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A study on protein sugar interactions: implications for bioprotection

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A STUDY
ON
PROTEIN SUGAR INTERACTIONS
IMPLICATIONS
FOR
BIOPROTECTION

BY E. CONSUELO LÓPEZ DÍEZ

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to my family

to Nacho

—without whom this thesis would have not been possible

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Summary

Hydration isotherm and dielectric measurements have been used to study the water interactions in biological materials and in particular the role of water in protein structure, dynamics and biological function. This study has employed these techniques in conjunction with enzymatic activity assays to investigate the relationship between protection of proteins by sugars against thermal and dehydration-induced denaturation, and the extent of direct interaction through hydrogen bonding and glass formation.

Two proteins with different structures and biological functions have been considered; β-lactoglobulin and trypsin. The preservative properties of three carbohydrates, trehalose, sucrose and 2-β-hydroxypropyl-cyclodextrin have been studied.

Data obtained in this work indicate that protein and sugars may interact through hydrogen bonding and that the structural state of the sugars (i.e. crystalline or amorphous) determines whether or not this interaction occurs. Trypsin formulations showed that hydrogen bond interaction is directly responsible for the preservation effect whereas the role of the glass forming properties of the sugars could not be related to the extent of the protection.

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I

BIOPRESERVATION

"What are we to think of an organism that loses practically all of its cellular water at ordinary temperatures and pressures and ceases to metabolize, but upon being rehydrated resumes all of the characteristics it previously exhibited?. In the dried state it cannot easily be considered 'alive' since we are told that the characteristics of living organisms are exhibited through the dynamics of their metabolism. On the other hand, we should be reluctant to call such an organism dead (unless we are willing to entertain the possibility of its resurrection) because it resumes an active life upon simple restoration of water. Thus either one considers life to be a discontinuous process, or the reversibly-dried organism presents to us a special level of biological organization, apart from any question of its being alive or dead". J.S. Clegg.

Preservation of biological activity and structure of biomaterials is of great interest and major importance in a number of different areas such as preservation of liposomes and proteins for drug delivery and in food technology. In particular, proteins are used increasingly in therapeutic and commercial applications, as part of novel controlled-delivery systems or as synthetic catalyst. However, isolation and purification of a protein is often detrimental to its structure and biological activity. Most proteins are only marginally stable at neutral pH and room temperature and therefore are readily denatured by increasing (or decreasing) temperature and pressure, pH changes, addition of chemical compounds, as well as by freeze-thawing and freeze-drying procedures. Consequently, much effort has been expended in order to identify means of effecting such preservation, to optimize the formulation procedure and to find suitable additives. In fact, several studies have indicated that the addition of solutes such as sugars, polyalcohols, salts and even proteins enhance stability in solution as well as decrease the damage produced by freezing and dehydration to cells and cellular components.

As long ago as 1961, the pioneering work of Shikama and Yamazaki (Shikama and Yamazaki, 1961) showed the protective action of a number of solutes (i.e. gelatine,

glycerol, glucose, sodium acetate, etc.) on the recovery of activity of catalase after freezing for ten minutes at temperatures ranging from -10 to $-200°C$ and subsequent thawing. In addition, other early studies reported recovery of activity of sarcoplastic reticulum (SR) upon rehydration after lyophilization in the presence of sucrose (Sreter et al., 1970), and van der Kloot (van der Kloot, 1969) found that sarcoplastic reticulum membranes were stabilized by poly-alcohols which may have prevented oxidation of sulfphydryl groups.

It was not until 1971 that the relevance of the study of anhydrobiosis[1] was emphasized (Crowe, 1971); the reversible interruption of life and the tolerance to desiccation could have a significant impact on induced bio-protection since these matters were seen to be of considerable importance to pharmaceutical, medical, food and agricultural research. Later, it was strongly suggested that biochemical investigations on organisms that usually survive complete dehydration could aid the search for suitable compounds for bio-protection (Crowe and Crowe, 1982).

Following on from this, the study of anhydrobiotic organisms uncovered certain entities that were able to persist in the dry state for many years and recover activity when contact with water was restored (i.e. resurrection plant, certain spores of fungi, dry active baker's yeast). These organisms were found to contain significant amounts of trehalose (as much as 20% of the dry weight) (Myrbäck and Örtenblad, 1936; Madin and Crowe, 1975; Loomis et al., 1980). Moreover, it was shown that nematodes of *A. avenae* which had been previously kept at high relative humidity (RH), produced large amounts of trehalose and glycerol upon induced dehydration and that the increase of these components was correlated with survival of anhydrobiotes when exposed to dry air (Madin and Crowe, 1975). An interesting observation from this study was that the longer the nematodes were kept at high RH the greater their ability to survive dehydration, clearly indicating the animal undergoes some sort of preparation whilst exposed at high RH.

These findings attracted attention to trehalose, as well as to other saccharides, resulting in extensive studies on the protective properties of sugars and to much

[1] anhydrobiosis defined as latent life induced by removal of water from the living system, where latent life is the state of an organism when it shows no visible signs of life and its metabolic activity becomes hardly measurable or comes reversibly to a standstill. (Crowe, 1971)

speculation about the mechanisms involved in protection in the solution and frozen states as well as in protection against desiccation damage which is implicated in long term storage.

Stabilization of proteins in solution by certain solutes has been successfully explained in terms of preferential hydration and preferential exclusion of the solute from the vicinity of the protein surface(Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983; Arakawa and Timasheff, 1984; Xie and Timasheff, 1997). Using the same preferential hydration concept, stabilization during freeze-thawing has been demonstrated (Carpenter and Crowe, 1988b). However, the mechanism of protein stabilization against freeze-drying and long-term stability are still being investigated. Results indicate that protection may be due to a combination of the glass-forming properties of the additive and its ability to form hydrogen bonds with biomaterials (Crowe et al., 1998; Allison et al., 1999; Allison et al., 2000). Details of the above mechanisms are given in the following chapter.

In this work, in order to further investigate the stabilizing effects of trehalose, moisture has been used to probe the interactions between protein and additives in the solid state following freeze-drying and subsequent storage at high temperature. The utilization of water of hydration as a probe is of particular interest since water has a crucial role in determining both the solid matrix integrity and the excipient-protein interactions.

Plasticization by water results in a phase transition from a "glassy" to a viscoelastic "rubbery" state at some temperature, Tg, defined as the glass transition temperature. This conversion to a more viscoelastic state results in significant changes in apparent viscosity and mobility within the system.

In protein structures, water moderates electrostatic interactions between charged and polar sites, therefore allowing the protein to both take up a biologically active conformation and exhibit the structural flexibility necessary for important intramolecular cooperative internal mobility. In protein-sugar complexes, water can be expected to moderate the interactions between the protein and sugar since these are most likely to be electrostatic in origin. In this case water will be a factor determining the mobility of individual molecules in the complex and will be instrumental in moderating structural

transitions and phase separations.

II

Searching for a bio-protectant

Stabilization of biological materials during processing either in solution or during freezing and drying is of great commercial importance, particularly to the pharmaceutical, medical, and food industries. Due to the complex nature of the damage that occurs during processing, much work has been done to develop protective compounds for biomolecules and cells.

Extensive research has elucidated the main factors which affect the stability of a given biomaterial. The mechanisms of protection and stabilization of protein structures by certain solutes, mainly sugars, in solution has been explain by Timanshef and co workers (Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983; Arakawa and Timasheff, 1984; Xie and Timasheff, 1997) using the concept of preferential hydration. Stabilization during freeze-thawing has been demonstrated (Carpenter and Crowe, 1988b) to also be due to preferential hydration. The mechanism of protein stabilization against freeze-drying and long term stability are still being studied, many studies pointing toward a combined effect, that is a combination of the glass properties of the additive and final product and the hydrogen bonding between biomaterials and additives (Crowe et al., 1998; Allison et al., 1999; Allison et al., 2000). The following review summarizes the most relevant works on the subject.

1 Solution state stability

From a series of measurements carried out by a dialysis equilibrium technique involving the binding of solvents to proteins Timasheff and co-workers (Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983; Arakawa and Timasheff, 1984; Xie and Timasheff, 1997) observed a deficiency of stabilizing solutes in the vicinity of the surface of proteins and developed this observation

into a thermodynamic theory. Briefly, the presence of these solutes in a protein solution creates a thermodynamically unfavorable situation, since the chemical potentials of both protein and the additive are increased, i.e. an entropically unfavorable state occurs when the solute molecules are preferentially excluded from contact with the protein. Consequently, the native structure of the protein is stabilised because denaturation would lead to a greater contact surface between the protein and the solvent and therefore, increase this thermodynamically unfavourable effect.

Protein denaturation can be described by a two state equilibrium between a native (N) and denatured form (D), if the equilibrium is shifted toward the native form of the protein due to a solvent additive (S),

$$N \overset{K(S)}{\rightleftharpoons} D; \quad K(S) = \frac{[D]}{[N]} \tag{II.1}$$

by definition, then the equilibrium constant, K, of the reaction will depend on the concentration of stabilizer. The effect of the stabilizer on K can be expressed by the Wyman linkage relation: Upon changing only the co-solvent concentration, the change in the equilibrium constant is given by the difference in the number of co-solvent molecules bound by the denatured and native molecules:

$$\frac{dlogK}{dloga_s} = \Delta\nu_s = \nu^D - \nu^N \tag{II.2}$$

where a_s is the thermodynamic activity of the additive, S, related to its free concentration by the activity coefficient; $\Delta\nu_s$ is the difference between the number of moles of solution component S bound per mole of protein (as measured by dialysis equilibrium or a similar technique) between the denatured and native states of the protein.

In the case of stabilization, the reaction is shifted to the left, so the equilibrium constant must decrease with an increase in concentration of the additive. In this case, $(dlogK/dloga_s)$ is negative, resulting in a negative $\Delta\nu_s$. Therefore, there must be less binding of the stabilizer to the denatured form of the protein than to the native one.

Experimentally, by a dialysis equilibrium technique of the binding of a number of stabilizing solvents to a variety of protein in the native state, it has been observed that

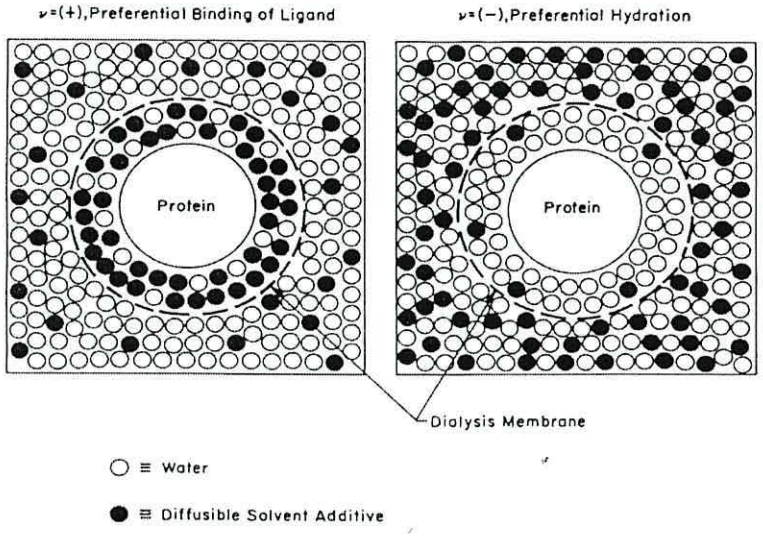


Figure II.1: Schematic representation of preferential binding (left) and preferential hydration (right), from (Timasheff and Arakawa, 1989). The dotted line represents the dialysis membrane

all the binding stoichiometries are negative ($\Delta\nu_s < 0$) (Timasheff and Arakawa, 1989)

What is the meaning of negative binding? A dialysis equilibrium experiment (which measures the amount of solute inside a dialysis membrane around the protein), can lead to two possible situations. Figure II.1 shows the two possible situations, on the left the solution inside the equilibrium membrane has a higher concentration of the additive than the bulk solvent. By contrast the right-hand diagram represents the solution inside the membrane having a lower concentration of the ligand than the bulk solvent. Now, binding is defined as

$$\nu = \frac{[L^{in}][L^{out}]}{[Protein]} \tag{II.3}$$

where $[L^{in}]$ and $[L^{out}]$ are the concentrations of ligand inside and outside the membrane. In fact, an equilibrium binding experiment measures not the total amount of ligand bound to the protein, but the relative affinities of the protein for ligand and water,

$$\nu = (\partial m_S/\partial m_P)_{T,\mu_W,\mu_S} = \nu_S - \frac{m_S}{55.56}\nu_W \tag{II.4}$$

where ν_S and ν_W are the total numbers of moles of ligand and the water interacting

with each mole of protein, m_i is the molal concentration of component i (mol/1000 g of water), μ_i is its chemical potential and T is the temperature in Kelvin. The number 55.56 is the molal concentration of pure water. The W, P, S, refer to water, protein and solvent additive, such as an stabilizer.

The binding parameter, ν, therefore refers to preferential binding, that is, it is the expression of the excess of binding of ligand over water relative to the bulk solvent composition. A negative value of ν signifies that there is an excess of water in the domain of the protein, that is, protein is preferentially hydrated, or there is preferential exclusion of the ligand from the protein domain.

The following question arises; how does preferential exclusion of a ligand lead to stabilization of a protein? Binding is a thermodynamic quantity which reflects the perturbation of the chemical potential of the protein by the ligand,

$$\nu = (\partial m_S/\partial m_P)_{T,\mu_W,\mu_S} = -(\partial \mu_P/\partial m_S)_{T,P,m_P}/(\partial \mu_S/\partial m_S)_{T,P,m_P} \qquad \text{(II.5)}$$

where P is pressure. Equation II.5 shows that a negative value of ν means a positive value for $(\partial \mu_P/\partial m_S)$ that is, addition of stabilizer increases the chemical potential of the protein and, thus the free energy of the system. This is a thermodynamically unfavourable situation. If, in the course of the denaturation, the chemical nature of the interactions between protein and stabilizer do not change, the situation should become even more unfavourable in the unfolded state of the protein.

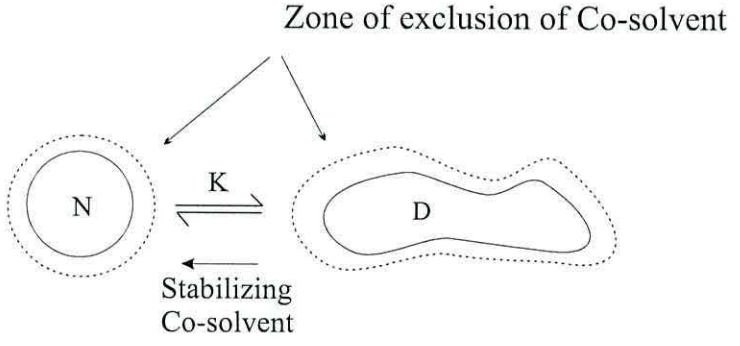


Figure II.2: Schematic representation of a protein denaturation reaction in the presence of stabilizing additive. The zone of exclusion of the stabilizing additive becomes greater as the protein solvent interface increases during denaturation, from (Timasheff and Arakawa, 1989)

Exclusion of the solute from the surface of the protein is clearly unfavourable thermodynamically since it results in a decrease in the entropy of the system. If the protein were to unfold, thus exposing more surface area, which presumably would preferentially exclude more solute, the already thermodynamically unfavorable situation would become even more unfavourable. As a result, the protein does not unfold and is stabilized in its native conformation. Conversely, if the solute preferentially binds to the protein (as in cases of urea and guadinine hydrochloride), unfolding of the protein, thus exposing more binding sites, is thermodynamically favoured. Consequently, the protein becomes more denatured.

2 Solid state stability

The preceding section has discussed the stabilization of protein in solution. Although stabilization is greatly enhanced by the addition of additives the concentration of these necessary to produce a significant effect, is relatively high. Moreover there are many cases in which the use of these additives is insufficient to stabilize proteins completely, especially when the aim is to store a protein for a long period of time.

Freeze-thawing and freeze-drying are often used in the preparation of protein products that are not sufficiently stable for long-term storage as aqueous solutions.

a *Freeze-thaw stability*

Freezing and freeze-thawing stresses may arise, accidentally or not, during the processing storage and shipping of proteins. For example, some proteins are frozen for long-term storage and then thawed before use, lyophilized proteins may be rehydrated, divided into aliquots and stored frozen.

The most critical stresses to which a protein is exposed during freezing are due to low temperatures and formation of ice. Cold denaturation is well documented (for instance see (Shikama and Yamazaki, 1961)). As ice is formed, the concentration of all solutes increases dramatically since freezable water is removed from the system; if some of the solutes are destabilizing, then the concentration effect can lead to denaturation. Note also that the duration of exposure of a protein to the perturbing conditions can influence how much damage occurs.

In general, any factor that alters protein stability in non-frozen aqueous solution will tend to have the same qualitative effect during freeze-thawing. If solution conditions (e.g. pH) are chosen to give maximum stability in solution, then the protein is expected to have maximum ability to withstand a given stress during freeze-thawing. However, even under the best initial conditions, many proteins are still denatured to a high degree, and in this case cryoprotectants may be used.

Many compounds have been employed to protect proteins against freeze-thaw stress, the most common being sugars, although amino acids and salts have also been used (reviewed in (Carpenter and Crowe, 1988b; Arakawa et al., 1993)). Carpenter and coworkers (Carpenter et al., 1986; Carpenter and Crowe, 1988b) noticed that there was a excellent agreement between compounds which stabilize proteins in non-frozen solution and during freeze-thawing. They therefore suggested that there was no need to invoke a more complex mechanism than that which operates in protein solution (Carpenter and Crowe, 1988b), that is, the preferential hydration theory described in the previous section.

b Freeze-drying stability

The marginal stability of proteins can limit their development for pharmaceutical and industrial use. Theoretically, protein stability may be increased by drying, since water participates in the physical and chemical degradation mechanisms leading to loss of biological activity and generation of non-native protein aggregates.

The freeze-drying process (or lyophilization) results in the removal of water from a given product and includes three phases, freezing, primary drying (sublimation of ice) and secondary drying during which residual unfrozen water is removed from the product rendering it self-stable. Freeze-drying may present stabilization problems due to conformational instability of proteins when exposed to freezing and desiccation stresses (Carpenter et al., 1994). The protein must be stabilized against both stresses; therefore on many occasions additives are added to the protein solution prior to freeze drying.

The election of a suitable additive and the mechanism by which certain protectants enhance the protein stability have been the subject of several studies.

Protection against the initial stage of lyophilization can be attributed to the preferential hydration effect (Carpenter and Crowe, 1988b; Crowe et al., 1990; Carpenter et al., 1993). However, its been argued that this theory cannot account for preservation against desiccation when all the bulk water is removed since, as dehydration progresses, it is clear that the solute concentration may rise to a point where the solute penetrates the hydration shell of the protein. At that moment the requirements for stabilization become very specific; solutes that can effectively protect protein in solution and during moderate desiccation stress have been proved not to be so effective when extreme dehydration takes place, which suggests that a specific, direct interaction between the solute and the protein may be required during extensive dehydration (Carpenter and Crowe, 1988a).

The initial hypothesis explaining protection against dehydration was proposed more than twenty years ago from studies on anhydrobiotes. It was suggested that trehalose might act as a water replacement agent as desiccation occurs (Crowe and Clegg, 1973). Ten years later, this theory was supported by studies on membranes and phospholipids which showed that the most likely mechanism for inhibition of structural and functional damage was a direct interaction of trehalose with phosphate head groups which had the effect of increasing the spacing between the head groups (Crowe et al., 1984a). Such possible interaction between trehalose and membranes was further investigated by infrared spectroscopy (IR) which showed that trehalose-phospholipid mixtures had similar spectra to that of phospholipids in the presence of water. Furthermore, IR spectra were interpreted as indicating that the interaction involved hydrogen bonds between OH groups of trehalose and phosphate head groups.

To explore the possibility of this hypothesis, Carpenter and Crowe used infrared spectroscopy to characterize protein-sugar formulations. This study provided evidence not only of hydrogen bond interaction between sugar and dried protein but also that such binding is necessary for protein preservation. The authors suggested that sugar molecules could satisfy the hydrogen bond requirement of the polar groups of the protein, thus replacing water molecules in the dried protein (Carpenter and Crowe, 1989). These conclusions were made on the basis of the following observations: (1) the spectrum of trehalose dried in the presence of bovine serum albumin was very similar to that of hydrated trehalose, (2) freeze-dried lysozyme in the presence of trehalose shows a

spectrum similar to that of the hydrated protein.

Other researchers have suggested that the protection against freeze-drying is related to the glass-forming properties of the additive and the physical state of the final product.Green and Angell have indicated a relationship between the glass transition temperature and the preservative action of some compounds. In several studies trehalose was compared to other saccharides and it was always concluded that trehalose was the most effective bio-protectant, followed by lactose, maltose, cellobiose, sucrose, glucose, fructose sorbitol, myo-inositol and glycerol (see for instance (Mouradian et al., 1984; Crowe et al., 1984b)). Green and Angell (Green and Angell, 1989) found that this order was exactly that of the glass transition temperatures and concluded that the preservative effect was a reflection of the viscosity of the system. Furthermore, trehalose was suggested to be anomalous; it had a significantly high glass transition temperature compared to any other of the dissacharides they tested. These authors also noticed that in trehalose systems, at water contents less than approximately two water molecules per glucose ring, samples were in the glassy state under ambient conditions, strongly indicating that the anhydrobiotes which contain high levels of trehalose might be in the vitreous state when in suspended animation. In relation with the other sugars, it seems that the closer their glass-forming properties to that of trehalose, the more efficient they are as preservatives. In fact, Leopold and co-workers (Williams and Leopold, 1989; Bruni and Leopold, 1991) determining the glass transition of a variety of anhydrobiotic organisms at different water contents using different techniques (i.e. differential scanning calorimetry (DSC) and spin-probe ESR[1] measurements), showed that these organisms are in a glassy state at physiological temperatures when the water content is below a certain threshold, $0.10 - 0.12$g/g.

In accordance with this, based on a materials science approach, Franks (Franks et al., 1991) proposed that regardless of the drying method, the crucial factor determining the shelf-life of a dried protein is the product's final physical state. He stressed the importance of the amorphous/glassy state[2]; when a liquid (protein solution) is supercooled a glass is formed within a narrow temperature interval and the flow rate of

[1] electron spin resonance

[2] a glass could be described as a liquid that flows only millimetres in a century (Franks et al., 1991)

the liquid decreases dramatically from 10 to 10^{-14} $m \cdot s^{-1}$. Thus, in a glassy state, the rate of chemical and biochemical changes that might affect the shelf life of a protein are decreased to a considerable extent, so that protein stability might be maintained if the product remains in the glassy state. Therefore, with respect to the addition of stabilizers to protein solutions, Franks stated that the addition of good glass-formers prior to drying will enhance protein stability. Furthermore, such stabilizers must be chemical compatible with proteins in solution, and also reluctant to phase-separate (crystallize) during the drying process (Franks et al., 1991; Aldous et al., 1995).

The importance of the amorphous state may be illustrated by what happens to proteins during dehydration in the presence of compounds that crystallize. For example, mannitol readily crystallizes during freeze-drying but the degree of crystallization can be manipulated by altering the protein:additive ratio (Carpenter et al., 1993). In the concentration range where it remains mostly amorphous, mannitol has been shown to protect enzymes during freeze-drying in a concentration-dependent manner. A relative high mass-ratio of protein:mannitol serves to inhibit mannitol crystallization whereas with excess mannitol, crystallization and loss of stabilization arise.

Nevertheless it was noted that, although necessary, glass formation is not sufficient to prevent drying-induced damage to bio-materials (Crowe et al., 1994; Carpenter et al., 1994). Dextran is a very good glass-former, with a high glass transition temperature (even higher than trehalose's) and therefore, according to the hypothesis proposed by Green and Angel (Green and Angell, 1989), can be expected to be an efficient stabilizer for biomolecules. However, it is not effective in preventing liposome leakage during drying, although it does inhibit fusion between liposomes. This effect is probably due to its glass-forming properties since it did not show evidence of direct interaction with the headgroup phosphates.

Direct interaction between the protectant and the biological material was further supported by Tanaka and co-workers (Tanaka et al., 1991) who examined the protection of catalase activity during freeze-drying by titrating various concentrations of enzyme with different saccharides. The results indicated that the parameter that directly correlated with the protection of enzyme activity was the weight ratio of the excipient not the bulk concentration of protectant agent. This result argues that a direct

interaction between the excipient molecules and the protein is the basis for protection against lyophilization-originated damage. If direct interaction were not a requisite factor and the mechanism involved glass formation, the bulk concentration of the additive would correlate with protection. In addition, that direct interaction through hydrogen bonds was required was also supported by the study of Lippert and Galinski (Lippert and Galinski, 1992), who found that the stabilization of phosphofructokinase (PFK) and lactase dehydrogenase by ectoines[3] during freeze-drying was dependent on the presence of hydroxyl groups on these molecules which were implicated in hydrogen bond interactions with the proteins. However, there is no clear correlation between the ability to biopreserve and the number and type of hydroxyl groups in carbohydrates, and not all carbohydrates that have the potential to form hydrogen bonds are found preserve biomaterials (Crowe et al., 1983; Carpenter et al., 1994).

Despite evidence appearing to indicate that hydrogen bonding is responsible for the protection effect, the mechanism by which this interaction results in the preservation of proteins and enzyme activity had not been completely elucidated. One of the most comprehensive works on mechanisms of protein preservation is attributed to Prestrelski and co-workers (Prestrelski et al., 1993a). Fourier-transform infrared spectroscopy (FTIR) allowed them to study proteins of all conformational classes, and their work concluded that after lyophilization, for all proteins, the secondary structure is perturbed, and that upon rehydration, three types of behaviour were identified. Some proteins do not undergo conformational changes during dehydration and retain their native structure upon rehydration. Others can unfold during dehydration but may recover their native conformation upon rehydration, and finally, some proteins are denatured in the dry state and remain denatured after rehydration. Therefore, it seems the stability of a protein and its ability to survive dehydration is clearly related either to its ability to retain its conformation during dehydration or its capacity to refold when rehydrated.

They further showed that dried proteins lyophilized in the presence of stabilizers had spectra similar to the fully hydrated protein and upon rehydration protein-sucrose mixtures resulted in spectra identical to those before lyophilization. What is more, the

[3]ectoines are hygroscopic low molecular mass, nitrogen containing compounds produced and accumulated by extremely halophilic and halotolerant bacteria for stress protection in a low water environment

retention of the enzymatic activity of rehydrated proteins is correlated to the ability of the stabilizer to preserve the native structure during preservation. Therefore, it seemed that the mechanism by which sucrose preserves protein activity is by preventing unfolding during lyophilization.

Additional results from the model polymer poly-L-lysine (Prestrelski et al., 1993a) suggested that protein dehydration-induced conformational changes could be due to the loss of hydrogen bonding interactions with H_2O during dehydration and the effect of the stabilizer would be to compensate for this loss. Poly-L-lysine assumes different conformations in solution; at neutral pH the molecule is a disordered peptide while at pH 12.2, it adopts an α-helix conformation. When poly-L-lysine is dried, it adopts a β-sheet conformation regardless of its initial conformation in solution. This appears to be a way of compensating for the loss of hydrogen bonding interactions with water since the β-sheet allows for the highest degree of hydrogen bonding in the dried peptide. If poly-L-lysine is dried in the presence of sucrose, the transition to the β-sheet conformation is inhibited and the solution structure is retained in the dried state. Therefore it was suggested that sucrose serves as a water replacement and makes the adoption of the β-sheet conformation unnecessary. These mechanism may be extrapolated to proteins, although several different types of interactions (i.e. hydrophobic interactions) that are present in proteins do not figure in the model polymer and therefore the effects of dehydration on proteins are more complex that in the selected peptide.

In contrast to the hydrogen bond hypothesis and to the IR results presented by Carpenter and Crowe (Carpenter and Crowe, 1989), a similar IR and Raman study (Belton and Gil, 1994) on lysozyme-trehalose mixtures did not find any marked difference between the dried trehalose spectra and that of dried protein/trehalose. Although their study clearly indicated that trehalose affects protein structure and that the protein in turn has an effect on trehalose, the authors considered their results insufficient to support Carpenter and Crowe's conclusions. Instead, they pointed out that the water content of the samples must be considered when analyzing IR and Raman spectra and that observed changes in dried protein-trehalose mixtures with respect to their dried state could 'simply' be due to trapped water in the system. As a result,

Belton and Gil proposed that the preferential hydration theory could also be applied to the dried state as follows: in solution sugar is excluded from the protein surface, so when the system is dried the effect of the sugar, in this case trehalose, is to concentrate the water molecules which remain close to the protein. That is water is trapped in the protein-sugar matrix.

A study on restriction endonucleases (Uritani et al., 1995) not only corroborated the fact that disaccharides are capable of protecting biomaterials (trehalose being the most effective compared to sucrose or maltose) but also attributed such protection to an interaction (not specified) which allowed entrapment of water molecules. This conclusion was based on the observation of the physical state of the samples. While endonucleases or disaccharides dried on their own appeared (to the naked eye), to be fully dried, the formulation containing both endonucleases and disaccharides were somewhat wet. This result is clearly in agreement with the entrapment of water hypothesis proposed by Belton and Gil (Belton and Gil, 1994).

Nevertheless, the IR and Raman study carried out by Belton and Gil (Belton and Gil, 1994), and their proposed hypothesis was challenged by Allison and colleagues (Allison et al., 1999). Also using IR, they showed that unprotected lyophilised proteins, can not only lose their native conformation but also that the loss of conformation coincides with the loss of hydrogen bonds, as indicated by the absence of the band assigned to hydrogen-bonded protein carboxylate groups (1580 cm^{-1}). In addition, it was observed that for dried protein-sugar samples in which a high level of native protein was retained, the apparent moisture predicted by the IR spectra was greater than the actual moisture content measured by coulometric Karl-Fischer titration, which strongly indicated that the preservation effect of sugars is related to hydrogen bonding rather than to its ability to trap water around the protein, as opposed to the theory proposed by Belton and Gil.

In the same study, Allison and colleagues also reported an important observation. Sucrose appeared to hydrogen-bond to the protein to a greater extent than trehalose does, which they suggested, could be due to the greater propensity of trehalose to be repulsed from the polymer when freezing (Izutsu et al., 1996), an effect that could be carried through to the dry state.

Again in the same study, the effect on dried protein spectra of a dextran, a

well known cryoprotectant and good glass-former, which has been reported to fail to protect biomaterials against dehydration (Crowe et al., 1994), was investigated. Results indicated that there were no hydrogen bonds between protein and dextran, presumably due to steric hindrance. Glucose was also investigated and even though it interacted with the protein through the formation of hydrogen bonds, it did not protect the protein during freeze-drying. The addition of polyethyleneglycol (PEG), a cryoprotectant, to a glucose-protein formulation resulted in protein-structure preservation. Together these results importantly seemed to indicate that *hydrogen bonding is necessary but not sufficient* to protect proteins during lyophilization. It appears to be the case that, in order to obtain optimal protection during freeze-drying, the protein must be protected against both freezing and dehydration-induced unfolding, as shown by Carpenter and Crowe (Carpenter and Crowe, 1988b).

The latest work supporting the hydrogen bond hypothesis is due to Souillac and collaborators (Souillac et al., 2002a; Souillac et al., 2002b). In their investigation, interaction was first identified by measuring the enthalpy of solution. It was observed that a solution of lyophilized mixtures were less exothermic than mechanically prepared samples, indicating that more energy is required to dissolve the interacting mixtures. This indicated that there was some interaction between protein and sugar, presumably, given the nature of the sugar studied (sucrose and trehalose), through hydrogen bonds (Souillac et al., 2002a). Diffusive reflectance FTIR showed that trehalose and sucrose were equally effective in minimizing the secondary structure changes on recombinant human deoxyrybonuclease upon freeze-drying.

c Long term shelf stability

Again two hypotheses have been suggested to be responsible for long-term protein preservation, hydrogen bonding and glass formation related to glass physical stability.

Sun et al. (Sun et al., 1996) reported a direct relationship between the glassy state transition and solute leakage of dry liposomes. When samples were stored at temperatures near and above the T_g of the systems, the degradation of the liposomes increased exponentially, while when the samples were kept at temperatures below the T_g, the solute leakage from liposomes was extremely slow, indicating that the glassy

state reduced liposomes damage.

Crowe and co-workers (Crowe et al., 1996) in their study on the role of trehalose in preserving liposomes at different water contents, observed that in liposome-trehalose samples, the T_g never fell too low, regardless of the plasticization by water, so the samples remained in the glassy state, whereas liposome-sucrose samples were found to sorbe water during storage which resulted in a significant decrease of the T_g. Furthermore, in the trehalose formulations the proportion of the dihydrate form increased with time of storage at high temperature and high relative humidity. These two observations were consistent with an interpretation of the preserving action of trehalose suggested by Aldous et al. (Aldous et al., 1995) who studied the properties of trehalose during drying and after drying from solution. They observed that prolonged heat treatment of partially dry trehalose solutions (8% w/w residual moisture) promoted crystallization, although the formation of a dihydrate crystal provided additional desiccation by removing water from the amorphous state, thereby increasing the T_g of the system and the structural stability of the majority of the sample throughout storage.

In addition, Crowe and co-workers in the study mentioned above (Crowe et al., 1996) investigated the possibility of trehalose being anomalous in relation to its glass transition temperature as proposed by Green and Angel (Green and Angell, 1989). They constructed a phase diagram (glass transition temperature vs. water content) and found that anhydrous trehalose has indeed a high T_g ($\sim$ 120°C) but that it is not anomalous at all, as it lies at the end of a continuum of the other dissacharides in this regard.

The role the glass transition in protein stability was further studied by Chang et al. (Chang et al., 1996). Several protein and protein:additive formulations were freeze dried and their T_g determined by Differential Scanning Calorimetry (DSC). Degradation was assessed by rehydrating the samples after storage and quantifying deamination by cation exchange high-performance liquid chromatography and aggregation by turbidity determinations. Protein degradation, by both deamination and aggregation, was greatly accelerated at temperatures above T_g whilst rates of degradation were greatly reduced at temperatures below T_g. However, in several cases storage below T_g did not ensure stability.

It is known (Crowe et al., 1998) that a pure protein alone will form an amorphous

phase in the final dried solid, having a relatively high transition temperature ($\sim 150 - 200^{o}$C) and yet simply keeping an unprotected protein sample below its T_g is adequate for complete storage stability. It has been suggested that in addition to storage below T_g, long term stability may be dependent on retaining the native structure in the dried solid[4] (Prestrelski et al., 1993b; Prestrelski et al., 1995). Studies on interleukin-2 showed that the protein, when stored unfolded, was less stable than when stored in its native form. Even so, degradation of the native protein was still significant. Additionally, Chang and colleagues (Chang et al., 1996) noted by IR Spectroscopy that structural changes arising during lyophilization led to further damage during subsequent storage even if the storage temperature was less than the T_g of the system.

Several studies have extensively investigated the relationship between the glass transition and the thermal stability of dry proteins in carbohydrate glassy matrices (Mazzobre et al., 1997; Cardona et al., 1997; Terebiznik et al., 1997). Briefly, they found that T_g might not be the best parameter for predicting protein stability since decay of enzyme activity was observed at temperatures below the T_g of the samples. It was proposed that instead of using T_g as the stability threshold, the difference between storage temperature, T, and the T_g of the sample, is $T - T_g$ should be employed; the more negative the difference the more efficient the protection (Cardona et al., 1997; Mazzobre et al., 1997).

