my sincere gratitude to those people without whose selfless help this research work would not have been possible. First of all I thank God who blessed me with this learning opportunity. Secondly and most importantly I would like to thank my research advisor, Dr. Wilson, who had been a continuous source of inspiration. I would than like to thank my committee members, Dr. Stephen Foster and Dr. Steven Gwaltney. Special thanks to my research group, family, and friends for all their well wishes and support. Finally, I would like to thank Poonam and Rahul Hardikar for emotional and moral support.

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LIST OF SYMBOLS, ABBREVIATIONS, AND NOMENCLATURE

B_{22} second virial coefficient $(\mathrm{mol.mL/g^2})$.

M molecular weight, also Mw (g/mol or Daltons).

K optical constant for light scattering $(\mathrm{cm^2.mol/g^2})$.

R_{90} Rayleigh ratio for light scattering $(\mathrm{cm^{-1}})$.

π osmotic pressure.

R gas constant (8.314 J/K.mol).

c concentration, also c_2 (mg/mL or g/mL).

WCS Wilson crystallization slot.

S protein solubility (mg/mL).

ESA equine serum albumin.

HSA human serum albumin.

AS ammonium sulfate.

SLS static light scattering.

DLS dynamic light scattering.

N native state protein conformation.

D denatured or non-native protein conformation.

K_{eq} equilibrium constant.

TS^* transition state.

B aggregates.

D diffusion coefficient $(\mathrm{cm^2/s})$.

Γ decay constant $(\mathrm{s^{-1}})$.

q scattering vector $(\mathrm{cm^{-1}})$.

$\mathrm{R_h}$ hydrodynamic radius (nm).

λ wavelength (nm).

λ_0 wavelength in vacuum (nm).

θ scattering angle.

RALLS right angle laser light scattering.

η_0 solvent refractive index.

$\mathrm{dn/dc}$ refractive index increment.

$\Delta \mathrm{G^{\circ}_{unfold}}$ free energy of unfolding at constant temperature and pressure.

CHAPTER I

INTRODUCTION

1.1 Motivation

The number of protein-containing therapeutic products is growing. The main issues in developing these products are shelf life / stability and solubility. The protein formulation products, when used for medicinal purposes, should be safe to use and physically, chemically, and biologically stable [4]. Products that are unstable may be denatured, and become aggregated. If these products do not meet safety standards, administration of such products may be deleterious.

Proteins in a body exist in a surrounding environment of complex cosolvents. To find a solution in which proteins used for medicinal purposes are stable, and administrable is very difficult [5]. Maintaining protein conformation and colloidal stability is difficult because, the proteins are marginally stable, and susceptible to both physical and chemical degradation [6].

Timasheff and his coworkers [7] have dedicated several years to understanding the role of cosolvents in the complex protein-water-cosolvent induced interaction. Choosing a proper cosolvent is the key for obtaining a stable protein formulation. But choosing a cosolvent, which could induce protein stability, is a difficult task.

The osmotic second virial coefficient (B_{22}) is a thermodynamic parameter that predicts the extent of protein interaction with other protein and solvent molecules in a dilute solution [5, 8, 9, 10]. It is believed that at negative B_{22} values the intermolecular forces are attractive, and they are repulsive when B_{22} values are positive. B_{22} accounts for contributions from electrostatic,van der Waals interactions, excluded volume, hydration forces, and hydrophobic effects. These parameters play important roles in protein stability and solubility [5, 8-10].

Static light scattering (SLS) has been historically used for measuring B_{22} values of protein solutions. Wilson and coworkers have pioneered the area of measuring B_{22} values for proteins using static light scattering methods [8-15]. The primary requirement for performing B_{22} measurements using SLS includes dilute protein solutions with concentration ranging from $\sim$ 1 to 7 mg/mL [10-12]. In this age, when nanotechnology is advancing, this amount is considered to be large, and several efforts have been made to reduce the amount of sample required for measuring B_{22}. Self-interaction chromatography (SIC) is an upcoming technique used for measuring B_{22}. Advantages offered by this method will be realized in the coming decade [11, 12].

In this study we have used SLS as a tool for measuring B_{22} for lysozyme, ovalbumin, and human serum albumin (HSA) in the presence of cosolvents such as NaCl, ammonium sulfate (AS), sucrose, trehalose, glucose, mannitol, and dextran150.

The main objective is to show that the addition of cosolvents or excipients to the protein solution increases B_{22}, which leads to increased stability of the protein in that solution. This can be rationalized in terms of increased protein solubility.

1.2 Protein Stability

If in a particular solution condition the protein maintains its native conformation, and is colloidally stable then it is said to be stable. Protein stability can also be defined as avoiding "denaturation" and "aggregation". "Denaturation is disorganization of the natural protein molecule, the change from the regular arrangement of a rigid structure to the irregular, diffuse arrangement of the flexible open chain" [13]. Aggregation takes place when two protein molecules come close to each other, and their hydrophobic patches join together. Aggregation can be of two types (1) the protein molecules come together, and form long chains in an irregular manner, resulting in amorphous precipitates also known as "praggs", and (2) the protein molecules come together in an orderly fashion, and form crystals, also known as "craggs" [14]. Both biologically active (native state) and biologically inactive protein molecules can be involved in precipitation, while only biologically active proteins are involved in crystallization.

Protein stability accounts for both colloidal stability and conformational stability of the protein in the surrounding environment [15]. In this study, emphasis is given to colloidal stability of the proteins. Thermodynamically, conformational stability of a protein's native state can be given in terms of free energy. The free energy of native conformation is only

5-6 kcal/mol less than the free energy of non-native conformations which are biologically inactive [6, 7]. The balance between large stabilizing forces and large destabilizing forces results in this net conformational stability. The free energy of folding arises due to contributions from electrostatics (charge repulsion and ion pairing), hydrogen bonding, van der Waals interactions, and hydrophobic interactions [6, 7].

The osmotic second virial coefficient (B_{22}) predicts the colloidal stability of the protein in a particular solution condition. B_{22} accounts for both short and long range interactions, such as electrostatic interactions, van der Waals interactions, excluded volume, and hydrophobic interactions. [5, 13].

A positive B_{22} indicates that the protein-protein interactions are repulsive; consequently, in a solution the protein molecules stay apart from each other, and prevent crystallization and aggregation. Crystallization and aggregation require the protein molecules to come together to from a nucleation center [9-12, 17] In other words, a positive B_{22} indicates that the protein in a solution is colloidally stable. A negative B_{22} suggest attractive protein-protein interactions, a condition favorable for crystallization/ aggregation. Thus the solutions for which, B_{22} values are negative are said to be colloidally unstable [9-12, 16].

External variables like temperature, pH, salt, cosolvents, surfactants, etc. can cause denaturation and aggregation of the proteins by altering the extent of the above-mentioned interactions. Any change in interactions will cause a change in the free energy of unfolding and in B_{22} [6, 7, 16, 17].

1.3 Effects of External Variables on Protein Stability

Protein stability is affected by external variables such as temperature, ligands and cosolvents, pH of the solution, preservatives and surfactants. We will take a detailed look at each of these factors in the following subsections.

1.3.1 Temperature

The role of temperature in protein stability is complex and controversial, though it is clear that it has a significant effect on the conformational and chemical stability of the protein. At high (e.g., $50°\mathrm{C} - 100°\mathrm{C}$) and low (e.g., less than $10°\mathrm{C}$) temperatures the free energy of unfolding $(\triangle \mathrm{G}^{\circ}_{\mathrm{unfold}})$ becomes negative, suggesting that denaturation/unfolding of the protein will occur [4, 6, 7].

Hydrophobic effects are considered to be the primary cause of thermal denaturation. Increase in temperature increases the entropy of the solution, which causes unfolding of the protein molecules. Exposed hydrophobic patches of unfolded protein molecules join together, and aggregation takes place. Hydrophobic effects weaken at low temperature, due to increased solvation of hydrophobic groups. As a result, dissociation of the proteins containing subunits takes place, resulting in its denaturation [4, 6, 7].

1.3.2 Ligands and Cosolvents

The protein in a solution is in equilibrium between its native conformation and its non-native conformation. Ligands and cosolvents, when added to the solution, shift

the equilibrium towards native or non-native conformations. Ligands such as polyanions effect the protein stability by binding to the native state, and shift the equilibrium toward the native state. However, binding of polyanions alters the structure of the protein, and renders it of no use for the desired medicinal purposes.

Sugars, polyols, and denaturants interact weakly with the native and the non-native conformation [4, 18, 19]. It is known that sugars and polyols are preferentially excluded from the surface of the protein, and hence the proteins are preferentially hydrated. Addition of sugars and polyols increase the surface tension of water, resulting in exclusion of additives from the water-protein interface. Since this is a high-energy state according to the Le Chatelier's principle, the system should reduce its energy by adopting a low energy state, which is obtained by minimizing the area of the water-protein interface. As a result, the protein adopts the more compact native conformation, and the equilibrium shifts towards native conformation on addition of such additives [18, 20, 21].

Denaturants do exactly the opposite, and bind preferentially to the unfolded state. This shifts the equilibrium towards the non-native conformation causing the protein denaturation [6].

1.3.3 The pH of the Solution

Protein stability largely depends on the pH of the solution, which imparts the overall positive or negative charge on the protein molecules, thus affecting electrostatic interactions. At extreme pH, non-specific electrostatic repulsion results due to the presence

of charged groups on the protein molecules. This disturbs the folded conformation due to the increase in charge repulsion within the protein molecule. An increase in the number of charged groups also gives rise to specific charge interactions, such as salt bridges (i.e., ion-pairing), which leads to conformational stability. At the isoelectric point where there are equal numbers of positive and negative charges on the protein, increase in dipole moment takes place, which makes the protein-protein interaction attractive leading to aggregation. The protein-protein interactions are repulsive when the protein molecules are highly charged, which stabilizes the protein solution [6, 22, 16].

1.3.4 Preservatives

Preservatives are required to prevent bacterial growth in the protein formulations used for medical purposes. Preservatives like phenol, m-cresol, and benzyl alcohol are known to cause the protein aggregation. The phenomenon by which these preservatives cause aggregation is still unknown, and requires more research work to be done [6].

1.3.5 Surfactants

Surfactants adsorb at air/water and solid/water interfaces reducing the adsorption of the protein, and interface induced aggregation. These surfactants can bind preferentially to the unfolded state, resulting in reduced protein stability, or can bind to the folded state, increasing protein stability [6].

1.4 Thermodynamics of Protein Stabilization

In a solution, the proteins exists at equilibrium between the (1) Native (biologically active) and (2) Non-Native denatured (biologically inactive) conformation.

$$N \overset{K_{eq}}{\rightleftharpoons} D \tag{1.1}$$

where K_{eq} is the equilibrium constant and is defined as

$$K_{eq} = \frac{[D]}{[N]} \tag{1.2}$$

The extent to which the protein will maintain its native conformation is given by $\triangle \mathrm{G}^{\circ}_{\mathrm{unfold}}$ which is defined as

$$\triangle G^{\circ}_{unfold} = -RTlnK_{eq} \tag{1.3}$$

The colloidal stability that consists of repulsive and attractive interactions between the protein molecules is measured by the osmotic second virial coefficient (B_{22}) [13, 20, 23].

The addition of excipients that cause a decrease in $\triangle \mathrm{G}^{\circ}_{\mathrm{unfold}}$, decreases the conformational stability of the protein. Urea, alcohols, amides, formamides, guanidium salts, glycols, sodium dodecyl sulfate, etc. are a few examples of destabilizers. On the other hand, excipients that increase the $\triangle \mathrm{G}^{\circ}_{\mathrm{unfold}}$, increases conformational stability.

Excipients like sugars, polyols, amino acids, polymers, etc. are known to increase $\triangle \mathrm{G}^{\circ}_{\mathrm{unfold}}$, hence they are used as stabilizing agents.

Another way to explain the same phenomenon is in terms of preferential exclusion or negative binding of excipients to the proteins. Stabilizers are preferentially excluded from

the protein surface because addition of stabilizers to the solution is found to be thermodynamically unfavorable. The addition of stabilizers increases the surface tension of the water, which results in exclusion of stabilizers from the protein-water interface. Since this is thermodynamically unfavorable, the system reduces its energy by adopting a more compact geometry and avoids expansion of the protein molecules, which would increase the surface free energy of the water. Thus, the overall process favors the native state, that is less exposed to solvent, and is thermodynamically preferred.

It is a very well established fact that addition of excipients like sugars, polyols etc., conformationally stabilize the protein in a solution [5, 6, 16, 20, 22, 23, 25-28]. Much work has been done showing that addition of stabilizers increases the conformational stability, but not much work has been done to see what effect addition of stabilizers have on the colloidal stability. In this study we have measured the effect of excipients on the colloidal stability of the protein in the solution by measuring B_{22} in solution using light scattering methods.

1.5 The Second Virial Coefficient (B_{22})

The Second virial coefficient is a dilute solution parameter, which gives the extent of the protein-protein interactions. There are three common ways in which the second virial coefficient is determined, namely osmometry, static light scattering (SLS), and self-interaction chromatography (SIC) [24].

Osmometry works on the principle, that a difference in osmotic pressure will exist between two solutions separated by a semipermeable membrane. In the case of ideal solutions the osmotic pressure is given by van't Hoff's law as

$$\frac{\pi}{c_2} = \frac{RT}{M_2} \tag{1.4}$$

For a dilute solution of macromolecules, the osmotic pressure is given by the virial expansion of van't Hoff's Law given as

$$\frac{\pi}{c_2} = \frac{RT}{M_2} + B_{22}RTc_2 + \ldots \tag{1.5}$$

where,

- $\pi =$ the osmotic pressure.
- $\mathrm{c}_2 =$ concentration of the protein/solute (g/mL).
- $\mathrm{R} =$ the gas constant (8.314 J/k.mol).
- $\mathrm{T} =$ temperature (K).
- $\mathrm{M}_2 =$ molecular mass (g/mol).
- $\mathrm{B}_{22} =$ the osmotic the second virial coefficient (mol.mL/g^2).

The contribution from higher order terms is small, and generally are negligible. If we plot $\frac{\pi}{\mathrm{c}_2}$ versus c_2, the intercept of the plot is $\frac{\mathrm{RT}}{\mathrm{M}_2}$, so that the molecular weight of the molecule can be determined. From the slope $(\mathrm{B}_{22}\mathrm{RT})$ the second virial coefficient can be determined. An example plot of $\frac{\pi}{\mathrm{c}_2}$ versus c_2 is shown in Figure 1.1 for various protein solutions [1].

The second approach used to determine B_{22} is by using static light scattering (SLS). This method requires less protein than that required for osmometry. In this method the

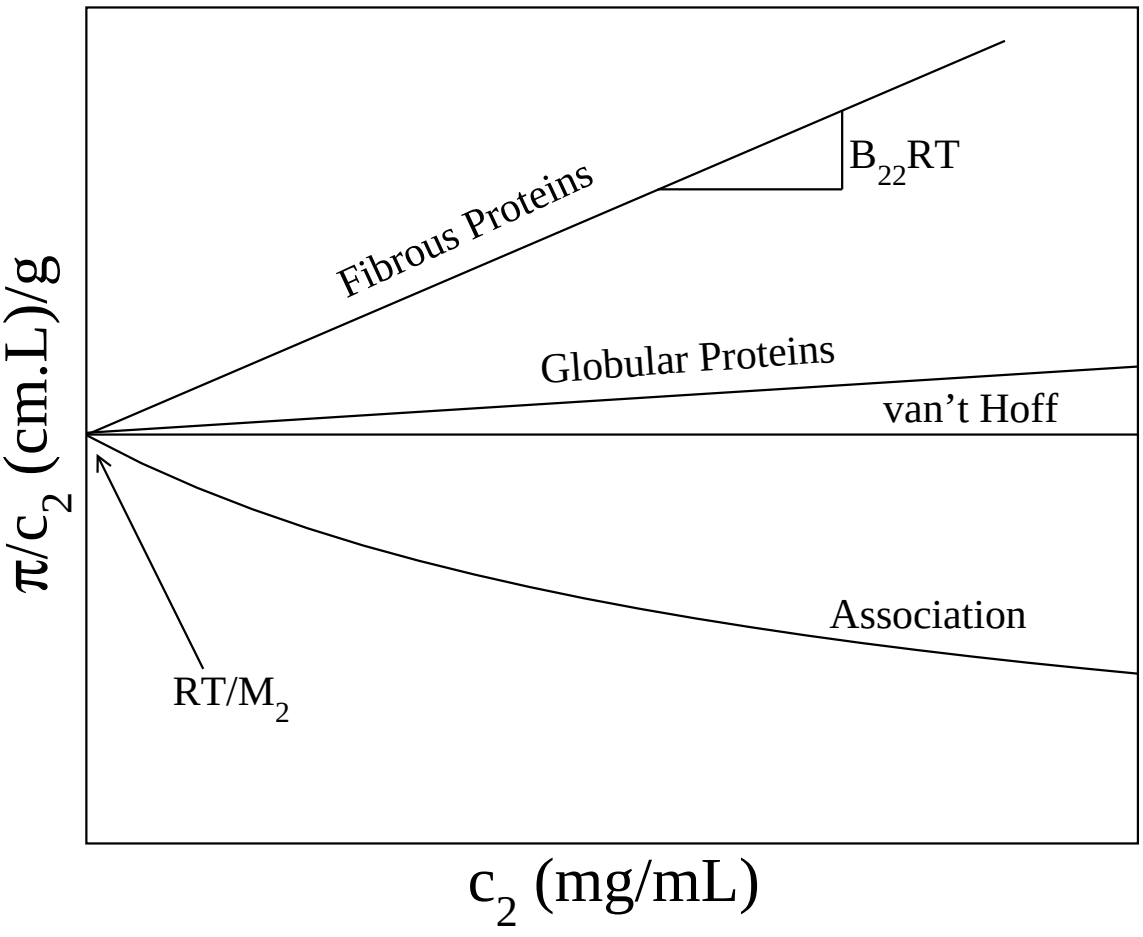

Figure 1.1

Plot of $\frac{\pi}{c_2}$ versus c_2 [1]

light scattered due to the protein molecules in excess of background (i.e., solvent, crystallizing agent, excipient, or dust particles) is measured as a function of increasing protein concentration [8-15, 17]. The data acquired are interpreted by the working equation given by Kratochvil as [10]

$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2B_{22}c \tag{1.6}$$

where K is an optical constant given by

$$K = \frac{4\pi^2\eta_\circ^2\left(\frac{dn}{dc}\right)^2}{N_A\lambda^4} \tag{1.7}$$

- c = protein concentration (g/mL).
- $\eta_\circ =$ refractive index of the solvent.
- $\frac{\mathrm{dn}}{\mathrm{dc}} =$ the refractive index increment.
- $\mathrm{N_A} =$ Avogadro's number $(6.023 \times 10^{23}\mathrm{mol}^{-1})$.
- $\lambda =$wavelength of incident light (nm).