Studies mentioned above have shown that biological degradation is indeed possible even when materials are in the glassy state. This might be, at least in part, because, although molecular mobility may be significantly decreased in the glassy state, the protein molecule is still mobile enough to lead to denaturation, resulting in loss of enzymatic activity. As a matter of fact, there is evidence (Hancock et al., 1995) which shows that there is molecular mobility in a glassy matrix and what is more, that at temperatures as much as 50 K below T_g there can still be significant molecular mobility and that mobility is only completely restricted below the Kauzmman temperature, T_K. Therefore, it has been suggested that in order to achieve long-term stable products, the storage temperature has to be kept below T_K (Hancock and Zografi, 1997; Shamblin

[4]note some proteins although unfolded in the dried state, after being freeze-dried, can regain the native conformation when rehydrated (Prestrelski et al., 1993a)

et al., 1999; Hancock and Shamblin, 2001).

It important to note that although the glass state might not be sufficient to prevent degradation it appears to be necessary; it has been observed (Cardona et al., 1997; Terebiznik et al., 1997) that crystallization of additives had a deleterious effect on enzyme activity, i.e. when trehalose was allowed to crystallise either by exposing the samples to high relative humidities or high temperatures the protective effect was lost as trehalose formed a separate phase no longer associated with the protein.

Evidence appears to strongly support the theory that the hydrogen bond interaction is responsible for protection against freeze-drying, however it has been shown that an amorphous phase is required for effective preservation (Carpenter et al., 1993; Cardona et al., 1997; Terebiznik et al., 1997). Therefore, the concept that both glass formation and hydrogen bonding are essential for effective preservation has been invoked (Crowe et al., 1998); the importance of the amorphous behaviour of an additive is that it allows for effective hydrogen bonding between the additive and the protein. A glassy additive that does not have this interaction will not protect the protein against dehydration.

Recent studies are focusing on the relationship between both hypotheses and how the initial conformation after lyophilization can affect the protein storage (Allison et al., 2000), on the optimization of storage stability through the combination of additives (Allison et al., 2000; Davidson and Sun, 2001) and on testing various proportions of additive to protein (Cleland et al., 2001).

Allison and colleagues (Allison et al., 2000) using FTIR and calorimetric techniques have reported that the storage stability of a dry protein (actin) depends on the structure of the dried protein, as well as on the storage temperature relative to the glass transition temperature of the dried preparation. They observed that the slight increase in the glass transition temperature of trehalose formulations compared to sucrose mixtures did not result in appreciable increase in the storage stability. Interestingly, it was shown that the addition of dextran to protein samples containing sucrose or trehalose did not have an effect on the extent of the hydrogen bonding between protein and sugars but increased T_g of the systems and improved the storage stability.

The optimum protein:additive ratio has been assessed by a number of techniques (i.e. size exclusion cation-exchange hydrophobic interaction chromatographies, IR and DSC) (Cleland et al., 2001). Trehalose and sucrose were found to be equally effective protecting recombinant humanized monoclonal antibody and that for both formulations a 360:1 molar ratio was needed to confer maximum protection, a ratio exceeding the latter did not inhibit further formation of aggregates.

3 Does Trehalose have unique properties?

Most of the papers reviewed earlier have shown that trehalose appears to be the most effective additive in protecting bio-materials. What is it that makes trehalose so effective?.

The effectiveness of trehalose in preservation has been recently attributed to a high glass transition temperature (Green and Angell, 1989), combined with the ability to form significant hydrogen bonding interactions with the protein (Allison et al., 1999). However, this appears not to be consistent with measurements on raffinose, a trisaccharide, which is relatively ineffective in preserving protein structures but which possesses a glass transition temperature comparable with trehalose (Saleki-Gerhardtand et al., 1995; Wolkers et al., 1998) together with a hydrogen bonding potential reported to be greater than that exhibited by trehalose (Gaffney et al., 1988).

It has been suggested that the superior performance of trehalose is due to a combination of some particular properties of this molecule; Aldous et al (Aldous et al., 1995) reported the ability of trehalose to maintain a high glass transition temperature despite the addition of small amounts of water, Sun and Leopold (Sun and Davison, 1998) mentioned the dynamic properties (such as low free volume and restricted molecular mobility) of the trehalose glass, and Schebor and co-workers (Schebor et al., 1999) put forward the unique combination of water replacement properties, relatively high glass transition temperature and chemical inertness.

The hydration characteristics of trehalose are also thought to be related to its protective properties; it could be that the effect of trehalose may be due either to modification of the water structure and dynamics around the biomaterial as a glass is formed or/and direct stabilization of the biological structures to be protected.Studies

on trehalose-water interactions in solution are somewhat controversial.

Experimental evidence shows that saccharides strongly affect the water structure. It has been reported (Kawai et al., 1992) that trehalose restricts the rotational motion of water molecules next to it more than maltose or sucrose and that trehalose solutions seem to have a greater amount of unfrozen water per residue and a higher hydration number.

Despite this experimental evidence, a molecular dynamic simulation based on a semirigid trehalose molecule, in which the only free rotation was about the glycosidic bond, concluded that the hydrogen bond network and water dynamics is only slightly altered by trehalose, thus suggesting that trehalose's protective action was due to direct interaction with the biological material rather than modification of the water network surrounding the biomolecules (Donnamaria et al., 1994).

By contrast, Sakurai (Sakurai et al., 1997) also using computational procedures but with a flexible trehalose molecule showed not only that trehalose modifies the water network to a great extent, but that it has a higher hydration ability than maltose. On the other hand, Liu and colleagues (Liu et al., 1997) from a calculation of an adiabatic, Ramachandran-like conformational energy map for trehalose concluded that the extensive solvent perturbation on the water network exerted by trehalose does not appear to be unique, which would be consistent with the suggestion made by Donnamaria (Donnamaria et al., 1994), that is, the effectiveness of trehalose must be due to its direct binding to biological materials rather than from unique solution properties.

Conrad and de Pablo (Conrad and de Pablo, 1999) developed a molecular model for trehalose which successfully reproduced experimentally-determined values of the physical properties of trehalose solutions, such as density and molar volume. Utilizing this model they investigated trehalose solution properties near the glass transition and water-trehalose interactions as well as intramolecular trehalose interactions. They confirmed the formation of an internal hydrogen bond at high trehalose concentrations. Interestingly they observed an apparent trapping of water molecules in the trehalose matrix as the system approaches T_g.

In later work, the same group studied sucrose (Ekdawi-Server et al., 2001) in a similar way to trehalose. They found that the sucrose was, at all concentrations, less

hydrated than trehalose, that water diffusion coefficients in sucrose matrices are higher than in trehalose and that sucrose showed more intramolecular hydrogen bonding. They concluded that, by binding water molecules more tightly, it was likely that the glass formed by trehalose hinders molecular motion more effectively, possibly leading to its superior cryo-and lyo-protection.

Also through molecular modelling Engelsen and Perez (Engelsen and Pérez, 2000) studied and compared the hydration pattern of trehalose with that of sucrose. No striking difference between the disaccharides was found, although results suggested that trehalose appeared to be almost completely hydrated in the dihydrate solid state, i.e. the water molecules of the dihydrate solid state structure are largely capable of satisfying the hydration requirements of the solute. The particular hydration-apparent compatibility between the solution state and the solid state of trehalose, the authors suggests, may indicate that trehalose can change state (at $2H_2O$ per trehalose) with practically no consequences for trehalose structure but where two water molecules are trapped. This observation, it is then proposed, may explain the trehalose glass transition 'anomaly' and the potential of trehalose to retain internal waters, both mechanisms playing a key role in the ability of trehalose to protect biological life/activity against dehydration and freezing. In the light of these findings, they suggest that the water replacement theory should rather be the trehalose water-trap theory (supporting Belton and Gil (Belton and Gil, 1994)), as trehalose, unlike other sugars is extraordinarily reluctant to lose the last two waters. Moreover, the position of these two water molecules need not be highly localized to satisfy the hydration "needs" of trehalose, thus preserving some water activity.

The solid state structure and physical stability of trehalose is also believed to be relevant to bio-protection. Molecular rearrangements resulting from the dehydration of trehalose dihydrate have been studied by FT-Raman (Taylor et al., 1998). In this study the dihydrate structure was compared to (1) that of the amorphous lyophilized form and (2) an anhydrous crystal form obtained by heating the dihydrate at 120°C for five minutes as well as to (3) a trehalose solution. It was shown that the conformation of both solution and amorphous phases is similar to that found in the crystalline states. The minor spectral differences observed between trehalose dihydrate and the anhydrous

forms were attributed to subtle changes in the conformation around the glycosidic linkage and in the formation of new hydrogen intramolecular bonds between hydroxyl groups in the anhydrous form as a result of the removal of water.

Polymorphic amorphous and crystalline forms of trehalose have been studied by Sussich and colleagues (Sussich et al., 1998; Sussich et al., 1999a; Sussich et al., 1999b). In addition to the dihydrate crystal the authors reported the existence of three different anhydrous crystals, t_β, t_α and t_γ, which can be obtained by thermal dehydration of the dihydrate crystal (Sussich et al., 1998) by keeping the dihydrate form under vacuum at 85^{o}C for 4 hours, at 130^{o}C for 4 hours and by heating the dihydrate crystal at 5-20 K min^{-1} respectively. That these forms corresponded to different structures of crystal was shown by their thermal properties, e.g melting temperature. Furthermore, the form t_γ was shown had two phases, a crystal dihydrate form and an amorphous anhydrous form (Sussich et al., 1999a; Sussich et al., 1999b). This observation is consistent with previously reported results (Aldous et al., 1995) and the authors suggest that it may play a relevant role in anhydrobiosis.

Background Theory

III

PROTEINS

Proteins are the major functional molecules of life whose properties are so useful that we employ them as therapeutic agents, catalysts and materials.
A. Fersht.

Proteins play a vital role in all living systems. Almost every property that characterizes a living organism is affected by proteins.

Life forms make use of many chemical reactions to supply themselves continually with chemical energy and to use it efficiently, but by themselves these reactions could not occur fast enough under physiological conditions (aqueous solution, 37°C, pH7, atmospheric pressure) to sustain life. The rates of these reactions are increased by many orders of magnitude in organisms, by the presence of enzymes, which are proteins.

Proteins store and transport a variety of particles ranging from macromolecules to electrons. They guide the flow of electrons in the vital process of photosynthesis; as hormones, they transmit information between specific cells and organs in complex organisms; some proteins control the passage of molecules across membranes that compartmentalize cells and organelles; proteins function in the immune systems of complex organisms to defend against intruders (the best known are the antibodies); and proteins control gene expression by binding to specific sequences of nucleic acids, thereby turning genes on and off. Proteins are the crucial components of muscles and other systems for converting chemical energy into mechanical energy. They also are necessary for sight, hearing, and the other senses. And many proteins are simply structural, providing the filamentous architecture within cells and the materials that are used in hair, nails, tendons and the bones of animals.

Also it should be noted that many diseases stem form mutations in proteins that cause them to lose function. In some cases, catalytic activity may be impaired,

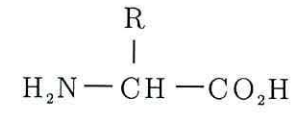
and so metabolic pathways may be altered. In other cases, structural properties may be impaired, leading to a loss of physical function (e.g. muscular dystrophy). Creutzfeld-Jakob disease and other transmissible encephalopathies result from proteins changing their shape and forming polymers (for reference see (Fersht, 1999)). Similarly, diseases result from amyloidosis, in which proteins gradually convert into long chains of polymerized β sheets and precipitate to form fibrils(for reference see (Fersht, 1999)). Many cancers result form mutations in proteins. Enzymes and receptors are the usual targets of drugs, either to restore function or to destroy infectious agents or cancers.

In spite of their diverse biological functions, proteins are a relatively homogeneous class of molecules. They all belong to the same type of linear polymer, built of various combinations of the same 20 amino acids. They differ only in the sequence in which the amino acids are assembled into polymeric chains. The secret to their functional diversity lies partly in the diversity of the amino acids but primarily in the diversity of the three-dimensional structures that these building blocks can form, simply by being linked in different sequences. The awesome functional properties of proteins can be understood only in terms of their relationship to their three-dimensional structure.

1 THE PRIMARY STRUCTURE OF PROTEINS

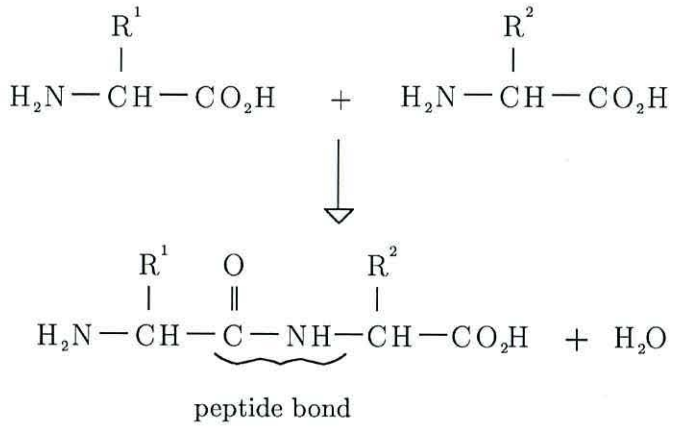
Natural proteins are linear, unbranched and have precise lengths and exact sequences of amino acids. Indeed, it is only the differences in length and sequence that distinguish one protein from any other, and that make possible a diversity of structures and functions. They may be as long as 25000 amino acid residues or as short as 50, although most proteins contain between 200 and 500 residues. With 20 different amino acid residues possible at each of $200-500$ positions, the number of possible sequences is astronomical, thus it is not surprising that virtually every protein has a unique amino acid sequence. Even though each protein sequence is unique, the 20 amino acids are used with similar frequencies in virtually all proteins.

Of the 20 amino acids usually found in nature, 19 have the general structure

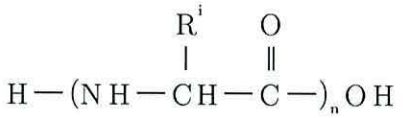


and differ only in the chemical structures of the side chain R. The $20th$ natural amino acid, proline is similar, but its side chain is bonded to the nitrogen atom.

The 20 amino acids are linked into proteins by the **peptide bond**, two amino acids link as follow:



Generally, between 50 and 3000 such amino acids are linked in this way to form a typical linear polypeptide chain. The **polypeptide** backbone is a repetition of the basic unit common to all amino acids. When the side chain is included, this unit is described as an amino acid **residue**:



All proteins and polypeptides have this basic structure. Proteins only differ in the number of amino acids linked together (n in eq) and the sequence in which the various amino acids occur. The sequence of amino acids, called the primary structure, identifies a protein unambiguously, determines all its chemical and biological properties and specifies, indirectly, the higher levels of protein structure (secondary, tertiary and quaternary).

a The polypeptide backbone

The backbone of the linear polypeptide chain consists of a repeated sequence of three atoms of each residue in the chain, the amide N, the C^{α} (the central carbon atom in the following expression), and the carbonyl C:

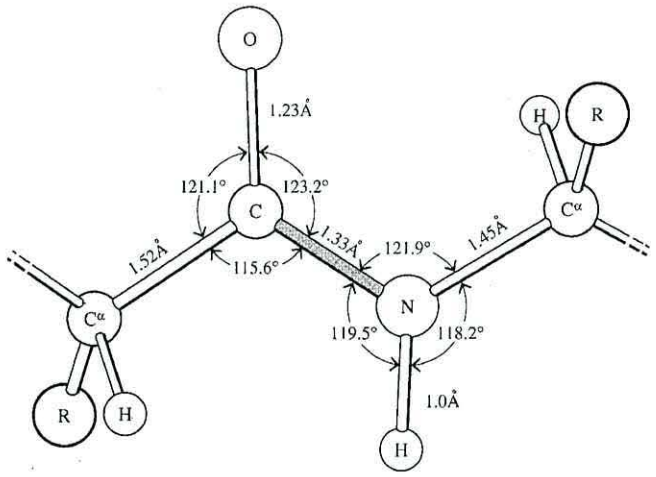
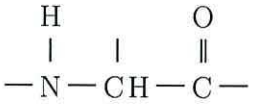


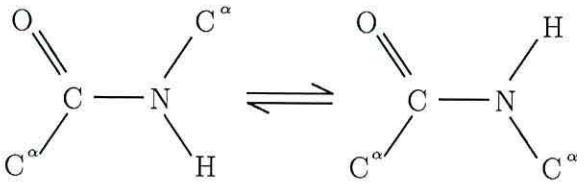
Figure III.1: The geometry of the peptide backbone (from (Creighton, 1993))



These atoms are generally represented as N_i, C_i^{α} and C_i', respectively, where i is the number of the residue, starting from the amino end of the chain.

X-ray diffraction studies of the crystals of small peptides have not only shown that the peptide bond is planar but also that the same structure has been found for all peptide bonds in proteins, with a few rare exceptions. This planarity results from a considerable delocalization of the lone pair of electrons of the nitrogen onto the carbonyl oxygen. The C-N bond is consequently shortened (1.33 Å as opposed to 1.45 Å), and it has a double-bond character. Twisting of the bond breaks it and loses 75 to 90 kJ/mol (18 to 21 kcal/mol) of delocalization energy.

Two configurations of the planar peptide bond are possible, one in which the C^{α} are *trans (anti)*, the other in which they are *cis*:



The *trans* form is intrinsically favored energetically, probably owing to fewer repulsions between non-bonded atoms. If the residue that follows the peptide bond is proline, its cyclic chain diminishes the repulsions between atoms, and the intrinsic stability of the isomer *cis* is comparable to that of the *trans* isomer.

Resonance of the peptide bond tends to redistribute its electrons and the polypeptide backbone is correspondingly polar. The H and N atoms appear to have, respectively, positive and negative equivalent charges of 0.20 electron, whereas C and O respectively have positive and negative equivalent charges of 0.42 electron. This gives the peptide bond a substantial permanent dipole moment of about 3.5 Debye units. Note that the polypeptide backbone of each residue contains one potent hydrogen bond donor, -NH- and a hydrogen bond acceptor, carbonyl -CO-.

2 Physical interactions that determine the properties of proteins

The section above has briefly described the primary structure of proteins in terms of their covalent/chemical structure. In order to fully understand the many special properties of proteins it is necessary to bear in mind that the large size of the polypeptide chain enables proteins to fold on themselves so that many simultaneous interactions take place among different parts of the molecule. As a result of such interactions, proteins assume a complex, three-dimensional structure which gives them their specific biological function. Furthermore, biological activities of proteins are also mediated by their interactions with their environment: with water, salts, other proteins, etc. The interactions arise from a number of non-covalent forces but with many variations; the liquid-water environment in which proteins naturally occur constantly change these interactions among molecules. Since the native structure of a protein is the direct result of the interaction of the polypeptide chain with water and the general distribution of side chains, the three-dimensional structure of globular proteins appears to be the direct consequence of being in the water environment, therefore it is of extreme importance to understand the physical basis of these interactions. Another structural feature of proteins of importance is described by the so called oil-drop model, which can be summarized in the general rule 'non-polar in, polar out' This means that the non-polar

residues are mainly buried in the interior of the protein whereas the polar are outside the surface in contact with the aqueous medium.

Intermolecular forces constrict macromolecules like enzymes, proteins, and DNA into the shapes required for biological activity.

a *Non-covalent interactions*

(i.) short range repulsions: The most important, energetically and structurally, interatomic/intermolecular interaction between atoms and between molecules is the repulsion that take place between them as they approach each other. As they come near enough for their electron-orbitals to overlap, the repulsion increases enormously because the electrons on the different molecules cannot be in the same part of space at the same time, as stated by the Pauli Exclusion Principle. The repulsive energy varies exponentially with the inverse of the interatomic distance.

(ii.) electrostatic forces, point charges and dipoles: All forces between atoms and molecules are electrostatic in origin although the electrostatic term is usually reserved for interactions between charged or dipolar atoms and molecules. Electrostatic interaction between charged particles is given by Coulomb's law: in a vacuum the potential energy of interaction (or electrostatic potential energy) between two atoms A and B separated by a distance r is given by

$$\Delta E = \frac{Z_A Z_B e^2}{r} \tag{III.1}$$

where e is the charge of an electron and Z the number of charges on each atom. If the interacting charges are of opposite sign, the energy decreases, thus the interaction is favourable whereas if the charges are of the same sign there is repulsion between them. For other environments the electrostatic interaction can be affected by other interactions, in a homogeneous medium the interaction is reduced by the dielectric constant, ϵ, so

$$\Delta E = \frac{Z_A Z_B e^2}{r\epsilon} \tag{III.2}$$

A molecule may not have net charge, but electron density can be localized if constituent atoms have different electronegativities. The separation of charge in a

molecule determines its *dipole moment*, μ, which is given by the product of the magnitude of separated excess charge Z and the distance d by which is separated:

$$\mu = Z \cdot d \tag{III.3}$$

One electron unit of positive and negative charge separated by 1 Å has a dipole moment of 4.8 Debye (D) units. The dipole moment of a peptide bond is 3.5 D and of a water molecule is 1.85 D. The dipole moment has a direction as well as a magnitude and is usually depicted as a vector along a straight line from the negative to the positive charge. The dipole moment of the peptide bond can be represented as

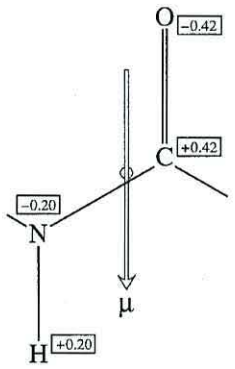


Figure III.2: Dipole moment of the peptide bond

Dipoles interact with point charges, with other dipoles and with more complicated charge-separations (quadrupoles, octupoles ...). The interactions among the four partial charges of two dipoles being analogous to those between two bar magnets, i.e. two side-by side dipoles repel each other when parallel, whereas there is an equivalent attraction between them when they are antiparallel. As a result, maximum interactions occur in a head-to-tail orientation. Dipolar interactions are, however, weaker than those between ions because both attraction and repulsion occur between the two separated charges of the dipoles.

Interaction involving dipoles can also modify the nature of the dipole charge distribution in the interacting molecules. Being simply an unequal distribution of electrons, that distribution is easily perturbed. For example, a nearby charged group can

induce a dipole moment in a given molecule, a phenomenon called induced-polarization[1]

The multiple interactions that take place among isolated charges and dipoles on a number of atoms are mutually dependent and turn the very simple relationship of Coulomb's law into a very complex phenomenon. In turn, although the electrostatic interactions among molecules in a homogeneous liquid can be averaged and expressed as a simple dielectric constant of the liquid, the calculation of electrostatic interactions in proteins is very difficult because of the local hetereogeneity in their structure and the presence of the protein-water interface, which causes the dielectric constant to vary widely.

(iii.) van der Waals interactions: *van der Waals interaction* is an attractive weak close-ranged interaction which exists between all atoms and molecules. Van der Waals forces are much weaker than chemical bonds, and random thermal motion around room temperature can usually overcome or disrupt them. The forces operate only when molecules pass very close to each other, during collisions or near misses.

Van der Waals interactions include interactions between two permanent dipoles, between a permanent and an induced dipole and interactions between two mutually induced dipoles (*London or dispersion forces*)

London forces arise when electron clouds oscillate in step on two molecules at close range. Bond vibrations in molecules may produce the oscillations or they may be triggered by random, instantaneous pile-ups of electrons in atoms. The electron-rich and electron-poor regions on the molecule may not persist for more that 10^{-14} or 10^{-15} seconds, but if they can polarize the electron distribution on an adjacent molecule, electron clouds on the two molecules may begin to oscillate cooperatively with each other. The dipoles are transitory but aligned, and a net attractive force pulls the molecules together. At closer range, the oscillation becomes even more effective.

Since all molecules have electron clouds that can oscillate, London forces always contribute to intermolecular attractions. However, they are usually weaker than the permanent forces so that they are usually only invoked to explain intermolecular forces between non-polar molecules.

[1]polarization processes are further discussed in chapter VI.

(iv.) hydrogen bonds A particularly important bond in biological systems is the hydrogen bond. A hydrogen bond occurs when two electronegative atoms compete for the same hydrogen atom:

$$-D - H \cdots A- \tag{III.4}$$

The hydrogen bond is formally bonded covalently to one of the atoms, the *donor* D, but it also interacts favourably with the other, the *acceptor* A.

The main component of the hydrogen bond is an electrostatic interaction between the dipole of the covalent bond, in which the hydrogen atom has a partial positive charge and a partial negative charge on the electronegative atom

$$-D^{\delta-} - H^{\delta+\cdots\delta-}A- \tag{III.5}$$

Hydrogen bonds are abnormally strong dipole-dipole attractions. When a bonded electronegative atom pulls electrons away from the hydrogen atom, the positive charge that results makes the hydrogen atom intensely attracted to atoms on other molecules.

The lengths and strengths of hydrogen bonds depend on the electronegativities of the acceptor and donor, the greater their electronegativities, and the shorter the distance between them, the stronger the hydrogen bond. The optimal configuration of the hydrogen bond is linear, but bending causes only small energy losses. The energies of hydrogen bonds have been shown to be between 12 and 38 kJ/mol ($3 - 9$ kcal/mol). Bonds of this strength are of particular importance since they are stable and strong enough to provide significant binding energy but sufficiently weak to allow rapid dissociation.

b Some properties of water, polarity and cohesiveness

As previously mentioned, in the section on protein structure, it is necessary to consider the effect of water on the electrostatic, van der Walls interactions. Two properties of water are especially important in this regard.

(i.) water is a polar molecule The shape of the water molecule is triangular rather than linear, and so there is an asymmetrical distribution of charge. The oxygen nucleus

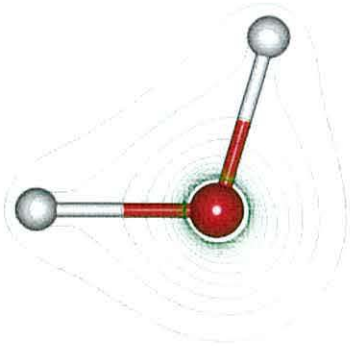


Figure III.3: A water molecule.

draws electrons away from the hydrogen nuclei which leaves the region around them with a net positive charge. The water molecule is then an electrically polar structure with a large dipole moment (about 1.85 D units).

(ii.) water molecules are highly cohesive Water molecules have a high affinity for each other. In a group of water molecules clustered together, a positively charged region in one molecule tends to orient itself toward a negatively charged region in one of its neighbour molecules. Each of its two electronegative regions attract a proton of a neighbour molecule and each of its own protons attracts the oxygen end of a neighbour. The tetrahedral arrangement of these bonds gives rise to an extensive three dimensional structure.

The polarity and hydrogen bonding capabilities of water make it a highly interactive molecule. As a consequence water weakens electrostatic and hydrogen bond interactions between other molecules. Water diminishes the strength of electrostatic interaction by a factor of about 80, the dielectric constant of water (compared to these interactions in vacuum). The high dielectric constant of water is an expression of its polarity and its capacity to form an oriented solvent shell around an ion. This attenuates the electrostatic interactions of one ion with another.

c The hydrophobic interaction

The *hydrophobic interaction* is said to be one of the most important driving forces for protein folding. Interactions between water and non-polar molecules are particulary

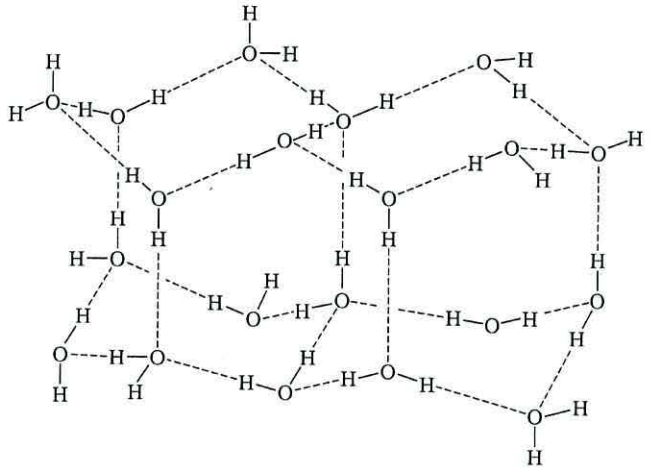


Figure III.4: Structure of normal ice. Each water molecule is involved in four hydrogen bonds (from (Creighton, 1993))

unfavourable. In fact as discussed below, the relative absence of interaction between water molecules and non polar molecules causes interactions among non-polar groups themselves.

The hydrophobic interaction can be easily pictured if a non-polar molecule is introduced into water; as a result a cavity is created. Such a cavity disrupts some of the hydrogen bonds between water molecules. The displaced water molecules then reorient themselves to form a maximum number of new hydrogen bonds. However, this has an energy/entropy cost; the number of ways of forming favourable hydrogen bonds in the cage of water around the non-polar molecule are much fewer than in pure water. The water molecules around the non-polar molecules are much more ordered than elsewhere in the solution, and so the entropy of the solution decreases. Now consider two non-polar molecules in water; it has been observed these two molecules come together. This association is driven by the release of some of the oriented water molecules around the separated non-polar molecules. The basis of the hydrophobic interaction is the increase in entropy resulting from the enhanced freedom of released water molecules. Thus non polar solute molecules are driven together in water, not primarily because they have a high affinity for each other, but because water bonds strongly to itself.

3 THE THREE-DIMENSIONAL STRUCTURE OF PROTEINS

The three-dimensional aspect of structure is especially important for macromolecules, in which many bonds can rotate and can adopt numerous conformations. Macromolecules tend to be very flexible. Proteins have used this flexibility to adopt relatively fixed conformations that are determined by non-covalent interactions among atoms that are distant in the covalent structure.

The complex three dimensional structure of proteins can be described by four levels of structure: *primary structure* is the amino acid sequence; *secondary structure* refers to regular local structures of linear segments of polypeptide chains; *tertiary* is the overall topology of the folded polypeptide chain and *quaternary* is the aggregation of separate polypeptide chains into the functional protein.

a The secondary structure

The secondary structure of a segment of polypeptide chain is defined as the local spatial arrangement of its main-chain atoms, without regard to the conformation of its side chains or to its relationships with other segments.

The secondary structure of a region of a protein is defined by the conformation of the polypeptide backbone. The conformation of the polypeptide backbone is defined by the location in space of the three sets of atoms that are linked together; the alpha carbon, carboxyl carbon and amide nitrogen atoms. In practice therefore, the secondary structure is defined by the rotation of the planar peptide units around the bonds connecting them to the alpha carbon atoms.

Sometimes the secondary structure is periodic in nature, giving rise to well defined structure such as α helix and β sheet.

α-helix The α-helix is a rod like structure. The tightly-coiled polypeptide main chain forms the inner part of the rod, and the side chains extend outward in a helical array (see figure III.5). The α-helix is stabilized by hydrogen bonds between the NH and CO groups of the main chain. The CO group of each amino acid is hydrogen-bonded to the NH group of the amino acid that is situated four residues ahead in the linear sequence. Thus all the main-chain CO and NH group are hydrogen-bonded. Furthermore, the dipoles of

hydrogen-bonding backbone atoms are in near perfect alignment. Each residue is related to the next by a translation of 1.5 Å along the helix axis and a rotation of 100°, which gives 3.6 amino acid residues per turn of helix. Thus amino acids spaced three and four apart in the linear sequence are quite close to one another in an α-helix. In contrast, amino acids two apart in the linear sequence are situated on opposite sides of the helix and so are unlikely to make contact. Note that although an α-helix can be right-handed or left-handed, only right-handed ones are found in proteins. This single-stranded alpha helix is rather short, a variation of the alpha-helical theme is used to construct much longer rods; two or more alpha-helices can entwine around each other to form a cable.

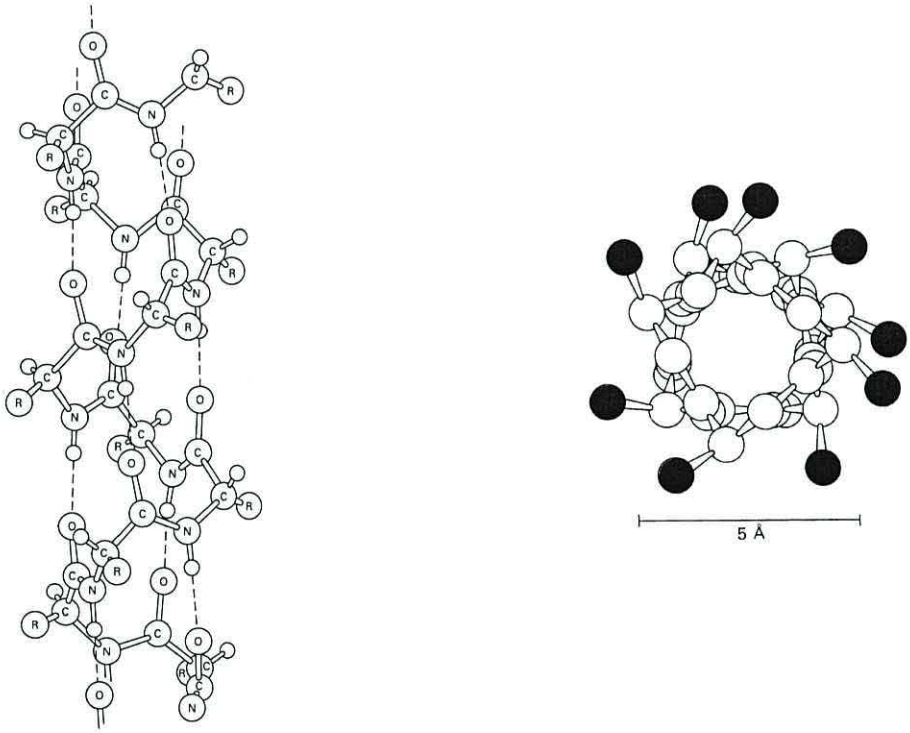


Figure III.5: Right handed α helix, longitudinal (from (Creighton, 1993)) and cross sectional view, side chains in black (from (Stryer, 1981))

β-sheet The β-pleated sheet differs markedly form the α-helix in that it is a sheet rather than a rod. The polypeptide chain in the β sheet is almost fully extended rather than being tightly coiled as in the α helix. The axial distance between adjacent amino acids is 3.5 Å, in contrast with 1.5 Å for the α helix. Another difference is that the β

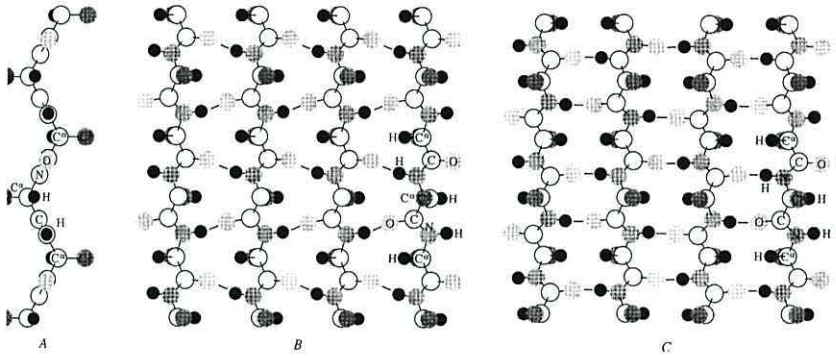


Figure III.6: A single β strand (A) and its incorporation into flat parallel (B) and antiparallel (C) β sheet from (Creighton, 1993)

pleated sheet is stabilized by hydrogen bonds between NH and CO groups in different polypeptide strands, whereas in the α helix the hydrogen bonds are between NH and CO groups in the same polypeptide chain. Adjacent strands in a β sheet can run in the same direction (parallel β-sheet) or in opposite directions (antiparallel β-sheet). Structural units comprising from two to five parallel or antiparallel β strands are especially common.

b The tertiary and quaternary structure

The folded structures of most small proteins are roughly spherical and remarkably compact, with very irregular surfaces. The structures of most proteins that have more than about 200 residues appear to consist of two, three or more structural units, usually referred to as domains. The domains of a protein molecule interact to a varying extent, but less extensively than do structural elements within domains.

Many proteins exist naturally as aggregates of two or more polypeptide chains, either identical or different. Different polypeptide chains can be called subunits, monomers or promoters. The overall organization of the subunits is known as the *quaternary structure.* This refers to the arrangement of the subunits in space and the ensemble of subunits' contacts and interactions, without regard to the internal geometry of the subunits. The three-dimensional structure of the subunits is still referred to as its *tertiary structure.* A change in the quaternary structure means that the subunits move relative to each other. The subunit may be identical polypeptide chains, or they may be chemically different.