R_{90} is the Rayleigh factor at a scattering angle of 90° measured as a function of protein concentration.

It can be seen from Eq. (1.7), if we plot $\mathrm{Kc}/\mathrm{R}_{90}$ versus c, a straight line is obtained. The intercept of this straight line (1/M) gives the molecular weight of the protein, and its slope ($2\mathrm{B}_{22}$) gives B_{22} of the protein in that particular solution.

The third method used to determine B_{22} is self-interaction chromatography (SIC), which is a fairly new technique [6, 12]. This method requires a smaller amount of protein when compared to osmometry and SLS.

The extent of intermolecular interaction in the protein-water-cosolvent system can be judged by the obtained B_{22} values. If negative B_{22} values are obtained the intermolecular attractions are attractive, i.e at high negative values the proteins will come together to form aggregates or precipitate, and the solution is said to have "crashed". At moderately negative B_{22} values the protein-protein interactions are attractive, and protein crystals may be obtained. The solvents in which negative B_{22} values are obtained are referred to as "poor" solvents [8, 10, 25, 26].

The protein-water-cosolvent system is said to be at a "Theta" state when B_{22} is zero. At this state there is no net intermolecular attraction or repulsion. This solution is said to be an "ideal" solution [8, 10, 25, 26].

Positive B_{22} values indicate net repulsive intermolecular forces. It is observed that as B_{22} becomes more positive, the solubility of the protein in that solution increases when compared to solubility of the protein in solution with negative B_{22}. The solvent is referred as a "good" solvent when it has positive B_{22} values [5, 7-9].

1.6 Solubility (S)

In terms of chemical potential, solubility can be defined as the concentration of the protein in a solution when the system is at equilibrium between the two phases, i.e the crystalline phase/amorphous phase and the solution phase [26, 27].

$$\mu_2 (sol) = \mu_2 (xtal) \tag{1.8}$$

Knowing the solubility of a protein in a particular solution works to our advantage. To study the structural properties of the protein, crystals are required. To get good crystals a saturated protein solution is required, which can be made easily if the solubility is known [24].

Solubility can also play an important role when searching for a suitable solvent for developing a stable protein formulation. Avoiding saturation while developing a stable protein formulation is important to minimize crystallization/aggregation.

Determining solubility is not easy and is labor intensive. Solubility data for most of the proteins besides lysozyme, horse serum albumin, and concanavalin A do not exist in available literature [9].

1.7 Correlation between S and B_{22}

George et al. developed a correlation between solubility and the second virial coefficient in 1997 [8, 10, 25, 28]. They determined B_{22} values for horse serum albumin using SLS in 50 mM sodium acetate with 45 % (w/v) saturated ammonium sulfate (SAS) at pH 5.5 at temperatures ranging from $10°C$ to $25°C$ and compared it with available solubility data. A systematic trend was observed between horse serum albumin solubility and the second virial coefficient. In the same study, B_{22} for lysozyme in 100 mM sodium acetate with 2.5 % (w/v) sodium chloride at pH 4.2 at temperatures varying from $10°C$ to $20°C$ were measured and than compared with available solubility data, a correlation was found between B_{22} and S values [9].

In 1999, Guo et al. measured B_{22} for lysozyme and ovalbumin in about 20 different solution concentrations [10]. Solubility at these conditions from the literature was then compared with B_{22} to make sure that a correlation existed between B_{22} and S values. This study led to the development of a theoretical model relating B_{22} and S, known as the Haas-Drenth-Wilson model [25].

Another study was done in 2002; Demoruelle et al. measured B_{22} and S values for Equine Serum Albumin (ESA) using SLS and the sitting drop method. A plot of B_{22} versus S was made and a good correlation existed between these values [26].

From these studies the conclusion was drawn that the dilute solution parameter (B_{22}) indicates the behavior of a protein at saturation. Thus B_{22} can be used to predict the solubility behavior of the protein in a particular solution [9].

CHAPTER II

EXPERIMENTAL

Static light scattering (SLS) was used to measure the second virial coefficient (B_{22}) values for Lysozyme, Human Serum Albumin (HSA), and Ovalbumin (purchased from Calbiochem) in buffer solution containing various excipients such as sodium chloride, ammonium sulfate (AS), mannitol, trehalose, sucrose, glucose, and dextran150. Solubility of the lysozyme was then estimated in the same buffer solution containing different excipients.

2.1 Lysozyme

Lysozyme is a globular protein, having a molecular weight of 14.4 kDa and a diameter of 4 nm. Lysozyme is obtained from hen egg white and was discovered by Alexander Fleming during a search for medical antibiotics in 1922 [24]. It is known that lysozyme is effective against bacterial infections since it destroys the structural integrity of the bacterial cell wall. Lysozyme in hen egg white protects the proteins and fats that feed the developing chick in the egg. Much research has been done on lysozyme and sufficient physical and chemical information is available for lysozyme compared to other proteins. Hence, lysozyme is used as model protein for most protein related studies.

2.2 Ovalbumin

Ovalbumin is a glycoprotein found in egg white. It protects the egg yolk and nurtures developing baby in the egg. It has an approximate molecular weight of 45 kDa.

2.3 Human Serum Albumin

Human serum albumin is a protein found in the human blood plasma. It is produced in the liver and has an approximate molecular weight of 67 kDa. Low levels of albumin in blood leads to malnutrition, liver disease, nephritic syndrome, etc.

2.4 Excipients

Excipients can be defined as cosolutes or additives, which interact weakly with the protein molecules causing an increase or decrease in protein stability. Ligands, salts, sugars, and polyols are a few examples of excipients. The protein in an ideal solution should exist at equilibrium between the native and the non-native protein conformation. Addition of excipients to the protein solution can either shift the equilibrium towards the native conformation, which would stabilize the protein in that particular solution, or it could shift the equilibrium towards the non-native conformation, and lead to the aggregation or denaturation of protein in that particular solution condition [6, 18, 29].

Excipients used in this study include sodium chloride (NaCl), AS, mannitol, glucose, trehalose, sucrose, and dextran150.

2.4.1 Sodium Chloride (NaCl)

Salt can interact in several ways with the protein molecule depending on the pH of the solution and the salt concentration. Different types of salts have different effect on the protein stability and solubility in a particular solution. Salt at low concentration shields the charge on the protein molecule, causing a decrease in electrostatic interactions, which results in conformational stability and colloidal stability. At high concentrations of salt, binding of salt ions with the protein takes place, which can increase electrostatic repulsion within the molecule, and can lead to unfolding of the molecule, which results in protein denaturation, and disrupts the colloidal stability. The overall charge on the protein molecule is dictated by the pH of the solution; hence the effect of salt on the protein in a particular solution is highly pH dependent [4, 6, 7, 16, 17, 30].

2.4.2 Ammonium Sulfate $(NH_4)_2SO_4$

Ammonium sulfate, also known as Mohrs salt, is a commonly used fertilizer. It is acidic in nature, and its addition to soil maintains the pH balance of the soil. It is a commonly used reagent for precipitating proteins out of the solution. It has a molecular weight of 132 g/mol.

2.4.3 Mannitol, Glucose, Trehalose, and Sucrose

Mannitol (a) is a polyol having molecular weight of 182 g/mol.

Glucose (b) is a monosaccharide and has an empirical formula $C_6H_{12}O_6$ and a molecular weight of 180 g/mol. It is a common sugar found in plants and animals, and is the major source of energy in animal metabolism. Glucose has been used to stabilize the proteins, and due to its ease of digestion and administration, it is a sugar of interest for pharmaceutical companies.

Sucrose (c) is a disaccharide made up of two molecules, namely glucose and fructose. having a molecular weight of 342 g/mol. It is a commonly used sweetener, is used widely in the pharmaceutical industries for maintaining the activity of the protein molecules, and is effective against denaturation of the proteins used for medical purposes.

Trehalose (d) is a disaccharide made up of two glucose molecules. It has a molecular weight of 378 g/mol, and is extensively used for maintaining the activity of the protein during freeze-drying. Structural formulas of the sugars used are shown in Figure 2.1.

The exact phenomenon by which these sugars stabilize the proteins in aqueous solution is controversial and complicated. After spending more than a decade researching the mechanism by which these cosolutes affect the stability of the protein molecules, Timasheff and coworkers [29] concluded that these excipients are preferentially excluded from the surface of the protein molecules due to increased surface tension resulting in hydration of the protein. In terms of free energy, it can be said that the free energy of unfolding is increased upon addition of these excipients; hence the native or folded state is favored over the unfolded state.

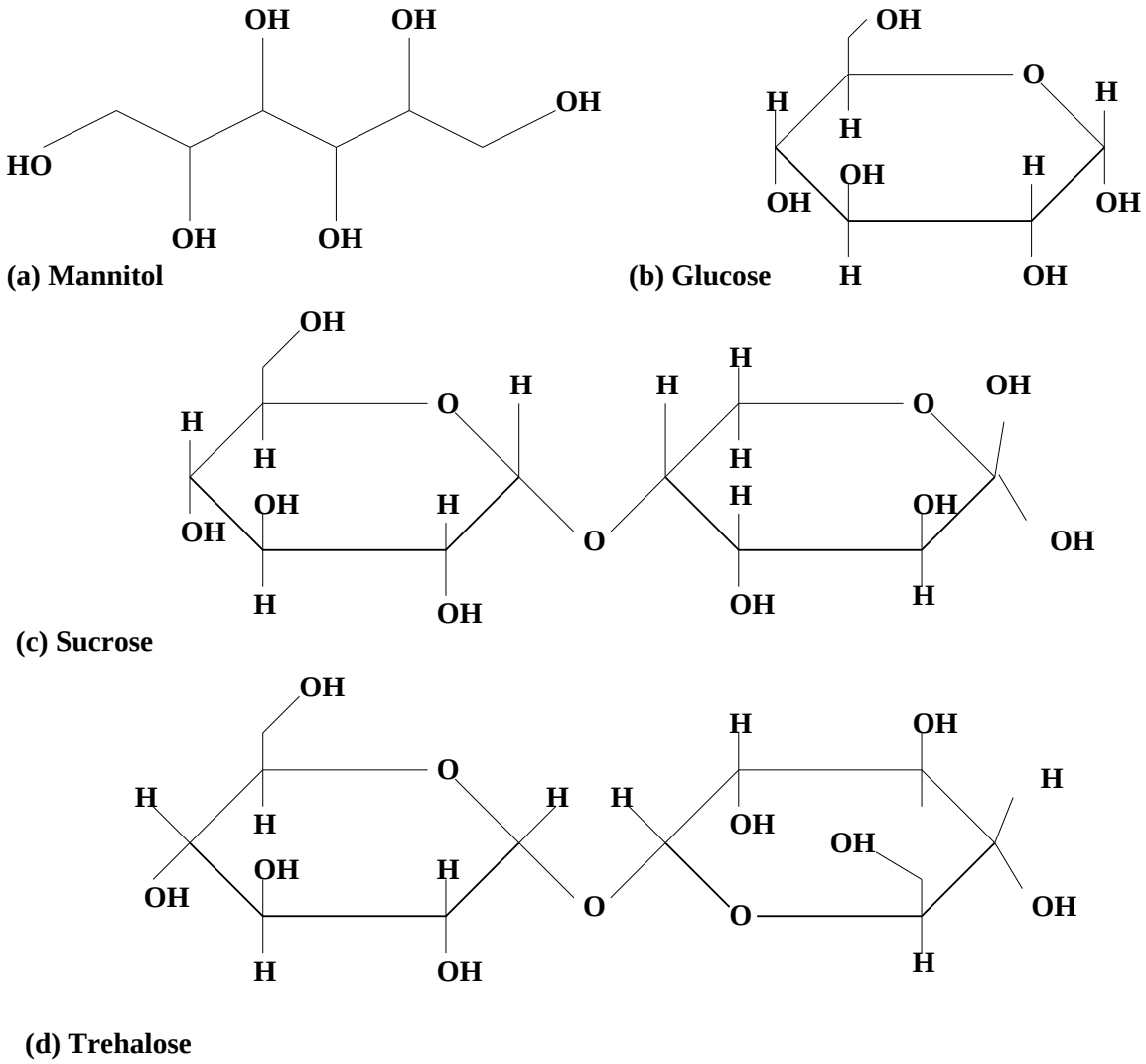

Figure 2.1

Structural formula of (a) Mannitol, (b) Glucose, (c) Sucrose, and (d) Trehalose

2.5 The Static Light Scattering Method for B_{22} Measurement

A commonly used method to measure B_{22}, which is a thermodynamic parameter related to the protein interactions with other protein and solvent molecules, is static light scattering (SLS).

Materials used and their sources are as follows: Hen egg white lysozyme (Calbiochem, Lot # B58915), ovablumin (Calbiochem, Lot # B60780), human serum albumin (Calbiochem, Cat # B65145), sodium chloride (Sigma Lot # 942836), sucrose (Sigma, Lot # 90H0679), mannitol (Sigma Lot # 023K0178), trehalose (Sigma Lot # 114K7020), ammonium sulfate (Fisher Scientific, Lot # 991875A), sodium phosphate monobasic (Fisher Scientific, Lot # 912830A), sodium phosphate dibasic (Fisher Scientific), glacial acetic acid 99.7 % (Fisher Scientific, A38), sodium hydroxide 97 % (Fisher Scientific), sodium azide (Fisher Scientific). Dionized water used for experimental purposes was prepared using a QUANTUM EX ultrapure organex cartridge (Millipore).

Experiments with lysozyme were carried out in sodium acetate buffer, which was prepared by weighing 6 g of glacial acetic acid into a 1 L beaker containing $\sim$ 800 mL of DI water and adding 50 g of NaCl to make a 0.1 M NaAc buffer with 5 % (w/v) NaCl. The solution was titrated against 0.1 M NaOH to adjust the pH to 4.5 and then filled to the 1 L mark in a volumetric flask. To prevent bacterial growth, 0.05 % w/v sodium azide (NaN_3) was added to the buffer. Buffer solutions with different excipients were prepared by adding the appropriate amount of the excipient into a volumetric flask of desired volume and slowly adding 0.1 M NaAc buffer with 5 % (w/v) NaCl and 0.05 % (w/v) NaN_3

so that all the excipients were properly dissolved and then filled to the mark. This buffer solution was then filtered using a 0.45 μm Millipore filtration system.

Lysozyme stock solution was prepared by dissolving lysozyme into the buffer solution containing excipient. Dissolving this protein into buffer was difficult due to lower solubility at room temperature; hence it was dissolved in a $40°$C water bath. The stock solution was filtered manually with a syringe using 0.22 μm, 4 mm Millex Millipore disposable filters, and the final concentration was determined spectrophotometrically using A (1 %, 1 cm, 280 nm) = 26.3 for lysozyme [7].

Experiments with ovalbumin were performed in 0.1 M sodium phosphate, pH 7.0 buffer. Sodium phosphate buffer was prepared by weighing 13.5 g of sodium phosphate monobasic into a 1 L beaker containing $\sim$ 800 mL of DI water and 0.05 % (w/v) sodium azide to prevent bacterial growth and weighing 14.9 g of sodium phosphate dibasic in a separate beaker containing $\sim$ 800 mL of DI water. Each solution was transferred into a separate 1 L volumetric flask and filled to the mark. Then $\sim$ 500 mL of sodium phosphate dibasic was titrated with sodium phosphate monobasic until the desired pH was obtained.

Ovalbumin was purified by preparative size exclusion chromatography. Monomer fractions were collected and concentrated using an Amicon 8010 concentrator with a Millex Millipore membrane, 42 mm, Molecular weight cut off 3,000 g/mol. The stock solution was filtered with a syringe using 0.22 μm, 4 mm Millex Millipore syringe filters. The stock concentration was determined spectrophotometrically using A (1 %, 1 cm, 280 nm) = 7.0 for ovalbumin [7].

Human serum albumin experiments were carried in 0.1 M sodium phosphate buffer, 2.6 M AS at pH 7.0. Sodium phosphate buffer was prepared as described earlier and appropriate weight of AS was added to make final AS concentration to be 2.6 M. HSA was purified and concentrated as mentioned for ovalbumin. Stock concentration was determined spectrophotometrically using A (1 %, 1 cm, 280 nm) = 5.3 for HSA [9].

The second virial coefficient was measured by Right Angle Laser Light Scattering (RALLS) Detector model 600 ($\lambda = 670$ nm) from Viscotek. In this method, the average intensity of light scattered by a protein solution of four to five known concentrations is measured in excess of that scattered by background sources such as solvents, excipients, salts, stray light, etc. A block diagram of the SLS setup is shown in Figure 2.2.

The data obtained is analyzed using the working equation given by Kratochvil as described in chapter 1.

2.6 The Solubility Measurement for Lysozyme

The solubility of lysozyme was estimated in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 buffer, 0.05 M, 0.2 M, 0.25 M and 0.3 M mannitol in 0.1 M NaAc, 5 % (w/v) NaCl buffer at pH 4.5, 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M trehalose in 0.1 M NaAc, 5 % (w/v) NaCl buffer at pH 4.5; and in 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M sucrose in 0.1 M NaAc, 5 % (w/v) NaCl buffer at pH 4.5.