As a rule, the contact interfaces of the subunits are as closely packed with each other as are the interiors of proteins. Also, any charged or hydrogen-bonding group on one surface is paired with a complementary partner on the other.

4 Enzyme catalysis

Chemical reactions in biological systems rarely occur in the absence of a catalyst, these catalysts are specific proteins called *enzymes.*

Some of the most important features of enzymes are: Enzymes accelerate reactions by factors of at least a million. They are highly specific both in the reaction catalyzed and in their choice of reactants which are called *substrates.* An enzyme usually catalyzes a single chemical reaction or a set of closely related reactions, furthermore enzymes do not alter reaction equilibria; an enzyme accelerates the forward and reverse reaction by precisely the same factor thus it accelerates the attainment of equilibria but does not shift their positions.

A chemical reaction

$$A \rightleftharpoons B \tag{III.6}$$

goes through a transition state that has a higher energy than either A or B. The rate of the forward reaction depends on the temperature and on the difference in free energy between that of A and the transition state, which is called the Gibbs[2] free energy of activation and symbolized ΔG,

$$\Delta G = G_{transition\ state} - G_{substrate} \tag{III.7}$$

Enzymes accelerate reactions by decreasing ΔG, the activation barrier. A factor responsible for slowing down multimolecular reaction in biological systems is the requirement of the bringing together of many molecules in the transition state. This is itself an unfavourable event because it requires the right number of molecules simultaneously colliding in the correct orientation.

[2]The Gibbs free energy defined as $G = H - TS$, where H is entalphy, S entropy and T temperature in Kelvin

The most outstanding characteristic of enzyme catalysis is that the enzyme specifically binds its substrates with the reaction taking place in the confines of the enzyme-substrate complex (ES). The substrate is bound to a specific region of the enzyme called the *active site.* Although enzymes differ widely in the structure-specificity and mode of catalysis, a number of generalizations concerning their active sites can be stated; the active site takes up a relatively small part of the total volume of the enzyme and it is a three-dimensional entity. Forces between enzyme and substrates are relatively weak. Water is usually excluded from the active site (unless it is a reactant), and although the active site contains polar residues that are essential for binding and catalysis it has a general non-polar character which enhances the binding of the substrate. The specificity of binding depends on the precisely defined arrangement of atoms in an active site, a substrate must have a matching shape to fit into the site.

a Enzyme kinetics

When an enzyme is mixed with a large excess of substrate, there is a minimum period, known as the *pre-steady state*, during which the concentration of the enzyme-bound intermediates build up to their *steady state* concentrations. During this steady state period the reaction rate changes relatively slow with time, thus, it is then when the rates of enzymatic reactions are traditionally measured. Steady state kinetics is important for the understanding of metabolism, since it measures the catalytic activity of an enzyme in the steady-state conditions in cells.

In other words, it has been found experimentally that, for many enzymes, the rate of catalysis, v, is proportional to the concentration of the enzyme $[E]_0$. However, v follows saturation kinetics with respect to the concentration of the substrate, [S]; at a fixed concentration of enzyme, v is almost linearly proportional to [S], up to moderate concentrations, whereas at high [S], v is nearly independent of [S].

In 1913, Leonor Michaelis and Maud Menten proposed a simple model to account for the enzyme kinetic characteristics

$$E + S \overset{k_s}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P \tag{III.8}$$

The catalytic reaction is divided in two processes. An enzyme E combines with the

substrate S to form an ES complex with a rate constant k_s. This step is thought to be rapid and reversible with no chemical changes taking place; the enzyme and the substrate held together by non-covalent interactions. The complex ES can then dissociate to E and S, with a rate constant k_s or it can form the product P. The second step of the reaction occurs with a first-order rate constant k_{cat}, the *turnover number*. The rate equations are solved to give the velocity of the reaction v, as follows: from equation III.8

$$k_s = \frac{[E][S]}{[ES]} \tag{III.9}$$

and the velocity of the catalytic reaction

$$v = k_{cat}[ES] \tag{III.10}$$

Also the total enzyme concentration $[E]_o$ and that of the free enzyme [E] are related by

$$[E] = [E]_o - [ES] \tag{III.11}$$

thus the concentration of the enzyme-substrate complex is

$$[ES] = \frac{[E]_o[S]}{k_s + [S]} \tag{III.12}$$

and finally

$$v = \frac{[E]_0[S]k_{cat}}{k_s + [S]} \tag{III.13}$$

The Michaelis-Menten mechanism assumes that the enzyme-substrate complex is in thermodynamic equilibrium with free enzyme and substrate, this would be only true if considering the following scheme, $k_2 \ll k_{-1}$

$$E + Sk_1k_{-1}\rightleftharpoons ES \xrightarrow{k_2} E + P \tag{III.14}$$

which gives

$$v = \frac{[E]_0[S]k_{cat}}{k_M + [S]} \tag{III.15}$$

where

$$k_M = \frac{k_2 + k_{-1}}{k_1} \tag{III.16}$$

To summarize, the catalytic constant, k_{cat} is a first-order rate constant that refers to the properties and reactions of the enzyme-substrate, enzyme-intermediate and enzyme-product complexes. This constant is often called the *turnover number* of the enzyme because it represents the maximum number of substrate molecules converted per active site per unit time, or the number of times the enzyme turns over per unit time whilst the k_M is a measure of the amount of enzyme that is bound in any form to the substrate.

b Determining enzyme activity. Absorption measurements

The activity of an enzyme is the amount of product that a certain amount of enzyme will produce in a specified period of time. The activity may be determined by measuring the amount of product or the amount of substrate used up per unit of time under saturation conditions of substrate. Therefore activity can be considered essentially as the measurement of the initial reaction velocity under conditions that make it the maximum velocity.

This is usually accomplished by performing a chemical analysis of the product or substrate. For example tyrosinase activity can be assayed by monitoring the oxidation of 3,4-dihydroxyphenylalanine resulting in the red-coloured dopachrome which absorbs maximally at 453 nm, the concentration of which can thus be determined spectrophotometrically.

A spectrophotometer is used to measure absorbance. The instrument produces ultraviolet or visible light which has a certain intensity I_o at wavelength λ, such light impinges on a sample for a path length of l cm. The light that is not absorbed by the sample emerges with intensity I.

Consider a infinitesimal path length, dl, then the fraction of light absorbed, $-dI/I$ should be proportional to the number of absorbing molecules, therefore

$$-dI/I = C\epsilon' dl \tag{III.17}$$

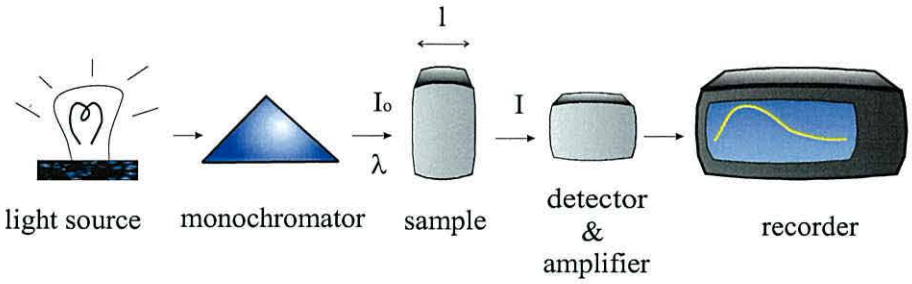


Figure III.7: Schematic diagram of a conventional spectrophotometer

where $\epsilon^{'}$ is a proportionality constant called the molar extinction coefficient. This is independent of the absorption concentration and it contains the wavelength dependence of the absorption spectrum. If equation III.17 is integrated over the entire sample (integrating the left-hand side from initial intensity I_o to final intensity I and the right hand side from zero to l) then

$$ln(I_o/I) = C\epsilon^{'}l \tag{III.18}$$

and converting to log base 10 the Beer-Lambert law is obtained:

$$Abs(\lambda) \equiv log(I_o/I) = C\epsilon(\lambda)l \tag{III.19}$$

where $\epsilon = \epsilon^{'}/2.303$. The quantity Abs is called the absorbance. Note the most accurate measurements of Abs are obtained in the range of 0.1 to 2. Smaller values mean that only a tiny fraction of the incident light is absorbed; larger values mean that only a small fraction of the incident light reaches the detector.

If an activity assessment involves an absorption measurement, that is the absorbance of a given product result of the reaction catalyzed by the enzyme then enzyme activity can be redefined as the increment in absorbance per minute, $\Delta Abs/min$.

5 Protein stability

Protein-folded conformations are only marginally stable under the best conditions and can often be denatured by changing their physical or chemical environments. The most common methods are by heating; adding a chemical denaturant; changing the

pH to acidic or alkaline conditions; or applying high pressure. Many proteins denature reversibly, they regain their native structures spontaneously when returned to conditions that favour folding. Many other proteins denature irreversibly, they often aggregate and precipitate after the denaturant had been removed or hot solutions are cooled.

In terms of thermodynamic stability, denaturation of a protein that unfolds reversibly in a cooperative, simple two state mechanism can be described as

$$N(ative) \rightleftharpoons D(enatured) \tag{III.20}$$

where the equilibrium constant, K_u, is the rate of unfolding.

In this case the stability of a protein is simply the difference in Gibbs energy, ΔG between the native and the denatured states. The only factors affecting stability are the relative free energies of the native state, G_N and the denatured, G_D states.

$$\Delta G_D = G_D - G_N \tag{III.21}$$

The larger and more positive ΔG_D the more stable is the protein to denaturation. The free energy difference, ΔG_D is typically small, of the order of $5 - 15$ kcal/mol for a globular protein (compare to $30 - 100$ kcal/mol for a covalent bond)

Irreversible loss of protein native structure is represented by

$$N \rightleftharpoons D \xrightarrow{k_i} I(nactive) \tag{III.22}$$

where k_i is the rate constant for some irreversible inactivation process.

From the point of view of physical biochemistry, stability is defined as above, whilst from the point of view of biotechnology, which is more concerned with the practical utility of the enzyme the question is: *is the protein stable enough to function under harsh conditions of temperature or solvent?*. The answer to this question may lie in thermodynamic stability but it can also simply lie in the reversibility of the protein or, for irreversibly denatured proteins, in the kinetic stability, that is in how rapidly a protein denatures.

6 KINETICS OF ENZYME DEACTIVATION

Detailed knowledge of deactivation kinetics is required for possible biotechnological applications of enzymes as well as for mechanistic interpretations of the enzyme deactivation phenomena. Deactivation kinetics allows the prediction of the useful life of an enzyme and could help to develop stabilization procedures.

Enzyme deactivation can be due to loss of the native conformation, with no rupture or formation of covalent bonds or to chemical modifications of functional groups of the active site. This thesis/section will only deal with the former, thermal deactivation.

The most common interpretation of enzyme deactivation assumes that loss of activity involves only two different enzyme forms: native(active) N and denatured (fully inactive); $N \rightarrow D$. In that case, the decay in activity is expected to follow first order kinetics with respect to the native enzyme concentration i.e.

$$-\frac{d[N]}{dt} = k[N] \tag{III.23}$$

Upon integration

$$[N] = [N]_0 e^{-kt} \tag{III.24}$$

where $[N]_0$ is the initial concentration of native enzyme. Thus the overall specific activity[3], A_t, (referred to the enzyme concentration),

$$A_t/A_o = exp(-k \cdot t) \tag{III.25}$$

where A_0 is the activity of the fully active enzyme.

However enzyme thermal deactivation often deviates from first order kinetics which is reflected by convex log(activity)vs. time curves. This suggest a more complex kinetic pattern is followed, other than a two-state transition.

Departure form first-order kinetics is usually explained by the occurrence of one or more of the following phenomena: protein aggregation, enzyme hetereogeneity and the appearance of partially denatured forms of enzyme whilst deactivation occurs (biphasic scheme).

[3]activity is usually defined as the mmol of substrate reacted per minute and per mg of enzyme.

a Aggregation

According to several authors (Cleland et al., 1993; Toscano et al., 1994) aggregation among different enzyme molecules is a major cause of irreversible deactivation. Aggregation can be described as a two-stage process. The first stage consists in more than one elementary step and it involves only reversible conformation and oligomer association equilibria. The second stage is intrinsically polymolecular and irreversible, aggregates are stabilized by covalent bonds (intermolecular disulphide bridges) or by non-covalent interactions (hydrophobic interactions, electrostatic forces, hydrogen bonds).

Since aggregation is a polymolecular event and if aggregation were the controlling phenomena of enzyme deactivation, the rate of deactivation should increase with enzyme concentration. Thus loss of activity with time is described by

$$A(t) = A_0(1 + (n-1)k_n[N]_0^{n-1}t)^{1/1-n} \tag{III.26}$$

b Enzyme hetereogeneity

Enzyme hetereogeneity, i.e. occurrence of isoenzymes or micro-heterogeneity has been also considered to account for deviation from first-order kinetics of enzyme deactivation curves. A model based on simultaneous, irreversible, first order deactivation of two isoenzymes with different initial activities and stabilities leads to the following expression

$$A = \alpha e^{-k_1 t} + \beta e^{-k_2 t} \tag{III.27}$$

where α and β are the activities of each isoenzyme times the initial weight-fractions in the native mixture.

c 'Biphasic' model

Enzyme loss of activity can also be explained by a serial mechanism involving more than one intermediate, partially deactivated forms;

$$N \rightarrow D_1 \rightarrow D_2 \rightarrow ... \rightarrow D_n \tag{III.28}$$

which yields an activity-time dependency of the form:

$$A = c_1 e^{-k_1 t} + c_2 e^{-k_2 t} + ... + c_n e^{-k_n t} \quad \text{(III.29)}$$

Note that if only one intermediate is considered, then a bi-exponential equation such as III.27 results, although now the pre-exponential factors α and β will have a different meaning.

Toscano and co-workers (Toscano et al., 1994) have proved none of these cases to be entirely true for the enzyme acid phosphatase. This, together with the fact that different mechanism can yield the same expression for the decay of enzyme activity (Aymard and Belarbi, 2000; Toscano et al., 1994) indicates that enzyme deactivation mechanisms are not fully understood. Furthermore, they suggest that the deactivation process cannot be resolved into separate steps but should be described as a continuous conformational change of the enzyme macromolecule in which the catalytic activity is gradually lost. During the process the number of active sites is not reduced, but the activity of each site decreases because of the time-progressive loss of the original conformation (Toscano et al., 1994).

Aymard and Belarbi (Aymard and Belarbi, 2000), have suggested the use of a bi-exponential, three parameter equation[4] as a model-free one, even if further analysis of the variation of its parameters with experimental settings could allow some conclusions to be made about the deactivation mechanism. The use of the bi-exponential, three parameter equation is justified since it facilitates analysis of the data and reduces the risk of cross-correlation, i.e. the possibility of finding several sets of parameters with equivalent goodness of the fit.

7 β-LACTOGLOBULIN

Two different proteins have been employed in this body of work to study and model sugar preservative properties, β-lactoglobulin and trypsin. In the present and following section a brief description of their main properties is given.

β-lactoglobulin is the major whey protein (about 6 g/l)in the milk of ruminants and some non-ruminants, such us the pig, horse, dog, dolphin, kangaroo, cat and whale.

[4]III.27 where $\beta = 1 - \alpha$

However, milk from humans and rodents appears to be devoid of β-lactoglobulin. It comprises 10% of the total milk protein or about 58% of the whey protein. It was first isolated by Palmer in 1934 but its amino acid composition was not determined until the $1960's$. It is usually formed by two monomers, each containing 162 amino acids with a molecular weight of about 18300. Below pH 3 and above pH 8 it exists as a monomer, but under physiological conditions it tends to be found as a dimer, see figure III.8

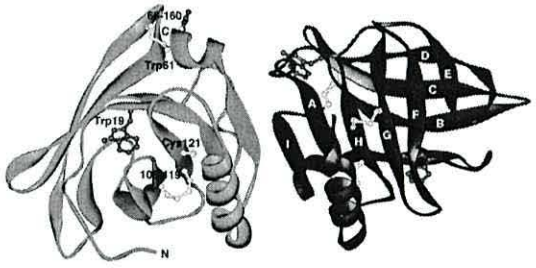


Figure III.8: Schematic picture of dimeric β-lactoglobulin A.

Although this protein was first isolated long ago, no function has been definitely ascribed to β-lactoglobulin. X-ray studies have indicated that the β-lactoglobulin structure is similar to that of a retinol-binding protein, which suggests they may be involved in similar processes such as the transport of small hydrophobic substances.

It is known that β-lactoglobulin is responsible for the ability of whey protein to form a gel on heating. In fact, its technological applications are related to its gelling, emulsifying and foaming properties which are of considerable relevance and much interest to the food industry, where it is currently being used as an emulsifying, foaming, gelling or dispersing agent when it is added as a spray-dried powder to a variety of food products.

8 TRYPSIN

It has already been discussed that the function and biological activity of proteins is directly related to their three-dimensional structure. For example, lysozyme becomes fully active when, spontaneously, it acquires a characteristic 3D form (Stryer, 1981). In contrast, there are other proteins that are initially synthesised as inactive precursors, called *zymogens*, which upon cleavage of one or more specific peptides become active.

Trypsin is part of a group of digestive enzymes, often called proteolytic enzymes, whose function is to hydrolyse proteins and are synthetised as zymogens in the stomach and pancreas. Trypsin is initially synthesized in pancreatic cells as an inactive precursor called trypsinogen[5], which, after cleavage of a single peptide is converted to the active enzyme.

Trypsin from beef pancreas was among the first proteolytic enzymes isolated in pure form in amounts sufficient for exact chemical and enzymological studies and it has been also isolated from the digestive systems of other vertebrates, e.g. humans, turkey, shark, etc. It has 223 amino acids and has a molecular weight of about 24000.

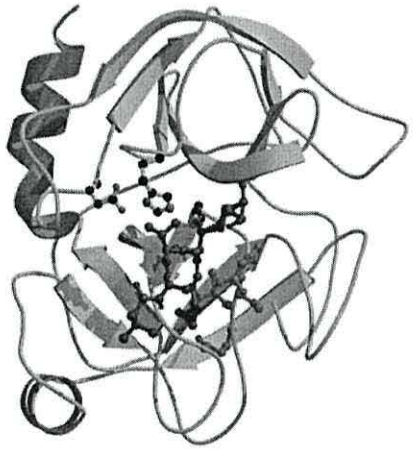


Figure III.9: Three dimensional structure of trypsin.

It has been found that trypsin is like chymotrypsin in many aspects, such as amino acid sequence (40% of their sequence is identical), tertiary structure and catalytic mechanisms. Nevertheless they differ in specificity; chymotrypsin breaks up a protein at a site where an aromatic or bulk polar side-chain is present, whilst trypsin requires a lysine or arginine residue. The remarkable property of trypsin is it specificity, that is, it is active only against the peptide bonds in protein molecules that have carboxyl groups donated by arginine and lysine. Trypsin is the most discriminating of all the proteolytic enzymes in terms of the restricted number of chemical bonds that it will attack. Good use of this fact has been made by chemists interested in the determination of the amino

[5]The other digestive enzymes and their respective zymogens are: pepsin-pepsinogen, chymotrypsin-chymotrypsinogen, carboxypeptidase-procarbopeptidase and elastase-proelatase (Stryer, 1981).

acid sequence of proteins; trypsin is widely employed as a reagent for the orderly and unambiguous cleavage of such molecules.

Trypsin catalyses the hydrolysis of polypeptide or ester bonds in two stages and follows the reaction:

$$E + S \rightleftharpoons ES \rightarrow ES' + P_1 \rightarrow E + P_2 \tag{III.30}$$

To picture the hydrolysis process consider the ester Nα-Benzoyl-L-Arginine Ethyl (BAEE) whose chemical structure is shown in figure III.10. BAEE has been used in this body of work to assay trypsin activity. The first stage of the hydrolysis called acylation, involves the formation of the enzyme substrate complex, ES. The ester bond of the substrate is cleaved, one of the products is then released, whereas the acetyl group of the substrate becomes covalently attached to the protein. During the second step, known as deacylation, water attacks the acetyl-enzyme intermediate, ES', to yield a carboxylic acid.

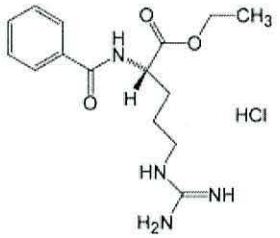


Figure III.10: The chemical structure Nα-Benzoyl-L-Arginine Ethyl (BAEE)

IV

Protein hydration

It has long been recognized that water plays a crucial role in determining the physical and chemical properties of proteins, properties which are ultimately responsible for the unique biological activity of these molecules. Apart from this fundamental importance, knowledge of processes occurring during hydration or dehydration of proteins is also important in biotechnological applications of proteins, such as their use as catalysts in anhydrous organic solvents, and in the stabilization of protein preparations for pharmaceutical use and in food preservation.

1 Sequential hydration of proteins.

The study of sequential hydration of proteins from dry powder to the solution phase is of particular interest when trying to define the levels of water at which significant changes in protein conformation, function, flexibility and stability may be encountered.

Many techniques have been used to study protein-water interactions and the physical properties of the water molecules interacting with proteins, such as dielectric relaxation spectroscopy, infrared and NMR spectroscopy and calorimetric and water sorption measurements[1]. Most methods estimate an amount of interacting water which has properties different from those of bulk water. This is the so-called 'bound' water fraction, which has been found to vary depending on the technique employed (see (Gregory, 1995)). Furthermore, there has been some debate as to whether the term 'bound' is entirely correct since it carries connotations of immobile water molecules and thermodynamic states of water that might not accurately describe the water molecules around the protein (Rupley and Careri, 1991; Levine and Slade, 1992).

[1] results obtained from these and other methods have been extensively reviewed elsewhere (Kuntz and Kauzmann, 1974; Rupley and Careri, 1991; Gregory, 1995)

The picture of sequential hydration presented here is based on that developed by Rupley and Careri (Rupley et al., 1983; Rupley and Careri, 1991); hydration of a dry protein occurs roughly in three stages, corresponding reasonably well with the three regions of the hydration isotherm (see next sections).

a - *Stage* **1**. *Hydration level from* **0** *to* $\sim$ **7%** *w/w water content*

The first stage of hydration involves water absorption and its primary interaction with charged groups. The first detectable event, as shown by thermodynamic and IR spectroscopy measurements is the ionization of COO^- and NH_3^+.

Below 7% water content, the denaturation temperature is much lower that in the solution state, protein motions are said to be frozen, enzymatic activity is negligible and the heat capacity of sorbed water is between that of ice and liquid water.

In addition, at $6\% - 8\%$ water content, roughly the knee in the hydration isotherm, there is a transition in surface water, from disordered to ordered and from a dispersed to clustered state associated with completion of charged group hydration. There is also a discontinuity in the apparent specific heat capacity of the protein which has been related to the transfer of protons from carboxylate to other protonatable groups for the lightly hydrated protein.

b - *Stage* **2**. *Hydration level from* $\sim$ **7** *to* **25%**

The hydration range from $6-8\%$ to $20-25\%$ corresponds to the plateau in the hydration isotherm. It has been observed that the denaturation temperature decreases strongly with increased hydration and that the heat capacity of sorbed water is greater compared to that of bulk water. Furthermore, increases in hydration result in condensation or clustering of water around the already hydrated charged groups and cooperative interaction of water with other polar groups such as the backbone and side-chain amide groups.

A potentially important percolation transition, rather than phase transition, occurs at approximately 15% water content (Rupley and Careri, 1991). This percolation transition reflects a change in the connectivity within a system. The importance of such an interconnective path for rapid transfer of a proton may have significant implications in bioactivity and in potential acid-catalyzed degradation reactions.

Internal protein motion, as shown by hydrogen exchange increases greatly (from 1/1000 to full solution rate at 15%), not surprisingly at 10-15% many enzymes such as chymotrypsin develop activity (lysozyme shows signs of activity at about 20%).

c - Stage **3**. *Hydration level from* $\sim$ **25** *to* **38%**

This last stage coincides with the strong upswing in the hydration isotherm.

At $20-25\%$ water content, a second transition occurs as evidenced by heat capacity measurements (Rupley and Careri, 1991) and saturation of hydrogen bonding sites. Above 25% water condenses onto the weakest binding sites of the protein surface to complete the hydration process.

Throughout this hydration range, there is a increase in enzymatic activity as well as greater water mobility with increasing hydration.

At 38%, full hydration, defined as the point at which major changes in thermodynamic properties are complete, is said to occur. At this point, the denaturation temperature is close to that of the solution state and mobility of most hydration water is close to that of bulk water. In addition, full internal motion of the protein is established and the enzymatic activity of lysozyme is approximately 1/10th of the solution value.

It is not until hydration of $30-40\%$, significantly greater than the BET monolayer[2], that the true monolayer (true monolayer understood as the full hydration shell) of the protein globule actually occurs. These levels of $30-40\%$ water are those generally measured as "unfreezable"[3] water in the low-temperature calorimetric and NMR studies.

2 Protein hydration and glass transition

Prior to describing the sorption isotherms and interpretations is worth highlighting the fact that, under certain conditions, such as those occurring during lyophilization, proteins may enter a rigid glassy state[4] in which large scale segmental motions of the polymer chain no longer occur and also the effect of water on the glass transition temperature of proteins.

[2]for an explanation of this concept see the following sections

[3]fraction of water surrounding the protein that does not freeze at temperatures below the bulk water freezing point

[4]a brief review on the main properties of glasses and amorphous state can be found in the Appendix A

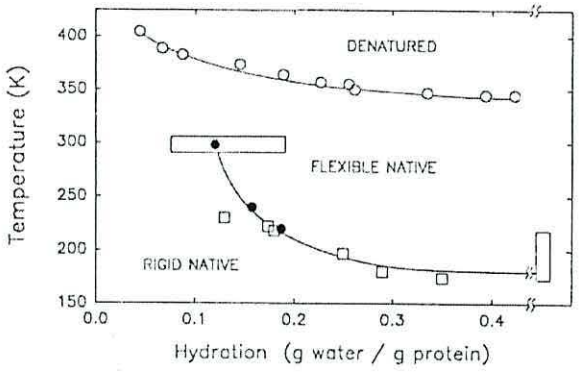


Figure IV.1: The hydration dependence of the glass transition temperature for lysozyme (open squares), the hydration dependence of the denaturation temperature (open circles) (Gregory, 1995)

Figure IV.1 shows the hydration dependence of the glass transition temperature of lyzozyme. The curve clearly shows the plasticizing effect of water, that is that T_g decreases with increasing amount of water.

Note that for hydration values lower than 10% moisture, the curve has not been determined. It is expected that at such low moisture content the protein is frozen into a distribution of conformational states and that mobility is restricted to group rotations and small amplitude vibrational and torsional motions, which makes the measurement of T_g extremely difficult. In fact there are only few studies which have reported T_g for fully dried proteins (Sochava, 1997).

It can be seen that at about 25% moisture content, the curve saturates indicating that further sorption of water does not have an effect on the glass transition of the system. It is likely that it is at this point that the transition from glass to rubber occurs.

In the sorption isotherm context, the plasticization of water is reflected by the transition above $20 - 25\%$ water content. This transition can be equated to a transition from a "glassy" to a viscoelastic state; that is, the water content has reached a level sufficient to reduce the T_g below the isothermic temperature of the surroundings (Oksanen and Zografi, 1990). Beyond this $20 - 25\%$ water content, the enhanced mobility and greater free volume of the polymer may then allow for uptake of significant amounts of water due to a decreased energy barrier for sorption into the now

viscoelastic system. Interestingly, it has been shown that the water content at which the glass transition of the polymer is reduced to the experimental temperature, v_g, is to some extent correlated to the hydration level corresponding to the 'knee' in the isotherm, suggesting that the initial shoulder in the isotherm is also linked to the glassy nature of the protein and its plasticization by water (Hancock and Zografi, 1993).

3 Sorption of water vapor by proteins: The sorption isotherm

The study of water vapour sorption[5], mentioned above, has been extensively used to investigate both biopolymer-water interactions and the effect of water on biopolymer physico-chemical properties. In addition, water sorption studies, in conjunction with other techniques such as Time Domain Reflectometry (Bone, 1987) and NMR (Lam et al., 2002) have been proved to be extremely useful in understanding the role of water in protein flexibility and mobility , which are known to be closely related to protein biological activity and stability.

Typically, a sorption experiment consists of measuring the water uptake by the dry protein under conditions of controlled relative humidity at a constant temperature. Hydration levels are often measured by isopiestic equilibration of protein samples against concentrated salt solutions of known water vapour pressure (Bull and Breese, 1968). Other methods include the use of relative humidity (RH) sensors to measure the vapour pressure and mass transducers to determine the sample weight (Wilkinson et al., 1976) and the use of a resonating quartz crystal microbalance (Gascoyne and Pethig, 1977) which measured changes in the resonating frequency of the crystal due to variation of the sample mass. In this work, a computer-controlled Sartorius vacuum microbalance together with a RH sensor was used to accurately obtain the hydration isotherms[6].

One of the earliest and more complete works on protein hydration isotherms is due to Bull and Breese (Bull and Breese, 1968) who, by an isopiestic method, determined the water sorption curve for several proteins (e.g. lysozyme and bovine serum albumin).

[5]Adsorption and absorption terms have been used to described the uptake of water by biopolymers but, as Kuntz and Kauzman noted (Kuntz and Kauzmann, 1974), none of them fully explain the protein hydration process, therefore throughout this work the more general term sorption will be used.

[6]for further details on experimental procedure used in this work see chapter VII

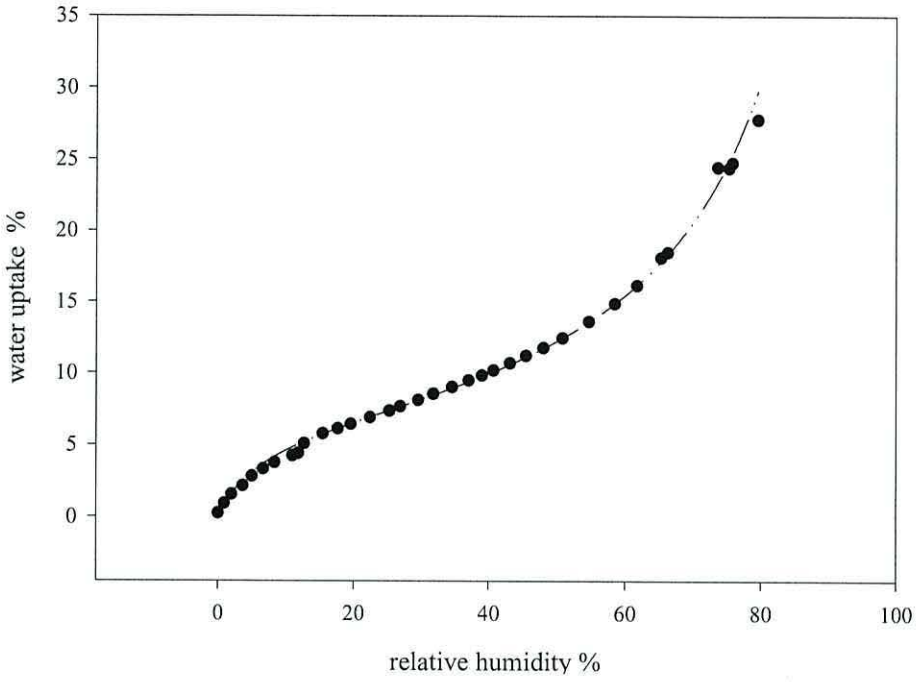


Figure IV.2: Hydration isotherm of β-lactoglobulin

Other studies have investigated the dependency of the water uptake on the state of the protein, native or unfolded and in the form of powers or films (Benson et al., 1950; Rüegg et al., 1975; Bone, 1994). Hydration isotherms have also been collected to study the theoretical aspects of the sorption process(Hoover and Mellon, 1950; Gascoyne and Pethig, 1977; Gascoyne, 1979).

All of these studies found that the hydration isotherms for proteins and many other biomolecules (as an example see figure. IV.2) have many common features:

i. Most of them show a sigmoidal shape. Up to 5% relative humidity, the water content increases rapidly. At $10-30\%$ RH a distinct 'knee' occurs in the isotherm, after which the water content increases fairly steadily with vapour pressure. At $80-90\%$ RH the water uptake rises sharply approaching infinity at 100% RH, if the protein shows water solubility.

ii. Pronounced hysteresis is always noted below 80% RH, the desorption curve lying $10-30\%$ above the sorption curve (Bull and Breese, 1968; Kuntz and Kauzmann, 1974). This is thought to be due to irreversible structural changes during dehydration.

iii. Furthermore, the amount of water sorbed by the protein depends on its state (native, denatured, etc.) and on the type of protein. In contrast, the uptake of water by the protein is independent of the surface area of the protein powder i.e. the same amount of water is absorbed regardless of how finely divided is the protein powder (Kuntz and Kauzmann, 1974)

Hydration isotherms can not only provide, an estimation of the number of water molecules which bind to the protein but can also estimate, through the van't Hoff and Clausius-Clayperon relationships, the heat of sorption, enthalpy and entropy of sorption (Bull and Breese, 1968; Kuntz and Kauzmann, 1974; Bone, 1996). The use of hydration isotherms for the determination of these thermodynamic values has been severely criticized (Franks, 1982; Zografi, 1988; Levine and Slade, 1992) since sorption does not appear to be an equilibrium process which is reflected by hysteresis.

a Sorption hysteresis

Protein water sorption isotherms exhibit pronounced hysteresis, for which the sorption curve may lie below the desorption characteristic. A number of explanations have been proposed to account for hysteresis, including capillary condensation (Morozov et al., 1988) and the existence of metastable states associated with the phase change within the adsorbate. In addition, it has been suggested that sorption hysteresis is a molecular phenomenon related to conformational changes in the protein molecule which are associated with the region of low hydration (Cerofolini and Cerofolini, 1980). For example, if it is assumed that all protein molecules have the same conformation at low relative humidities, but undergo a slow conformational change at some intermediate relative humidities and the conformational change is sufficiently slow that the new equilibrium position is not reached in the time required for the sorption experiment, the adsorption isotherm should be reversible below the vapor pressure range over which the conformational change take place, while desorption should be reversible above this range.

Hysteresis has an important consequence. Conversions of the isotherm information into free energies of transfer of water to the protein surface, and of the temperature dependence of the isotherm into enthalpies of transfer are done under the assumption

that the isotherm reflects an equilibrium state. If there is hysteresis, the equilibrium state may not be defined well enough for thermodynamic analysis. However, it is possible, and generally believed, that the high reproducibility of an isotherm justifies the extraction of thermodynamic values from data that show hysteresis, although hysteresis would still be a source of systematic error in the values.

In any case the isotherms have been proven to be a powerful tool to compare the hydration behaviour of different proteins (Bull and Breese, 1968) as well as, in conjunction with other techniques, to determine the populations of water molecules which are relevant for protein dynamics(e.g (Bone, 1987)).

4 Sorption theories. Mathematical models.

Theoretical treatments of the sorption process for protein are generally undertaken with the intent of explaining the sigmoidal shape typical of protein isotherms. Van der Berg and colleagues (van den Berg and Bruin, 1981) have listed more than seventy five equations which have been proposed and used to fit sorption isotherms. As they pointed out, from a mathematical point of view, it is reasonably easy to fit any number of experimental data points to a sigmoidal curve. Nevertheless, it is desirable to find a relatively simple equation describing the water sorption with a limited number of parameters, with the parameters, having a clear physical meaning.