Lysozyme stock solutions were prepared in the above mentioned buffers. Supersaturated stock solutions were prepared to obtain phase separation because solubility is defined

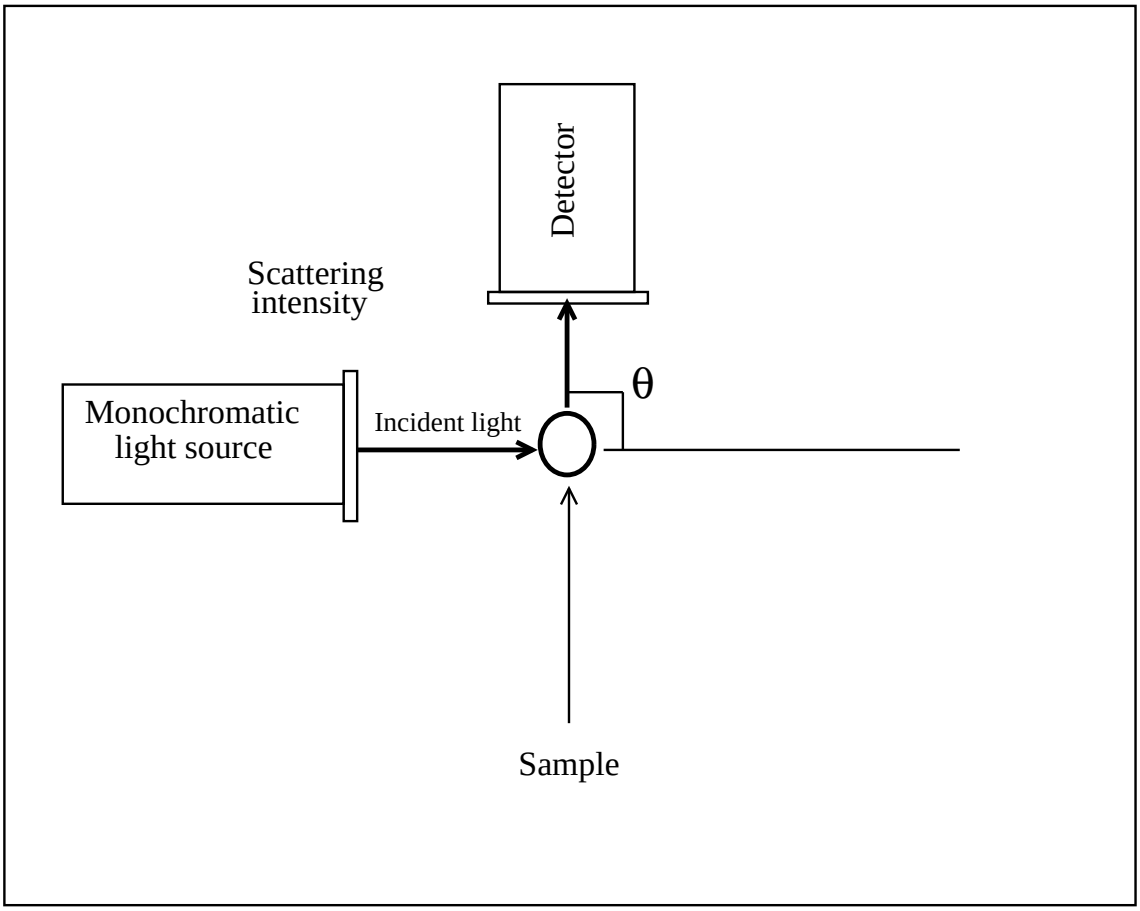

Figure 2.2

Block diagram of a light scattering instrument

as the protein concentration at which the solution is at equilibrium between the protein in the solution phase and the protein in crystalline phase or amorphous phase. Protein was dissolved in the buffer solutions at room temperature, and then incubated at 25°C for 48 hours. Before measuring the concentration of the protein spectrophotometrically the tubes containing protein and amorphous precipitate were centrifuged at 6,000 rpm for 20 minutes, and then 30-50 μL of supernatant was diluted, and the absorbance was measured at 280 nm. The obtained solubility values are reported in the results section .

CHAPTER III

RESULTS AND DISCUSSION

Predicting protein stability in a particular solution condition is a sensitive issue. With the increasing number of protein therapeutic products in the market, an effective way to determine protein stability should be developed. According to Krishnan, et al [23] protein stability consists of (1) conformational stability and (2) colloidal stability of the protein in the solution [13, 20, 23]. To develop a stable protein formulation both conformational and colloidal stability of the protein in the solution should be achieved. The conformational stability is measured by $(\triangle \mathrm{G}^{\circ}_{\mathrm{unfold}})$ and the colloidal stability is measured by the osmotic second virial coefficient (B_{22}) in a solution [13].

In this study, the effect of addition of excipients to the protein solution on the colloidal stability, i.e., stability of the protein in a solution in terms of crystallization and aggregation, is measured and the results are presented in the following sections.

3.1 B_{22} Measurement for Lysozyme in Buffer Containing Cosolvents

The second virial coefficient B_{22} was measured for lysozyme monomer. Lysozyme was dissolved in a 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 buffer and filtered into quartz cells of volume 45 μL or 12 μL and dynamic light scattering (DLS) experiments were performed to check for the presence of oligomers like dimers, trimers, or aggregates and

dust particles. Figure 3.1 shows the percentage intensity of light scattered by each species present in the solution.

The presence of only lysozyme monomer, having an average hydrodynamic radius of 1.8 nm is shown in Figure 3.1. The molecular weight estimated from the $\mathrm{R_h}$ value was $\sim$ 14 kDa, calculated using

$$D = \frac{\Gamma}{q^2} \tag{3.1}$$

where, $\mathrm{D}\ (\mathrm{cm}^2/\mathrm{s})$ is the diffusion coefficient, $\Gamma\ (\mathrm{s}^{-1})$ is the decay constant obtained from the correlation function, q^2 is the magnitude of the scattering vector, given as

$$q = \frac{4\pi\eta_\circ}{\lambda_\circ \sin(\frac{\theta}{2})} \tag{3.2}$$

where $\eta_\circ$ is the solvent refractive index, $\lambda_\circ$ is the vacuum wavelength of the incident light, and θ is the scattering angle.

Using the Stokes-Einstein equation, the hydrodynamic radius can be calculated

$$R_h = \frac{kT}{6\pi\eta D} \tag{3.3}$$

where k is Boltzmann's constant, T is the temperature in Kelvin, and η is the solvent viscosity $(\mathrm{cm}^3/\mathrm{g})$.

From the hydrodynamic radius, the molecular weight of the protein can be estimated using the empirical relationship developed from years of work in Wilson's lab:

$$M = \left(\frac{R_h}{4.9 \times 10^{-2}}\right)^{2.64} \tag{3.4}$$

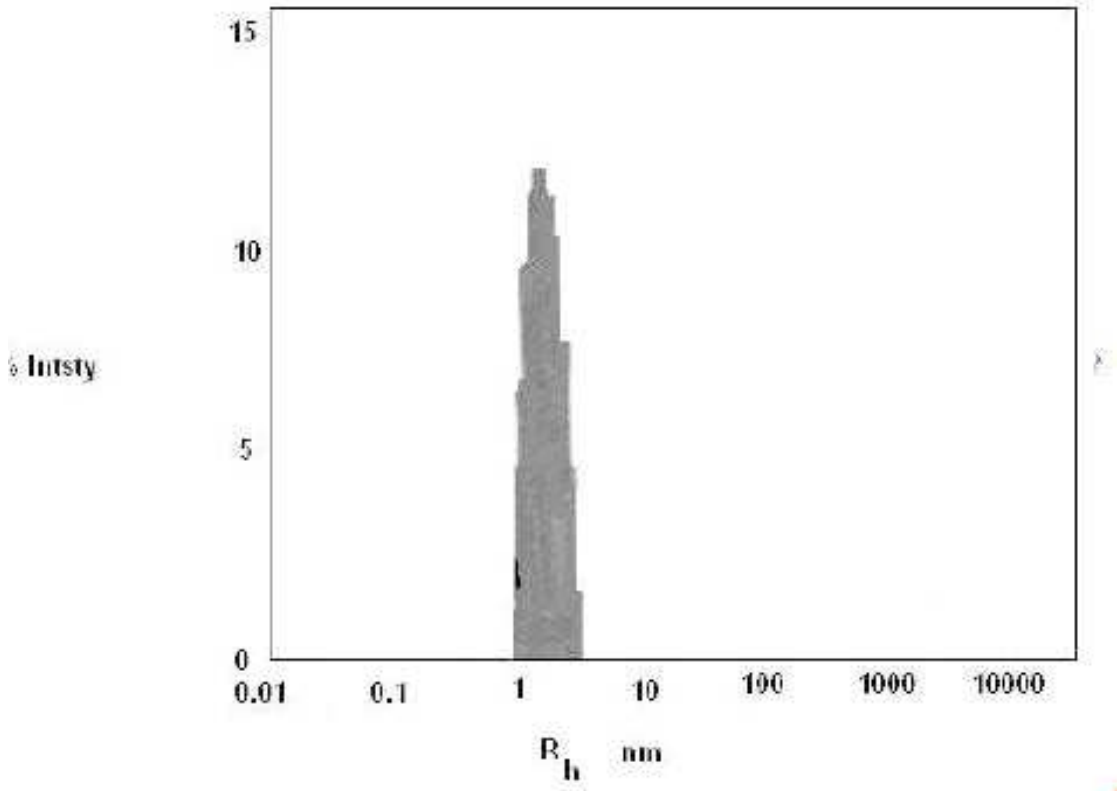

Figure 3.1

DLS histogram for lysozyme

where M is the molecular weight of the protein and R_h is in nm.

The DLS results confirmed that no further purification of the commercial lysozyme was required other than membrane filtration to remove dust.

Static light scattering (SLS) was used to determine B_{22} for lysozyme. Right Angle Laser Light Scattering was used for performing light scattering experiments. Protein solutions of several known concentrations ranging from $\sim$ 1- 8 mg/mL were injected directly into a 10 μL scattering cell using the "SIPPER" method. In this method protein solution in a small well is sipped into the cell with the help of peristaltic pump. All the solutions were filtered using 0.22 μm, 13 mm Millex Millipore disposable filter.

The light scattering profile of the buffer (baseline) and five different lysozyme concentrations is shown in Figure 3.2. The lysozyme stock solution was diluted to give protein concentrations ranging from 1 mg/mL to 5 mg/mL. The solutions were injected starting from lower concentration to higher.

From the intensity of the light scattered by buffer (background) and the light scattered by the protein solutions going from lower to higher concentration, B_{22} can be calculated based on Eq. (1.7). A plot of $\frac{\mathrm{Kc}}{\mathrm{R}}$ versus c is shown in Figure 3.3. The slope of the line gives B_{22} as -7.2×10^{-4} $(\mathrm{mol.mL/g^2})$.

3.1.1 B_{22} *and Solubility Measurement for Lysozyme in Buffer Containing Sucrose*

The second virial coefficient of lysozyme was measured in 0.1 M NaAc, 5 % (w/v) NaCl, pH. 4.5 containing different sucrose concentrations. Figure 3.4 shows the $\frac{\mathrm{Kc}}{\mathrm{R}}$ versus

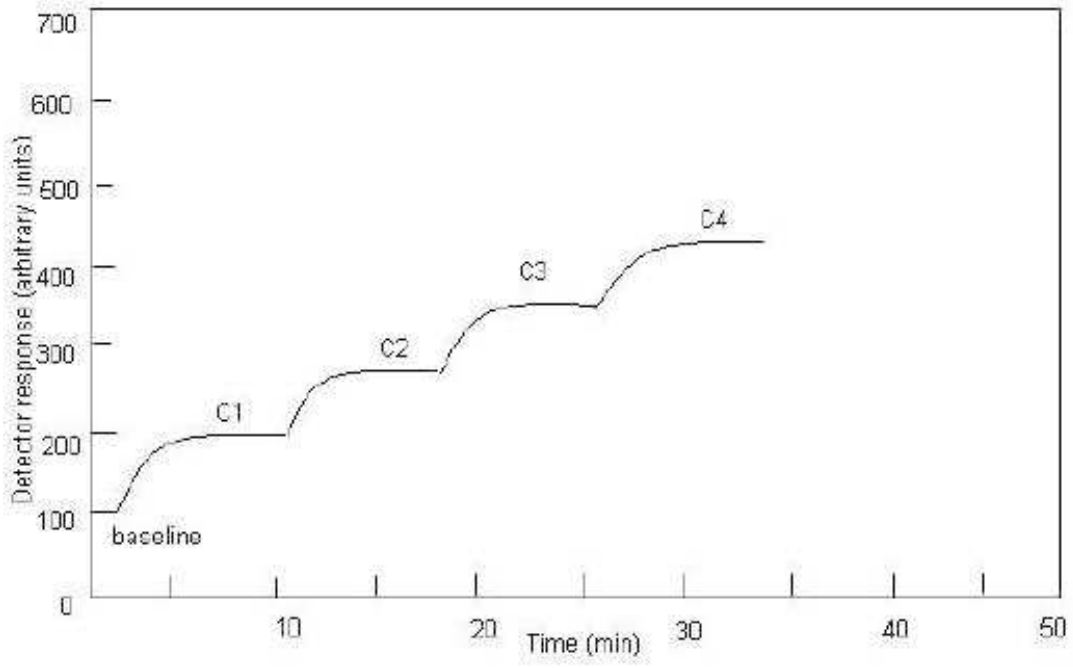

Figure 3.2

Light scattering profile of lysozyme in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

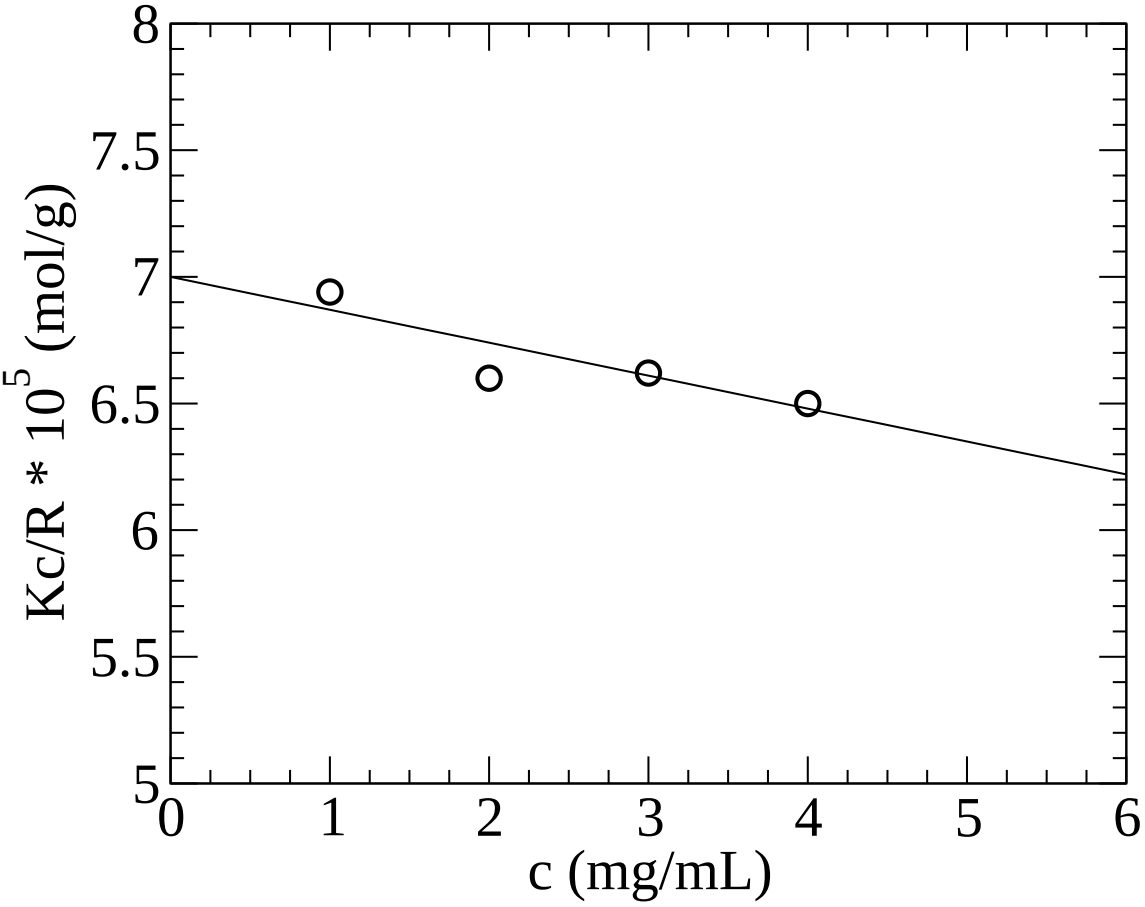

Figure 3.3

$\frac{Kc}{R}$ versus lysozyme concentration, c, in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

lysozyme concentration, c, in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5, $25^{\circ}\mathrm{C}$ at 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 M sucrose. The tabulated B_{22} values in Table 3.1 were calculated from the slopes of the plots in Figure 3.4.

B_{22} values increased with increasing sucrose concentration ranging from -7.2×10^{-4} at 0.0 M sucrose to -0.7×10^{-4} $(\mathrm{mol.mL/g^2})$ at 0.5 M sucrose. At 0.0 M sucrose, B_{22} value of -7.2×10^{-4} $(\mathrm{mol.mL/g^2})$ suggests that the protein-protein interactions are attractive. At the maximum sucrose concentration studied here B_{22} value increases to -0.7×10^{-4} $(\mathrm{mol.mL/g^2})$, which means that the protein-protein interactions have become less attractive. A plot of B_{22} versus sucrose concentration is shown in Figure 3.5. The data points in Figure 3.5 represent average of at least two B_{22} values, and error bars represent standard deviations of the average B_{22} values.

B_{22} values listed in Table 3.1 are average values with the standard deviation of the average B_{22} values.