It has been very common to fit water sorption data to equations originally derived for adsorption processes. By far the most popular approach has been the use of the well-known BET equation. The use of the BET equation, its extensions (i.e. GAB equation) and other models (i.e. solution theories) for evaluation of water sorption by amorphous solids has been extensively reviewed (Ling, 1972; Kuntz and Kauzmann, 1974; Adamson, 1982; Gregory, 1995). Therefore, in the following summary it is intended to give a broad view of the sorption theories and only a few of the most relevant models will be considered and described.

a Surface models

Surface models generally consider a fixed number of binding sites to which vapour-phase water first attaches forming a tightly-held monolayer followed by multilayers of much

more loosely held water. The simplest of these is the Langmuir theory (Langmuir, 1918), which is based on molecular kinetic considerations. It has two basic assumptions, firstly, the water vapour molecules are bound at active centres on the surface of the solid to form a single monolayer and secondly, there are no interactions between sorbed molecules. The sorption is then of a saturation-type and is described by:

$$\frac{x}{v(x)} = \frac{x}{h_m} + \frac{1}{h_m C} \tag{IV.1}$$

where x is the vapour partial pressure, $v(x)$ is the amount of gas sorbed, h_m is the amount adsorbed when all sites are occupied, and C is a proportionality constant related to the energies of sorption and desorption of the vapour molecules from the binding sites.

Brunauer, Emmett and Teller (Brunauer et al., 1938) extended Langmuir's approach to multilayer adsorption by taking into account additional sorption layers which bind more weakly than the first layer. Again no lateral interactions were considered and it was assumed that the sorption energy of the molecules in the first layer is constant. The second and higher adsorbed layers were considered to have the evaporation-condensation properties of the liquid state. This analysis lead to:

$$v(x) = \frac{a v_m x}{(1-x)(1+(a-1)x)} \tag{IV.2}$$

where v_m is the monolayer hydration value corresponding to the hydration of the primary binding sites, and a is a constant related to the energies of sorption and desorption in the first monolayer. The BET theory provided the first widely accepted description of the sigmoidal curves and has been commonly used despite its poor predictability above 40% relative humidity.

Anderson (Anderson, 1946) and Guggenheim (Guggenheim, 1966) proposed a more complex case where the second and subsequent layers of water molecules are considered to have a sorption energy different from that of the bulk condensed vapour, the total amount of water absorbed $v(x)$ is then given by the G.A.B equation which has been found to model the data successfully over the entire hydration range (Gascoyne and Pethig, 1977),

$$v(x) = \frac{abv_m x}{(1-bx)(1+b(a-1)x)} \tag{IV.3}$$

where ab is the activity of the water absorbed in the first layer and b is the activity of all subsequent layers relative to that of bulk water.

It is interesting to note that the Langmuir, BET, and GAB equations may be derived from statistical mechanics (Hill, 1946a; Hill, 1946b; Guggenheim, 1966), the grand partition function for N_s identical primary sorption sites is given by

$$F(x) = (1 + a_1 x + a_1 a_2 x^2 + a_1 a_2 a_3 x^3 + ...)^{N_s} \tag{IV.4}$$

where x is the activity of the sorbed gas and may be identified with the relative vapour pressure. The coefficients a_i refer to the activity of the sorbed molecules in the i^{th} layer. The partition function for a single site is

$$f(x) = F(x)^{1/N_s} \tag{IV.5}$$

and the total sorption, $\theta(x)$ will be the sum of the occupations of all layers of sites and has been shown to be

$$\theta(x) = x\frac{xf'(x)}{f(x)} \tag{IV.6}$$

It has also been shown (Gascoyne and Pethig, 1977) that this expression leads to the totally general sorption equation

$$v(x) = v_m x \frac{\partial}{\partial x}(lnf(x)) \tag{IV.7}$$

Substitution for $f(x)$ allows the corresponding sorption to be established, i.e. setting the activities of the sorption sites above the monolayer to zero leads to the partition function $f(x) = 1 + ax$, which yields the Langmuir equation (eq. IV.1). The statistical mechanical treatment of sorption is completely general, the only assumptions being that sites in any one layer are non-interacting and are identical.

b Solution theories

Solution theories are based on the assumption that the sorption process can be treated as a solution process. Perhaps the best know and most used is the Flory-Huggins (Flory, 1942) model. Statistical-mechanical analysis of polymer solutions using a lattice model allowed Flory and Huggins to find an expression for the free energy, G, of a penetrant (i.e. water) in a system, which in terms of vapour pressure results in an isotherm of particularly simple form,

$$p/p_o = \phi_1 exp[(1 - 1/x)\phi_2 + \chi\phi_2^2] \qquad \text{(IV.8)}$$

where ϕ_1 and ϕ_2 are the volume fraction of solvent and polymer respectively, p/po is the partial vapour pressure of the solvent, x is the ratio of molar volumes of the solvent and the polymer, and χ is the Flory-Huggins polymer-solvent interaction parameter. χ is a measure of polymer-solvent compatibility and reflects the nonideality of the system. A large value of χ indicates poor interaction whereas a low values indicates strong interaction.[7]

It has been shown (Hancock and Zografi, 1993) that considering x =infinity (i.e. for polymer mixed with water, the polymer concentration is much larger than the water concentration) the Flory-Huggins equation predicts a positive deviation from linearity at high vapour pressure but no shoulder at low pressures. In contrast, if the parameter χ is allow to vary with solute concentration, the Flory-Huggins model is capable of giving an approximate description of the experimental data for the whole water-vapour sorption isotherm but it is least accurate in the region of interest for most pharmaceutical and food industry applications, that is at low partial vapour pressures.

Other attempts have been made to explain sorption isotherms by considering solution processes. The Hailwood and Horrobin (Hailwood and Horrobin, 1946) model assumed the sorbed gas exists in two different phases. The first sorbed gas molecules are considered to form a layer which is tightly bound to the sorbate while the following layers form an ideal solid solution. This argument has much in common with the BET

[7] χ values can be measured by viscometric, osmotic, or light scattering measurements and have been tabulated for a large number of different polymer-solvent combinations (see (Hancock and Zografi, 1993) for references

equation (eq. IV.2) and yields a similar equation.

D'Arcy and Watt (D'Arcy and Watt, 1970) proposed three separate processes, strong and weak monolayer bonding and the formation of multilayers which adds a new parameter to the Hailwood-Horrobin equation, the resulting four-parameters equation is in better agreement with the experimental data but it is not clear if much understanding of the sorption process is gained.

c Other models

Sorption in glassy polymers Recent work (Vrentas and Vrentas, 1991) concerned with sorption and volumetric behaviour of glassy polymer-penetrant systems has shown that sorption could be explain by taking into account structural rearrangements of the polymer matrix. They showed that the nonideal change of volume as water is sorbed can be estimated if these changes are related to the variation of the glass transition temperature of the polymer-penetrant mixture

$$\Delta V = -(dT_{gm}/dw_1)w_1w_2V_2^{o}{}_{T_{g2}}(\alpha_2 - \alpha_{2g}) \tag{IV.9}$$

where ΔV is the excess specific volume of the polymer-penetrant mixture, dT_{gm}/dw_1 is the change in T_g of the mixture with varying penetrant mass-fraction, w_1, w_2 are the penetrant and the polymer mass fraction, $V_2^{o}{}_{T_{g2}}$ is the specific volume of the pure polymer at its T_g, α_2 is the thermal expansion coefficient of the rubbery polymer, and α_{2g} is the thermal expansion coefficient of the glassy polymer.

As a consequence of the non-ideal changes that occur on mixing the glassy polymer and the penetrant (water) the sorption of a penetrant below the glass transition temperature of the polymer can be described by the following expression

$$p/p_o = \phi_1 exp[(1 - 1/x)\phi_2 + \chi\phi_2^2]expf \tag{IV.10}$$

where

$$f = [M_1w_2^2(C_{pg} - C_p)(dT_{gm}/dw_1)((T/T_{gm}) - 1)]/RT \tag{IV.11}$$

where w_1, w_2 are the penetrant and polymer mass fraction, respectively and C_{pg} and C_p are the specific heat capacities of the mixture in the glassy state and in the rubbery state.

Equation IV.10 is very similar to the Flory-Huggins equation (eq. IV.8), but contains one additional term, $exp(f)$. In this term are all those factors that reflect the changing structure of the polymer-penetrant mixture as the penetrant is sorbed. Note that each of these terms can be obtained independently of vapour sorption experiments, so the model can be considered to be a predictive one.

It has been demonstrated (Hancock and Zografi, 1993) that the term dT_{gm}/dw_1 is the one which determines the shape of the isotherm. A significant change in its value would produce a large shoulder in the sorption isotherm whereas a constant relationship between T_{gm} and w_1 would produce no shoulder at all. Therefore, Hancock and Zografi (Hancock and Zografi, 1993) argued that it is the solid-water interactions that determine the efficiency of the plasticization process which are primarily responsible for both deviations from linearity in the water vapour sorption isotherms of amorphous solids. The initial extent of water uptake by the solid is directly related to the strength of the water-solid interactions at low water pressures, and the shoulder on the isotherm occurs as a result of the changing interaction between the solid and the aqueous phases as the vapour pressure increases. Eventually, although the level of interaction becomes reduced, the solid gains enough energy from the sorption process to undergo a phase change from the glassy to the rubbery state and this is accompanied by a significant increase in the efficiency of water uptake into the solid.

Site heterogeneity and conformational perturbations A completely different approach has been considered by Cerofolini (Cerofolini and Cerofolini, 1980) to account for the site heterogeneity and conformational perturbation of the protein as the sorption process takes place. Two factors are responsible for protein surface heterogeneity. First, the water binding sites which are chemically diverse and include a variety of charged groups and uncharged groups. Second, the protein conformational states which give rise to a distribution of local environments for each water-binding site. In Cereoflini and Cerofolini's work, it is proposed that this heterogeneity may be measured by the

distribution of sorption energies, q, through the probability density function $\phi(q)$, so that $\phi(q)dq$ is the fraction of sites with adsorption energy between q and $q + dq$. The probability that a water-binding site with adsorption energy q is exposed is denoted by $P(q|W)$, a function which accounts for the influence of hydration on the conformational state of the protein. The hydration level at a vapour pressure p is given by

$$W(p) = \int_0^\infty \Theta(p,q)P(q|W)\phi(q)dq \qquad \text{(IV.12)}$$

where $\Theta(p,q)$ is the local adsorption isotherm describing the uptake of water at sites with adsorption energy q at a vapour pressure p. If the BET equation (eq. IV.2) is considered then the water uptake $W(p)$ may be rewritten as

$$W(p) = (1 - \frac{p}{p_o})^{-1} \int_0^\infty [p + p_L exp(-q/KT)]^{-1} P(q|W)\phi(q)dq \qquad \text{(IV.13)}$$

This equation appears to be more realistic that the conventional isotherms since, for instance, it considers site heterogeneity, but obviously the difficulty of using it lies in defining the functions $P(q|W)$ and $\phi(q)$.

d Summary

Almost any mathematical model attempting to explain a physico-chemical event can be questioned. It might be that a particular model or equation describes well a given experimentally-determined curve, but one can find that the parameter(s) obtained from such a model are difficult to interpret and might even lack any clear physical meaning.

In this respect, the models presented here are similar. The applicability of surface models to proteins has been extensively discussed. Kuntz and Kauzmann cite a number of reasons for preferring the solution models, among them that no phase transition is observed in the sorption isotherm over the entire composition range, despite the change in sample appearance from solid to liquid. Others (Gregory, 1995) have argued that the characterization of the monolayer sites by a single sorption energy is not very realistic given the variety of polar groups found at the protein surface. Nor it is reasonable to expect the protein conformation to be unaffected by binding water. It has also been pointed out (Levine and Slade, 1987; Zografi, 1988) that water induces changes in the

properties of the solid (such as the transition form the glassy state to the rubber and crystal states) which are not considered in these models.

Solution models have been found to work well at high relative humidities but fail to describe the low hydration end of the isotherm, which is the region in the isotherm of most interest especially for the stability of food and pharmaceutical products. The use of the Vrentas model does not seem to be widespread, probably due to the number of parameters that are included in the equation provided, and Cerofolini's expression for the isotherm, although realistic, clearly does not facilitate the analysis of experimental data.

All of these facts together have made the BET equation and its extensions, by far, the most commonly used model, clearly due to the 'simplicity', easy applicability and the 'straightforward' meaning of their parameters, v_m and a (v_m, ab and b in the GAB equation). The monolayer value, v_m regarded as a measure of the primary binding sites and a, ab and b values related to the energies of sorption of the first and subsequent layers.

In view of the success of the BET equation, a recent work (Zhang and Zografi, 2000) has attempted to find a more meaningful interpretation of the BET parameters when applied to amorphous solids, by studying the relationship between the parameter obtained from the former equation and those from the Flory-Huggins and Vrentas models. It was concluded that v_m is a reflection of the extent of water binding to the molecules making up the solid and, therefore, is related to the Flory-Huggins χ parameter. The parameter a was found to be related to the plasticizing effect (f) in that it is correlated directly with the free energy changes associated with structural relaxation effects due to sorbed water.

5 Water sorption and solid state stability of proteins

It is a common practice in the pharmaceutical and food industries to prepare dry formulations (dry powders) to increase the stability of proteins. For instance, it has been shown that the removal of water from the vicinity of the protein results in up to a 1000-fold increase in thermostability; however even in the absence of water, proteins still denature.

The most important variables dictating the stability of proteins in the amorphous solid state are moisture content, temperature and powder composition. These interacting variables determine the dynamic mobility of the system and thus the stability of the system and the components within it.

The effect of water content on the solid-state stability of proteins results from different effects; changes in dynamic activity of the protein, participation of water as a reactant or medium for mobilization of reactants. Moisture impacts on several deleterious reactions such as deamination, oxidation, or disulphide cross-linking, and these have been reviewed by Hageman (Hageman, 1992) and Towns (Towns, 1995). As an example of how water content affects the protein stability, the conformational stability, discussed in terms of the thermodynamic stabilization of folded versus unfolded structures may be considered; It has been observed that the denaturation temperature, T_D decreases with increasing hydration until approximately equal to that of the protein in solution (Zaks, 1992).

The role of additives, in particular sugars, on the solid state stability of proteins is currently a main concern for many researchers. Many techniques, including Fourier transform infrared spectroscopy, differential scanning calorimetry and computational simulations have been employed to study the stability of proteins in the solid state and the effect of sugars on this stability. A few recent papers have reported water sorption studies on protein sugar systems. Costantino and co-workers (Costantino et al., 1998) examined the moisture sorption isotherms of proteins (recombinant human insulin-like growth factor I (rhIFG-I), recombinant human growth hormone (rhGH) and recombinant humanized monoclonal antibody (rhuMAb)) co-lyophilized with either manitol, sucrose or trehalose. The analysis of the isotherms, in terms of the BET monolayer value of the mixtures compared to that expected based on the contributions of the pure protein and additive, showed that manitol had no effect on the hydration properties of the three proteins considered. On the other hand, the monolayer of recombinant human growth hormone-sucrose/ trehalose samples appeared to be lower than expected. This observation was taken to represent an interaction between the proteins and the sugars which reduced the availability of water binding sites. In addition, the same study reported that the presence of the protein inhibits sucrose and trehalose hydration-induced crystallization. This latter effect was further investigated

on rhIFG-I/rhGH/rhuMAb-sucrose and crystallization was found to be retarded to a different extent by the three proteins considered. Furthermore, retardation of crystal formation was shown to be due to the interaction between the protein and sucrose in a co-lyophilised sample since inhibition of crystallization was not observed when studying mechanically mixed protein and sucrose. Tzannis and Prestrelski (Tzannis and Prestrelski, 1999) studied spray-dried trypsinogen-sucrose formulations. In agreement with the study by Costantino and colleagues (Costantino et al., 1998) mentioned above the monolayer value of the mixtures was observed to decrease with sugar content, crystallization of sucrose was inhibited and the hysteresis loop decreased with increasing sucrose concentration, indicating the decreased plasticization of amorphous solid by water. The authors concluded that these observed effects were due to interaction between protein and sugar which was likely to be through hydrogen-bonding. A saturation of the effects appeared to coincide with the loss of protein activity.

V

CARBOHYDRATES

1 GENERAL FEATURES/DESCRIPTION

Carbohydrates comprise one of the major groups of natural compounds. They form the major constituents of the shells of insects and the supporting tissue of plants, and are also present in the cell walls of plants and bacteria. In addition, they are utilised as food by man, other animals or micro-organisms, being the central source for the supply of energy needed for mechanical work and chemical reactions.

Carbohydrates were thought originally to be formed solely by carbon, hydrogen and oxygen atoms, thus their name. However, nowadays, it is well known that other elements and functional groups can be included in the carbohydrate structure, yet their main structure can be described by the general formula $C_x(H_2O)_y$. All of them having C=O and -OH groups.

Classification of carbohydrates can be performed on the grounds of different criteria; if the size of the base carbon chain is considered, they may be called *trioses* (three carbons), *tetroses* (four carbons), and so on. Depending on the location of the C=O groups, they are named either *aldeoses* or *ketoses*. Stereochemistry divides carbohydrates which have at least one asymmetric carbon[1] in $D-$ or $L-$ isomers. Stereo-isomers have the same order and types of bonds but different spatial arrangement, hence a pair of isomers $D-$, $L-$ are mirror images that can't be overlapped. $D-$ and $L-$ can be distinguished by studying how they rotate polarized light, whilst $D-$ isomers rotate light to the right (*dextrorotatory*) L- isomers rotate light to the left (*levorotatory*).

Carbohydrates can also be classified according to the number of basic units they contain; traditionally, they are considered in three groups, mono-, oligo- and polysaccharides. Monosaccharides are the basic units, the most simple which cannot

[1] i.e. a carbon with four different 'things' attached to it

be hydrolysed to smaller molecules and from which the other two groups are obtained by formation of a *glycosidic linkage*, i.e. a covalent bond formed through a dehydration reaction between two monosaccharides, and composed of an oxygen atom linking two carbon atoms [2]

Sugars also form ring structures which, for many of them is the common form. The process by which the ring form is achieved is called *cyclization.*

based on the position of the c-1 OH

2 TREHALOSE

α, α-Trehalose (α-D- glucopyranosyl$(1-1)$-α-D-glucopyranoside) also called mushroom sugar or mycose, is a unique, naturally occurring disaccharide of glucose. It was probably first isolated from ergot of rye, in 1832 by Wiggers (see(Birch, 1963) for references). It is widespread in nature and commonly found in yeast, fungi, bacteria, the blood of insects and in seaweed and lichens as well as in higher plants such us the resurrection plant (Selaginella Lepidophylla) [3]. Although its main functions are related with transport and storage of carbohydrates, it has also been found that many organism that contain trehalose can remain in a state of suspended animation in harsh condition such as at temperatures of more that 50^oC in the day time when there is insufficient moisture for survival. Once the water content of their tissues is replenished, they can function normally again. Furthermore, it has been reported that the integrity and functions of its membranes and proteins are maintained through freeze-drying processes when they are treated together with trehalose (Crowe et al., 1983; Prestrelski et al., 1993a).

Trehalose has the empirical formula $C_{12}H_{22}O_{11}$, and a molecular weight of 342.23. α, α-Trehalose is relatively inert compared to many carbohydrates due to its non reducing character/nature (two anomeric carbons are joint through the glycisidic bond) and stable glycosidic linkage (Schebor et al., 1999).

[2] a glycosidic bond is formed between the hydroxyl group on the anomeric carbon atom of one monosaccharide and any hydroxyl group on another monosaccharide.

[3] Selaginellae (Selaginella) The genus Selaginella is widespread in the moist tropics, and the temperate rain forests. In contrast, some Selaginella species are also found in arid lands, where they successfully desiccate. Most plants are tightly coiled when desiccated. One Selaginella is sold as the resurrection plant because it can be revived when watered, photosynthesize for a bit, and when water is unavailable, desiccate for several years until revived again.

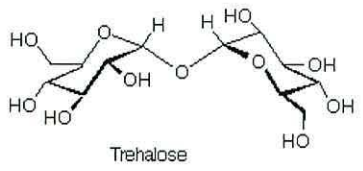


Figure V.1: Three dimensional structure of Trehalose

In the solid state, trehalose is commonly found as a dihydrate crystal although some researches have reported the existence of anhydrous forms which may be obtained through different dehydration treatments(Taylor et al., 1998; Sussich et al., 1998; Sussich et al., 1999a; Sussich et al., 1999b).

Anhydrous trehalose a glass transition temperature has been determined to be approximately 105^o although this value vary slightly from 95 to 120, depending on technique employed and residual moisture present in the sample.

3 Sucrose

The best known sugar is sucrose ($\alpha - D$-Glucopyranosyl-$(1-2)\beta - D$-fructofuranoside), also named after the natural sources it comes form, cane sugar, beet sugar or maple sugar. Sucrose is a disaccharide widely distributed through out the plant world and is the main soluble carbohydrate reserve and energy source as well as being an important dietary material for humans.

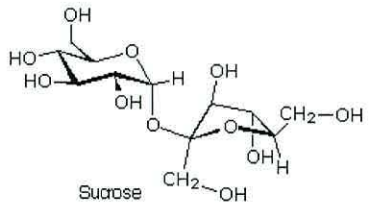


Figure V.2: Three dimensional structure of sucrose

Sucrose has the same empirical formula (i.e.same molecular weight) as trehalose but differs from it in structure (see figures V.3 and V.2). In is found in nature as a white

anhydrous crystalline solid and it is, as trehalose, a non-reducing sugar but with a less stable glycosidic bond (Schebor et al., 1999).

The glass transition temperature has been measured to be between 65 and 80. The variance of reported dry glass transition temperatures for both sucrose and trehalose is most likely due to considerable plasticization effect small amounts of moisture and on the technique used.

4 2-HYDROXYPROPIL-β-CYCLODEXTRIN

Cyclodextrins (CDs) are cyclic oligosaccharides formed by the enzymatic modification of starch.

Structurally, CDs consist of 6, 7, or 8 (α, β, and γ respectively) D-glucopyranosyl units connected by alpha-(1,4) glycosidic linkages. The most stable three dimensional molecular configuration for these non-reducing cyclic oligosaccharides takes the form of a toroid with the upper (larger) and lower (smaller) opening of the toroid presenting secondary and primary hydroxyl groups, respectively, to the solvent environment. The cyclodextrin structure provides a molecule with an exterior hydrophilic surface and interior hydrophobic cavity. On account of their relatively hydrophobic interiors, CDs have the ability to form inclusion complexes with a wide range of substrates in aqueous solution. This property of CDs has led to their application in areas as varied as enzyme mimics, catalysis and the encapsulation of drugs.

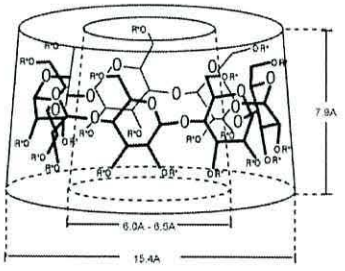


Figure V.3: Cyclodextrin structure

The hydrophilic surface generates good water solubility for the cyclodextrin and the hydrophobic cavity provides a favorable environment in which 'to fit' the drug molecule.

This association isolates the drug from the aqueous solvent and may increase the drug's water solubility and stability

Many different chemical moieties may be introduced into the CDs molecules by reaction with the hydroxyl groups lining the upper and lower ridges of the toroid; for example, methyl, hydroxypropyl, carboxymethyl, and acetyl. 2-Hydroxypropyl-β-cyclodextrin (hp-β-cd) is one of the most used chemically modified cyclodextrins due, mainly, to its physicochemical properties, i.e. the addition of two hydroxypropyl groups enhances the solubility of β-cyclodextrin, hp-β-cd is non-hygroscopic and does not change the surface tension of water as much as other CDs. Additionally, hp-β-cd has been reported to preserve protein under certain conditions (Branchu et al., 1999a) although it may be found in the published literature that hp-β-cd has deleterious effects on protein stability.

VI

Dielectric relaxation spectroscopy

Dielectric studies have been traditionally of interest to physicist and electrical engineers. However it has been shown that the dielectric properties of biomaterials are of great importance and that dielectric spectroscopy can be used to aid the understanding of biological mechanisms and function.

This chapter presents a brief description of the dielectric theory as well as an introduction to Time Domain Reflectometry, the high frequency dielectric technique employed in this work.

1 Fundamental electrostatic relations

a *Electrostatic interaction*

All forces between atoms and molecules are electrostatic in origin, although the term electrostatic is usually reserved for interactions between charged or dipolar atoms and molecules. The electrostatic interaction between two charged particles is given by Coulombs law which states that

> *the electric field interaction between two charged particles at rest or in very slow relative motion is proportional to their charges and its direction is along the line joining the two charges*

It may be expressed by

$$\mathbf{F} = \frac{qq'}{4\pi\epsilon_o \mathbf{r}^2} \tag{VI.1}$$

where r is the distance between charges q and q', $\mathbf{F}$ is the force acting on either charge and ϵ_o is the vacuum permittivity.

b Electric field

It is usually said that there is an *electric field* in any region where an electric charge experiences a force. The force is due to the presence of other charges in that region. The intensity of the electric field at a certain point is defined as the force per unit charge placed at that point

$$\mathbf{E} = \frac{\mathbf{F}}{q} \tag{VI.2}$$

so the electric field at a distance r from a point charge q is

$$\mathbf{E} = \frac{q}{4\pi\epsilon_o \mathbf{r}^2} \tag{VI.3}$$

when several charges are present the resultant electric field is the vector sum of the electric fields produced by each charge.

c Dipole moment

An electric dipole, μ, consist of two equal and opposite charges $+q$ and $-q$ separated by a distance d. Although in a dipole, the two charges are equal and opposite, giving to a zero net charge, the fact that they are slightly displaced is enough to produce a non vanishing electric field. The dipole moment is defined by

$$\mu = q\mathbf{d} \tag{VI.4}$$

where $\mathbf{d}$ is the vector distance between the negative and the positive charges.

In many substances, like H_2O, the centres of the positive and negative charges of the molecule are slightly separated and thus resemble electric dipoles; in this case the dipole moment is given by the product of the magnitude of separated excess charge Z and the distance d by which it is separated

$$\mu = Z\mathbf{d} \tag{VI.5}$$

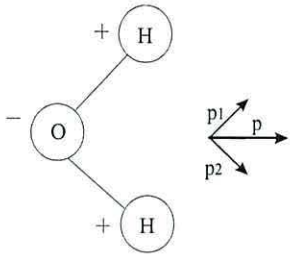


Figure VI.1: Dielectric dipole of a water molecule

2 Electric polarization of matter

In isolated atoms, because of their spherical symmetry, the centre of mass of the electrons coincides with the nucleus. Therefore atoms do not have permanent electric dipole moments. However, when atoms are placed in a electric field, they become polarized, acquiring an *induced electric dipole moment* in the direction of the external field. This dipole moment results from the perturbation of the motion of the electrons produced by the electric field. Molecules may also acquire an induced electric dipole moment in the direction of the field. Therefore, if an insulator is placed in an electric field, its atoms or molecules become electric dipoles oriented in the direction of the applied field. However, many molecules also have a permanent dipole moment. Such molecules are called polar (e.g. HCl, H_2O). In a molecule such as water the two H-O bonds are at angle of 105^o, the electrons try to crowd around the oxygen atom, which becomes slightly negative relative to the H atoms. Each H-O bond thus contributes to the electric dipole moment, whose resultant, because of symmetry, lies along the axis of the molecule and has a value equal to $6.2 \cdot 10^{-30}$ Cm (see figure VI.1).

In the absence of an external field the dipole moments of polar molecules are in general oriented at random and no macroscopic or collective dipole moment is observed. Nevertheless, when a static electric field is applied it tends to orient all the dipoles along the direction of the field (see figure VI.2)

When the dipoles in a piece of matter become aligned either spontaneously or under the action of an external electric field, it is said that the substance is *polarized.* Different polarization mechanisms will be discussed in more detail below (section 3).

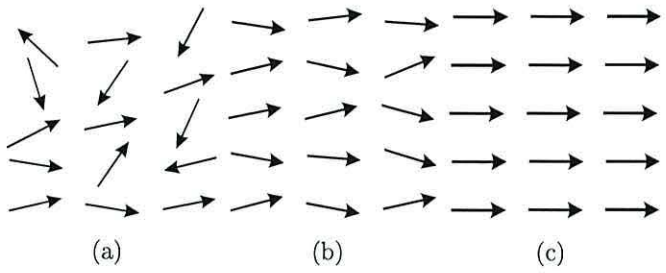


Figure VI.2: Orientation of electric dipoles in an electric field. (a) Thermal motion orients the dipoles at random. (b) An electric field produces a partial orientation. (c) At very low temperature the orientation is complete

a *The polarization vector*

A non conducting medium that can be polarized by an electric field is called a *dielectric.*The polarization gives rise to a net positive charge on one side of the piece of matter and a net negative charge on the opposite side. The molecules (or atoms) of the dielectric become electric dipoles oriented in the direction of the electric field, because of either distortion of the electronic motion or the orientation of the permanent dipoles.

The polarization $\mathbf{P}$ of a material is a vector defined as the dipole moment of the medium per unit volume and is proportional to the applied electric field. That is, if $\mathbf{p}$ is the dipole moment induced in each atom or molecule and N is the number of atoms or molecules per unit volume the polarization is given by

$$\mathbf{P} = N\mathbf{p} \tag{VI.6}$$

In terms of the electric field the polarization is usually written as,

$$\mathbf{P} = \epsilon_o \chi_e \mathbf{E} \tag{VI.7}$$

where χ_e is the electric susceptibility of the material. χ_e describes the response of a medium to the action of an external field, it is related to the properties of the atoms and molecules of the medium. The induced electric susceptibility due to the distortion of electronic motion in atoms or molecules is temperature-independent, since it is an effect related to the electronic structure of the atoms or molecules and not their thermal motion. On the other hand, the electric susceptibility due to the orientation of polar molecules varies inversely with temperature because, as the temperature increases, the

thermal disorder more effectively offsets the ordering effect of the electric field, resulting in a smaller polarization of the substance.

b Dielectric displacement. Permittivity.

Often the concept of the dielectric displacement, **D** is introduced to better understand the response of a material to an electric field, which in turn introduces the dielectric permittivity.

Consider a dielectric material inside a capacitor, i.e. two conducting parallel plates. Let $+\sigma_{free}$ and $-\sigma_{free}$ be the surface change density in each plate, these charges will produce an electric field that polarizes the dielectric. The polarization charges having an opposite sign to that on the plates. Given that **P** is the polarization in the dielectric, the surface charge density on the 'faces' of the dielectric are $\sigma_{pol} = -P$ or $\sigma_{pol} = +P$. Therefore the effective net charge on the plates surface will be

$$\sigma = \sigma_{free} + \sigma_{pol} \tag{VI.8}$$

or

$$\sigma = \sigma_{free} - P \tag{VI.9}$$

since the electric field created by a conductor is $E = \sigma/\epsilon_o$ then electric field in the dielectric is given by

$$E = \frac{1}{\epsilon_o}(\sigma_{free} - P) \tag{VI.10}$$

thus it is possible to give an expression for the surface charge density on the plates

$$\sigma_{free} = \epsilon_o E + P \tag{VI.11}$$

from this expression the electric displacement is defined as a vector

$$\mathbf{D} = \epsilon_o \mathbf{E} + \mathbf{P} \tag{VI.12}$$

Dielectric displacement is charge per unit surface area. Dielectric displacement is charge displacement without charge transport.

Combining the expression for the polarization and the dielectric displacement

$$\mathbf{D} = \epsilon_o \mathbf{E} + \epsilon_o \chi_e \mathbf{E} = \epsilon_o \mathbf{E}(1 + \chi_e) = \epsilon \mathbf{E} \tag{VI.13}$$

where the coefficient

$$\epsilon = \epsilon_o(1 + \chi_e) \tag{VI.14}$$

is called the permittivity of the medium. The relative permittivity is defined as $\epsilon_r = \epsilon/\epsilon_o$ also called the *dielectric constant.*

The dielectric constant is a measure of the extent to which the electric charge distribution in the material is distorted or polarized by the application of an electric field.

c *Dielectric screening and polarization.*

An atom or molecule in an externally imposed electric field develops a nonzero net dipole moment; if the molecule already has a nonzero dipole moment, the field increases it further. Thus, the atoms or molecules in a material develop dipole moments when the material is exposed to an electrical field. The field generated by these induced dipoles opposes the inducing field. As a consequence, the overall field is weakened, as expected for a dielectric medium.

Figure VI.3 illustrates how a dielectric medium weakens the field due to a positive charge. The dipoles induced in the material are aligned with the inducing field. The charges at the heads and tails of the induced dipoles cancel each other except at the surface of the inducing charge. This leaves a net surface charge that produces a field opposite to that of the inducing charge.

The electric field produced by a point charge embedded in a dielectric is given by,

$$\mathbf{E} = \frac{q}{4\pi\epsilon_0\epsilon_r \mathbf{r}^2} \tag{VI.15}$$

and the electrostatic interaction between two charged particles in a dielectric is still given by the Coulomb's but it will weakened relative to that for the same charges in vacuum by a factor ϵ_r, the relative permittivity of the dielectric material.

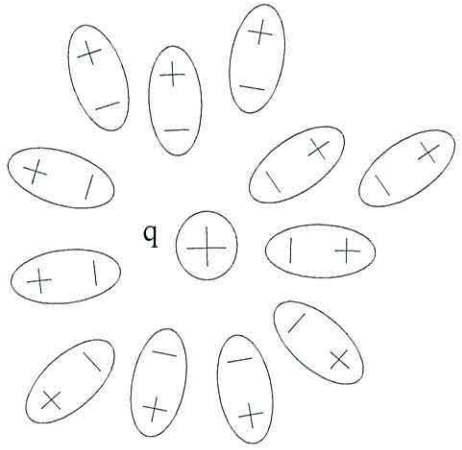


Figure VI.3: Orientation of polar molecules by a charge

It may be expressed by

$$\mathbf{F} = \frac{qq'}{4\pi\epsilon_o\epsilon_r\mathbf{r}^2} \tag{VI.16}$$

where r is the distance between charges q and q', $\mathbf{F}$ is the force acting on either charge and ϵ_o is the vacuum permittivity.

3 Polarization processes.

There are three main ways by which an atom or a molecule (a material) can develop a dipole moment in response to an inducing field: electronic, atomic and orientational polarization. In addition to the polarization mechanism mentioned above, other polarization processes need to be considered, such as *ionic polarization* and when dealing with heterogeneous systems composed of materials of different dielectric properties (e.g. solution containing biological materials), *Maxwell-Wagner interfacial polarization.*

a *Electronic and atomic polarization*

Electronic polarization occurs when the inducing field shifts the positively charged nucleus of an atom in the direction of the inducing field and the negatively charged electrons in the opposite direction (Figure VI.4, left), creating a dipole. Every atom can be polarized in this way. Electronic polarization rises linearly with the inducing field up to very high field strengths.

Atomic polarization takes into account the movement of ions of different charges relative to each other within the crystal lattice on the application of an external field.

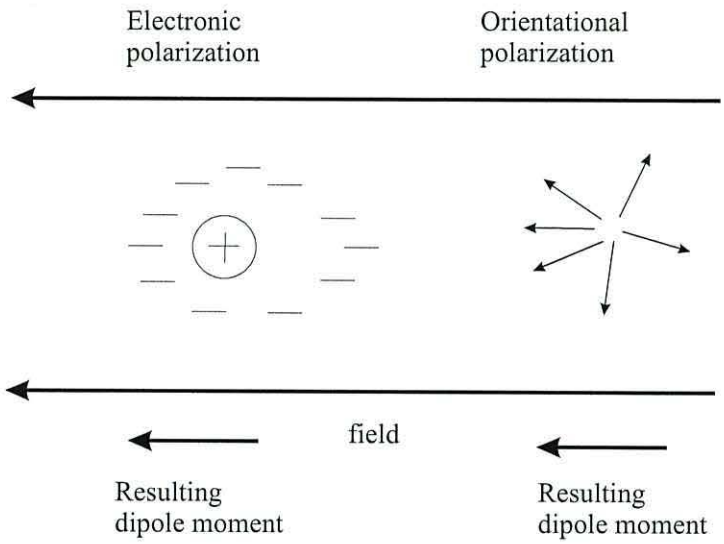


Figure VI.4: Left: Electric field displaces the nucleus of the atom with respect to its electron cloud, creating a nonzero dipole moment. Right: Electric field biasing of a thermally randomized polar molecule to form a non-zero time-averaged dipole moment.

b *Orientational polarization*

Orientational polarization is limited to molecules that have significant permanent dipole moments; i.e., molecules that have a dipole moment even in the absence of an inducing field. Water is a good example of such a molecule. In the absence of an electric field, a water molecule in liquid water tumbles randomly due to thermal motion. As a consequence, its time-averaged dipole moment is zero. (Remember that the dipole moment is a vector). However, in the presence of an electrical field, the tumbling of the molecule is biased in the direction of the field (Figure VI.4, right), leading to a nonzero time-averaged dipole moment along the field direction.