The solubility of lysozyme was estimated for some sucrose concentrations at which B_{22} was measured, and the results are presented in Table 3.2. To measure the solubility, lysozyme stock was prepared by adding lysozyme to $\sim$ 100 μL of buffer until the solutions turned turbid. This solution was stirred on a magnetic stirrer for at least 1 hr and then incubated at $25^{\circ}\mathrm{C}$ for 48 hrs. The stock solution was than transferred into a centrifuge tube and spun down at 6,000 rpm for 20 minutes. Then 30 μL - 50 μL of supernatant was removed and diluted with buffers, and its concentration was measured spectrophotomet-

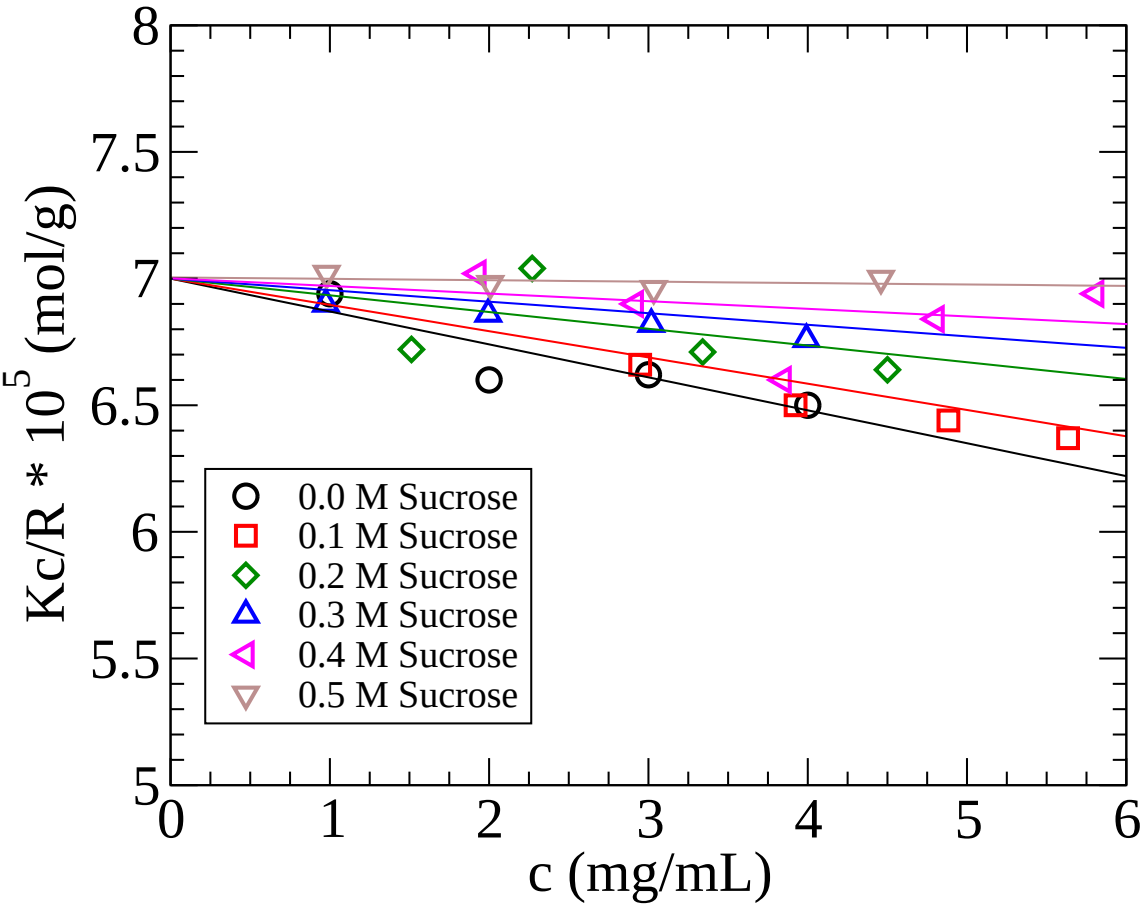

Figure 3.4

$\frac{Kc}{R}$ versus lysozyme concentration, c, at varying sucrose concentration in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

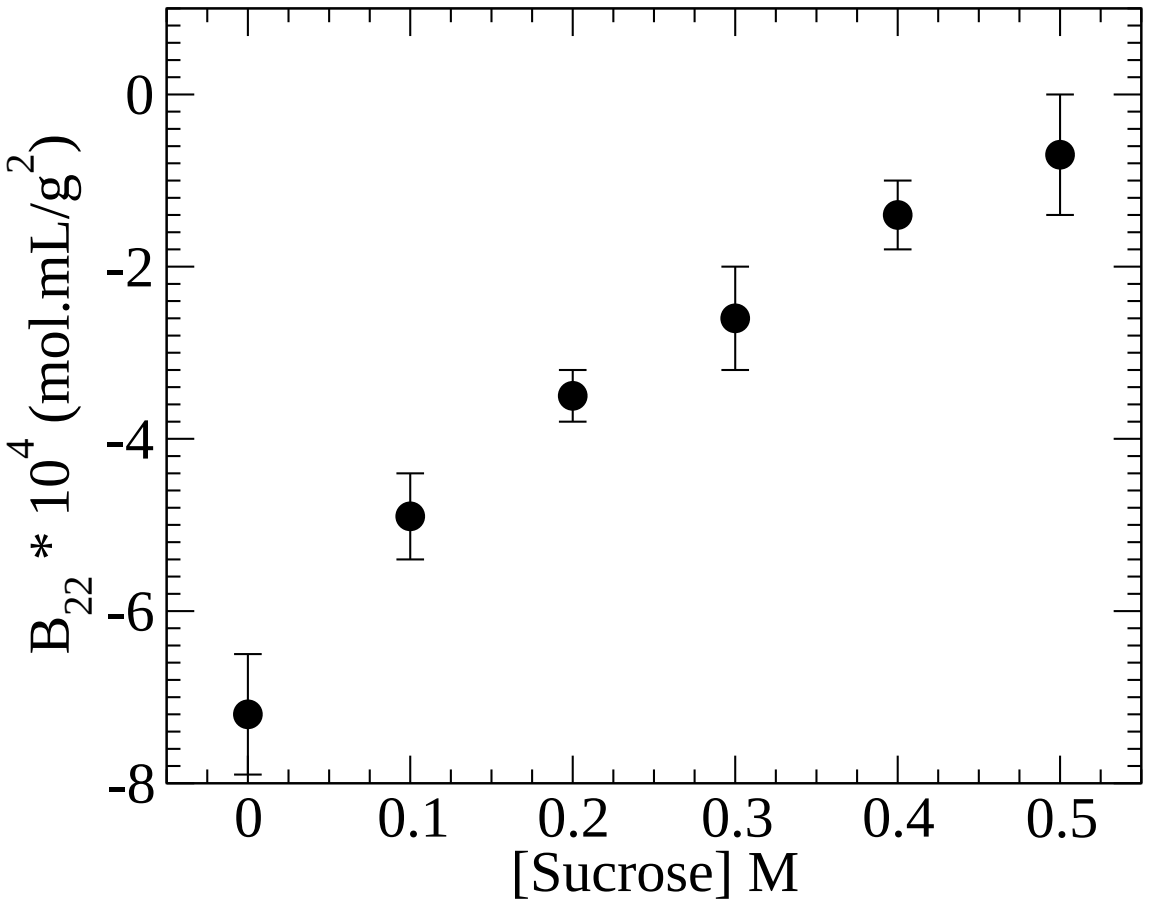

Figure 3.5

B_{22} versus sucrose concentration for lysozyme in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

Table 3.1

B_{22} for Lysozyme in Buffer Containing Sucrose

[Sucrose] M	$B_{22} \times 10^4$ (mol.mL/g^2)
0.0	-7.2 $\pm$ 0.7
0.1	-4.9 $\pm$ 0.5
0.2	-3.5 $\pm$ 0.3
0.3	-2.6 $\pm$ 0.6
0.4	-1.4 $\pm$ 0.4
0.5	-0.7 $\pm$ 0.7

rically. The obtained concentration values are the solubility values of the protein in that particular solution, and are reported in Table 3.2.

Table 3.2

Solubility of Lysozyme at $25°$C in Buffer Containing Sucrose

[Sucrose] M	Solubility $(\mathrm{mg/mL})$
0.0	4.5
0.1	–
0.2	9.3
0.3	–
0.4	12.9
0.5	18.5

An increase in solubility and B_{22} values was found with increase in sucrose concentration. Figure 3.6 shows a plot of lysozyme solubility versus obtained B_{22} values. The dashed line here does not imply any kind of theoretical fit but is a suggestive line.

The results obtained agree with the trend observed by George et al. [6, 9]. According to those results, solubility follows the trends shown by B_{22} values. If B_{22} increases, solubility

also increases and vice versa. The results in Figure 3.5 are consistent with the findings [9, 10, 25].

3.1.2 $\mathrm{B_{22}}$ *and Solubility Measurement for Lysozyme in Buffer Containing Trehalose*

The second virial coefficient B_{22} of lysozyme was measured in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 with and without trehalose, and the obtained values are listed in Table 3.3. The listed B_{22} values are average values of at least two SLS experiments along with the standard deviation of the average B_{22} values. With the addition of trehalose, an increase in B_{22} values was observed. Figure 3.7 shows $\frac{\mathrm{Kc}}{\mathrm{R}}$ versus lysozyme concentration in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5, $25^{\circ}\mathrm{C}$ at 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 M trehalose. B_{22} values obtained were determined from the slopes of the plots in Figure 3.7. With the addition of trehalose the slope of the lines becomes less negative, and so do B_{22} values.

B_{22} values obtained increased with the increasing trehalose concentration, ranging from -7.2×10^{-4} at 0.0 M trehalose to -0.7×10^{-4} $(\mathrm{mol.mL/g^2})$ at 0.5 M trehalose. At 0.0 M trehalose, a B_{22} value of -7.2×10^{-4} $(\mathrm{mol.mL/g^2})$ suggests that the protein-protein interactions are attractive, while addition of trehalose increases B_{22} values to -0.7×10^{-4} $(\mathrm{mol.mL/g^2})$, which means that the protein-protein interactions have become less attractive. A plot of B_{22} versus trehalose concentration is shown in Figure 3.8.

The data points in Figure 3.8 represent average B_{22} values obtained from at least two SLS experiments, and error bars represent the standard deviation of the average B_{22} values.

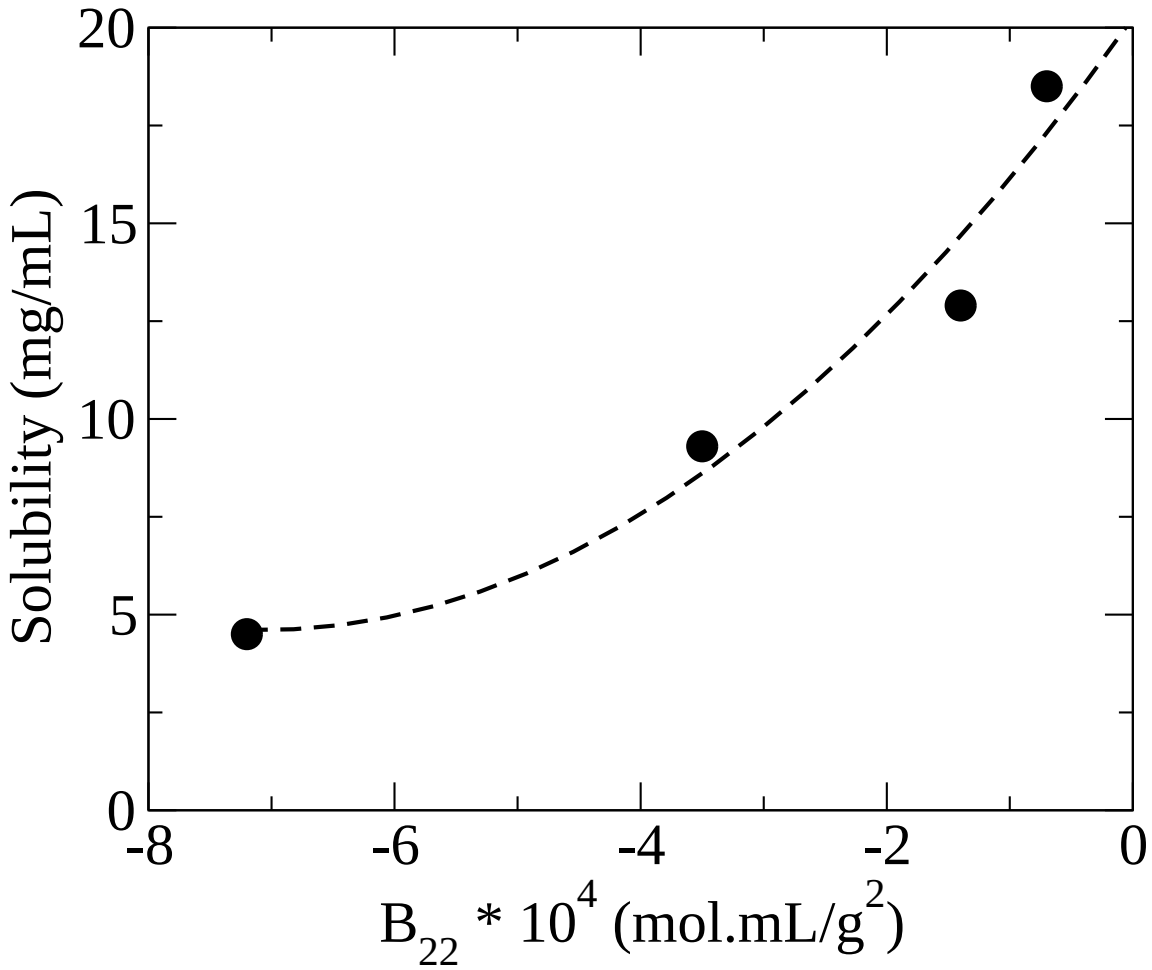

Figure 3.6

Lysozyme solubility versus B_{22} in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 at varying sucrose concentration

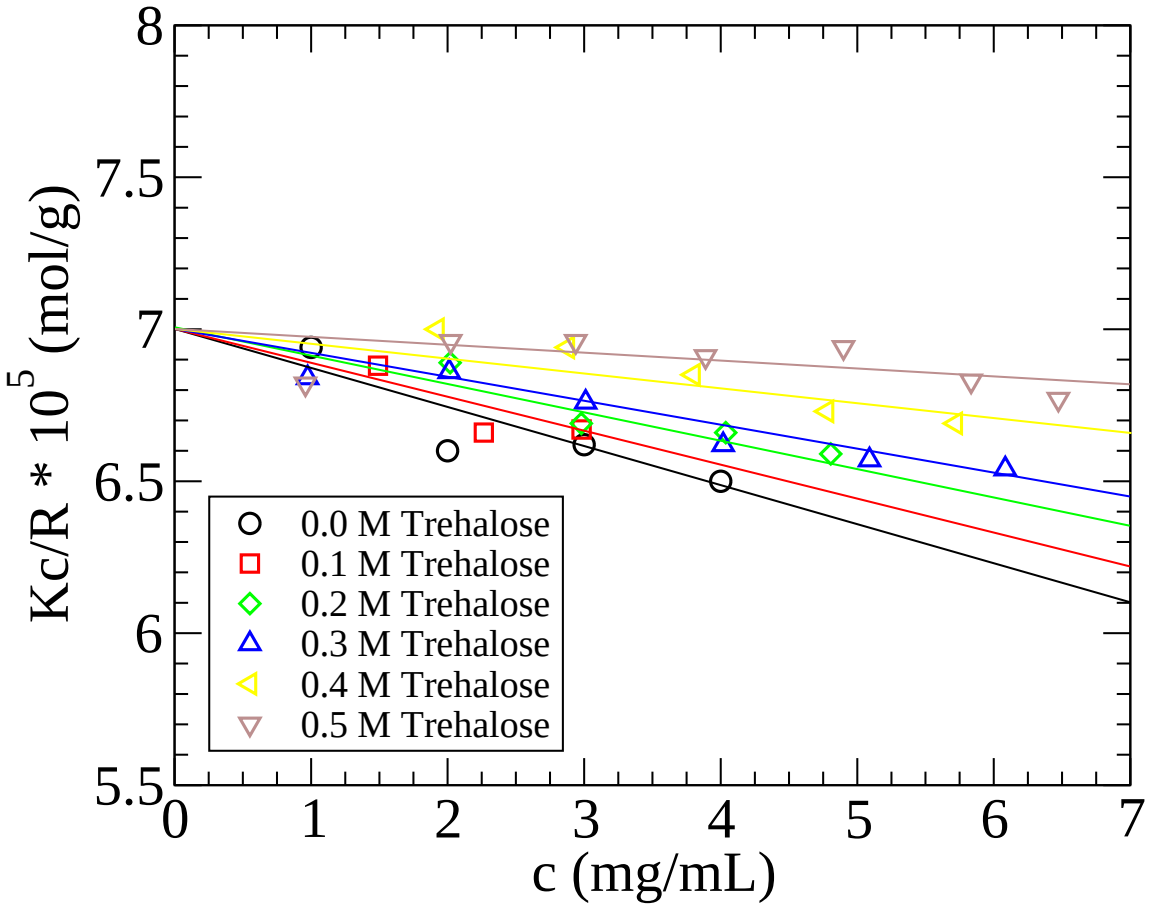

Figure 3.7

$\frac{Kc}{R}$ versus lysozyme concentration, c, at varying trehalose concentration in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