The average dipole moment of a tumbling dipole in an electric field is given by the Langevin function (for further details see next section on the local electric field). This function is linear for weak fields but saturates as the field becomes very strong: once a dipole is fully aligned with the field, its average dipole moment can increase no more. Note that the work of aligning an isolated, thermally agitated dipole is purely entropic

because the internal energy of the system does not depend upon the orientation of the dipole.

c Induced polarization

Maxwell-Wagner polarization Interfacial polarization arises as a result of the difference in dielectric properties throughout a heterogeneous system. Across the interface between materials there will be a non-uniform distribution of free electronic charges, in order to maintain current continuity across the whole system, a charge carrier discontinuity will give rise to the formation of charged double layers at the interface between the dissimilar materials. Such a build-up of charge gives rise to an induced frequency dependent dielectric relaxation.

Ionic polarization *Ionic polarization* is caused by ionic mobility. A charged molecule will attract counter-charges in order to maintain its electrical neutrality. It is often the case that the counter-charges are ions of the opposite polarity to the charged molecule. Such counter-ions will form an 'atmosphere' of charged particles around the molecule, creating an electric double layer. The counter ions associated with the double layer will experience electrostatic attraction to the charged particle. As a result these counter ions could be thought as located at the bottom of potential energy wells. On the application of an external electric field the counter ions my acquire enough energy to overcome the barrier and be free to move to a new site. It can be seen that the application of an external electric field to a system containing ionic charges can lead to a process of activated hopping of ionic charges along a charged surface, this in turn can lead to an induced polarization resulting from the displacement of the ionic charges by an externally applied field.

d Polarization and local electric field

The fact that a dielectric medium weakens an applied field has been discussed in a previous section. The field that is weakened is actually the average field, where the averaging takes place over the smallest volumes that still contain enough induced dipoles to make the averaging meaningful. The local fields in a material are strong and are complex in form.

It is also necessary to bear in mind that the calculation of the microscopic polarization or molecular dipole moment in terms of macroscopic polarization is not simply a matter of dividing by the molecular volume: the actual field experienced by the molecule is not in general the same as the applied macroscopic field **E**. Alternatively to equation VI.7, polarization can be expressed as

$$\mathbf{P} = N\alpha\mathbf{E_i} \tag{VI.17}$$

where N is the number of atoms per unit volume, α is the polarizability of atom and $\mathbf{E_i}$ the internal field acting on a particular molecule. So the electric susceptibility, χ can be rewritten as

$$\chi = \frac{N\alpha}{\epsilon_o}\frac{\mathbf{E_i}}{\mathbf{E}} \tag{VI.18}$$

It is possible to calculate the internal field $\mathbf{E_i}$ considering a microscopic spherical region surrounding the molecule, but large compared with it. The interaction between the molecular dipoles within the sphere can be calculated. Lorentz showed that for a cubic lattice of polarizable atoms the dipoles inside the sphere produce zero field. The total inner field therefore arises from the external contribution, which is the electric field inside the spherical region due to all sources except the polarization inside this region. He showed that the internal field could be approximated by

$$\mathbf{E_i} = \mathbf{E} + \frac{\mathbf{P}}{3\epsilon_o} = \frac{\epsilon + 2}{3}\mathbf{E} \tag{VI.19}$$

For non-polar molecules equation VI.7 should hold (permanent dipoles not present in atoms) so the combination of equations VI.18 and VI.19 results in

$$\frac{\epsilon - 1}{\epsilon + 2} = \frac{\alpha}{3}N \tag{VI.20}$$

which is the Clausius-Mossotti formula for the dielectric constant.

The polarization of a system or rigid polar molecules was first calculated by Debye (Debye, 1929). The average moment per dipole in the direction of the applied field is given by

$$\mu_{\mathbf{a}} = \mu\langle cos\theta\rangle \tag{VI.21}$$

where $\langle cos\theta\rangle$ denotes the mean value of the cosine of the angle of inclination of a dipole to the applied field. So then the polarization can be written as

$$\mathbf{P} = N\mu\langle cos\theta\rangle \tag{VI.22}$$

The dipole moments will be distributed about an applied field in accordance with Boltzmann's law.

The relative probability of finding a dipole oriented in an element of solid angle $d\theta$, is given by with the Boltzmann distribution law [1], from which Debye deduced that

$$\langle cos\theta\rangle = \coth\frac{\mu E_i}{kT} - \frac{kT}{\mu E_i} = L(\mu E_i/kT) \tag{VI.23}$$

which defines the Langevin function, which approximates to $\mu E_i/3kT$ when $\mu E_i/kT \ll 1$. Under these conditions, of applied fields much smaller than 10^4 Vm^{-1}, the apparent average moment of the molecule is proportional to the field intensity

$$\mu_a = \frac{\mu^2 E_i}{3kT} \tag{VI.24}$$

and the polarization is then given by

$$P = \frac{N\mu^2 E_i}{3kT} \tag{VI.25}$$

Combining equations VI.18, VI.19 and VI.25 the Debye expression for the static dielectric constant is obtained

$$\frac{\epsilon - 1}{\epsilon + 2} = \frac{N}{3}(\alpha + \mu^2/3kT) \tag{VI.26}$$

[1]The Maxwell-Boltzmann distribution is the classical distribution function for distribution of an amount of energy between identical but distinguishable particles. Besides the presumption of distinguishability, classical statistical physics postulates further that: There is no restriction on the number of particles which can occupy a given state. At thermal equilibrium, the distribution of particles among the available energy states will take the most probable distribution consistent with the total available energy and total number of particles. Every specific state of the system has equal probability.

Onsager developed a model for liquids in which a given molecule is represented as point dipole in a spherical cavity of molecular size. This representation yields the following expression for the dielectric constant

$$\frac{(\epsilon - n^2)(\epsilon + n^2)}{\epsilon(n^2 + 2)^2} = \frac{N\mu^2}{9kT} \tag{VI.27}$$

where n is the refractive index.

This results is superior to that of Debye in predicting the static dielectric constants of dense fluids. However, both expressions predict inner electric fields which increase without limit on increasing E. Both theories are also inadequate in that they take no account of the local forces between neighbour dipoles.

4 Complex dielectric permittivity

An alternating field of the appropriate frequency applied to a polar dielectric gives rise to a dielectric dispersion: the characteristic orientational motions of the dipoles result in a frequency variation of the dielectric constant, and the appearance of a dielectric loss over a broad band of frequencies. If the applied alternating field has sufficiently low frequency, then the dipoles are able to follow the changing electric field and the re-orientation of the dipoles transmits electromagnetic energy through the medium. The movement of the charges associated with each dipole produces a current in the dielectric which is termed the *displacement current*. At low frequencies when there is no lag between the orientation of the dipoles and the variation of the applied field, the displacement current is 90^o out of phase with the voltage producing the electric field. At this stage there is no component of the displacement current in phase with the voltage so the electromagnetic energy is transmitted without loss. As the frequency is increased, the molecular forces impeding the dipole orientation dominate, and the dipoles become unable to follow the changes. As a consequence of this, the displacement current acquires a component which is in phase with the voltage producing the electric field. As a result, Joule heating of the dielectric occurs and this phenomena is known as the *dielectric loss*.

In addition, since dipoles are becoming unable to follow the changing electric field, there is a fall in the amount of charge stored by the dielectric as the frequency increases and this is reflected as a decrease in the permittivity. As the frequency increases there

will be a moment at which the dipoles are completely unable to respond to the dielectric field. By this stage, the displacement current becomes constant resulting in a return to the zero loss condition and the charge storage capability becomes equivalent to that of a non polar dielectric.

This phenomenon is described by a complex representation of the dielectric constant:

$$\epsilon^* = \epsilon^{'} - i\epsilon^{''} \tag{VI.28}$$

The real part, $\epsilon^{'}$ represents the dielectric constant or relative permittivity discussed in the previous sections, whilst the imaginary part $\epsilon^{''}$ is known as the dielectric loss.

Describing the polarization process of a material exposed to an time dependent field of the form

$$E = E_o e^{i\omega t} \tag{VI.29}$$

it is possible to demonstrate that such as process is frequency dependent and find the frequency dependence of the complex permittivity.

Consider a step voltage applied to a dielectric material. As a result the dipoles within the dielectric will tend to align with the direction of the electric field. However, the dipolar response will not be instantaneous but time dependent at a rate proportional to the difference between the maximum and initial polarization; the final value being equal to $\chi\mathbf{E}$ (where χ is the electric susceptibility of the material). The process can be described by the following equation:

$$\frac{d\mathbf{P}}{dt} = \frac{1}{\tau}(\chi\mathbf{E} - \mathbf{P}) \tag{VI.30}$$

where τ is a time constant of proportionality. Solving the previous equation (eq. VI.30) using the substitution $e^{(t/\tau)}$ gives:

$$\mathbf{P}e^{(t/\tau)} = \chi\mathbf{E}e^{(t/\tau)} + C \tag{VI.31}$$

where C is a constant of integration. If the applied step voltage is zero at $t = 0$, and since $\mathbf{P}$ is zero at $t = 0$ then equation VI.31 gives the following expression for the time dependent polarization:

$$\mathbf{P} = \chi[1 - e^{(-t/\tau)}]\mathbf{E} \tag{VI.32}$$

Solving the equation VI.30 for electric field of the form described by equation VI.29 the polarization is given by

$$\mathbf{P} = \frac{\chi \mathbf{E}}{1 - i\omega\tau} \tag{VI.33}$$

Now consider a system in which the total polarization consist of two parts, P_1 and P_2. Assume P_1 arises from atomic and electronic displacements and P_2 arises from the much slower process of dipolar reorientation. For the frequency range of interest P_1 can be assumed to relax instantaneously and that the part P_2 lags behind the electric field, $\mathbf{E}$, so that any instant P_2 approaches to its final value of $\chi_2\mathbf{E}$ at a rate proportional to $(\chi_2\mathbf{E} - P_2)$ so that

$$\frac{d\mathbf{P_2}}{dt} = \frac{1}{\tau}(\chi\mathbf{E} - \mathbf{P_2}) \tag{VI.34}$$

therefore,

$$\mathbf{P} = \mathbf{P_1} + \mathbf{P_2} = (\chi_1 + \frac{\chi_2}{1 - i\omega\tau})\mathbf{E} \tag{VI.35}$$

Combining equations VI.7 and VI.35 leads to the expression for the complex permittivity, ϵ^*

$$\epsilon^* = \epsilon_\infty + \frac{\epsilon_s - \epsilon_\infty}{1 + i\omega\tau} \tag{VI.36}$$

so the real and imaginary parts are

$$\epsilon' = \epsilon_\infty + \frac{\epsilon_s - \epsilon_\infty}{1 + \omega^2\tau^2} \tag{VI.37}$$

and

$$\epsilon'' = \frac{\omega\tau(\epsilon_s - \epsilon_\infty)}{1 + \omega^2\tau^2} \tag{VI.38}$$

where ϵ_s is defined as the limiting low frequency permittivity, ϵ_∞ is the permittivity at sufficiently high frequency for the observed polarization to have disappeared (note that

when $\omega = 0$, the complex dielectric constant becomes ϵ_s), and τ is the characteristic relaxation time given by

$$\tau = \frac{1}{2\pi f_r} \tag{VI.39}$$

where f_r is the frequency of the maximum dielectric loss.

It has been shown (Frohilch, 1958) that using the principle of conservation of energy described by the first law of thermodynamics, and considering the energy stored by a dielectric material while in a alternating field, the dielectric loss, ϵ'' is a measure of the loss of energy from the field per cycle. On the other hand, the permittivity, ϵ' relates to the energy stored per cycle of the field. Figure VI.5 shows permittivity and dielectric loss as function of frequency for a system showing a relaxation behaviour resulting in a dielectric dispersion.

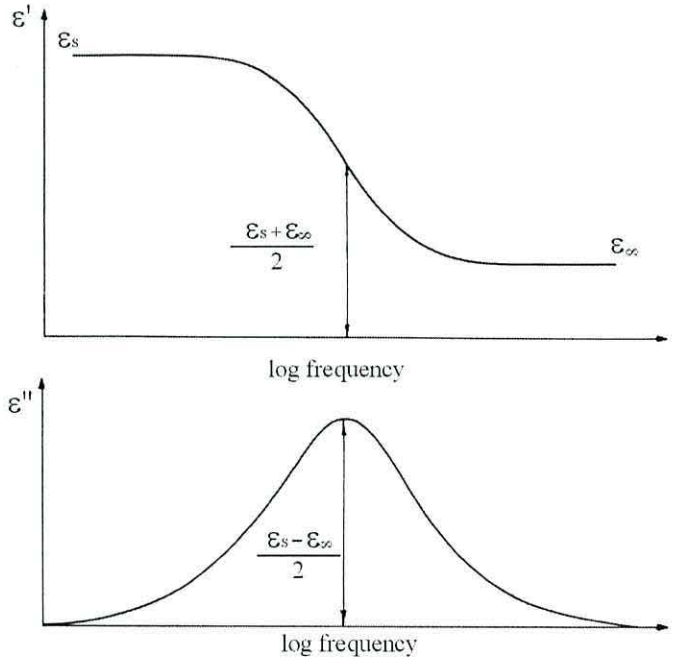


Figure VI.5: Dielectric permittivity and loss

In addition to the frequency plots shown in figure VI.5, dielectric data may be presented as the so-called Cole-Cole plots or complex permittivity plot in which the dielectric loss, ϵ'', is plotted against the permittivity, ϵ'. This plots take the form of a

semi-circumference (see figure VI.6) which results from the rearrangement of equation VI.37 and VI.38 to eliminate the term $\omega\tau^2$

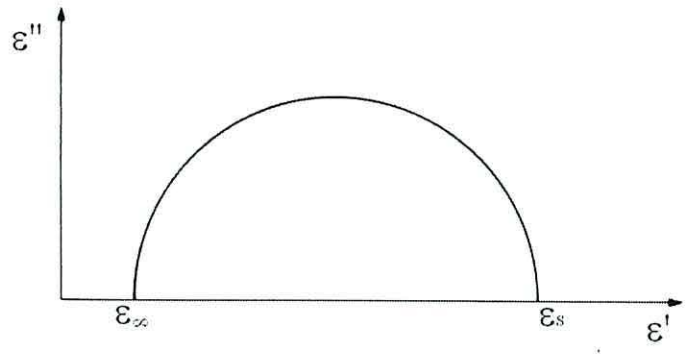


Figure VI.6: Cole-Cole plot

In addition to the relaxation time, an important parameter in dielectric spectroscopy is the dielectric increment, $\Delta\epsilon$, which is defined simple as the difference between the low-frequency and high-frequency relative permittivities,

$$\Delta\epsilon = \epsilon_s - \epsilon_\infty \tag{VI.41}$$

The experimentally-observed dielectric increment is related to the dipole moment of the polar molecules, μ, and the molecular weight, M, according to the equation

$$\Delta\epsilon = \frac{N\mu^2 g c_o}{2\epsilon_o M k T} \tag{VI.42}$$

where ϵ_o is the permittivity in vacuum, N is the Avogadro's number, and c_o is the concentration of the polar molecules. The parameter g, is introduced to account for local molecular interactions and correlation effects which occur, for example, between solute and solvent molecules. Such interactions can be very important in aqueous solutions of biological molecules where there may be extensive hydrogen bonding (e.g. water has a g value of 2.82).

[2]such rearrangement yields an equation of the form

$$r^2 = x^2 + y^2 \tag{VI.40}$$

5 Time domain reflectometry, TDR

The frequency-dependent dielectric constant ϵ' and loss factor, ϵ'' can be measured as a function of frequency over the range from 0.1 mHz to 10 GHz with the help of suitable experimental equipment such as ac bridges, network analysers and frequency response analysers. Measurements can also be carried out in the time domain using a technique known as time domain spectroscopy, which was used in this study in the form of time domain reflectometry, TDR.

Time domain reflectometry is an experimental technique which involves the application of a rectangular step-voltage pulse to a sample and the observing of the resultant time-dependent response of the material after the reflection from a section of co-axial line filled with the sample.

TDR is usually employed to study the frequency range 100 KHz to 10 GHz. The main characteristic that makes this technique so attractive is that a single record is sufficient to give information over a wide frequency range.

In a TDR experiment, a train of fast-rising voltage pulses is applied to a low loss coaxial line and the waveform in the line is observed with a suitable sampling system connected to a sampling oscilloscope. The sample of interest is inserted into the line which is terminated by either a short circuit, open circuit or matched section. The applied voltage pulse and reflected sample response are combined and transformed into the frequency domain to give the desired frequency response. By choice of suitable time windows and sample cells it is possible to cover the mentioned frequency range from 100 KHz to 10 GHz.

The most commonly used TDR methods to obtain dielectric data are the direct method and the precision difference method. Both methods usually employ a sample cell in the form of an open-ended, open-circuit coaxial air line which terminates a section of low-loss co-axial cable. In this case care must be taken to ensure that the appropriate lengths of cable are inserted between the pulse generator and the sampling head and between the sampling head and the sample so that unwanted reflections from these units are not recorded in the time window of interest.

a Direct method

In this method, two waveforms are required, the input step pulse and that of the sample's reflected response. Note, it is not possible to observe the incident step pulse at the sample cell position, the nearest approximation is obtained by acquiring the reflected pulse from an empty test cell with no inner electrode.

It can be shown (Cole, 1975a; Cole, 1975b) from transmission line theory that the basic equation for determining the complex permittivity ϵ^* of the sample is given by

$$\epsilon^*(\omega) = \frac{(v_o - r)c}{i\omega(v_o + r)d} f(z) \tag{VI.43}$$

where v_o and r are the Laplace transforms of the incident waveforms $V(t)$ and reflected waveform $R(t)$, d is the effective length of the sample cell and c is the speed of light. Propagation and multiple reflection effects in the sample and co-axial line are accounted for the function $f(z)$ where

$$f(z) = z \cot z \tag{VI.44}$$

where $z = \sqrt{(\omega d/c)\epsilon^*}$. Equation VI.43 is only an implicit solution for ϵ^* as z contains ϵ^*. However, the solution can approximated by a Taylor series expansion of $z \cot z$ in powers of z^2, this is valid if $z < 1$, therefore if d is chosen so that $z < 1$ then $f(z)$ can be approximated to 1.

Summarizing this method, the incident pulse and the reflected waveform are combined to give $V - R$ and $V + R$ which are then transformed into the frequency domain producing values of $v_o - r$ and $v_o + r$ which can be which can be applied to equation VI.43 to give dielectric permittivity and loss data.

b Precision difference method

The precision difference method is mostly used to investigated dilute solutions[3]. However, it has also been successfully applied to solid/powder samples. This methods in comparison to the direct methods adds the use of the time domain response of a

[3]This method was initially develop by Cole and co-workers (Cole et al., 1980) and Nakamura's group (Nakamura et al., 1982a; Nakamura et al., 1982b) for non conducting solution and by Bone (Bone, 1988) for the measurement of aqueous conducting solutions.

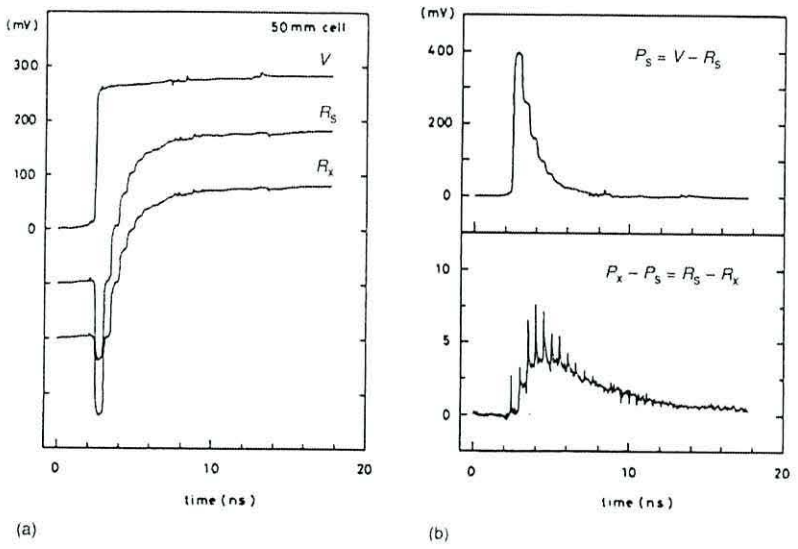


Figure VI.7: Time domain signals of the input signal, V, the reflected reference, R_r and the reflected sample signal, R_s

reference dielectric whose dielectric properties are known over the frequency range of interest.

For non-conducting samples, the reflected waveform of the reference dielectric,R_r, that from the sample R_x and the incident pulse V can be combined to give a very accurate measurement of small differences between the reference and the sample under investigation. The equation which gives the relation between the complex dielectric constant of the reference and sample is

$$\frac{\epsilon_x^*}{\epsilon_r^*} = \frac{(v - r_r) + (r_r - r_x)}{(v - r_r) - [\frac{i\omega d\epsilon_r^*(r_r - r_x)}{cf(z_r)}]} \tag{VI.45}$$

where ϵ_r^* is the complex dielectric constant of the reference dielectric and v, r_r, r_x are the Laplace transform of V, R_r and R_x respectively [4].

A simple summation can be used to obtain the Laplace transform of the given waveforms $F(t)$,

$$f(w) = \Delta \sum_{n=0}^{N} exp(-i\omega n\Delta)F(n\Delta) \tag{VI.46}$$

[4]For conductive samples as as solution of biological materials, additional terms are required to take into account of the effective d.c. conductivity of the solutions.

where Δ is the time interval between data points and N the number of terms , chosen so that aliasing and truncation errors are minimised. The Shannon sampling theorem states that aliasing and truncation errors are minimised if $f(w)$ has no Fourier components with frequencies greater than $1/2\Delta$.

If the applied pulse used has a rise time, T_r, then any high frequency component of the pulse will be reduce approximately to zero when:

$$f > \frac{1}{T_r} \tag{VI.47}$$

e.g. a pulse with a time rise of 50 ps would transform to zero at 20 GHz, so the maximum upper frequency may be limited to 10 GHz in this case to allow for adequate frequency content to be obtained form the pulse.

The length of the time window, indicated by NΔ, should extend over a long enough period to ensure that the difference waveform is adequately recorded. In general, NΔ = 4 to 5τ, is found to record enough of the waveform. Mathematical techniques can be utilised to fit an exponential tail to account for any part (e.g the last 10% or less) of the waveform that was not recorded by the chosen time window.

Equation VI.46 is suitable for non-conducting samples where $V - R$ approaches zero as t$\rightarrow \infty$. However for conducting samples, $V - R$ tend to finite value when t$\rightarrow \infty$ and equation VI.46 needs to be modified to avoid divergence of the transform as $(i\omega)^{-1}$ in the limit $\omega \rightarrow 0$. Transformations from the time into the frequency domain are then carried out using the Samulon modification of the Shannon sampling theorem,

$$i\omega f(\omega) = (\theta/\sin\theta)exp(i\theta)\sum_{n=0}^{N} exp(-i\omega n\Delta)[F(n\Delta) - F(n\Delta - \Delta)] \tag{VI.48}$$

where $\theta = \omega\Delta/2$ and Δ is the time interval between consecutive data points. It is usually arranged that the frequency range of interest is such that $\theta/\sin\theta = 1$ and $exp(i\theta) \simeq 1 + \theta$, in which case equation VI.48 can be rewritten as

$$i\omega f(\omega) = [1 + \theta]\sum_{n=0}^{N}[\cos(\omega n\Delta)\sin(\omega n\Delta)][F(n\Delta) - F(n\Delta - \Delta)] \tag{VI.49}$$

The single largest source of systematic error in all TDR methods is time shift errors. These arise mainly from drift of the tunnel diode pulse relative to the scanning sweep

and, if uncorrected, result in a time mismatch between individual traces. Errors of this time can become serious at high frequencies and with out time referencing, can produce erroneous permittivity data above 100 MHz. Various methods have been employed to reduce timing errors. The most commonly used time referencing technique involves extrapolation of the initially rising portions of the incident and reflected waveforms to the point where they cross the baseline. This then gives a common time reference point which can be used to align the appropriate waveforms. A method which produces better results is one where a marker impulse is used as a timing reference. The incident pulse is split into two, one pulse being used to to characterize the sample response while the other carries the marker. Very accurate time referencing can be achieved using this method but an oscilloscope with two synchronized sampling channels is required.

6 Dielectric spectroscopy and biological materials

A large number of works can be found in the literature which deal with the dielectric and conduction properties of bio-materials. In certain cases, dielectric measurements have been used to study biologically important structures such as the phospholipids in membranes (Shepherd and Bldt, 1978) and in many others it has contributed to a better understanding of the properties of water in biological materials (Pethig and Kell, 1987).

Amino acids The dielectric properties of amino acids are important to characterize, since they are the basic structural units of proteins. The general structure of amino acids was previously shown (chapter III, section 1). However, the charge distribution was not considered and, depending on the pH of the environment, amino acids can take different ionized states, as described by figure VI.8.

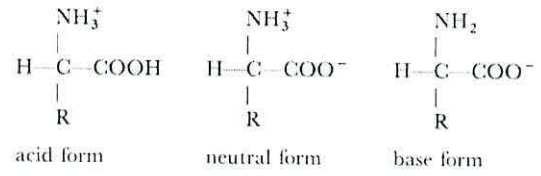


Figure VI.8: The pH dependent ionic forms of basic structure of the amino acids

Each amino acid has a different side chain, R, which has a particular charge distribution, therefore, according to charge asymmetry, amino acids can be classified as non-polar (e.g. glycine or alanine) or polar (e.g. lysine).

Solutions of amino acids generally exhibit two dielectric dispersions in the gigahertz region, one due to the amino acid and the second associated with bulk water. The first dispersion is usually attributed to the rotation of the ionized amino-carboxyl dipole which is a characteristic of the zwitterionic[5] form of the amino acids. The dipole moment of an amino acid can be simply calculated by multiplying the charge and the distance separating the amino and carboxyl groups (0.32 nm). This gives a value of the dipole moment of 15.3 Debye. Although the contribution of the amino carboxyl group to the overall dielectric dispersion is the most dominant, it is important to remember that the carbonyl and amino zwitterions may not be the only charged groups in the amino acid structure. The side chain may also be ionizable, thus, in such cases, the animo acid dipole moment is due to the vector addition of the contributions from both the side chain and the amino-carboxyl dipole. Electrostatic interactions between water molecules and the amino acid molecule may also contribute to the dielectric response of amino acids; by dielectric screening water molecules may lower the effective dipole moment of the amino acids.

Proteins Proteins are formed by the multiple combination of amino acids, which, when they link together by a condensation reaction involving amino and carboxyl groups, form what is called the peptide bond (see chapter III). Although the peptide bond has a polar character it only exhibits a small dipole moment. Nevertheless due to this, the protein can be considered as a string of connected dipoles, which, from a dielectric point of view, makes the protein appear as a conglomeration of interacting dipoles of various magnitudes and alignments. The conformation of the protein and the way in which the individual dipoles are aligned with respect to one another in the three-dimensional structure determines how the dipoles interact. In theory the resultant dipole moment

[5]In general Zwitterionic polymers are macromolecules with oppositely charged groups located on the chain ends of the form

$$R_1^- - [C_x H_y ...]_m - R_1^- \tag{VI.50}$$

where $-[C_x H_y ...]m-$ is a polymer with m repeat units and R_1 and R_2 are functional groups.

of the protein as a whole should be the vector addition of all of the constituent dipoles, see figure VI.9, but in practice, given the magnitude and number of constituent dipoles of a protein, the resultant dipole moment is not as large as expected.

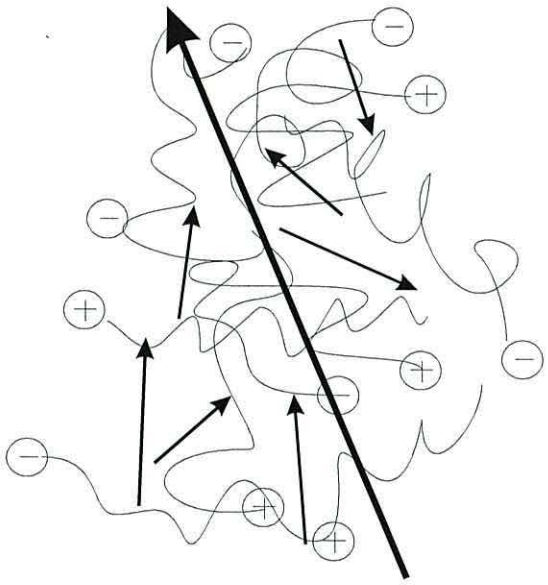


Figure VI.9: Protein dipole moment

The dielectric relaxation characteristics of protein solutions have been extensively studied (for reviews see for example(Grant et al., 1978; Grant, 1982)). Three dispersions have been identified. The *β-dispersion* centered in the low megahertz region, which is thought to be due to the rotation of the protein molecule in the electric field, the *γ-dispersion* which is centred at 17 GHz describes the relaxation of bulk water molecules and the *δ-dispersion* middle dispersion which extends over a wide frequency range and has been suggested is composed of two dispersions, one attributed to the orientation of polar side groups and peptide bonds and the second due to the rotation of water molecules bound to the hydrophilic sites of the protein molecule.

Studies of hydration of protein powders have provided further information on relaxation processes of proteins. The study of these in the solid state allows the measurement of very small dispersions since the two major dispersions seen in the solution spectrum (β and γ) disappear in the solid work.

In the low frequency range (i.e. $2 \cdot 10^{-5}$ to 10^5 Hz), studies on hydrated protein

powders have identify two main dispersions. For instance studies on bovine serum albumin (Eden et al., 1980) gave evidence of two dispersions designated as α and Ω dispersions. The Ω dispersion was seen to increase in magnitude with increasing hydration whereas the α-dispersion showed a hydration dependent relaxation time which decreased with water content and its magnitude was independent of hydration. No definite mechanism was attributed to these two dispersions but it was suggested that the α-dispersion could be related to hopping of charge carries over potential energy barriers whilst the Ω-dispersion was associated with polarisations occurring at the electrode-sample or crystalline interfaces.

Further investigation in the low frequency range (Hawkes and Pethig, 88) identified not only the previously studied α-dispersion but also a further weak dielectric loss named α_2. In this case α-dispersion was related to proton displacement in the protein structure and the α_2-dispersion was explained in terms of vibrational motions of the polypeptide backbone.

The dielectric properties of hydrated protein powders in the frequency range 1 MHz to 20 GHz has been the subject of many studies which have usually interpreted the dispersions observed in the frequency range in terms of 'bound' water molecules forming various hydration layers onto the protein surface.

Measurements at 9.4 GHz made at different temperatures (Kent, 1972) suggested that the dispersion measured at 9.4 GHz was related to bound water molecules to the protein, furthermore authors propose that correlation between the dielectric response and the water sorption indicates that water in both the primary and secondary hydration layers were similarly rotationally hindered. By contrast, further research at 9.9 GHz together with analysis in terms of multilayer sorption (Bone et al., 1977) concluded that only water bound to the proteins in the secondary hydration layers is able to contribute significantly to dielectric losses at this frequency.

Harvey and Hoekstra (Harvey and Hoekstra, 1972) studied the hydration dependency of the dielectric response of lysozyme powders in the frequency range 10 MHz to 10 GHz. At water contents below 30% (g water/g protein) a dispersion was found at 170 MHz and at higher hydrations a new dispersion was observed at about 10 GHz, consequently these dispersions were attributed to two different layers of absorbed water.

Kent and Meyer (Kent and Meyer, 1984) studied the frequency range 0.3-16 GHz on various proteins (e.g haemoglobin and casein). The temperature dependence of dielectric response of proteins in which the water content was high showed evidence of more that one state of multilayer water. Bone and Pethig (Bone and Pethig, 1982; Bone and Pethig, 1985), studying water binding to hydrated proteins including lysozyme, collagen and elastin over the frequency range 10 kHZ to 10 GHz, identified a dielectric dispersion which was also interpreted in terms of two different populations of water, singly and multiply hydrogen bonded to the protein.

Dielectric studies on the hydration properties and structural flexibility of chymotrypsin (Bone, 1987) identified a dispersion centered at 12 MHz which increased with increasing hydration and showed signs of a dispersion at higher frequencies (above 100 MHz) attributed to rotation of water molecules. Investigation of the higher frequency dispersion again identify two categories of protein bound water, the first being irrotationally bound to the protein and the second relatively weakly bound, having a freedom of motion comparable to that of normal bulk water.

Dielectric studies have been extensively performed on protein powders and recently attempts have been made to study the physico-chemical stability of protein based pharmaceutical formulations. Duddu and coworkers(Duddu and Sokoloski, 1995; Duddu and Monte, 1997a; Duddu and Monte, 1997b) carried out a series of works dedicated to the characterization of lyophilized formulations containing a monoclonal antibody, using dielectric spectroscopy in the frequency range from 10 Hz to 100 kHz. For protein-sugar formulations, the maximum of the dissipation factor $\tan\delta$ with temperature, measured at 10 Hz, was identified as being representative of the glass transition, that is the phase transition from the glassy to rubbery state. Furthermore, studies at temperatures below the glass transition temperature, T_g, appeared to indicate that a monoclonal antibody-sucrose formulation was more fragile[6] compared to a antibody-trehalose mixture.

[6]fragile glass is one that with little provocation from thermal excitation reorganize to structure that fluctuate over a variety of orientation

Results

VII

Gravimetric and dielectric measurements on proteins and carbohydrates

1 Introduction

It has been previously stated that the objective of the work presented here is to gain an insight into the protein sugar interactions which may be relevant to biological preservation. In order to do so, gravimetric and dielectric measurement together with enzyme activity assays have been employed to investigate various protein-additive formulations. As model biological structures to be protected, two different proteins which differ in structure and biological function have been considered, these are β-lactoglobulin and trypsin. The studied additives are three different carbohydrates, trehalose, sucrose and 2-β-hydroxypropyl-cyclodextrin.

The present chapter describes the hydration characteristics of the species on their own, a study of which will facilitate the comparison of the protein-additive mixtures' behaviour with that of the separate species. Consequently, the following chapters are dedicated to the protein-additive mixtures. Chapter VIII presents a preliminary study on β-lactoglobulin-trehalose/sucrose mixtures' interactions which set ups the basis of subsequent data analysis. Chapter IX describes a study on trypsin and trypsin-additives mixtures' long term stability and finally, chapter X studies further protein-additive interactions and the effect of long term storage on the latter.

2 Materials and methods

a Sample preparation.

β-lactoglobulin (from Bovine milk, 3x crystallized and lyophilized), trypsin (from Porcine Pancreas, crystallized and lyophilized), sucrose (99.5%) and trehalose (dihydrate, from Saccharomyces cerevisiae) were purchased from Sigma Chemical Company and used without further purification. 2-hydroxypropyl-β-cyclodextrin was obtained from Fluka Chemicals and was also used with no further purification.

β-lactoglobulin samples were prepared either by freeze or spray-drying, whereas trypsin samples were freeze-dried or freeze-dried and heated. Sugar samples[1] were either used as received from the supplier, prepared by freeze-drying, spray-drying or evaporated at room temperature from solution. Trehalose and sucrose were also studied after incubation at 77°C.