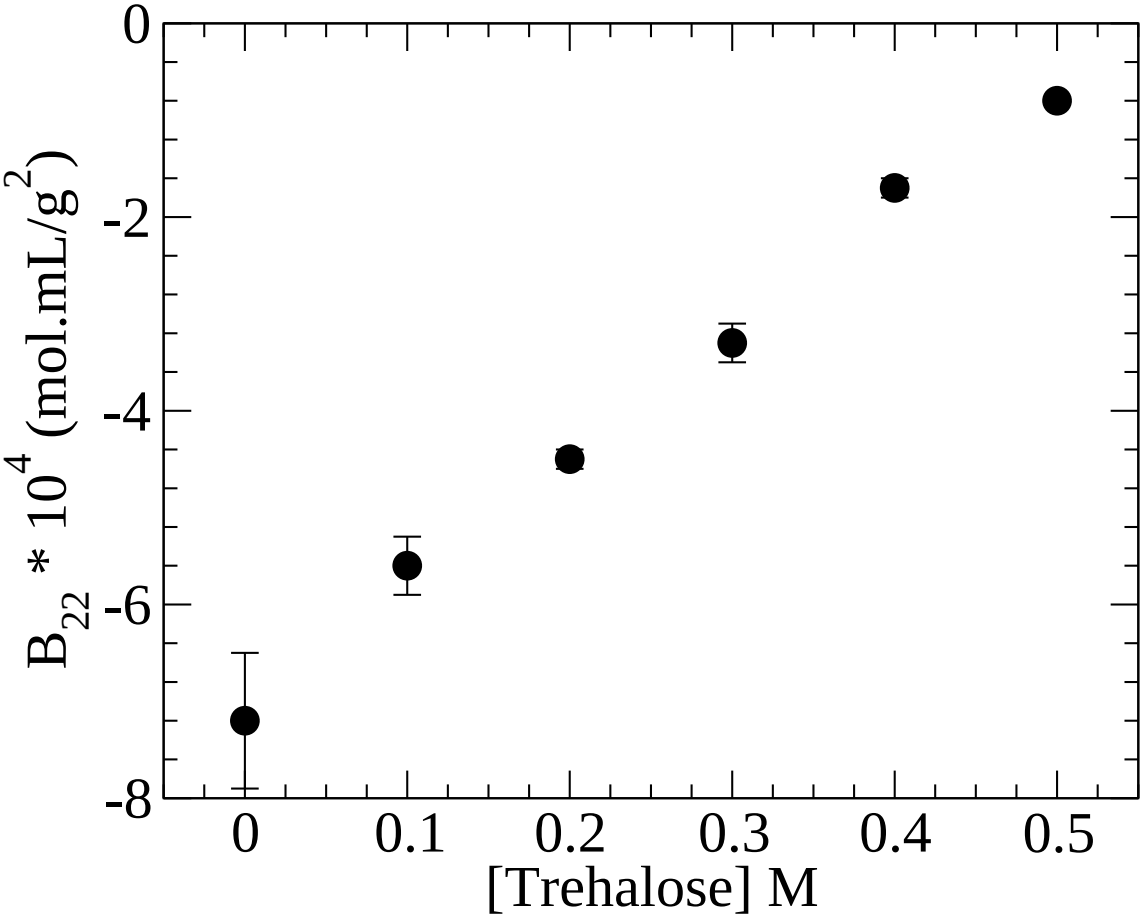

Figure 3.8

B_{22} versus trehalose concentration for lysozyme in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

Table 3.3

B_{22} for Lysozyme in Buffer Containing Trehalose

[Trehalose] M	$B_{22} \times 10^4$ (mol.mL/g^2)
0.0	-7.2 $\pm$ 0.7
0.1	-5.6 $\pm$ 0.3
0.2	-4.5 $\pm$ 0.1
0.3	-3.3 $\pm$ 0.2
0.4	-1.7 $\pm$ 0.1
0.5	-0.8 $\pm$ 0.0

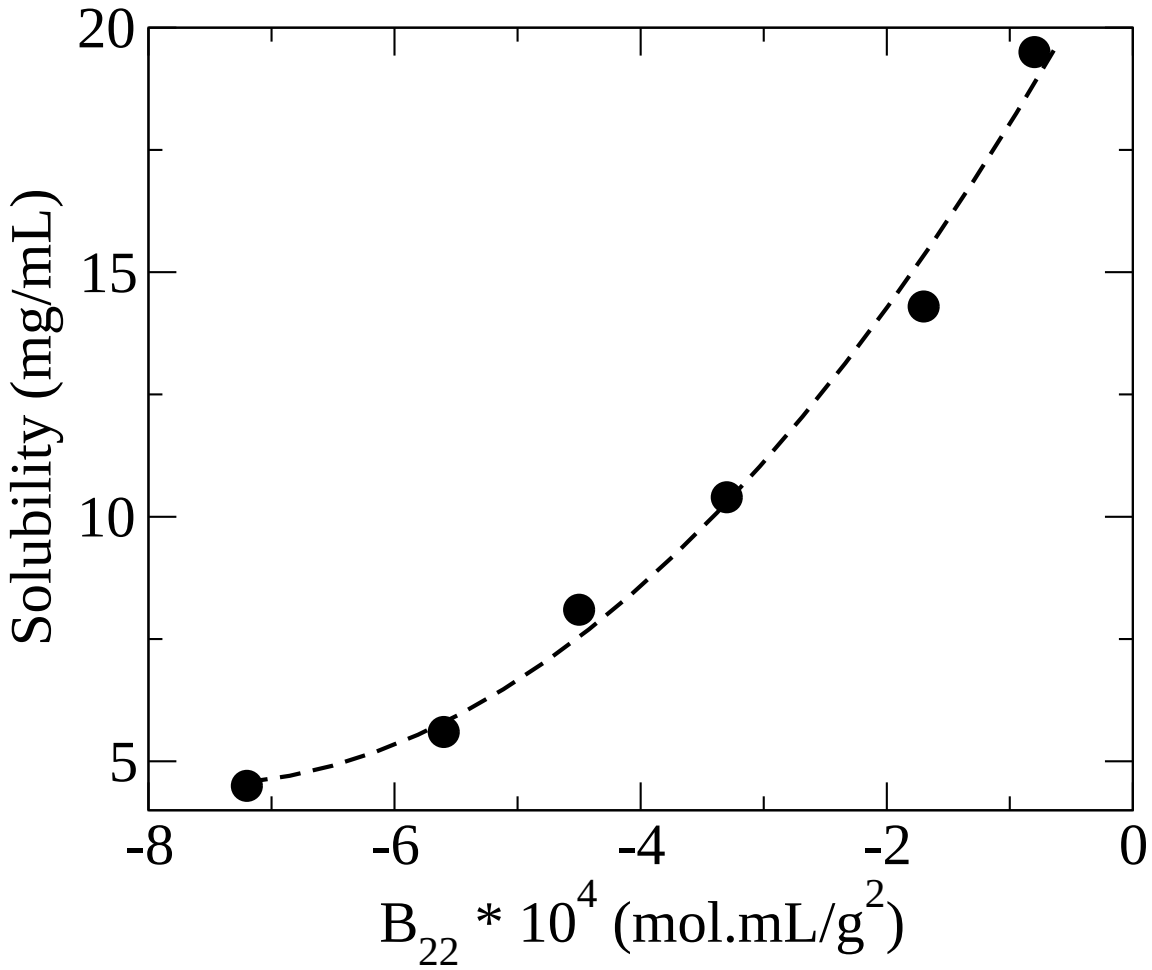

Figure 3.9

Lysozyme solubility versus B_{22} in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 at varying trehalose concentration

The solubility of lysozyme was estimated at each trehalose concentration at which B_{22} values were measured, and the results are presented in Table 3.4.

Table 3.4

Solubility of Lysozyme at $25°C$ in Buffer containing Trehalose

[Trehalose] M	Solubility (mg/mL)
0.0	4.5
0.1	5.6
0.2	8.1
0.3	10.4
0.4	14.3
0.5	19.5

An increase in solubility and B_{22} value was measured with increase in trehalose concentration. Figure 3.8 shows a plot of lysozyme solubility versus obtained B_{22} values. The dashed line here does not imply any kind of theoretical fit but is a suggestive line.

The results obtained agree with the trend observed by George et al [8]. According to those results solubility follows the trends shown by B_{22} values [9, 10, 25].

3.1.3 B_{22} *and Solubility Measurement of Lysozyme in Buffer Containing Mannitol*

The second virial coefficients B_{22} were measured at varying mannitol concentration in 0.1 M NaAc, 5 % (w/v) NaCl, pH. 4.5. Figure 3.10 shows the $\frac{Kc}{R}$ versus lysozyme concentration at 0.0, 0.05, 0.2, and 0.3 M mannitol. The tabulated B_{22} values were calculated from the slopes of the plots in Figure 3.10. B_{22} values obtained increased with increasing mannitol concentration ranging from -7.2×10^{-4} $(mol.mL/g^2)$ at 0.0 M mannitol to -1.5×10^{-4} $(mol.mL/g^2)$ at 0.3 M mannitol. Addition of mannitol increases B_{22} values to -1.5×10^{-4} $(mol.mL/g^2)$, which means that the protein-protein interactions has become less attractive. A plot of B_{22} versus mannitol concentration is shown in Figure 3.11.

The data points in Figure 3.11 represent average B_{22} values obtained from at least two SLS experiments, and error bars represent the standard deviation of the average B_{22} values.

B_{22} values listed in Table 3.5 are average values with the standard deviation of the average B_{22} values.

The solubility of lysozyme was estimated for some mannitol concentrations at which B_{22} values were measured, and the results are presented in Table 3.6. The procedure for estimating the solubility was the same as that used for estimating solubility of lysozyme in sucrose.

An increase in solubility and B_{22} was measured with increase in mannitol concentration. Figure 3.12 shows a plot of lysozyme solubility versus obtained B_{22} values. The dashed line here does not imply any kind of theoretical fit but is a suggestive line.

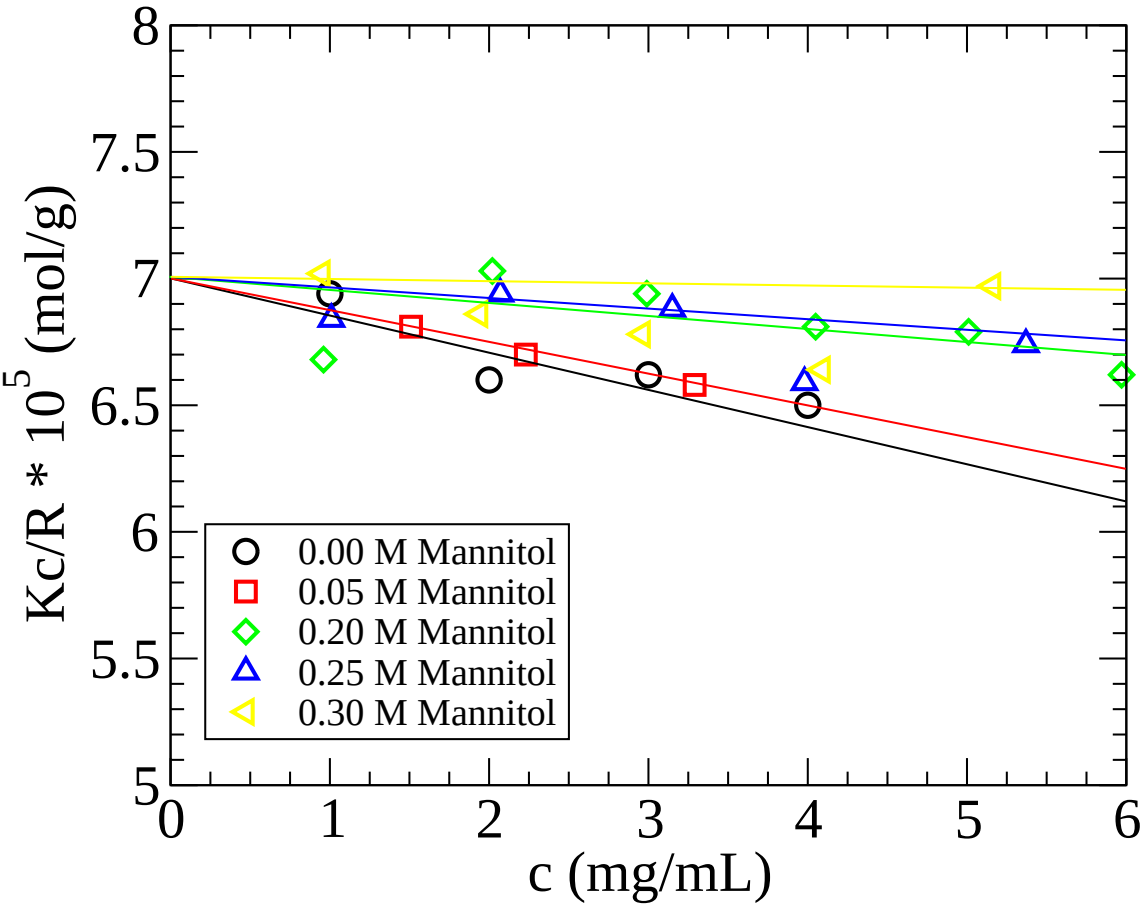

Figure 3.10

$\frac{Kc}{R}$ versus lysozyme concentration, c, at varying mannitol concentration in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

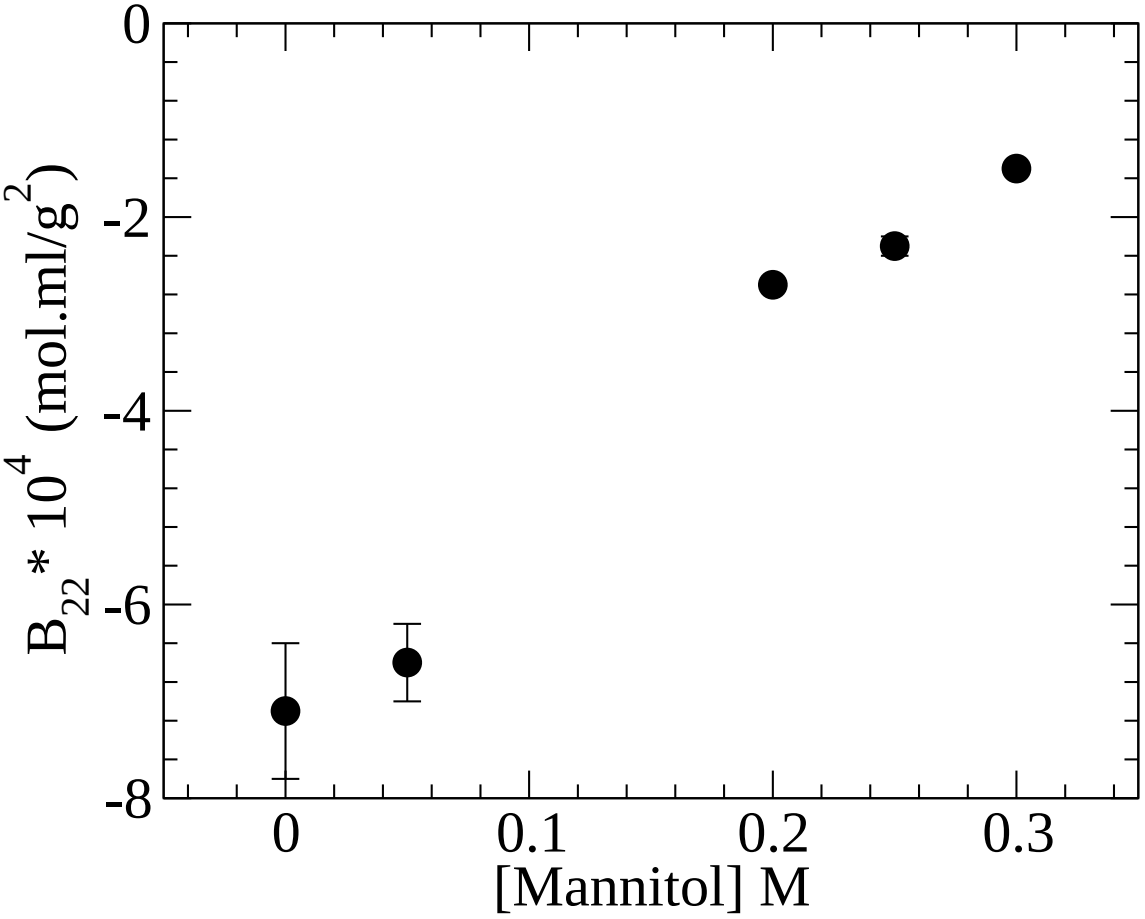

Figure 3.11

B_{22} versus mannitol concentration for lysozyme in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5

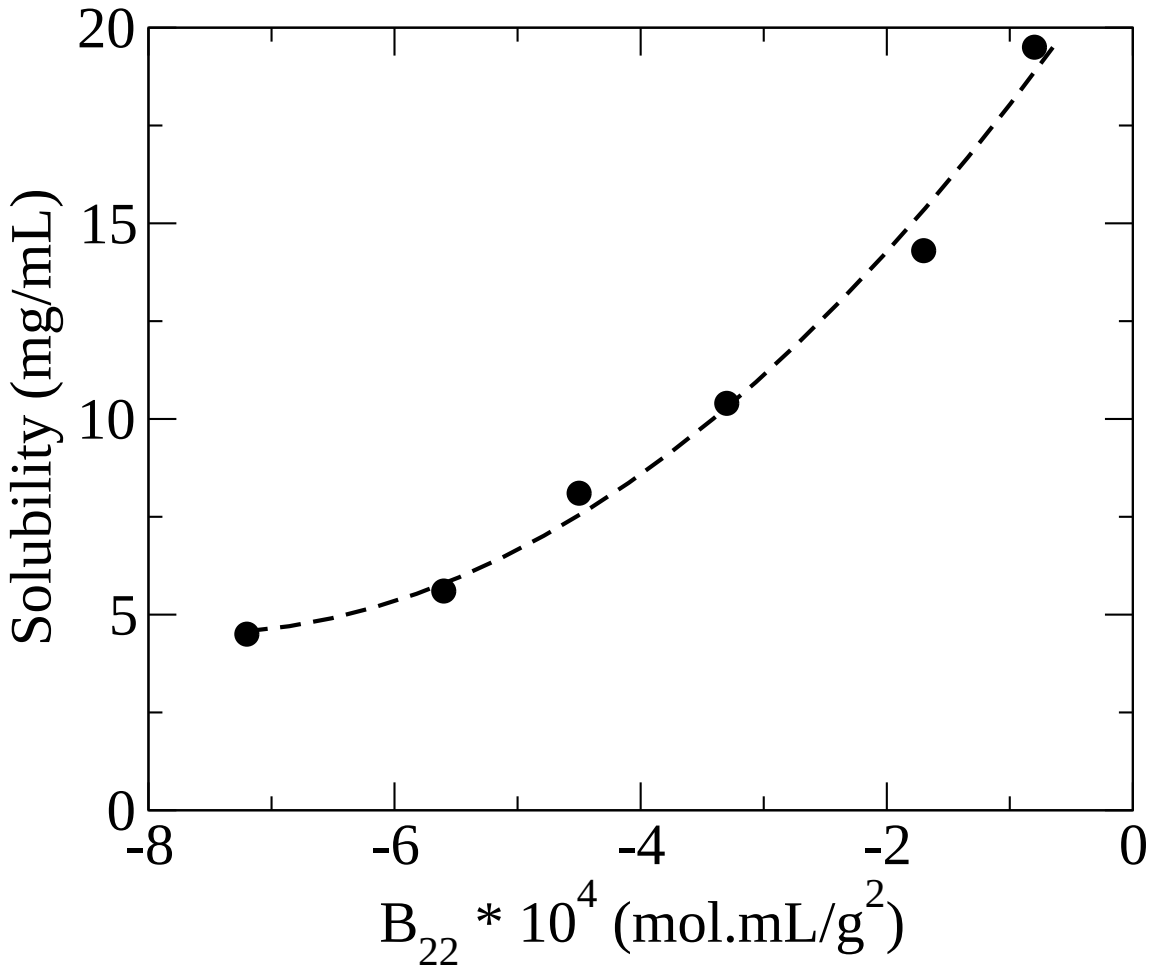

Figure 3.12

Lysozyme solubility versus B_{22} in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 at varying mannitol concentration

Table 3.5

B_{22} for Lysozyme in Buffer containing Mannitol

[Mannitol] M	$B_{22} \times 10^4$ (mol.mL/g^2)
0.00	-7.1 $\pm$ 0.7
0.05	-6.6 $\pm$ 0.4
0.20	-2.7 $\pm$ 0.0
0.25	-2.3 $\pm$ 0.1
0.30	-1.5 $\pm$ 0.0

Table 3.6

Solubility of Lysozyme at 25°C in Buffer containing Mannitol

[Mannitol] M	Solubility (mg/mL)
0.00	4.5
0.05	4.5
0.20	6.7
0.25	7.8
0.30	-

3.2 B_{22} Measurement for Ovalbumin Monomer

Commercial ovalbumin was analyzed by DLS to verify the presence of high molecular weight impurities other than monomer. It was difficult to run DLS experiments, due to automatic detector shut down caused due to the presence of high molecular weight species. This suggested the presence of larger species like oligomers, aggregates, or dust particles.

Ovalbumin stock at $\sim$ 30 to 35 mg/mL prepared in 0.1 M sodium phosphate buffer, pH 7.0 was loaded on a Superdex 75 HR (16 mm $\times$ 160 mm) size exclusion column (Pharmacia Biotech) connected to a BioCad SPRINT perfusion chromatography system and eluted with 0.1 M sodium phosphate, pH 7.0 mobile phase at a flow rate of 1.5 mL/min. The elution profile of ovalbumin is shown in Figure 3.13.

Peak A is ovalbumin monomer, which was confirmed by DLS, while peaks B and C may be lower order oligomers such as dimers or trimers. Peaks B and C were not collected due to our interest in ovalbumin monomer only. Peak D that eluted first can be attributed to high molecular weight impurities.

The monomer fraction was collected and concentrated using an Amicon 8010 concentrator with a Millex Millipore membrane, 42 mm, molecular weight cut off 3000 g/mol. Purified monomer was analyzed using DLS to verify molecular size homogeneity. The average hydrodynamic radius was 2.6 nm and the molecular weight estimated was $\sim$ 45 kDa calculated using Eq. (3.1-3.4). The final stock concentration after concentrating was $\sim$ 9.6 mg/mL.

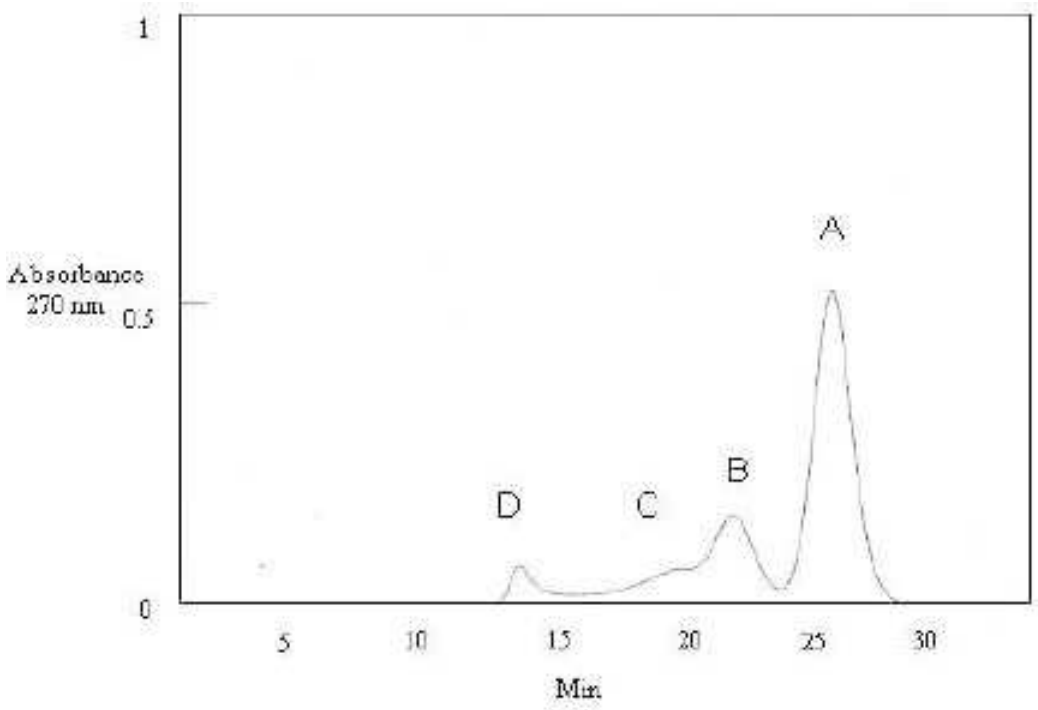

Figure 3.13

Elution profile of ovalbumin, A- ovalbumin monomer (45 kDa), B and C - lower order oligomers and D- high molecular weight impurities

The DLS histogram for the purified ovalbumin monomer is shown in Figure 3.13. DLS showed the presence of one species at $\mathrm{R_h} = 2.6$ nm, while some high molecular weight impurities like dust are present.

The second virial coefficient $\mathrm{B_{22}}$ for ovalbumin was measured in 0.1 M sodium phosphate buffer, pH 7.0, containing varying amount of sucrose. The obtained values are listed in Table 3.7. The listed $\mathrm{B_{22}}$ values are average $\mathrm{B_{22}}$ values along with the calculated standard deviation of the average $\mathrm{B_{22}}$ values. Figure 3.15 shows $\frac{\mathrm{Kc}}{\mathrm{R}}$ versus ovalbumin concentration in 0.1 M sodium phosphate buffer, pH 7.0 at 0, 5, 10, 15, 20, and 25 % w/v sucrose. $\mathrm{B_{22}}$ values listed in Table 3.7 were determined from the slope of the plots in Figure 3.15. The slopes become more positive as the sucrose concentration increases, and so does $\mathrm{B_{22}}$. With increasing concentration of sucrose, $\mathrm{B_{22}}$ values changed from -1.6×10^{-4} $(\mathrm{mol.mL/g^2})$ at 0 % w/v sucrose at which the protein-protein interactions are slightly attractive to 7.3×10^{-4} $(\mathrm{mol.mL/g^2})$ at 25 % w/v sucrose when the protein-protein interactions turn repulsive.

A plot of $\mathrm{B_{22}}$ versus sucrose concentration for ovalbumin monomer is shown in Figure 3.16. The data points on the plot represent average $\mathrm{B_{22}}$ values, and error bars represent the standard deviation of the average $\mathrm{B_{22}}$ values. The values obtained here agree completely with the values obtained in literature [2].

The relative solubility of ovalbumin as a function of sucrose concentration was obtained from the literature [2]. The purpose here is to obtain $\mathrm{B_{22}}$ values and solubility data of ovalbumin to show that solubility follows the trend shown by $\mathrm{B_{22}}$ values. In this case,

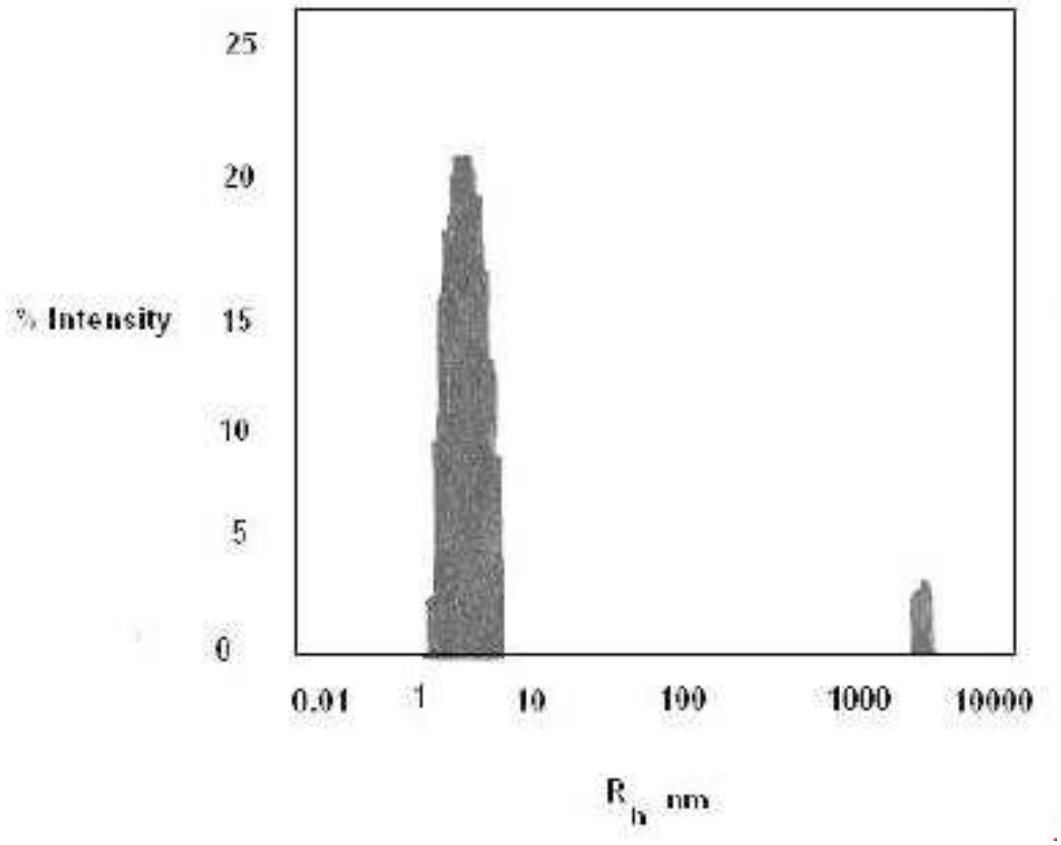

Figure 3.14

DLS histogram of ovalbumin

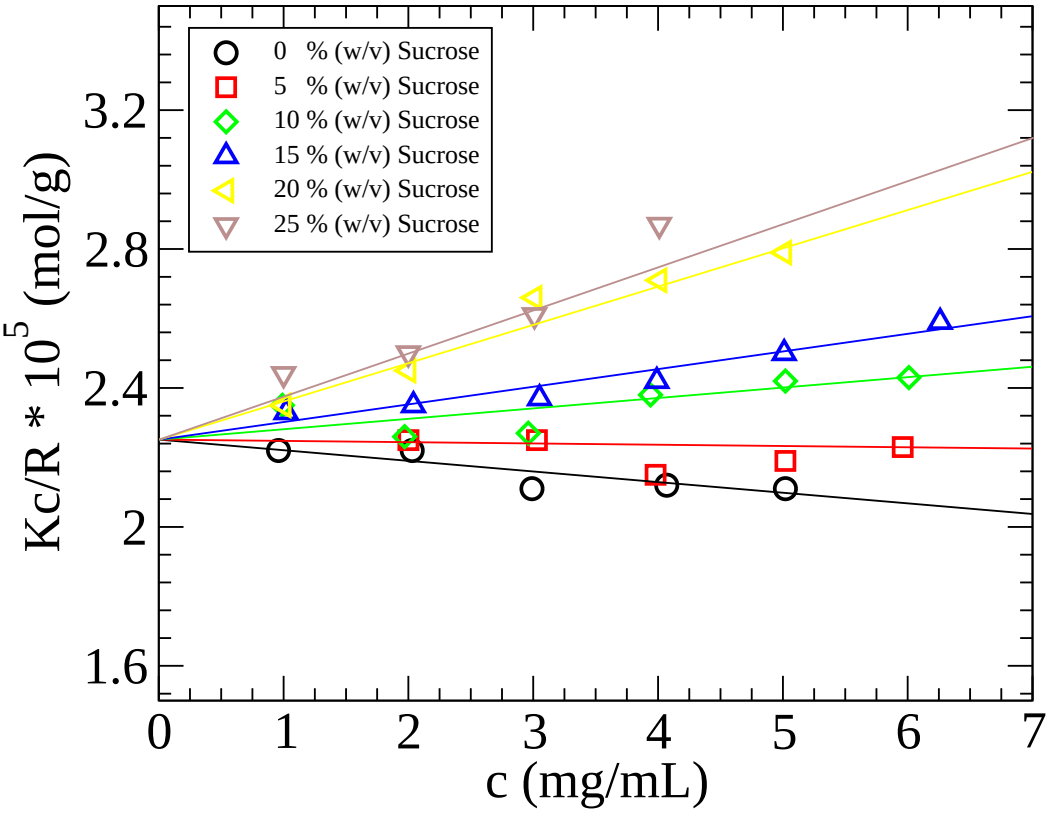

Figure 3.15

$\frac{Kc}{R}$ versus ovalbumin concentration, c, at varying concentration of sucrose in 0.1 M sodium phosphate, pH 7.0

addition of sucrose increases B_{22} values, and the same trend is observed for the solubility Figure 3.17.

Table 3.7

Measured B_{22} for Ovalbumin monomer in Buffer Containing Sucrose

[Sucrose] M	$B_{22} \times 10^4$ (mol.mL/g^2)
0	-1.6 $\pm$ 0.0
5	-0.5 $\pm$ 0.0
10	1.1 $\pm$ 0.5
15	2.3 $\pm$ 0.2
20	5.7 $\pm$ 0.0
25	7.3 $\pm$ 0.4

3.3 B_{22} Measurement for Human Serum Albumin (HSA)

Human serum albumin was checked for molecular homogeneity using dynamic light scattering. DLS were ambiguous for HSA due to the solution consisting of higher molecular weight impurities.

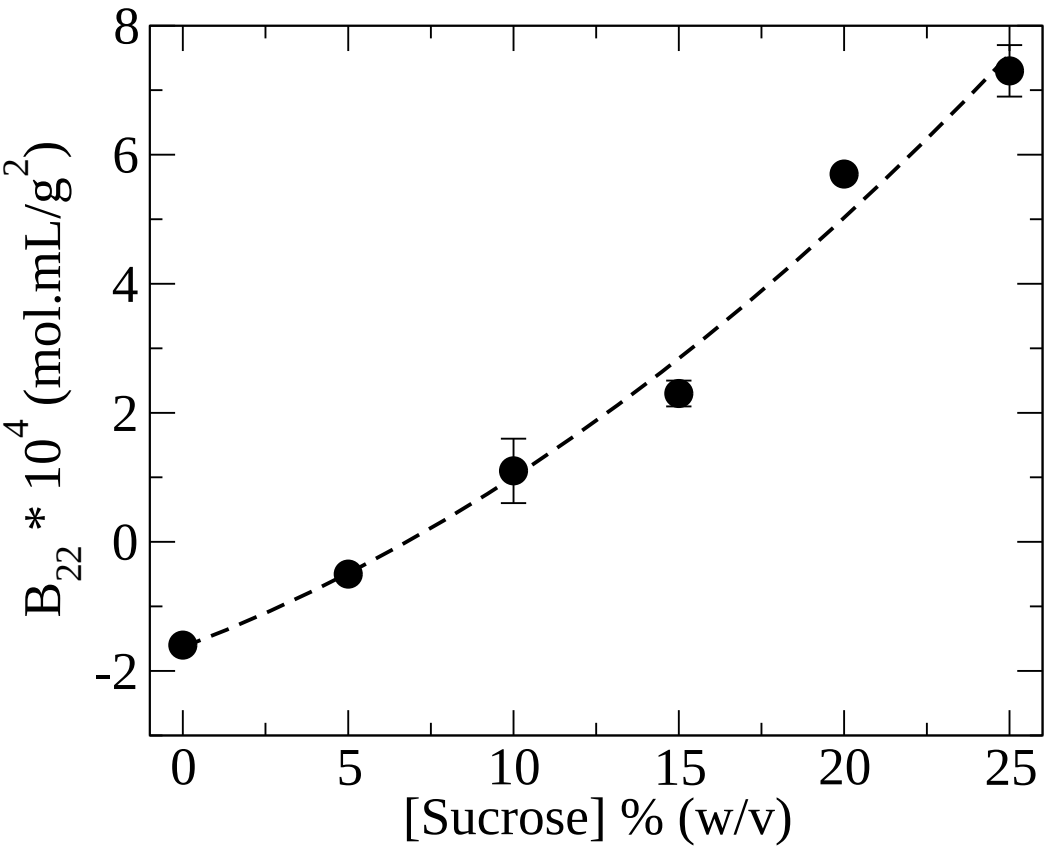

Figure 3.16

B_{22} versus sucrose concentration for ovalbumin in 0.1 M sodium phosphate, pH 7.0