Freeze-drying Freeze-drying also referred to as lyophilisation is the most common dry state stabilization method for many labile products. A complete lyophilization cycle results in the removal of water from a given product and includes three phases, freezing, primary drying (sublimation of ice) and secondary drying during which, residual unfrozen water is removed from the product rendering it self stable.

Throughout the work described here, the freeze-drying process was carried out in a Edwards freeze dryer 'modyulo' where the temperature of the cold trap was maintained at $-40°$C, at pressure of approximately 10^{-2} Torr.

Spray-drying Spray-drying is a widely used industrial process involving particle formation and drying.

Spray-drying entails the atomization of the liquid material into a spray of droplets and contacting the droplets with hot air in a drying chamber. The sprays can be produced by either rotary or nozzle atomizers, evaporation of moisture from the droplets

[1]Raman spectroscopy was applied to all sugar samples at room temperature (25°C) and ambient relative humidity (40%). As expected, commercial samples showed well-defined narrow peaks characteristic of crystalline structures while those freeze-dried exhibited broader bands which indicates a largely amorphous structure. Samples produced by evaporation showed spectra similar to that of their crystalline forms. However some subtle differences such as small changes in peak intensities or frequency shifts were observed (see appendix c).

and formation of dry particles is achieved under controlled temperature and airflow conditions.

Spray-dried samples investigated in this work were prepared at the Food Science Department of the University of Leeds. Spray-drying was performed on a Büchi B-191 mini spray dryer at an inlet/outlet temperature of 110 and 80°C degrees respectively. The spray dryer holds the dried material at the outlet temperature until all the solution has been atomised and dried (20 min for this temperature range). Detailed description of the experimental set up, atomizer and drying chamber can be found elsewhere (Murray and Liang, 1999).

Note both freeze and spray-drying procedures usually render glassy amorphous products (Hancock and Zografi, 1993; Angell, 1995a)

Evaporation and heating Sample preparation by evaporation simply consisted of the preparation of a solution of the chosen product and leaving it to lose water at ambient temperature until the product became completely/apparently dry.

Heated samples were obtained after incubating previously freeze-dried samples in the oven at 77°C for different periods of time.

b Experimental techniques.

Hydration isotherms A typical hydration isotherm consists of measuring the water uptake by the dry protein under conditions of controlled relative humidity at a constant temperature. The water uptake of the studied samples was measured for step increases in water partial pressure of approximately 2.5% over the hydration range 0 to 90%. The relative humidity level was controlled to within 0.1% and the temperature was maintained at 25 ± 0.1°C.

A computer controlled Sartorius vacuum microbalance, type 4332 was employed to obtain the hydration isotherms for the protein and saccharides. The experimental set up is depicted in figure VII.1. The microbalance is constructed in a vacuum system which also contains a reservoir of water (connected to the system through a solenoid valve), a pressure gauge and a humidity probe.

Samples of about 10 mg were loaded into one of the microbalance pans and the

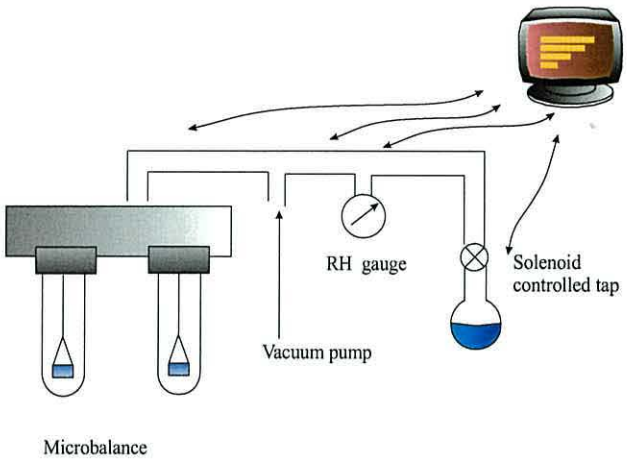


Figure VII.1: Microbalance. Experimental set up

system was evacuated to pressures of 10^{-2} Torr for at least 24 hours until the sample mass had reached a steady value.

Hydration experiments were divided in two different stages, firstly from 0% to about 15% RH and secondly form 15% to 90% RH. During the initial hydration stage, the partial pressure measurements were monitored using an oil manometer and sample mass readings were taken directly from the microbalance display. Once the mass of the sample was observed to be in equilibrium with the water content of the vacuum chamber (the equilibrium criteria being a mass increment of less than 5 μg in an hour) a small amount of water vapour, approximately equivalent to 2.5% RH, was allowed to enter the system. This procedure was repeated until 15% RH was reached. For measurements above this humidity level, data collection and control of the experiment was transferred to a computer. The computer read humidity values from a calibrated Humilab HL24D vacuum humidity probe, monitored the mass variation of the sample, determining when the solenoid valve connected to the water reservoir should be opened/closed and also ran BBC Basic software to process the data and evaluate when the equilibrium criteria was met. Sample mass at a certain relative humidity, m was referenced to the dry mass, m_o to determine the sample water uptake as $m/m_o \times 100\%$.

Dielectric measurements Dielectric measurements in the frequency range 0.1 MHz 1 GHz were performed at 25 $\pm$ 0.1^{o}C, using Time Domain Reflectometry (TDR)

measurement technique. The difference method for non-conductive samples was used to determine the complex permittivity of the samples of hydrated protein as a function of sample hydration level. A discussion of such a method has previously been given in chapter VI, section 5 .

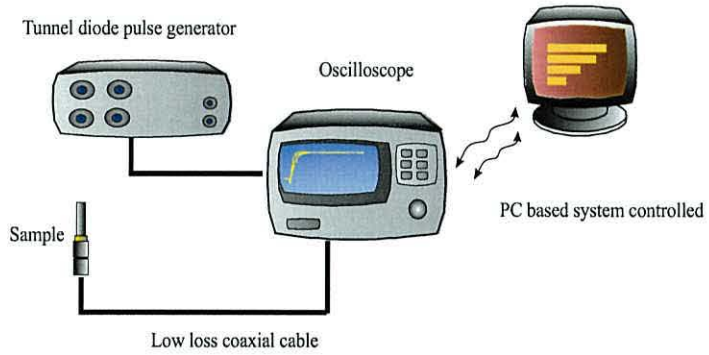


Figure VII.2: TDR. Experimental set up

The experiments were performed using a Hewlett Packard 54120A Digitising Oscilloscope Mainframe with a 54118A Trigger unit. The experimental set up is shown in figure VII.2. The oscilloscope was connected via a GPIB/IEEE-488 to a standard personal computer, such a connection made possible the downloading and storage of waveforms from the oscilloscope to the computer and viceversa. The computer also ran Matlab software to process the waveforms and perform the mathematical analysis required to obtain the dielectric data.

Figure VII.3 shows a diagram of the sample cell. For the measurements reported in this work, the sample cell was formed by a 7 mm diameter outer electrode and an inner electrode of diameter 5 mm and length 5 mm or 20 mm depending on the frequency of the dispersion process studied.

Care was taken to ensure that the choice of time window allowed a sufficiently long time period to ensure an adequate proportion of the reflected wave form was captured. For experiments investigating a dispersion centered about $\sim$ 3 MHz dispersion a 250 ns time window was used to record the reflected voltage waveforms from the sample cell, whereas for the dispersion of frequency approximately of $\sim$ 100 MHz the time window was 7 ns. As discussed previously (chapter VI section 5) the time window should, in general, extend over a period equal to 4 or 5 times that of τ_o. For the relaxation time of

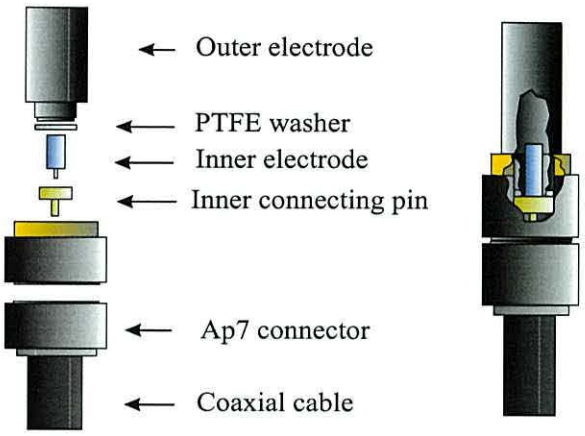


Figure VII.3: Sample cell construction, cell design and inner and outer electrodes

the lower frequency dispersion, this equates to 212 ns and 6 ns for the higher one. The oscilloscope generated an incident pulse with a rise-time, T_r, of 45 ps and the reflected waveforms were recorded using 500 points over the time window employed.

It is likely that the maximum practical frequency of the system was further reduced due to the commonly observed phenomenon of signal drift along the time axis. When very short time windows are used, timing errors in the sampling processes can be significant. Jitter in the observed signals is very rapid, but this can be reduced by smoothing and averaging. Drift is slow, asymmetric and very irregular and therefore cannot be corrected using averaging methods. It has been suggested (for references see (Lee, 1997)) that errors arising from signal drift could be compensated using an experimental set up involving two or more sampling oscilloscopes and a lock-in amplifier. It was not possible to implement such a compensation with our experimental set up, alternatively, to reduced such errors, equipment was left constantly on and manual adjustment of the waveform was performed on the oscilloscope.

Determination of the dielectric increment parameters from the permittivity and loss data was done by fitting the experimental data to a Cole-Cole plot.

Samples were packed into the sample cell and the density calculated. When the longer electrode was used, i.e. when studying the lower frequency dispersion all sample cells contained $\sim$ 0.2 g packed to a density of $\sim 0.50 \cdot 10^6$ g m^{-3}. The use of a shorter inner electrode required less mass; the sample cell contained $\sim$ 0.07 g which

resulted in a density for all samples $\sim 0.74 \cdot 10^6$ g m^{-3} Hydration of the sample was achieved by evacuating the cell to a pressure of $4.8 \cdot 10^{-3}$ Torr and then allowing the atmosphere to equilibrate with saturated salt solutions of known water partial pressure. The samples were left until equilibrium with the saturated salt was achieved, the process taking between 24 and 48 hours. Table VII.1 list the saturated salts used and their corresponding partial pressures.

Saturated salt	$p/p_o\%$
LiBr	6.35
LiCl	11.30
CaBr	16.30
KAc	22.40
MgCl	32.70
K_2CO_3	43.10
$Mg(NO_3)_2$	52.75
NaBr	57.45
SrCl	70.60
Nacl	75.30
KBr	80.80
KCl	84.30

Table VII.1: Saturated salts and their partial pressures

Error assessment

The reproducibility of the hydration isotherms was assessed characterizing 10 replicates of a given sample and studying the standard deviation at certain relative humidities. Error bars have only been plotted for the first isotherm presented here, see figure VII.4, in subsequent graphs the error bars have been omitted for clarity.

Similarly, the reproducibility of the estimated dielectric increment (i.e values of ϵ_s and ϵ_∞) was determined by collecting several reflected waveforms from the same sample at different relative humidities and analyzing the data as described in the previous section. Error bars showing the standard deviation have only been included in the first dielectric increment plot (figure VII.7).

3 RESULTS

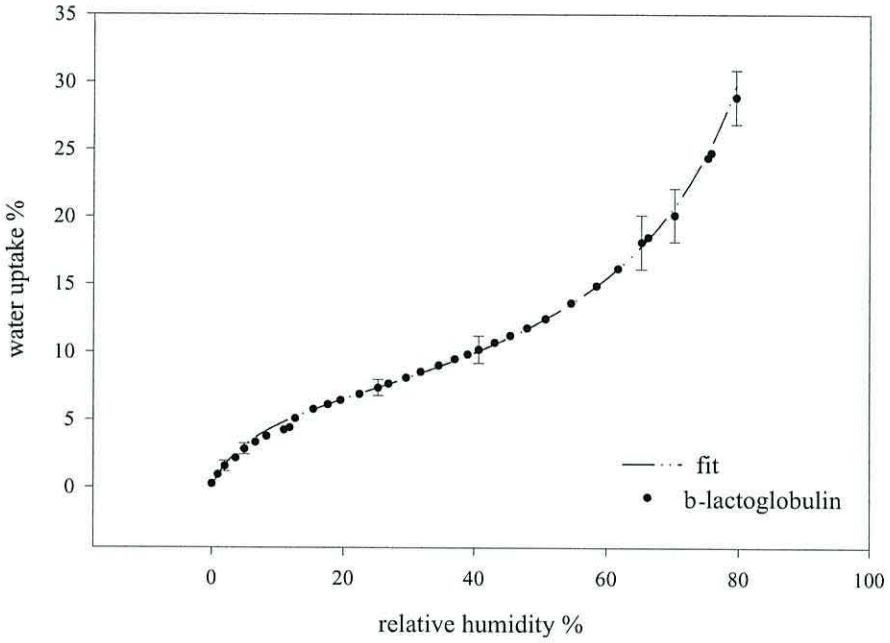


Figure VII.4: β-lactoglobulin hydration isotherm. (Error bars represent 2 standard deviation units)

a ***β-lactoglobulin.***

Figure VII.4 represents the hydration isotherm of native freeze-dried and spray-dried β-lactoglobulin at 25 ± 1^{o}C. As it can be seen both preparations show the same hydration isotherm, clearly indicating the preparation method does not have an effect on the hydration of the protein molecule. Furthermore, both isotherms were found to be in good agreement with that recorded by Rüegg (Rüegg et al., 1975) who studied a lyophilized form of β-lactoglobulin.

To analyse the protein isotherm the GAB equation IV.3 was considered, however it was found convenient to write it in the form

$$\frac{x}{v(x)} = A(1 + Bx - Cx^2) \tag{VII.1}$$

where $v(x)$ is water uptake, x is relative humidity, $A = (v_m ab)^{-1}, B = b(a-2)$ and $B = b^2(a-1)$ (Gascoyne and Pethig, 1977).

A least square routine of the experimental data to the modified GAB equation VII.1 was successfully employed ($R^2 = 0.99$) to obtain the values for the hydration parameters. Goodness of the fit can be evaluated by the close match between the modelled and the experimental isotherm to model the isotherm. The values ab and b, a measure of how protein interact with sorbed water molecules related to the free energies of sorption[2] were found to be equal to be 14.97 and 0.98 respectively. The closer the b value is to unity the weaker the interaction water-protein are, furthermore, when $b = 1$, the water molecules attached to the protein have the same properties as bulk water (Gascoyne and Pethig, 1977). Such a high b value therefore indicates that water molecules attached to the second and subsequent sorbed layers have the same properties as those of pure bulk water (Gascoyne and Pethig, 1977). The v_m value, related to the hydration capacity of the primary bindings sites was found to be 6.3% g/g dry matter. These values were in good agreement with previously reported ones, see for example (Gascoyne and Pethig, 1977)

Dielectric measurements The components of the complex permittivity ϵ^* ($\epsilon^* = \epsilon^{'} - i\epsilon^{''}$), the permittivity, $\epsilon^{'}$, and dielectric loss, $\epsilon^{''}$, of hydrated β-lactoglobulin were determined at 25 ± 0.1^oC, using the TDR technique described in chapter VI, section 5.

In the frequency range considered (1 MHz to 1 GHz) two dielectric dispersions were observed, the first one centered at approximately 3 MHz and the second at about 100 MHz. The two dispersions were found to increase in magnitude with increasing hydration but their relaxation frequencies appear to be independent of hydration. The hydration characteristics of both dispersions are shown in figures VII.6 and VII.8. The existence of these dispersions is consistent with previously published results, (for example, see (Harvey and Hoekstra, 1972; Kent, 1972; Bone et al., 1977; Bone, 1996)), the higher frequency peak usually being attributed to relaxation of dipolar water molecules bound to the protein, and the lower being interpreted in terms of side chain relaxations.

[2]for more details on these parameter see chapter IV

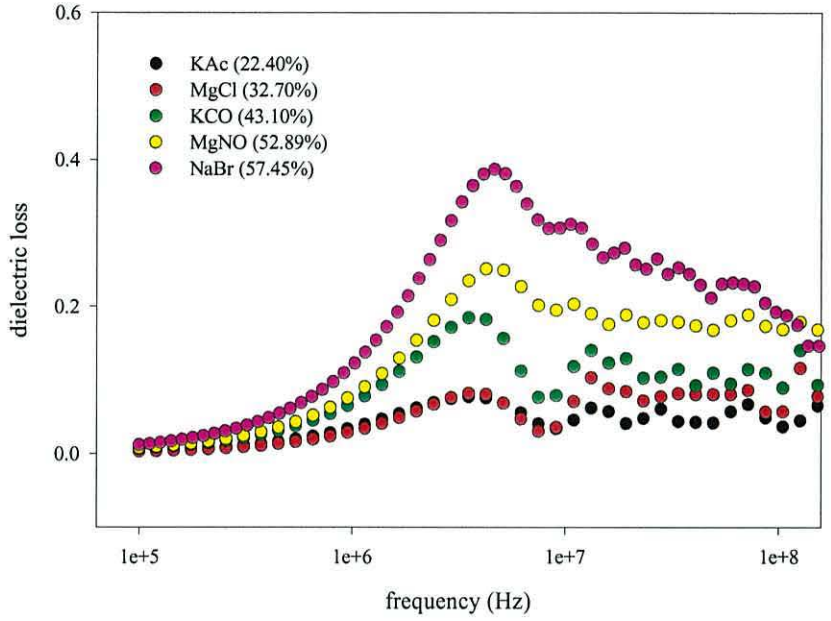


Figure VII.5: β-lactoglobulin dielectric dispersion centered at $\sim$ 3 MHz represented by the dielectric loss as a function of hydration

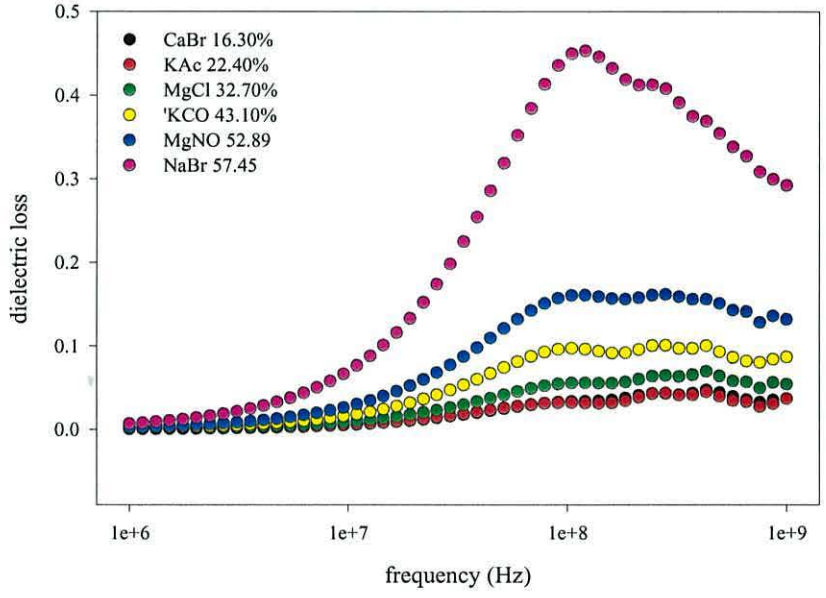


Figure VII.6: β-lactoglobulin dielectric dispersion centered at $\sim$ 100MHz represented by the dielectric loss as a function of hydration.

The effect of increasing water content on the dielectric response of the hydrated protein was monitored by plotting the dielectric increment, $\Delta\epsilon$, which is a measure of the magnitude of the dielectric dispersion and is given by

$$\Delta\epsilon = \epsilon_s - \epsilon_\infty \tag{VII.2}$$

where ϵ_s and ϵ_∞ are the low and high-frequency relative permittivities describing the relaxation process respectively (see chapter on Dielectric Relaxation, chapter VI).

Figures VII.8 and VII.7 show the variation of the dielectric increment with water uptake for the two studied dispersions. The hydration dependence of the dielectric increment of both dispersions observed here is in good agreement with previously published results (Bone, 1987; Bone, 1996). It can be seen that $\Delta\epsilon$ of the lower frequency dispersion (3 MHz) only increases significantly after the water uptake exceeds the 15% g/g dry matter. The $\Delta\epsilon$ of the higher frequency dispersion (100 MHz) appears initially to be quite insensitive to the addition of water molecules, but after a certain hydration level is reached, (6.5%) the magnitude of the dispersion rises with increasing hydration.

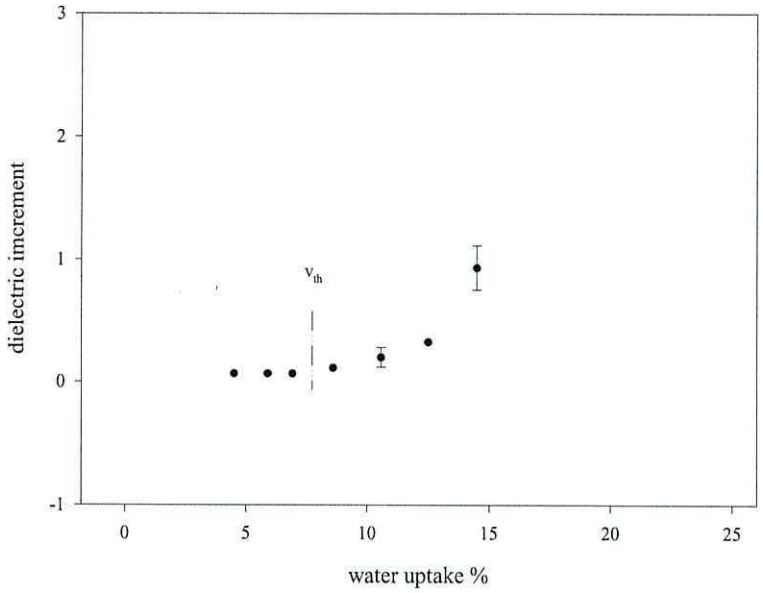


Figure VII.7: The dielectric increment of the higher frequency dispersion ($\sim$ 100 MHz) plotted against protein water uptake of β-lactoglobulin. (Error bars represent 2 standard deviation units)

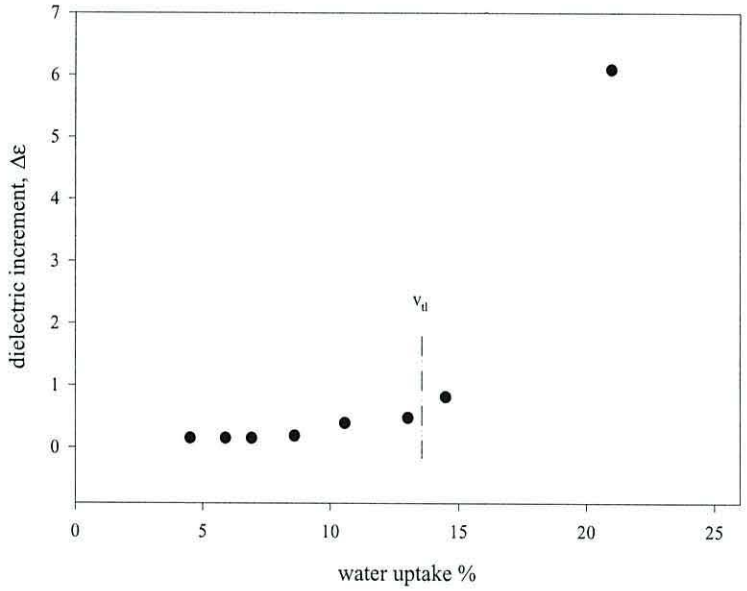


Figure VII.8: The dielectric increment of the lower frequency ($\sim$ 3 MHz) dispersion plotted against protein water uptake of β-lactoglobulin

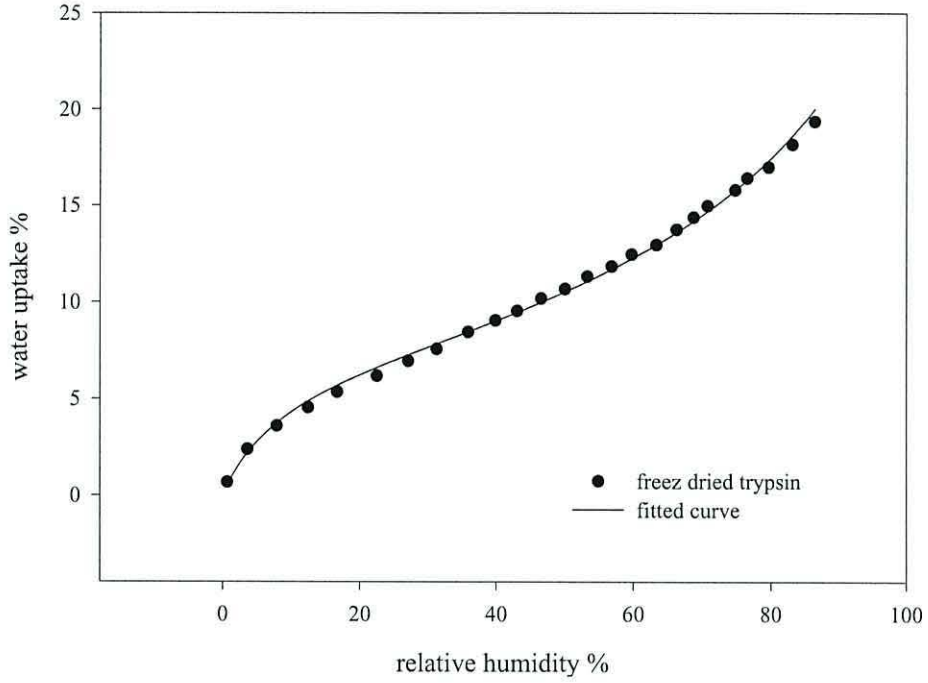


Figure VII.9: Trypsin hydration isotherm

b Trypsin.

Gravimetric measurements The hydration isotherm of native trypsin at 25 ± 1^{o}C is shown in figure VII.9.

The trypsin isotherm was fit to a modified GAB equation VII.1 and values for the hydration parameters were obtained. Goodness of the fit may be evaluated by the close match between modelled and experimentally determined isotherms (see figure X.1). The value of v_m, corresponding to a monolayer coverage of the primary, highly active binding sites, of native trypsin is 7.65%. The value of b, a measure of how protein interact with sorbed water in the second and subsequent layers is 0.71. Both values, v_m and b compare well with those found for β-lactoglobulin and by other workers (Gascoyne and Pethig, 1977).

Dielectric measurements The dielectric response of hydrated trypsin was studied, over the frequency range 1 MHz to 1 GHz. In this case one dielectric dispersion was observed at approximately 3 MHz, that is, the one attributed to side chain relaxations (Bone, 1987; Bone, 1994).

The dispersion was found to increase in magnitude with increasing hydration but its relaxation frequency appears to be independent of hydration (see figure VII.10).

The hydration dependence of the dielectric increment, $\Delta\epsilon$ is depicted in figure VII.11. As it can be seen the dispersion of trypsin shows the same pattern as β-lactoglobulin, i.e. $\Delta\epsilon$ appears to be relatively insensitive to water contents below a certain value, v_{tl}, approximately 12% which also seems to coincide with a subtle change of slope in the hydration isotherm.

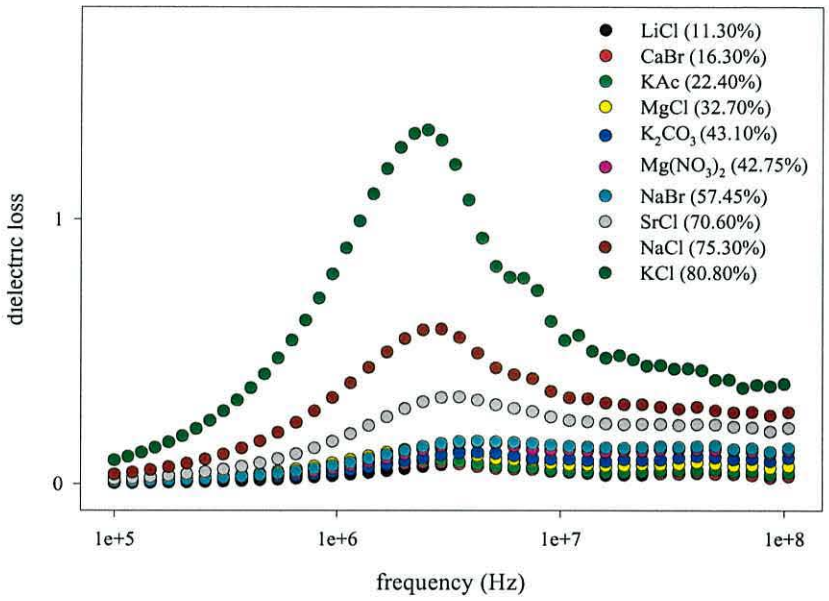


Figure VII.10: Hydration dependence of the trypsin dielectric dispersion represented by the dielectric loss as a function frequency

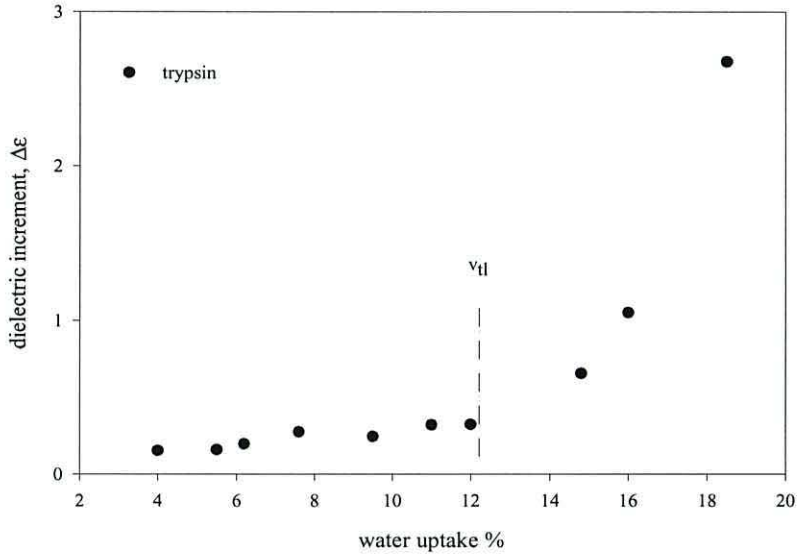


Figure VII.11: The dielectric increment of the lower frequency dispersion ($\sim$ 3 MHz) plotted against protein water uptake of trypsin

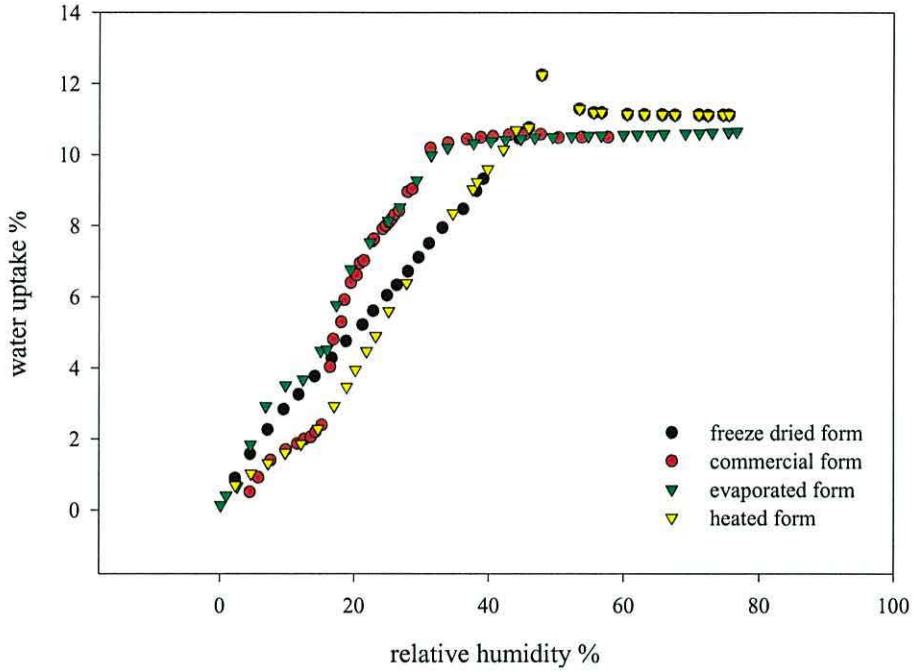


Figure VII.12: Trehalose hydration isotherms

c Trehalose.

Figure VII.12 shows the water sorption isotherms for freeze-dried, commercial (as purchased), evaporated and heated trehalose (freeze-dried trehalose incubated at 77°C for five days). The isotherms of commercial and evaporated trehalose are very similar with minor variation in the low hydration region (0 to 15% RH). However, there is considerable difference between these and the freeze-dried sample which absorbs significantly less water over a relatively wide range of relative humidity, from 15% to 40%. Interestingly, the heated trehalose sample, at low relative humidities (0 to 15% RH,) shows the same isotherm as the commercial form, whereas for the mid hydration region the water uptake follows the isotherm of the freeze-dried sample. Note that, for all samples, the hydration level saturates at 10.7% (g/g dry matter), from 40% RH upwards. The isotherm of freeze-dried form presented here is in good agreement with those previously reported (Iglesias et al., 1997).

Despite the fact that trehalose exhibited a saturation-type isotherm in all cases, it was found that the water adsorption data did not conform to the Langmuir adsorption

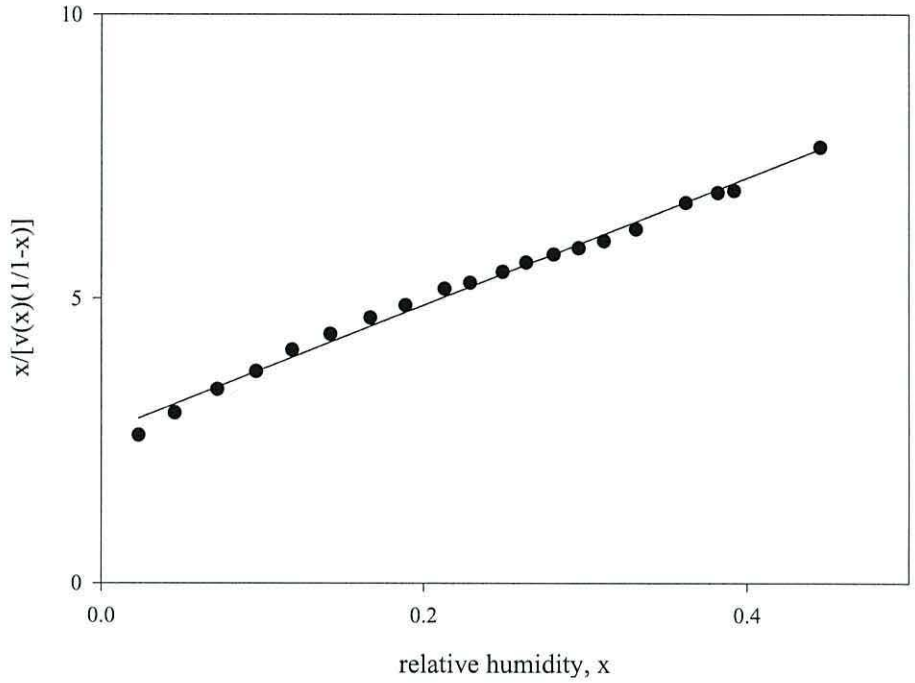


Figure VII.13: Plot of the function $x/[v(x)/(1-ax)]$ against relative humidity for trehalose

isotherm equation (eq. IV.1). It is likely that this is because of the complex nature of hydration of this molecule which undergoes hydration-induced structural changes. However, the BET equation (eq. IV.2) (only valid up to 40% RH) could be applied to the freeze-dried form of trehalose, which apart from the transition at about 45% RH shows no other transition. The BET equation may be rewritten as

$$\frac{x}{v(x)(1-x)} = \frac{1}{v_m a} + \frac{(a-1)x}{v_m a} \tag{VII.3}$$

so the monolayer value can be estimated from the offset and the slope of the plot $x/[v(x)/(1-ax)]$ against x, the relative humidity. Figure VII.13 shows such a plot; the monolayer value v_m being 7.2% g/g dry matter which compares well with previously estimated (Costantino et al., 1997; Costantino et al., 1997)

d Sucrose

In the case of sucrose (figure VII.14) very little water is absorbed by the commercial and evaporated samples. However, the freeze-dried sucrose, in agreement with previously

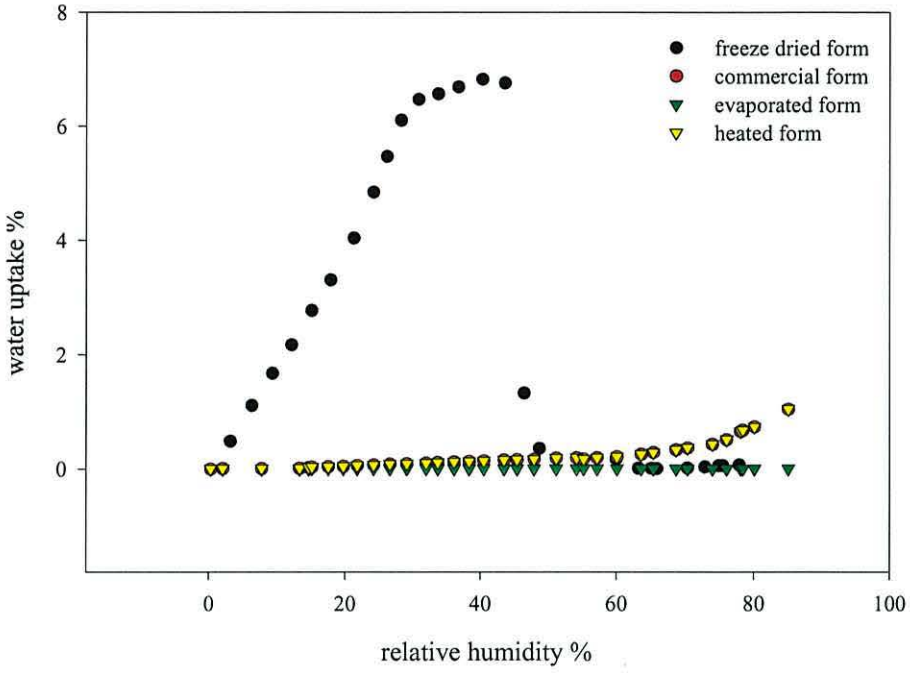


Figure VII.14: Sucrose hydration isotherms

reported results (Costantino et al., 1997; Hancock and Zografi, 1997; Costantino et al., 1998), absorbs water strongly up to 40% RH after which a transition appears to occur, followed by a sudden and spontaneous loss of water.