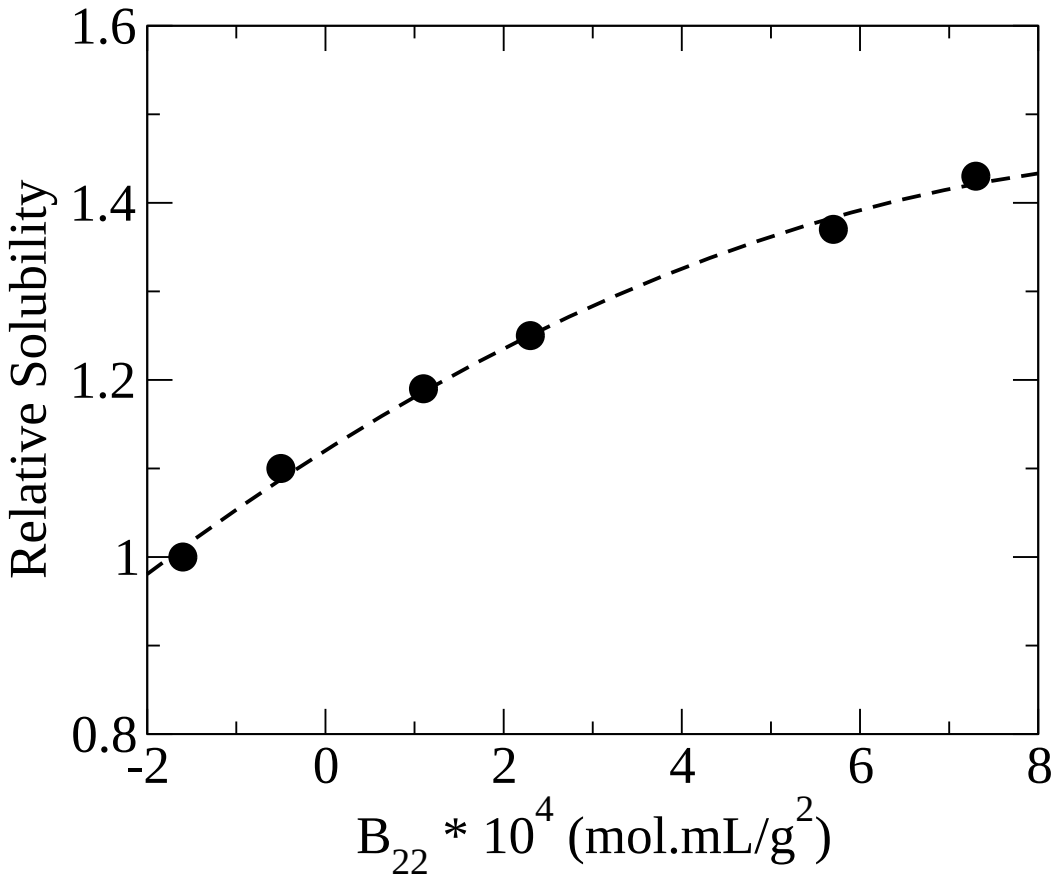

Figure 3.17

Relative solubility of ovalbumin in 0.1 M sodium phosphate, pH 7.0 at varying sucrose concentration [2]

A stock solution containing $\sim$ 30 to 35 mg/mL HSA was prepared in 0.1 M sodium phosphate buffer, pH 7.6. About 1 mL of the stock was injected in a Superdex 200 HR (16 mm $\times$ 160 mm) size exclusion column (Pharmacia Biotech) connected to BioCad SPRINT perfusion chromatography system and eluted with 0.1 M sodium phosphate, pH 7.6 mobile phase at a flow rate of 1.5 mL/min. The elution profile of HSA is shown in Figure 3.18.

Peak A is HSA monomer, which was confirmed by DLS, while peak B may be lower order oligomers such as dimers. Peak C that eluted first can be attributed to high molecular weight impurities. Peaks B and C were not collected due to our interest in HSA monomer only.

The monomer fractions were collected and concentrated using an Amicon 8010 concentrator with a Millex Millipore membrane, 42 mm, with Molecular weight cut off 3000 g/mol. Purified monomer was analyzed using DLS to verify molecular size homogeneity. The average hydrodynamic radius was 2.9 nm, and the estimated molecular weight was $\sim$ 68 kDa using Eq. (3.1-3.4). The final stock concentration determined spectrophotometrically after concentrating was $\sim$ 55 mg/mL.

The DLS histogram for the purified HSA monomer is shown in Figure 3.19. DLS showed presence of one species at $\mathrm{R_h}$ = 2.9 nm.

3.3.1 $\mathrm{B_{22}}$ *Measurement for HSA in Buffer Containing Glucose*

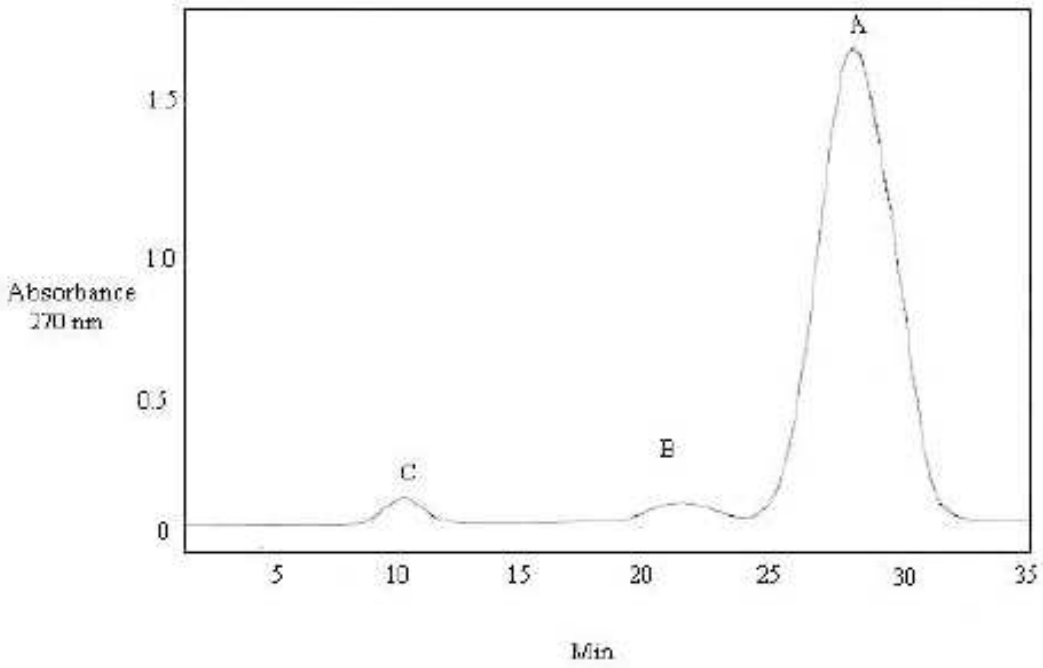

Figure 3.18

Elution profile of human serum albumin, A: HSA monomer (68 kDa), B: oligomers and C: higher molecular weight impurities

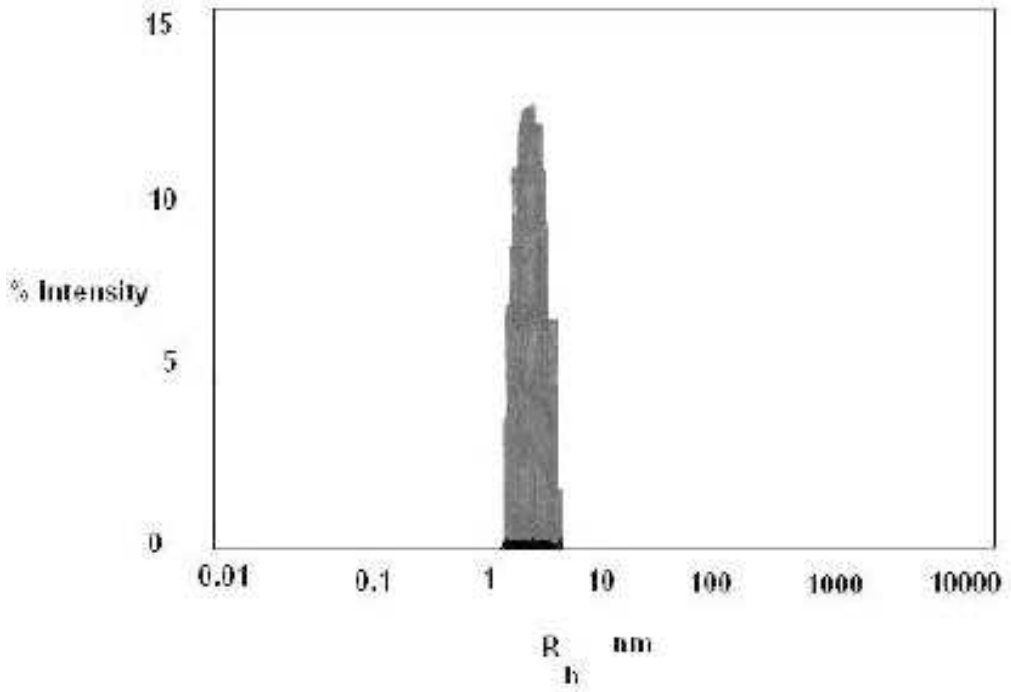

Figure 3.19

DLS histogram of the purified HSA

The second virial coefficient B_{22} for HSA was measured in 0.1 M sodium phosphate buffer, 2.6 M AS, pH 7.6, containing varying amounts of glucose. The obtained B_{22} values are listed in Table 3.8. The listed B_{22} values are average B_{22} values along with the calculated standard deviation of the average B_{22} values. Figure 3.20 shows $\frac{Kc}{R}$ versus HSA concentration, c, in 0.1 M sodium phosphate buffer, 2.6 M AS, pH 7.6 at 0, 20, 40, and 80 mg/mL glucose. B_{22} values listed in Table 3.8 were determined from the slope of the plots in Figure 3.20. The slopes become more positive as the glucose concentration increases, and so does B_{22}.

With increasing glucose concentration, B_{22} values changed from -7.8×10^{-4} at 0 mg/mL glucose to -3.0×10^{-4} $(mol.mL/g^2)$ at 80 mg/mL glucose. The B_{22} values measured for HSA monomer with varying glucose concentrations are listed in Table 3.8.

Table 3.8

Measured Solubility and B_{22} for HSA Monomer in Buffer containing Glucose

[Glucose] (mg/mL)	Solubility (mg/mL)	$B_{22} \times 10^4$ $(mol.mL/g^2)$
0	2.2	-7.8 $\pm$ 0.2
20	-	-6.5 $\pm$ 0.1
40	39.0	-5.4 $\pm$ 0.0
80	$\geq$55.0	-3.0 $\pm$ 0.3

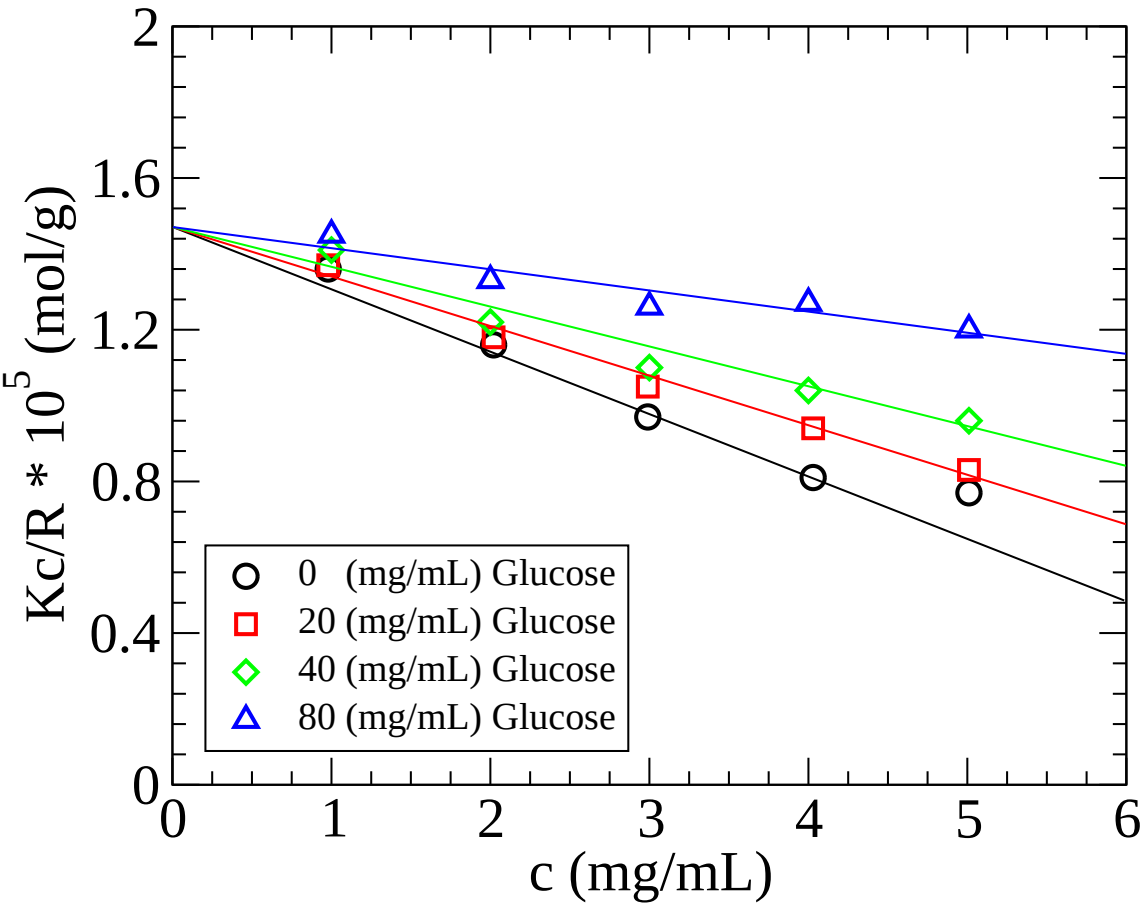

Figure 3.20

$\frac{Kc}{R}$ versus HSA concentration, c, at varying glucose concentration in 2.6 M AS, 0.1 M sodium phosphate, pH 7.6

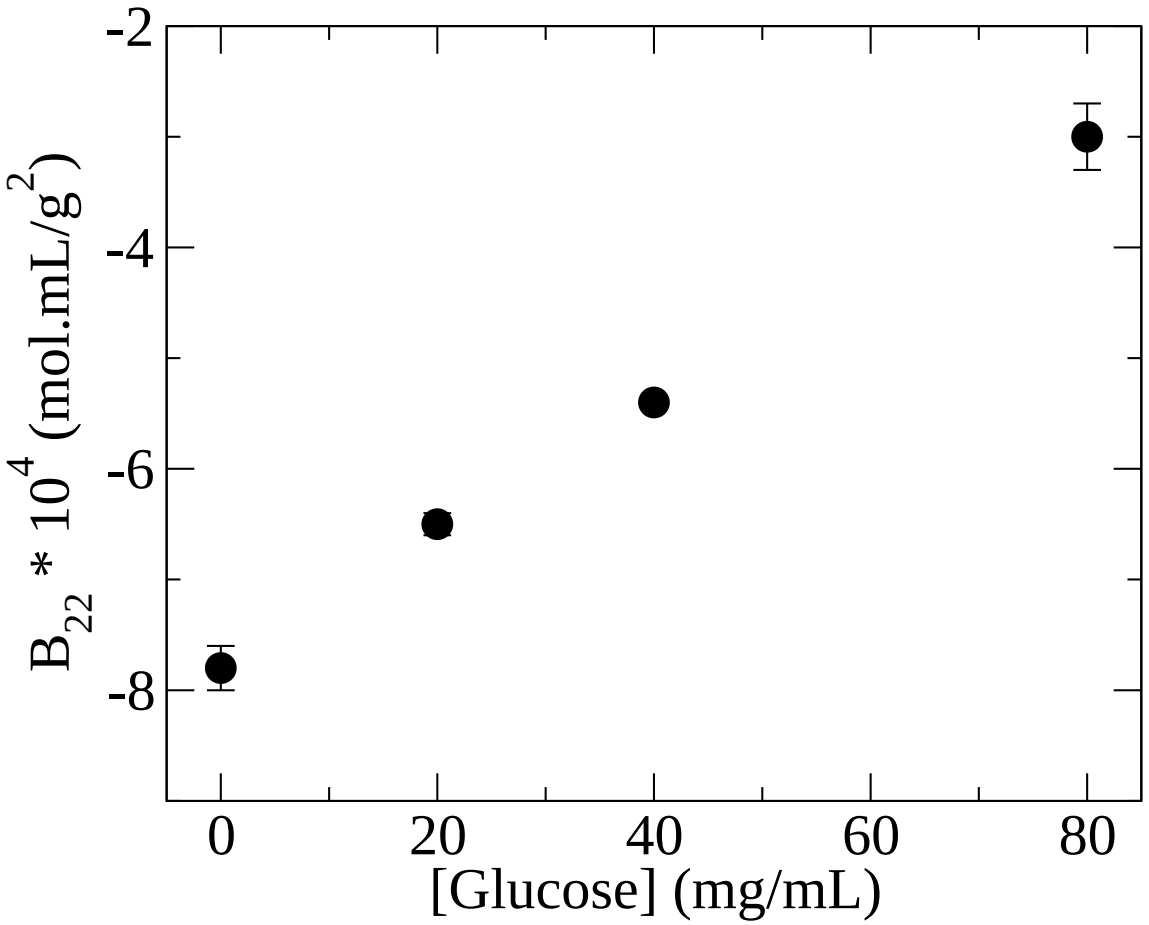

Figure 3.21

B_{22} versus glucose concentration for HSA in 2.6 M AS, and 0.1 M sodium phosphate, pH 7.6

3.3.2 B_{22} Measurement for HSA in Buffer Containing Dextran150

B_{22} for HSA was measured in 0.1 M sodium phosphate, 1.65 M AS, pH 7.6 at 0, 10, and 20 mg/mL dextran150 concentration. The average molecular weight of dextran150 is around 150,000 g/mol. In the presence of 0 mg/mL dextran150, B_{22} was found to be -0.4×10^{-4} $(mol.mL/g^2)$, which decreased at 20 mg/mL dextran150 to -3.3×10^{-4} $(mol.mL/g^2)$.