Sucrose isotherms may be interpreted in the same fashion as trehalose isotherms. Freeze-dried sucrose exhibits hydration properties likely to be associated with a glassy amorphous form; hydration smoothly increase as RH increases until crystallization takes place after which a constant, almost negligible hydration value is reached which suggest formation of anhydrous crystals. Commercial and evaporated forms, which hardly sorb any water throughout the whole hydration range, presumably as a result of intermolecular hydrogen bonding which is likely to occur in the anhydrous crystalline form.

The heated form of sucrose showed an identical isotherm to the commercial and evaporated forms, indicating that incubation at 77°C has induced a transition between the glassy amorphous freeze-dried form and a crystalline form.

The freeze-dried form of sucrose was found not to conform to any of the sorption

theories, this is likely to be due to the structural transitions which occur during sorption.

Dielectric measurements on trehalose and sucrose Dielectric studies on both sugars indicated that in the considered frequency range no appreciable dispersion could be observed. This may be due to the sugars not supporting any dielectric relaxation process at such high frequencies and/or to the fact that none of the forms, amorphous or crystal, absorb enough water for the dispersion to be visible. In the case of the amorphous forms, another effect could have hindered a dispersion, that is, crystallization; when sugars do absorb significant amounts of water for the dispersion to be measurable, crystallization is triggered therefore any dielectric relaxation is further hampered.

e **2-*hydroxypropyl-cyclodextrin.***

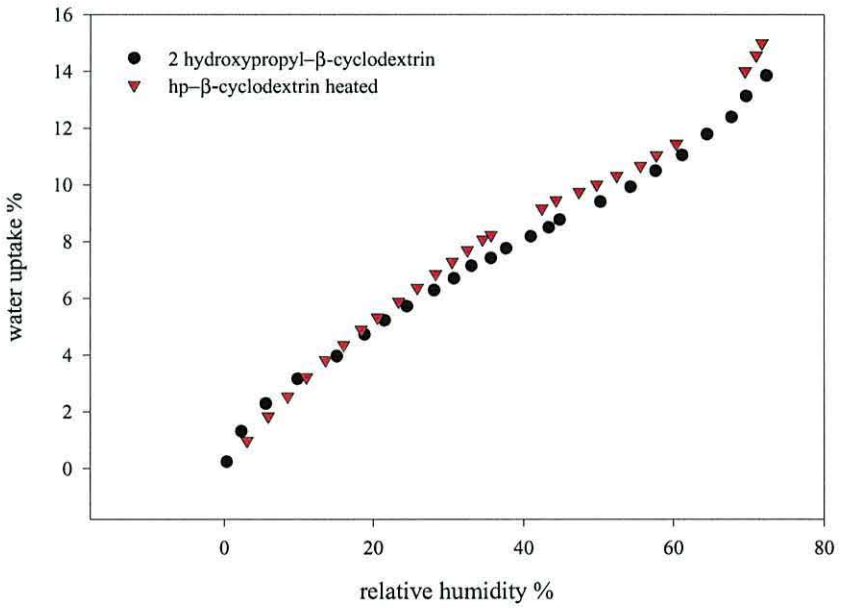


Figure VII.15: 2-hydroxypropyl-β-cyclodextrin isotherm

Hydration isotherms Both forms of 2-hydroxypropyl-β-cyclodextrin (h-β-CD), heated and freeze-dried, showed similar hydration isotherms: a sigmoidal shape isotherm (figure VII.15), as opposed to the isotherm of β-cyclodextrin (β-CD) isotherm which is of the saturation type (Bone S., personal communication). It seems likely that the addition of two hydroxy-propyl groups to the cyclodextrin molecule could disrupt the

network of hydrogen bonding around the β-CD rim; as a result of this the hydroxyl groups interact more strongly with water.

The monolayer for the freeze-dried form was calculated using a modified BET equation (eq. VII.3), the plot of the function (see figure VII.16) yielded a v_m of 5.47%. The monolayer value for the heated form was calculated using the same procedure resulting in 6.1%.

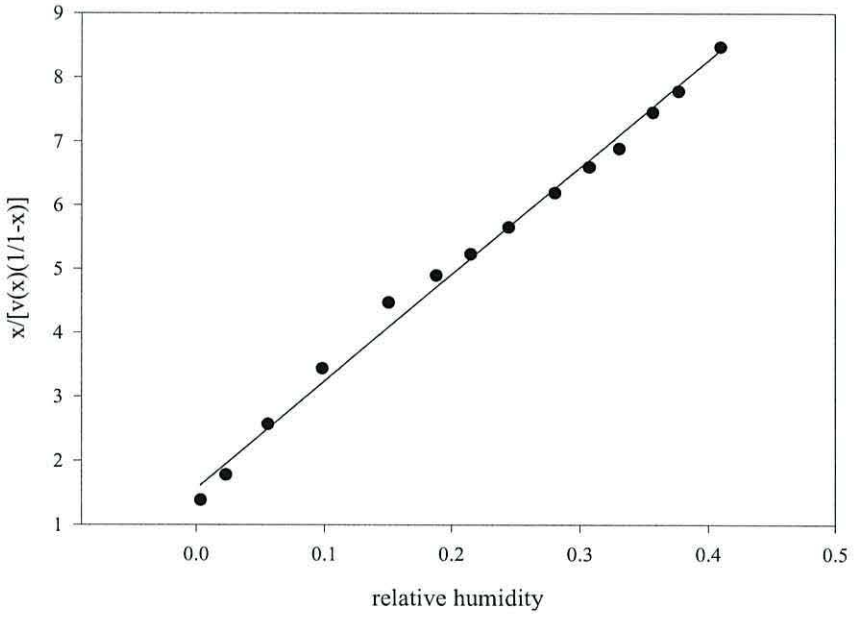


Figure VII.16: Plot of the function $x/[v(x)/(1-bx)]$ against relative humidity for 2-hydroxypropyl-β-cyclodextrin

Dielectric measurements 2-hydroxypropyl-β-cyclodextrin dielectric response was studied as a function of hydration. As shown in fig VII.17 a dispersion was found at $\sim$ 3 MHz, it can be seen that the magnitude of the dispersion increases with increasing hydration and that the relaxation frequency does not appear to be dependent on hydration.

To further look into the hydration dependence of the magnitude of the dispersion, the dielectric increment $\Delta\epsilon$ was graphically represented against the water uptake (figure VII.18). It can be seen that $\Delta\epsilon$ increases significantly after the water uptake exceeds 10%.

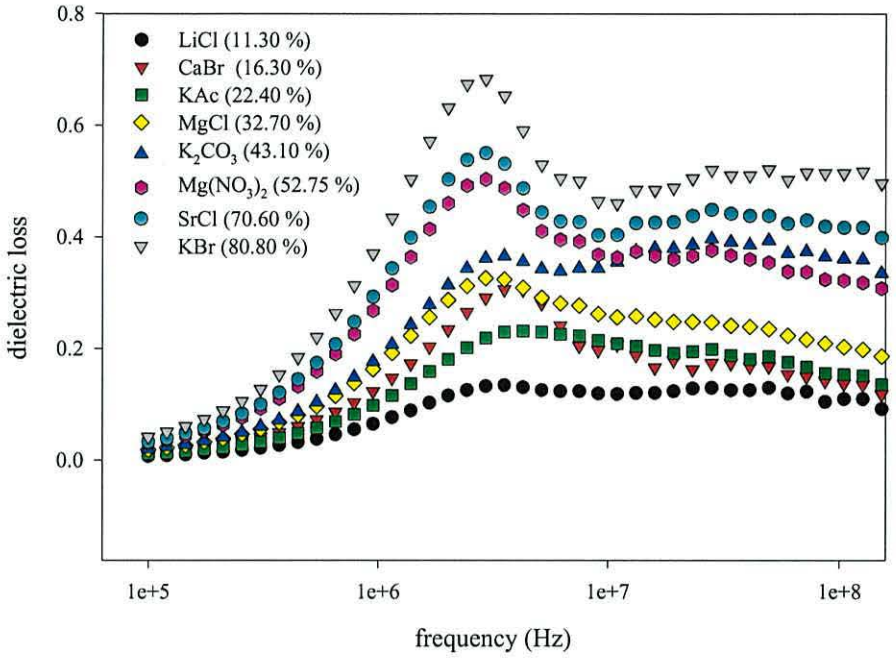


Figure VII.17: 2-hydroxypropyl-β-cyclodextrin dielectric response as a function of hydration

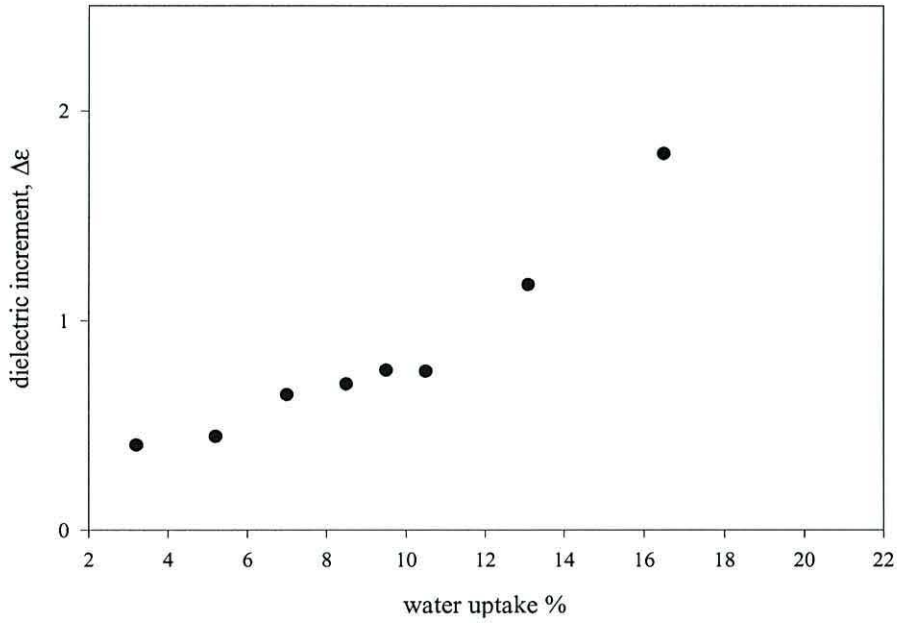


Figure VII.18: 2-hydroxypropyl-β-cyclodextrin dielectric increment vs. water uptake

4 Discussion

Proteins: β-lactoglobulin and trypsin The hydration isotherm of both proteins showed a sigmoidal shape that could be successfully modelled using the 3-parameter equation know as the GAB equation IV.3. Hydration parameters, v_m and b could therefore be calculated for these samples.

There has been considerable discussion about the physical meaning of v_m, the monolayer value. v_m has traditionally been regarded as the sorbate mass forming a complete monolayer on the solid surface, (see chapter IV), and is usually taken to represent the point or region on the isotherm where the initial shoulder occurs. In fact, at water contents in the region of v_m, a number of crucial effects of water on the properties of the solid occur, such as discontinuities in the apparent heat capacity[3]. However, the exact mechanism that gives rise to this initial shoulder is not well understood.

It has been suggested (Oksanen and Zografi, 1990), based on empirical observations, that, for any solid, at some water content that is a definitive multiple of v_m, a second critical point, where the isotherm deviates from linearity, should be present in the isotherm. For starch and cellulose this point corresponds to a water content of about three and five times v_m respectively (Oksanen and Zografi, 1990). In the case of β-lactoglobulin the second deviation from linearity in the isotherm corresponds to water content of about 15% which is interestingly also about three times v_m and for trypsin is about twice v_m. In fact, it is above this critical point, at approximately 20% water content in the protein hydration isotherm, where important changes in the protein-water system occur, as evidenced by, for example, heat capacity measurements and onset of enzymatic activity.

A series of works on poly(vinylpyrrolidone) (PVP) (Oksanen and Zografi, 1990; Hancock and Zografi, 1993) have shown that the deviation from linearity of the hydration isotherm at high relative humidities coincides with a water content, v_g, which is sufficient to lower the T_g of the system to that of its environment. The ratio v_g/v_m is approximately three, remaining constant with temperature. Together these facts, the authors suggest, indicate that hydration of an amorphous solid involves a close relation between water

[3]for more details and references see chapter IV

molecules and the physical state of the solid, suggesting that v_m is also linked to the plasticization of water on the amorphous polymer.

It is interesting to mention that early work on the dependence of the glass transition of a given polymer with water content has been also related to the monolayer value (for references see (Levine and Slade, 1992)). The steep decreases of T_g with moisture seems to stabilise after the hydration level reaches values similar to v_m.

Dielectric measurements in the frequency range 1 MHz to 1 GHz have shown the existence of two different dispersions, the first centered at about 3 MHz and the second at about 100 MHz.

The higher frequency dispersion has been related to the water molecules bound to the protein structure (Harvey and Hoekstra, 1972; Bone, 1987; Bone, 1996). Close inspection of the results obtained from gravimetric and dielectric measurements revealed good correlation between the v_m value, the traditionally considered monolayer capacity and the v_{th} value identified for the higher frequency dispersion, both approximately 6.5%. An important question arises as to the nature of the binding of the water molecules above and below this boundary value v_{th}. The irrotational nature of the water molecules attached to the protein below v_{th} has been suggested to be due to them being multiply hydrogen bonded to the protein ('tightly bound')[4] whereas the molecules above v_{th} are supposedly singly hydrogen bonded to the protein ('loosely bound') (Bone, 1987; Bone, 1994; Bone, 1996)[5].

As for the dispersion centered at $\sim$ 3 MHz, literature seems to indicate that protein-structural motions and side chain relaxations are responsible for lower frequency dispersion (Bone, 1987). Nevertheless other mechanisms such as proton/ion fluctuations have not been completely ruled out.

The hydration dependence of the dispersion revealed that dielectric measurements identify a threshold water uptake of magnitude v_{tl} after which the dispersions seems to increase. Again close inspection of the results obtained from gravimetric and dielectric measurements showed that the v_{tl} value is 2.4 times larger than v_m and v_{th}, thus is clearly

[4]Good correlation between v_m and v_{th} of β-lactamase (Bone, 1994) and lysozyme (Bone and Pethig, 1982) and the number of multiply hydrogen bonded water molecules identify by x-ray crystallography studies (Blake et al., 1983) support that scheme

[5]the effective dipole moment of bound water molecules in the hydration range above v_t was found to be within 10% of the dipole moment of vapour water molecules (Bone, 1987; Bone, 1994; Bone, 1996)

not possible to correlate v_{tl} with v_m, the monolayer value. However, it can be seen that at water content about v_{tl} the hydration isotherms of β-lactoglobulin and trypsin show a slope change, after which the water uptake increases more rapidly. The characteristic deviation from linearity of the hydration isotherm of polymers at high relative humidities has been shown to coincide with a water content, v_g, which is sufficient to lower the glass transition temperature, T_g, of the system to that of its environment (Oksanen and Zografi, 1990; Hancock and Zografi, 1993). This indicates that for water contents below v_g, the polymer is in a glassy state while above v_g the polymer is in a rubbery state in which mobility is greatly enhanced.

It may be, therefore, that the dielectric measurements characterizing the hydrated β-lactoglobulin and trypsin dispersion centered at about 3 MHz identify the same hydration value as the T_g measurements carried out on poly(vinylpyrrolidone) (PVP) (Oksanen and Zografi, 1990; Hancock and Zografi, 1993), both corresponding to the same point in the hydration isotherm. At this hydration, the amorphous solid looses its glassy properties to become a rubbery material. Therefore the lower frequency dispersion may reflect the glassy nature of the protein and its plasticization by water. It could be that at water contents below v_{tl} any relaxation process is hindered as a result of the high viscosity of the glassy matrix whilst above v_{tl}, the increase of the dielectric increment may be related to the increased mobility of the polymer in the less viscous rubbery state. If the dispersion is considered to be due to structural motion of the protein structure and relaxation of side chains, plasticization may be pictured as follows; as water is sorbed and penetrates into the amorphous solid it lowers the energies of interaction between polar entities, (i.e. side chain, peptide groups), possibly weakening electrostatic interactions, hence increasing molecular mobility of the protein, inducing, at water content $\sim v_{tl}$, the structural transition from glass to rubber.

The picture in which the the protein/polymer hydration process is understood in terms of bound, either tightly or loosely, water molecules has been argued by several authors (Levine and Slade, 1987; Zografi, 1988; Levine and Slade, 1992). The fact that water plasticizes the amorphous solid as it is sorbed lowering the T_g of the system has lead some authors to question the view in which sorption into amorphous materials is regarded as a 'tightly bound'-monolayer - 'loosely bound'-multilayer sorption

process (Levine and Slade, 1987; Zografi, 1988; Levine and Slade, 1992). It has been suggested that the change in apparent states of sorbed water in polymers arises from a change in the physical state of the polymer as plasticization occurs. Therefore, it has been argued that what has been traditionally referred to as the 'bound' state of water may simply arise from immobility due to the high viscosity of the glassy state. Likewise the 'solvent like' state of water at higher water contents may reflect the much less viscous rubbery state of the polymer above T_g. This suggests that the state of the polymer above and below its T_g (as reflected by its viscoelasticity) actually may contribute significantly to the amount of water sorbed at a particular relative humidity as well as to the shape of the isotherm.

Results presented here, indicate that there is a threshold number of water molecules, $v_{tl} \cong v_g$ which may be related to the hydration induced glass transition of the polymer. The fact that relaxation processes appear to be hampered below this threshold is consistent with a physical state transition, at this point, from glass to rubber. In this case it may be misleading to say that the water molecules below v_{tl} are tightly bound to the protein. However, the identified population of water molecules, $v_{th} \cong v_m$ related to water molecules 'bound' to the protein, appears to be in conflict with the 'glass state' view, that is that below v_g water is not bound but instead its mobility is retarded due to the high local viscosity of the glass. Mobility of water molecules above v_m and below v_g can not apparently be explained by the fact they are 'trapped' in a glassy matrix. However it is interesting to note the attempts made by some authors (Oksanen and Zografi, 1990) to correlate the monolayer value to the plasticization phenomena and the glassy nature of polymers.

Trehalose and sucrose Most of the various forms of trehalose and sucrose exhibited hydration characteristics which do not conform to any of the generally accepted models described in the literature. Nevertheless the monolayer value of the freeze-dried form of trehalose could be estimated using the BET equation (eq. IV.2). That the theoretical models fail to predict the sugar hydration isotherms may be due to the fact that the sugars undergo structural changes throughout the hydration range. This 'failure' highlights the already discussed questionable validity (see chapter IV) of the use of gas

sorption theory onto a solid surface for amorphous solids.

The isotherm of the freeze-dried form of trehalose clearly reflects the plasticization process discussed in the section *'Water as a plasticizer- the hydration dependence of* T_g in appendix A: as the relative humidity increases, the water uptake rises smoothly until the level of water in the sample is sufficient to lower the activation energy for crystallization. Above this level crystals are formed, and the isotherm reaches a constant hydration level, 10.7% (g/g dry matter), which is consistent with the formation of a dihydrate crystal. In more detail, as water penetrates into the glassy amorphous solid (freeze-drying renders a glass amorphous state), it enhances molecular mobility weakening and breaking intermolecular hydrogen bonds. Hence the T_g of the system is lowered and the glass becomes rubber and eventually as hydration progresses, trehalose molecules form crystals. Water not only lowers the T_g of the system but also reduces the crystallization energy barrier (Zografi, 1988) and, kinetically, is capable of controlling the transformation of the metastable states (water acting as a crystallizing plasticizer, i.e. promoting molecular motion until crystallization occurs). Following crystallization, it has been observed that the water content of the amorphous solid drops to some extent to later achieve a constant hydration value (Costantino et al., 1997; Hancock and Zografi, 1997; Iglesias et al., 1997; Costantino et al., 1998; Hancock and Dalton, 1999). Crystallization induces complete saturation of the available binding sites; therefore the solid is no longer capable of sorbing water, consequently the excess of water molecules is expelled and a constant hydration value is observed.

It has been reported that trehalose can exist in different amorphous and crystalline forms depending on the initial structure, dehydration rate, temperature at which dehydration take place and particle size [6] (Gil and Belton, 1996; Sussich et al., 1998; Taylor et al., 1998; Sussich et al., 1999a; Sussich et al., 1999b). Therefore it seems reasonable that the isotherms of commercial, evaporated and heated forms of trehalose may be explained in terms of different structural forms of trehalose.

Removal of the water molecules of the dihydrate crystal (commercial form) upon vacuum drying may result in a form which, as it can be seen in figure VII.12, absorbs a relatively low amount of water (up to $\sim$ 2% g/g dry mater) in the low hydration range

[6] these are described in chapter II

(up to $\sim$ 15% RH). This sorption of water may be indicative of the existence of packed form.

At 18% RH the water uptake rapidly rises from from 2% to 7% which may reflect a transition between packed form and a more loosely packed form which allows water molecules to induce crystallization, after which no further hydration occurs.

Dehydration of a trehalose solution at room temperature (evaporation) resulted in a form of trehalose which is presumably in a crystalline form[7]. Therefore, both formulations, commercial and evaporated, should be expected to show the same hydration isotherm. However their respective isotherms although very close from $\sim$ 15% RH onwards, differ to a great extent in the low hydration range with the evaporated form sorbing considerably more water than the commercial form. It could be speculated that evaporation yields a second crystalline form which when gently dehydrated under vacuum results in an anhydrous form less packed than that obtained from the commercial form.

The heated trehalose sample shows an interesting hydration isotherm which, in the first instance, follows the isotherm of the commercial form and from 15% RH upwards clearly resembles the isotherm of the freeze-dried form. Freeze-dried trehalose incubated at 77°C is expected to be structurally stable, since the reported glass transition temperature, T_g, $\sim$ 105°C (Shamblin et al., 1999) is well above the incubation temperature. Even allowing for the plasticizing effect of the residual water ($\sim$ 0.5%) remaining in the sample after freeze-drying, T_g (about 90°C) is still above the incubation temperature. However, incubation at high temperature may be expected to, in the first place, increase the T_g of the system by removal of residual water, causing the amorphous glassy solid to rearrange itself in a more compact form since the loss of water may provoke reduction of intermolecular spacing and free volume. As a result, the solid, in the initial stages of hydration only shows a low water uptake, but as sorption progresses, water 're-plasticizes' the system and the isotherm smoothly rises until crystallization takes place which produces a minor loss of water content followed by a constant hydration value.

[7]Raman spectra of both crystalline and evaporated forms were almost identical, (expect for minor differences in peak intensities and minor peak shifts), see appendix B

Unfortunately, the data available does not allow definite conclusions to be drawn about the possible forms adopted by trehalose nor correlate them with the already characterized forms by other authors (Gil and Belton, 1996; Sussich et al., 1998; Taylor et al., 1998; Sussich et al., 1999a; Sussich et al., 1999b).

2-*hydroxypropyl-β-cyclodextrin* Gravimetric measurements revealed that the hydration isotherms of freeze-dried and heated h-β-cd are very similar, indicating that the heat stress does not induce structural changes in the oligosaccharide.

Dielectric measurements on h-β-cd indicated the existence of a dispersion centered at about 3MHz with a hydration dependence similar to that of the proteins, i.e. its magnitude appears to increase above a certain hydration value (10%) of the amorphous solid. Furthermore, this sorption threshold also seems to coincide with the change of slope in the hydration isotherm related to the transition from glass to rubber.

Correlation between proteins and hp-β-cd dielectric response seems to supports the view that the dispersion centered at 3 MHz reflects the transition from glass to rubber, however does not clarify the nature of the dispersion, i.e. whether side chains relaxation or proton-ion fluctuations on the molecule surface are responsible for this dispersion.

5 Conclusion

In this chapter the experimental findings of a gravimetric and high frequency dielectric study on two model proteins, β-lactoglobulin and trypsin, two disaccharides, trehalose and sucrose and a polysaccharide, 2-hydroxypropyl-β-cyclodextrin were presented and discussed. The main conclusions can be summarized as folows:

- Hydration measurements on the two model proteins, in conjunction with dielectric experiments, have revealed that the two dispersions showing a hydration dependency which may be related to different events in the hydration process, (1) primary binding sites coverage and (2) plasticization resulting in a physical state transition from glass to rubber.

- Sugar molecules, depending on their physical state show different hydration characteristics (for example, crystalline sucrose absorbs little water in comparison

to its amorphous form). In the frequency and hydration ranges studied, neither trehalose nor sucrose appeared to show any dielectric relaxation.

- Dielectric and hydration characteristics of 2-hydroxypropyl-β-cyclodextrin are interestingly similar to those of the proteins which might indicate that the dielectric dispersion centered at about 3 MHz is of the same nature for proteins and hp-β-cd.

VIII

Preliminary studies on protein sugar interactions: β-lactoglobulin-trehalose/sucrose

1 Introduction

The preserving properties of sugar molecules on proteins against the denaturing effects of formulation preparation by either lyophilization or spray-drying and subsequent storage has been the object of many studies.

Reported results on β-lactoglobulin foaming properties (Murray and Liang, 1999; Murray and Liang, 2000; Murray et al., 2001) have shown that spray-drying of protein in the presence of trehalose and sucrose had an enhancing effect on its functional properties. Apparently, properties of protein dried on its own, such us surface tension, foamability and foam stability do not resemble those of the native protein whereas protein spray-dried in the presence of increasing amounts of sugar results in increasing similarity to the native protein. Furthermore, these results showed that protein-trehalose mixtures were more effective in retaining the native protein properties that those of sucrose.

This chapter describes the characterization of various β-lactoglobulin-sugar mixtures in an attempt to relate their hydration properties to the hypothesis (hydrogen bond interaction, glass formation and entrapment of water) which have been proposed to explain preservation.

2 Materials and Methods

a Sample preparation.

β-lactoglobulin (from Bovine milk, 3x crystallised and lyophilized), sucrose (> 99.5%) and trehalose (dihydrate, from Saccharomyces cerevisiae) were purchased from Sigma Chemical Company and used without further purification.

Samples of 1:2 β-lactoglobulin:sugar by weight were prepared in solution and were then either spray-dried at high temperature (110°C), freeze-dried in vacuum or evaporated at room temperature over a 24 hour period. Samples of 1:1 protein to sugar ratio were prepared by freeze-drying and 1:5 samples were spray-dried. For details on evaporation, freeze and spray-drying procedures see chapter VII.

b Experimental techniques.

Hydration isotherms Measurement of the hydration isotherms for β-lactoglobulin:sugar structures were performed using a computer controlled Sartorius vacuum microbalance (type 4332). Prior to each hydration isotherm experiment, samples were dried in vacuum ($1 \cdot 10^{-2}$ Torr) for at least 24 hours. The water uptake of the protein samples was measured for step increases in water partial pressure of approximately 2.5% over the hydration range 0 to 90%. The relative humidity level was controlled to within 0.1% and the temperature was maintained at 25 ± 0.1°C. The partial pressure measurements were made by manometer ($0 - 15\%$ relative humidity) and a calibrated Humilab HL24D vacuum humidity probe (10-90% RH)[1].

Usually at least two hydration runs were performed on each sample since hydration dependent changes were found to take place during the initial hydration which resulted in different subsequent hydration characteristics. Measurements on each sample were repeated to ensure reproducibility.

Desorption experiments were carried out on the evaporated samples; using the computer controlled Sartorius vacuum microbalance, samples were allowed to dehydrate over two days from 43% RH down to 0% mass readings were approximately taken in 7% steps of decreasing relative humidity.

[1]for further details see chapter VII

Dielectric measurements The hydration-dependent dielectric response of the protein-sugar samples was investigated using Time Domain Reflectometry (TDR) which covers the frequency range 100 KHz to 10 GHz[2].

As in the study of protein dielectric dispersions (chapter VII), care was taken to ensure that the choice of time window allowed an adequate recording of the observed waveforms. When investigating the lower frequency dispersion centered about $\sim$ 3 MHz a 250 ns time window was used whereas for the dispersion of frequency approximately of $\sim$ 1 GHz the time window was 7 ns long. Inner electrode length was 5 mm for the higher frequency dispersion and 20 mm for the lower one.

When studying the lower frequency dispersion (i.e. when the longer electrode was used), packing of samples into the sample cell resulted in measured densities values varying between 0.47 and $0.55 \cdot 10^6$ g m^{-3}. For the higher frequency peak , when using a shorter inner electrode, the density varied from 0.44 to $1.1 \cdot 10^6$ g kg m^{-3}. Hydration of the samples was achieved as previously described in chapter VII.

3 Results

a *Hydration isotherms*

Trehalose mixtures Figures VIII.1 and VIII.2 show initial (1st hydration run) and subsequent (2nd hydration run) water sorption isotherms for 1:2 β-lactoglobulin:trehalose mixtures which have been spray-dried, freeze-dried and evaporated from solution. As can be seen, there is a reasonably close coincidence of the three sets of data with the spray-dried, freeze-dried and evaporated mixtures exhibiting similar hydration characteristics.

Also shown in the graphs is a theoretical adsorption isotherm based on the same ratio of protein to sugar. The mathematical models traditionally used to fit protein hydration isotherms were successfully employed on the isotherms of β-lactoglobulin (see chapter VII). However, due to the dramatic hydration-induced structural changes in the sugars investigated, the protein-sugar mixtures did not conform to these models. Therefore, instead of fitting the isotherms to these mathematical models, a very simple analysis, involving comparison of the experimental isotherms with theoretical

[2]for further details see chapter VII and chapter VI, section VII.2

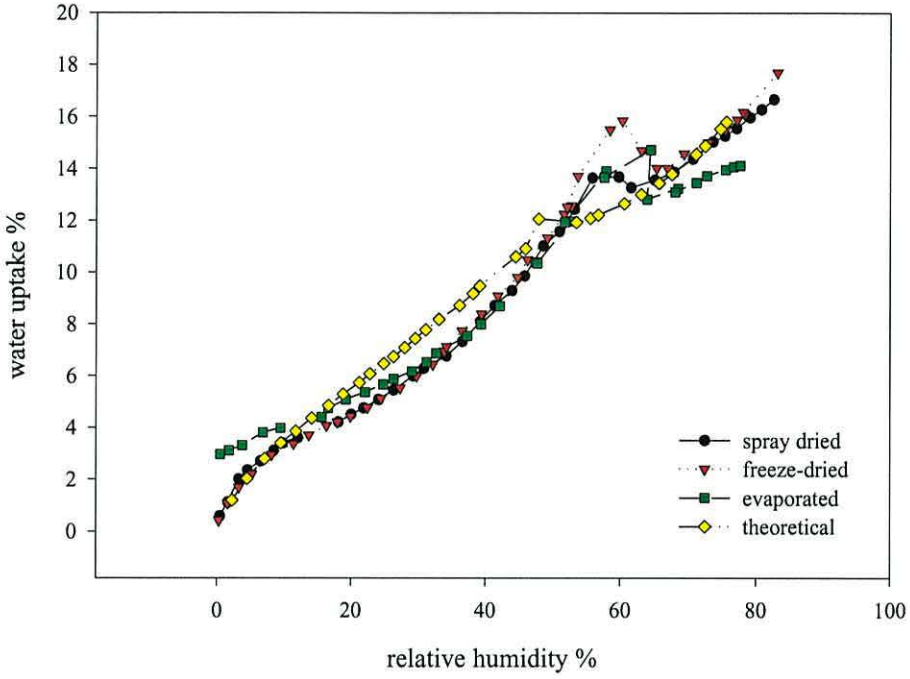


Figure VIII.1: 1:2 β-lactoglobulin:trehalose first hydration isotherms (spray-dried, freeze-dried, evaporated and theoretical isotherms)

characteristics has been performed. These theoretical isotherms are constructed from the experimental isotherms of the constituents on their own and assuming no interaction between protein and sugar molecules. Therefore a given theoretical isotherm was calculated by the addition of the isotherms of the components which had been previously weighted according to the proportion of each present in the sample.

For the initial hydration runs, the isotherms of freeze-dried β-lactoglobulin and trehalose were used to construct the theoretical isotherm. Comparison of the experimental and theoretical isotherms, shows that the amount of water adsorbed in the mid-hydration range is considerably less than that predicted by the theoretical isotherm. This indicates the likelihood that there is interaction between the β-lactoglobulin and trehalose and that a complex is formed between the two involving sites on the protein which would normally be involved in binding water molecules.