The $\frac{Kc}{R}$ versus HSA concentration is shown in Figure 3.22. B_{22} values tabulated in Table 3.8 are calculated from the slopes of the $\frac{Kc}{R}$ versus HSA concentration plots. The tabulated values are average values of at least duplicate sets of experiment, and together are the standard deviation of the average B_{22} values. A plot of B_{22} versus dextran150 concentration is shown in Figure 3.23.

The B_{22} values measured for HSA monomer in 0.1 M sodium phosphate, 1.65 M AS, pH 7.6 with varying dextran 150 concentrations are listed in Table 3.8.

Solubility values of HSA in dextran150 were obtained from the literature [3]. We found that B_{22} values decreased with increasing dextran150 concentration, and the same was true for solubility. This effect can be attributed to the big size of dextran150. As dextran150 concentration increases, the excluded volume available for the protein molecules decreases. Consequently, the protein molecules precipitate out of the solution, causing a decrease in the protein solubility. This phenomenon is also responsible for increased protein-protein interactions, which is reflected in B_{22}, as they become more negative with

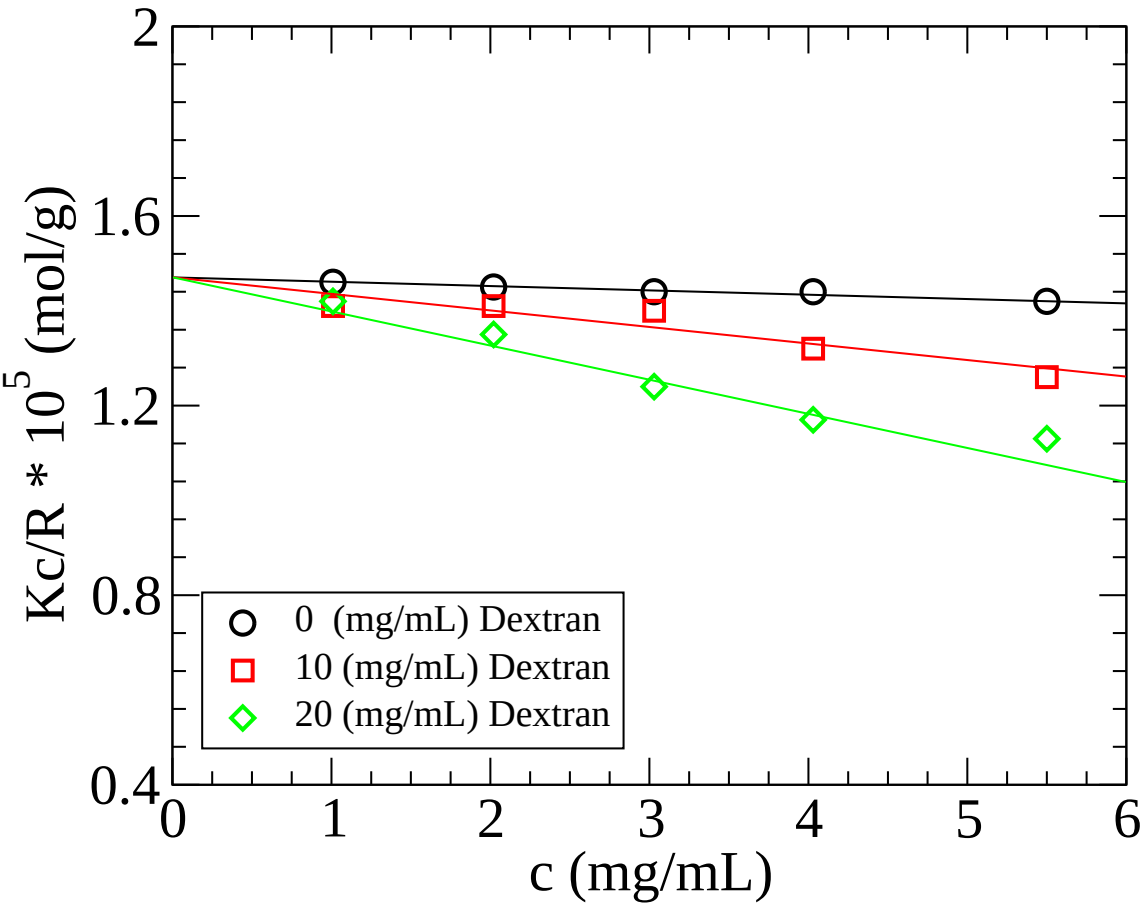

Figure 3.22

$\frac{Kc}{R}$ versus HSA concentration, c, at varying dextran150 concentration in 0.1 M sodium phosphate, 1.65 M AS, pH 7.6

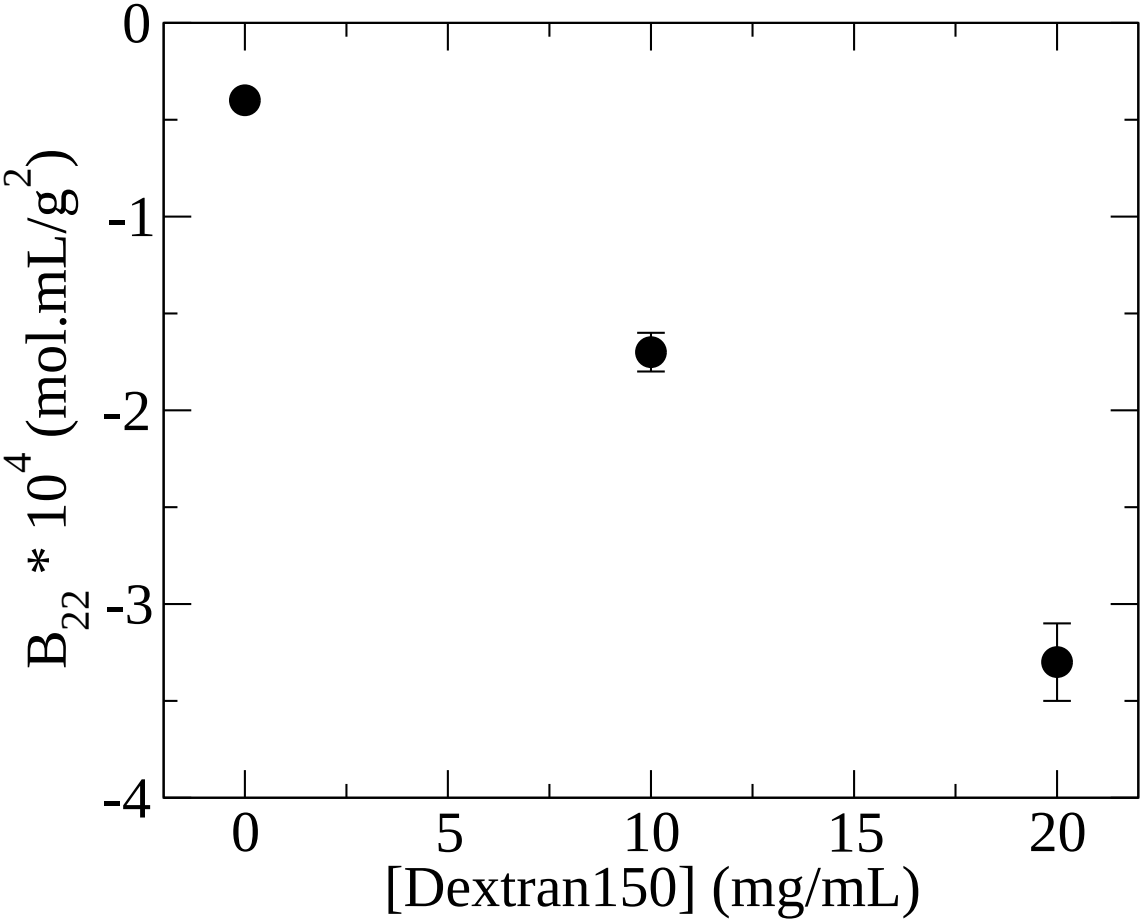

Figure 3.23

B_{22} versus dextran150 concentration for HSA in 0.1 M sodium phosphate, 1.65 M AS, pH 7.6

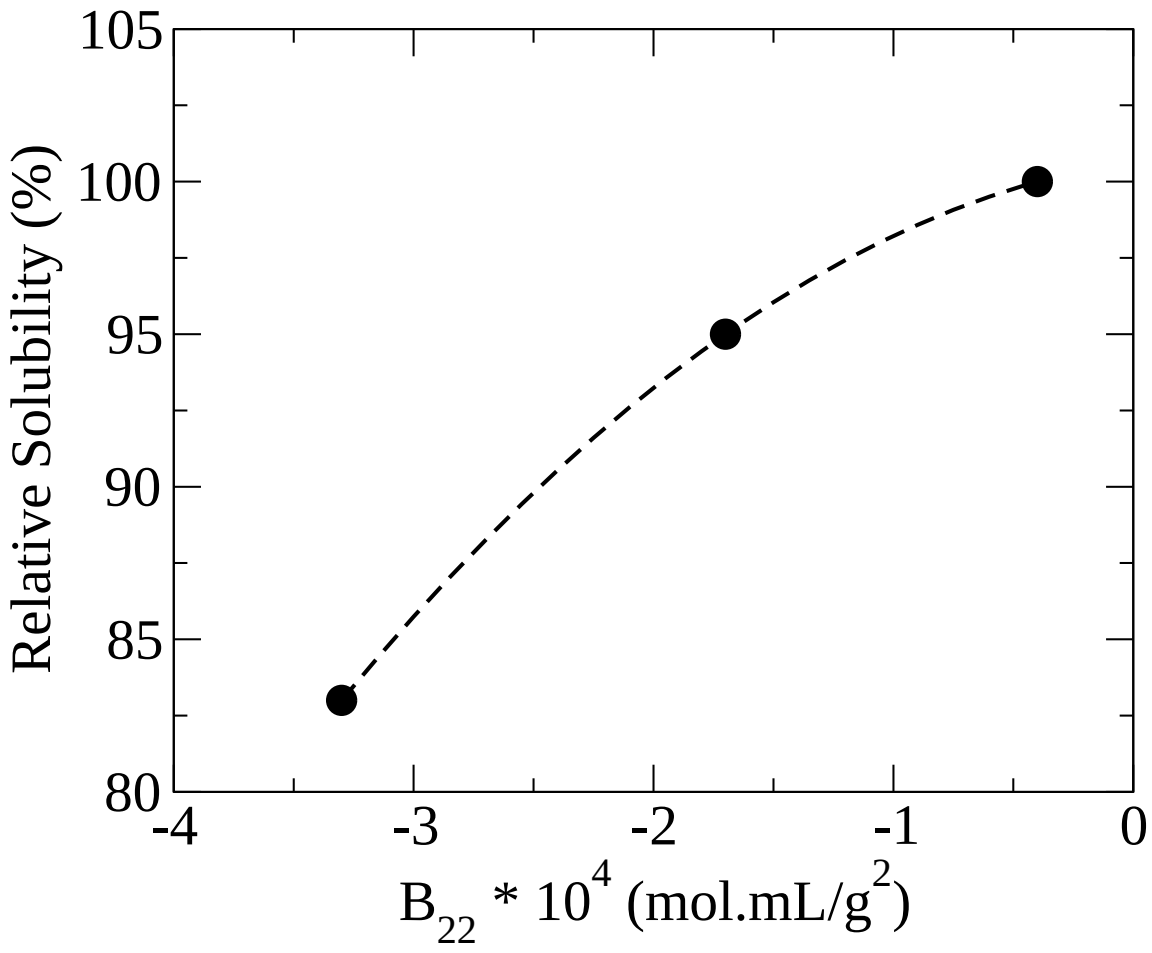

Figure 3.24

Relative solubility (%) for HSA versus B_{22} in 0.1 M sodium phosphate, 1.65 M AS, pH 7.6 at varying dextran150 concentration [3]

Table 3.9

B_{22} for HSA at varying dextran150 concentration

[Dextran150] (mg/mL)	$B_{22} \times 10^4$ (mol.mL/g^2)
0	-0.4 $\pm$ 0.0
10	-1.7 $\pm$ 0.1
20	-3.3 $\pm$ 0.2

addition of dextran150. A plot of HSA relative solubility versus B_{22} in buffer at varying dextran150 concentration is shown in Figure 3.24.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Static light scattering (SLS) was used to measure B_{22} of lysozyme, human serum albumin, and ovalbumin in buffer solutions containing various excipients. B_{22} values for lysozyme was measured in 0.1 M NaAc, 5 % w/v NaCl, pH 4.5 with 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 M sucrose, 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 M trehalose, and 0.0, 0.05, 0.2, 0.25, and 0.3 M mannitol at $25°C$. B_{22} obtained for lysozyme in 0.1 M NaAc, 5 % (w/v) NaCl, pH 4.5 was -7.2×10^{-4} $(mol.mL/g^2)$. Addition of salt shields the charge on the protein molecules, reduces long-range repulsive interactions, and increases hydrophobic effects. Small salt molecules penetrate the protein molecules, causing unfolding of the molecule, and exposing the protein hydrophobic patches. Since this is a thermodynamically high-energy state, the protein molecules come together, and join by hydrophobic patches, resulting into aggregation. B_{22} obtained suggests that the protein-protein interactions are attractive. Addition of sucrose changes B_{22} to -0.7×10^{-4} $(mol.mL/g^2)$ at 0.5 M sucrose concentration. B_{22} is still slightly negative, and the addition of sucrose shields one-protein molecule from other, and increases the hydration of the protein molecules, causing reduction in hydrophobic effects. Since the hydration around the protein molecules increase, the protein solubility is also increased. B_{22} values suggest that addition of sucrose makes

the protein-protein interactions less attractive at 0.5 M sucrose concentration. Similar effects were observed on addition of trehalose and mannitol. B_{22} values of -0.9×10^{-4} $(\mathrm{mol.mL/g^2})$ and -1.0×10^{-4} $(\mathrm{mol.mL/g^2})$ were observed at 0.5 M trehalose concentration and 0.3 M mannitol concentration respectively. The results obtained are in agreement with B_{22} values obtained by self-interaction chromatography (SIC) [5].

In the solubility study, the solubility of lysozyme was measured in the above-mentioned buffers. In the solution with a B_{22} of -7.2×10^{-4} $(\mathrm{mol.mL/g^2})$ lysozyme solubility of 4.5 mg/mL was obtained, while a solubility of 9.3 mg/mL was observed at 0.2 M sucrose concentration, and 18.5 mg/mL at 0.5 M sucrose concentration. For trehalose, solubility ranged from 5.6 mg/mL at 0.1 M trehalose concentration to 19.5 mg/mL at 0.5 M trehalose concentration. Solubility of lysozyme in buffer containing mannitol was 4.5 mg/mL at 0.05 M mannitol that changed to 7.8 mg/mL at 0.25 M mannitol concentration.

The measured B_{22} for ovalbumin in the presence of sucrose in 0.1 M sodium phosphate, pH 7.0 buffer was found to be -1.7×10^{-4} $(\mathrm{mol.mL/g^2})$ at 0 % w/v sucrose concentration and 7.3×10^{-4} $(\mathrm{mol.mL/g^2})$ at 25 % w/v sucrose concentration. These results are in agreement with the values in available literature [2]. Since measuring solubility is difficult, solubility values available in literature were used [2]. These results showed that with increasing sucrose concentration both B_{22} and solubility of the protein increased. At 25 % w/v sucrose concentration the protein-protein interactions become repulsive, and solution becomes colloidally stable.

The relative solubility of human serum albumin in presence of glucose in 0.1 M sodium phosphate, 2.6 M AS, pH 7.6, and in presence of dextran150 in 0.1 M sodium phosphate, 1.65 M AS, pH 7.6 were obtained from literature [3]. Solubility of HSA increased in glucose, which can also be predicted from increasing B_{22} values, which turned out to be -7.8×10^{-4} $(\mathrm{mol.mL/g^2})$ at 0 mg/mL glucose concentration, and -3.0×10^{-4} $(\mathrm{mol.mL/g^2})$ at 80 mg/mL glucose. While with dextran150, B_{22} values were found to be decreasing with increasing dextran150 concentration, as did the solubility. A B_{22} of 0.4×10^{-4} $(\mathrm{mol.mL/g^2})$ was determined at 0 mg/mL dextran150, which decrease to -3.3×10^{-4} $(\mathrm{mol.mL/g^2})$. The solubility values correspond well with B_{22}. The increased solubility with increasing excipient concentration can be attributed to the fact that excipients are preferentially excluded from the protein surface increasing the amount of water in the vicinity of the protein molecule, resulting in increased solubility.

Measuring solubility is very time consuming, and none of the methods used for determining solubility are efficient enough to predict accurate solubility value. But as seen in this study, solubility behavior of the proteins in buffer containing cosolvents can be predicted from B_{22}. The Haas-Drenth-Wilson model [10], used to predict solubility for the crystalline phase cannot be used to determine solubility in such cases, because this model does not account for the stabilizing, or destabilizing effect of the added excipients. An increase in B_{22} with the addition of cosolvents makes the protein-protein interactions repulsive, and increases the solubility. An increase in solubility by itself is enough to assert that excipients like sucrose, trehalose, mannitol, and glucose stabilize the protein in a

buffer solution. These excipients increase colloidal stability of the solution. An excipient like dextran150, which decreases B_{22} and solubility, has a destabilizing effect. It decreases colloidal stability, and at high enough concentration can cause precipitation of the protein molecules from the solution. B_{22} has been used to predict the solubility behavior when conditions are crystallizing. But from this study it can be said that they can also be used to predict solubility behavior in stabilizing conditions.

A correlation exists between B_{22}, S, and stability of the protein in a solution. B_{22} can be used to predict solubility, which in turn can be used to predict colloidal stability. Thus B_{22} is not only predictor of solubility behavior, it also predicts the protein stability. In future, this correlation can be used by pharmaceutical companies to measure the colloidal stability, and predict solubility of the proteins in the solution.

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