On reaching a hydration value corresponding to approximately 60% RH (~ 14% weight water), a discontinuity is evident in the initial adsorption isotherms. At hydrations above this value, the isotherms appear to follow that of the theoretical

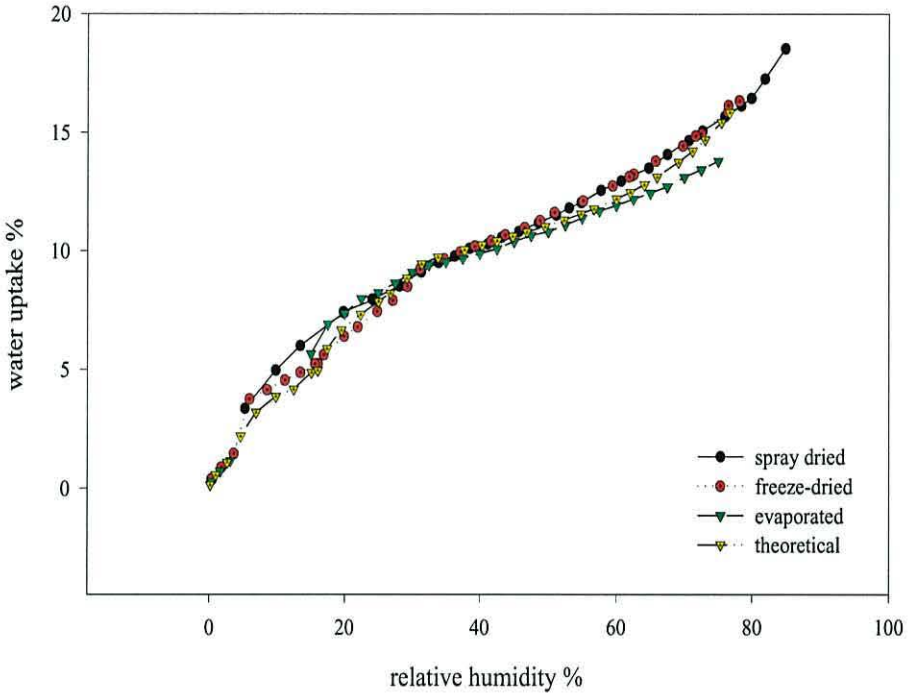


Figure VIII.2: 1:2 β-lactoglobulin:trehalose second hydration isotherms (spray-dried, freeze-dried, evaporated and theoretical isotherms)

characteristic for a non-interacting mixture.

After subsequent dehydration of the sample, the step-wise hydration was repeated, producing a considerably altered characteristic compared with the first hydration run. In this case, the sorption isotherm appears to closely follow the theoretical mixture characteristic which had been calculated using the sorption isotherms for β-lactoglobulin and evaporated trehalose. The closeness of the experimental and theoretical isotherms would seem to indicate a separation of the protein and sugar constituents with no significant interaction between the two species.

Note that the positions of the discontinuities in the initial hydration isotherms indicating phase separation transitions are not successfully predicted by the theoretical model, the transition for the complex occurring at a higher relative humidity and water content for the complex than the two non-interacting components.

The results for the mixture dried from solution at room temperature by evaporation exhibit some deviation from freeze- and spray-dried samples at very low hydrations. In this region it can be seen (figure VIII.1) that a significant amount of water, some 3% by

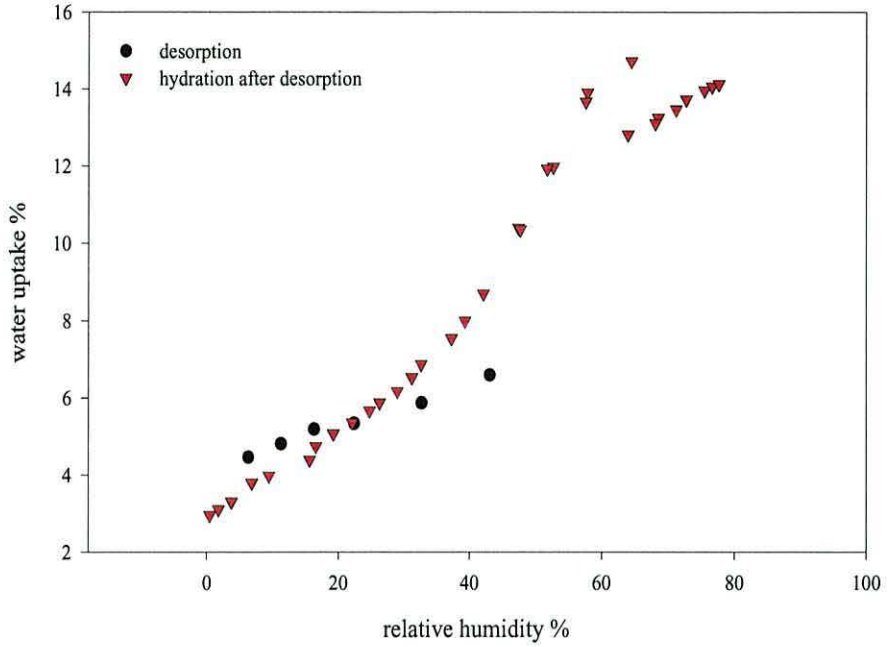


Figure VIII.3: Desorption isotherm for 1:2 β-lactoglobulin:trehalose, evaporated and subsequent hydration.

weight, appears to be "frozen" into the structure of the complex despite the fact that the relative humidity was held at zero for > 24 hours by evacuating the sample prior to the first hydration experiment. The first absorption isotherm, as a result, commences from this non-zero hydration value at 0% RH and from approximately 17% RH follows the experimental isotherm of spray-dried and freeze-dried samples. The second hydration of the sample started at the expected nil water content which indicated that the trapped water was apparently released when the complex undergoes the characteristic transition and phase separation above 60% RH.

In order to investigate further the retention of water by the evaporated β-lactoglobulin trehalose mixture i.e to rule out that the entrapment of water is an artifact due to vacuum dehydration, samples were allowed to dehydrate over two days from 43% RH down to 0% and a desorption isotherm was obtained (see figure VIII.3). The subsequent sorption isotherm on this sample was found to be similar to that of the sample which had been rapidly dehydrated prior to the initial isotherm characterisation and a similar amount of water was trapped in the structure of the complex.

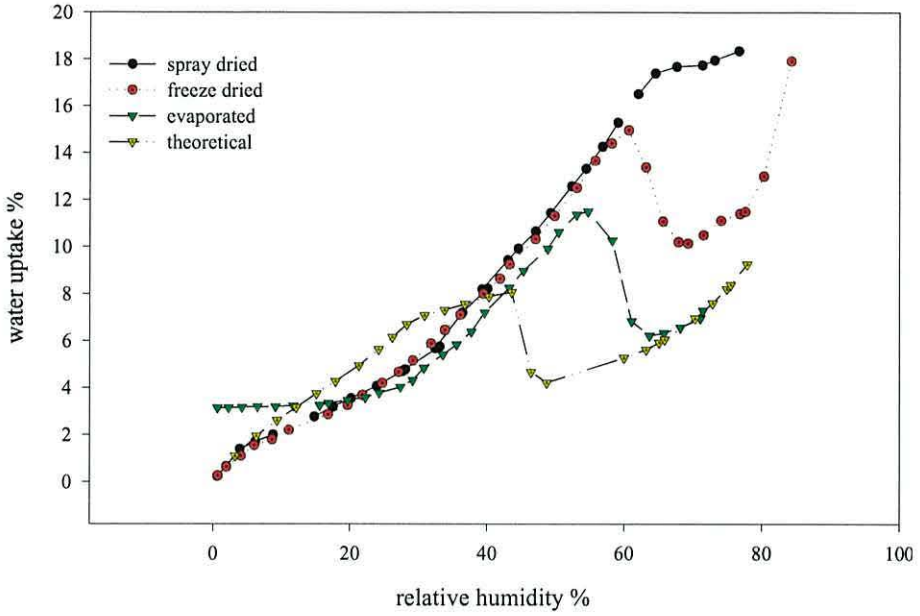


Figure VIII.4: 1:2 β-lactoglobulin:sucrose first hydration isotherms (spray-dried, freeze-dried, evaporated and theoretical isotherms)

Sucrose mixtures In figure VIII.4 initial and experimental isotherms and theoretical isotherms are shown for spray-dried, freeze-dried and evaporated β-lactoglobulin sucrose 1:2 mixtures.

These mixtures undergo dramatic structural transitions above 10% water content involving considerable loss of bound water. Furthermore, the position of the transitions in terms both of relative humidity and amount of water absorbed is dependent on the drying process with the evaporated mixture exhibiting a discontinuity in the isotherm at 54% RH (11.2% absorbed water) compared with the freeze-dried sample at 60% RH (14.4% water content) and the spray-dried sample at 66% RH (17.2% water). Note the transition predicted by the theoretical isotherm is considerably lower than any of these.

Over the hydration region 3 to 8% water content, comparison of the experimental data with the theoretical isotherm indicates that less water is bound to the protein-sugar complex than expected. As with the trehalose complex, it is likely that interaction between the sucrose and protein at sites normally engaged in water binding accounts for this discrepancy.

Following initial hydration, the isotherms for subsequent hydration runs (see

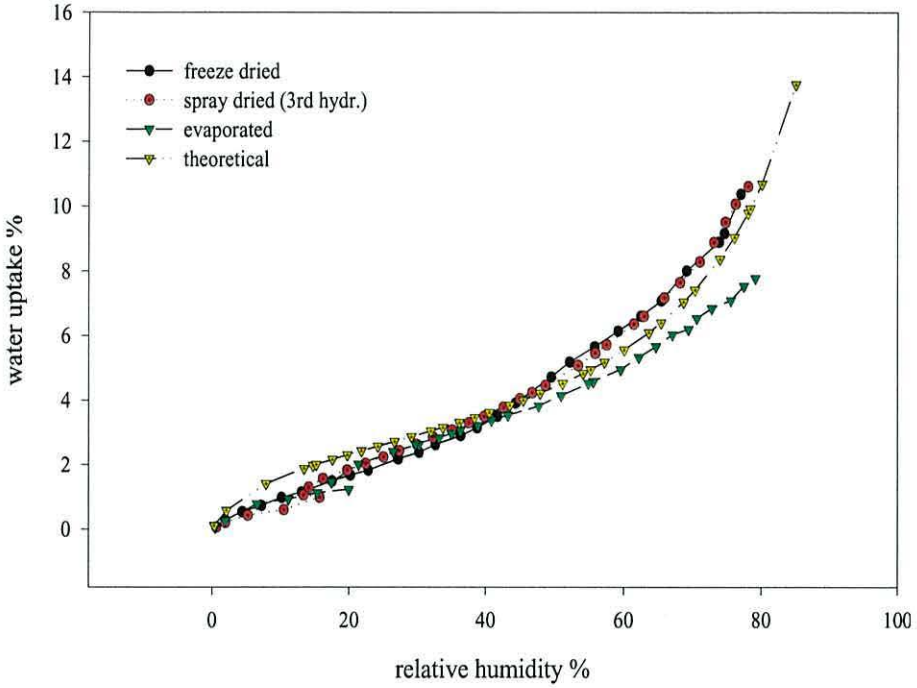


Figure VIII.5: 1:2 β-lactoglobulin:sucrose second hydration isotherms (spray-dried, freeze-dried, evaporated and theoretical isotherms)

figure VIII.5) all appear to follow the same characteristic which is similar to the theoretical isotherm composed from the protein and evaporated sucrose. As in the case of trehalose mixtures, the closeness of the experimental data to the theoretical isotherm theoretical isotherm seems to indicate that, following the transition during the first hydration process, the protein and sucrose separate into essentially separate species.

As with trehalose, the protein-sucrose complex prepared by evaporation from solution appears to trap water even under conditions of extreme dehydration where the sample is vacuum dried for > 24 hours. This is reflected by the deviation of the isotherm for this sample from the theoretical isotherm and from the isotherms of the freeze-dried and spray-dried samples which do not exhibit this property (figure VIII.4).

b Dielectric measurements

β-lactoglobulin-trehalose/sucrose mixtures The dielectric response of hydrated protein-sugar mixtures was studied, as described previously for β-lactoglobulin and trypsin (see chapter VII), in the frequency range 1 MHz to 10 GHz.

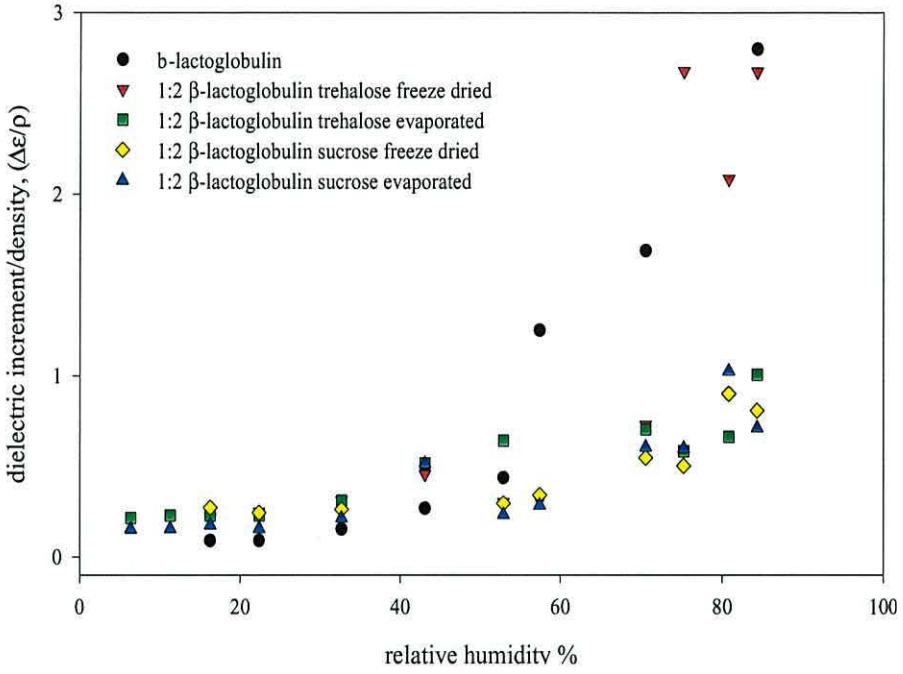
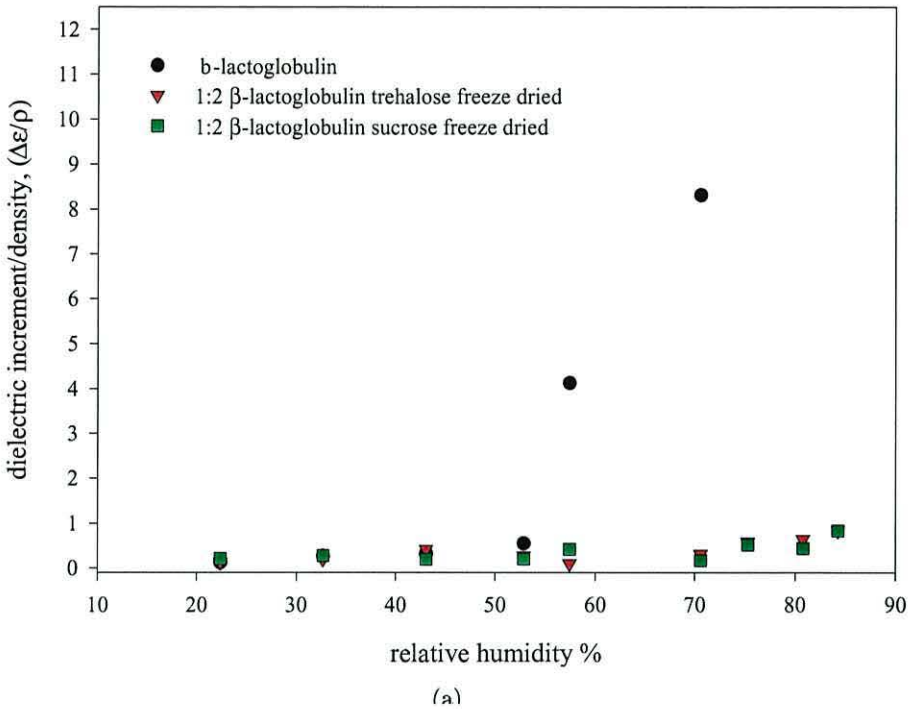


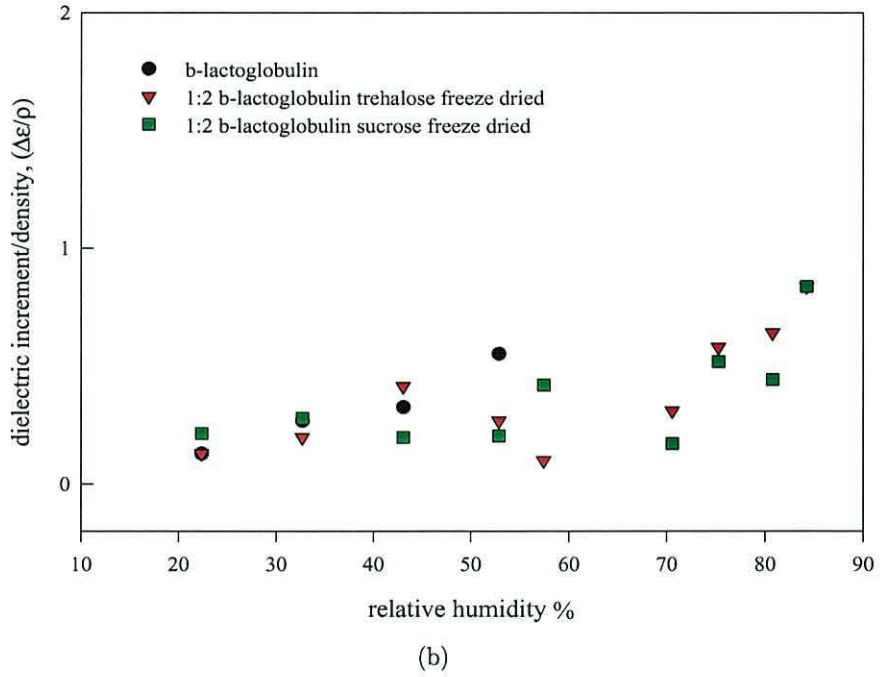
Figure VIII.6: The dielectric increment of the dispersion centered at 100 MHz plotted against relative humidity

In the frequency range considered, two dielectric dispersions were observed, the first at approximately 3 MHz and the second at about 100 MHz, which was consistent with the previously observed dispersions of β-lactoglobulin on its own. This seems to indicate that the presence of sugar does not have an effect on the relaxation frequency. It was found however that, whereas the frequency of both dispersions appeared to be independent of hydration, the magnitude of both dispersions was found to follow a complex hydration dependency, both increasing and decreasing over the hydration range studied.

This variable effect of increasing water content on the dielectric response of the hydrated protein can be monitored by studying the dependence of the dielectric increment, $\Delta\epsilon$, on the relative humidity (figures VIII.6 and VIII.7).



(a)



(b)

Figure VIII.7: (a) The dielectric increment of the lower frequency dispersion of 1:2 β-lactoglobulin trehalose and sucrose mixtures plotted against relative humidity. (b) A closer view

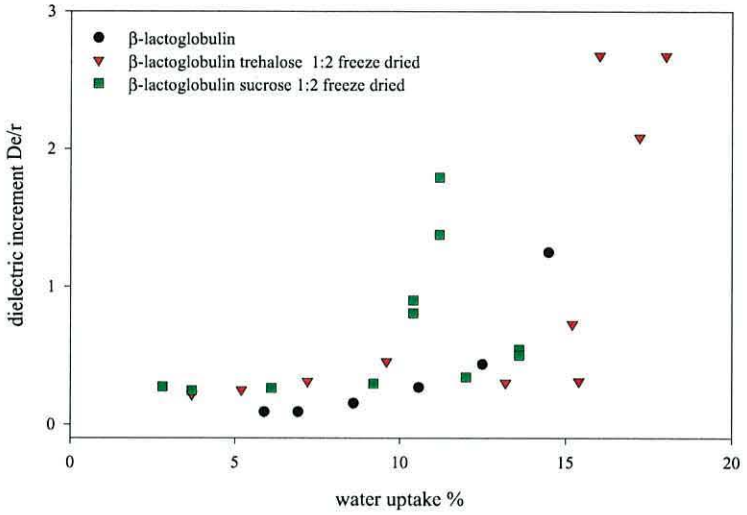


Figure VIII.8: The dielectric increment of the dispersion centered at 100 MHz plotted against protein water uptake of 1:2 β-lactoglobulin trehalose and sucrose mixtures

It can be seen that, over the whole relative humidity range, for both dispersions, the dielectric increment of the mixtures, in compared to that of the protein on its own, is relatively insensitive to the addition of water molecules. For both dispersions the same pattern is observed; up to a certain relative humidity the dielectric increment of the mixtures is close to that of the protein on its own. However at approximately 40-50 %RH for sucrose containing samples and 50-60 %RH for trehalose mixtures the dielectric increment shows a discontinuity, after which the dielectric increment of the mixtures' is completely different to that of the protein. Note that, at the mentioned relative humidities, the hydration isotherms of the mixtures show a marked transition.

The hydration dependence of dielectric increment can also be seen in figures VIII.8 and VIII.9, where the dielectric increment is plotted against the water uptake (for clarity only freeze-dried mixtures are shown). Up to 10% water content the mixtures seem to follow the protein characteristic, however, around this point mixtures present marked discontinuities. Note that at this hydration value a transition is seen in the sorption isotherms.

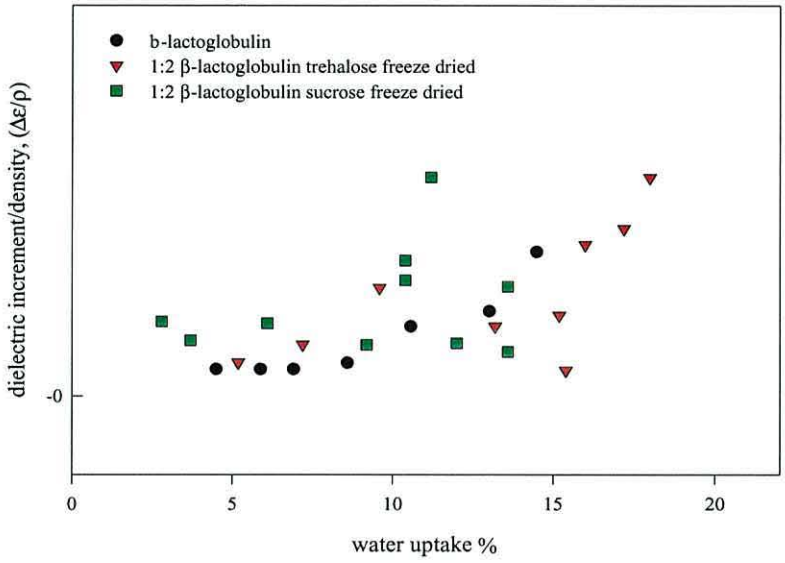


Figure VIII.9: The dielectric increment of the dispersion centered at 3 MHz plotted against protein water uptake, 1:2 β-lactoglobulin trehalose and sucrose mixtures

4 Discussion

The experimental procedures performed in this study have involved the characterisation of the water-binding properties of freeze-dried, spray-dried and evaporated β-lactoglobulin sucrose and β-lactoglobulin-trehalose samples over the hydration range 0 to 85% relative humidity.

Data collected on the protein-sugar samples indicate that, to a large extent, the water-binding trends of these complexes are independent of the drying method. This is especially true for the protein-trehalose samples but less so for β-lactoglobulin-sucrose samples, where the hydration value at which the glass to crystal transition occurs is somewhat dependent on the method of sample drying.

It is evident from comparison of measured isotherm data of protein-sugar complexes with that constructed from isotherms of the components, that there is significant interaction between the protein with both trehalose and sucrose.

If the difference between the experimental and modelled isotherm which assumes

non-interacting species, is considered to reflect the magnitude of the interaction, a parameter describing/quantifying the protein-sugar interaction can be defined. This parameter is taken to be the average, over the hydration range where interaction occurs, of the difference between the estimated and experimental water uptakes, i.e

$$I = \frac{w_t - w_e}{w_t} \tag{VIII.1}$$

where w_t and w_e are, respectively, the theoretical water uptake for non-interacting mixtures and the experimental water content.

This parameter is then related to the interaction between species present in a particular sample as follows; when I is equal to zero there is no interaction between species, when $I > 0$ (i.e. theoretical isotherm lies above the experimental) we can assume there is a positive interaction involving sites on the protein which would normally be involved in binding water molecules and that a complex is formed between the two species. When $I < 0$ the interaction between species is such that more sites are available to bind water molecules, in this case it is likely the interaction between species has damaged the protein structure resulting in partial protein unfolding.

Of course, it is possible that the positive interaction ($I > 0$) could be due to reversible protein structural changes rather than to direct binding between sugar and protein molecules. However, it can be seen that the difference between the theoretical and experimental isotherms seems to be restricted to the mid-hydration range (e.g. see figure VIII.1) where singly hydrogen bonded water can be assumed to be bound (Bone, 1987; Bone, 1994; Bone, 1996). It seems unlikely that any structural change would be limited to sites which singly-hydrogen bond water.

It is possible that the addition of solutes to the solution prior to freeze-drying could induce structural changes to the protein. However this possibility appears to be unlikely given that the concentration of sugar (solute) used here (1:2 or 1:1 protein to sugar) which is very low compared to the others reported (eg. 1:37) (Xie and Timasheff, 1997) to have a significant effect on the protein structure evidenced by greater preferential hydration[3] of the protein with increasing amounts of solute.

[3]preferential hydration induces a protein to be close to its folded state (for more details see chapter II)

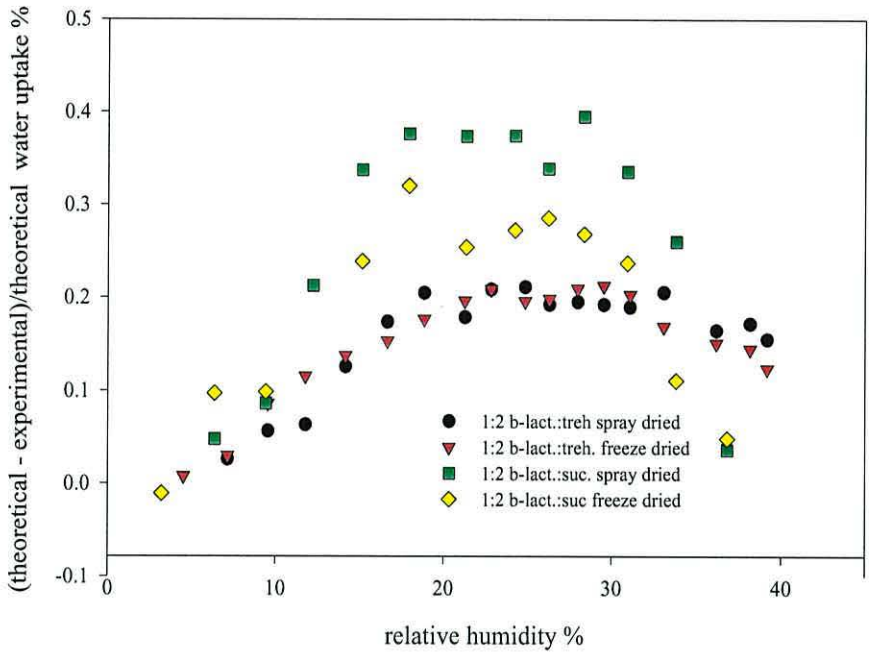


Figure VIII.10: Difference between theoretical isotherm for non-interacting protein:sugar and the experimental isotherms, (1:2 freeze and spray-dried samples).

Therefore, the assumption is made that $I > 0$ implies direct interaction between species. Furthermore, this interpretation is in agreement with previously published works on protein-sugar hydration isotherms. Note other authors (Costantino et al., 1998; Tzannis and Prestrelski, 1999), also studying protein sugar mixtures with the aid of gravimetric studies have reported lower water uptake by lyophilized and spray-dried mixtures in comparison to the water uptake based on the sorption of the separated species and have also interpreted this phenomena in terms of hydrogen bond interaction between the protein and sugar.

Figure VIII.10 describes the difference between the theoretical and experimental isotherms for 1:2 freeze and spray-dried samples; table VIII.1 shows the values of the interaction parameter. It can be seen that for both sugars, the early hydration characteristics of the complexes are almost identical to the modelled data indicating that the interaction may not affect the number of primary bound water molecules which are likely to be tightly bound to the protein by multiple hydrogen-bonds. The interaction appears to be restricted to higher hydrations involving sites where more mobile, singly

hydrogen-bonded water can be assumed to be bound (Bone, 1987; Bone, 1994; Bone, 1996). In addition, it can also be seen that the extent of the interaction seems slightly greater for sucrose than for trehalose.

	spray-dried	*freeze-dried*	*evaporated*
β-lactoglobulin:trehalose (1:2)	0.2	0.18	0.20
β-lactoglobulin:sucrose (1:2)	0.27	0.27	0.27
β-lactoglobulin:trehalose (1:1)	–	0.18	–
β-lactoglobulin:sucrose (1:1)	–	0.26	–

Table VIII.1: Estimated interaction parameter for β-lactoglobulin:trehalose/sucrose mixtures

The fact that hydrogen bonding interactions between the protein and sugar exist has been previously reported (Prestrelski et al., 1993a; Allison et al., 1999; Lopez-Diez and Bone, 2000). For instance Allison and colleagues (Allison et al., 1999) have shown, in their FTIR study, that trehalose and sucrose bind to lysozyme carboxyl groups, as indicated by the appearance of the carboxylate band at 1580 cm^{-1} in freeze-dried mixtures. It was observed that, in the absence of sugar, this band disappeared due to the reduction in hydrogen bonding to these groups as a result of the removal of water. This appeared to indicate that sugar molecules replace water bound to these groups. That sucrose forms more hydrogen bonds with the protein than trehalose, i.e interact more effectively, is also consistent with previous findings (Allison et al., 1999). Allison and coworkers (Allison et al., 1999) suggested that such an effect could be due to trehalose being more excluded from the polymer's surface in frozen solutions (Izutsu et al., 1996), an effect that could have continued in the dried state.

The protection mechanism describing the effect of sugars on proteins in solution is well established (Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1983); additives that are (more) excluded from the protein surface are those which are (more) effective in stabilizing the protein structure (a detailed description of the mechanism can be found in chapter II). This mechanism has also been invoked to explain protection against freezing (Crowe et al., 1984b). Moreover, it has also been suggested that, in order to be a good protectant during freeze-drying, an additive has to be first an effective cryo-protectant (Crowe et al., 1990; Prestrelski et al., 1993b). Thus, as trehalose and sucrose are good protectants in solution they are

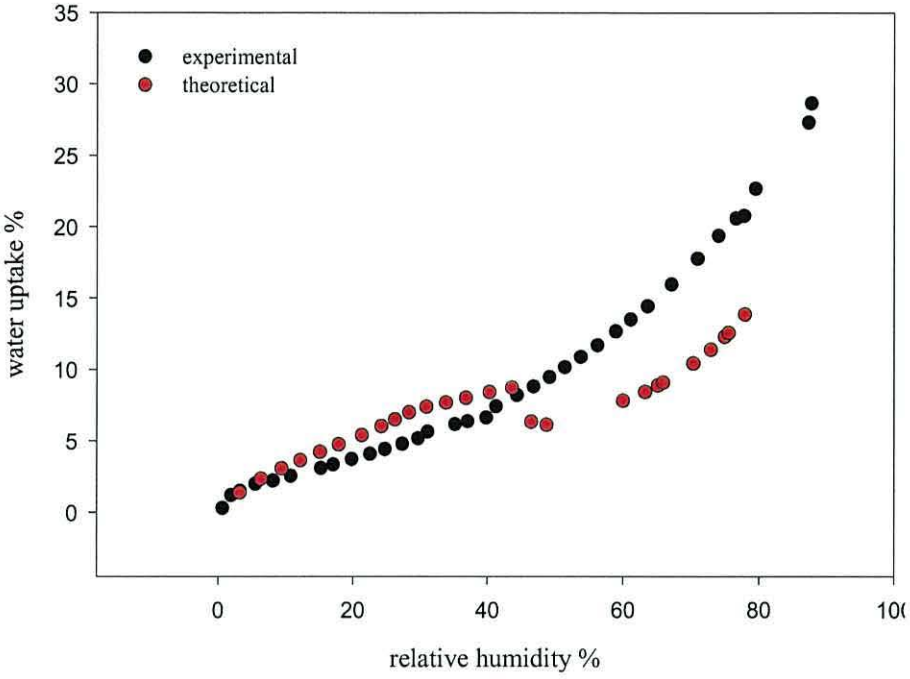


Figure VIII.11: β-lactoglobulin-trehalose hydration isotherms, freeze-dried 1:1 weight ratio

excluded from the protein surface (Arakawa and Timasheff, 1982; Xie and Timasheff, 1997). This effect may continue when the solution is frozen. Furthermore, Izutsu and co-workers (Izutsu et al., 1996) have demonstrated that, in frozen solutions, interactions between polyethyleneglycol (PEG) and trehalose are more repulsive that those of PEG and sucrose. This would seem to indicate that increased repulsive interactions in frozen solutions may result in less hydrogen bonding between protein and trehalose in the dried state.

To further support the results obtained, i.e that β-lactoglobulin positively interacts with the sugars and that sucrose seems to hydrogen bond to the protein to a greater extent that trehalose, freeze-dried 1:1 weight ratio, protein to sugar samples were also gravimetrically studied. For these samples, only the first hydration isotherms were recorded (figures VIII.11 and VIII.12).

The estimation of the interaction between species again showed that this interaction was restricted to the mid-hydration range and that sucrose seemed to form more hydrogen bonds with the protein compared to trehalose. Figure VIII.13 shows the

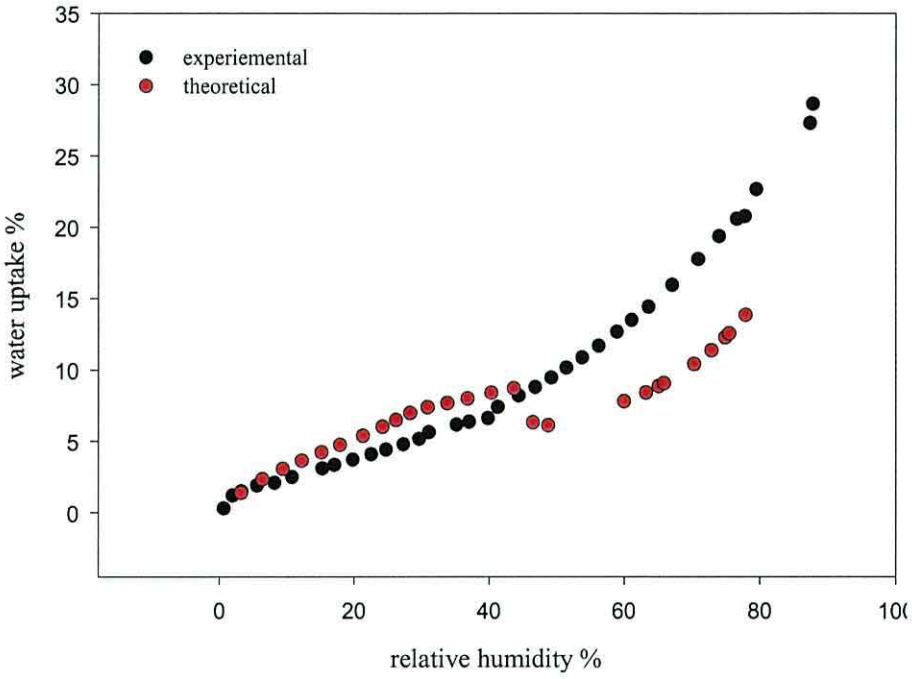


Figure VIII.12: β-lactoglobulin-sucrose hydration isotherms, freeze-dried 1:1 weight ratio

extent of this interaction as a function of relative humidity and table VIII.1 lists the estimated interaction parameters, I. Interestingly, the extent of the interaction for both sugars seemed to be the same as with 1:2 samples, which appears to indicate that the hydrogen bond interaction is saturated at levels higher than 50 sugar molecules per protein molecule, equivalent to a 1:1 weight ratio.

On reaching a sufficiently high water content, all protein-sugar samples except the 1:1 protein-sucrose freeze-dried mixture experience a transition which probably reflects crystallisation of the sugar component and phase separation leading to considerable changes in the hydration level. This observation is in agreement with previously published works on trypsinogen-sucrose mixtures (Tzannis and Prestrelski, 1999) and insulin-like growth factor I, recombinant human growth hormone (Costantino et al., 1998).

It has been previously shown, in this work, (chapter VII), and by others (Iglesias et al., 1997; Costantino et al., 1998; Saleki-Gerhardt and Zografi, 1994; Hancock and Dalton, 1999; Tzannis and Prestrelski, 1999) that isothermal hydration of sucrose

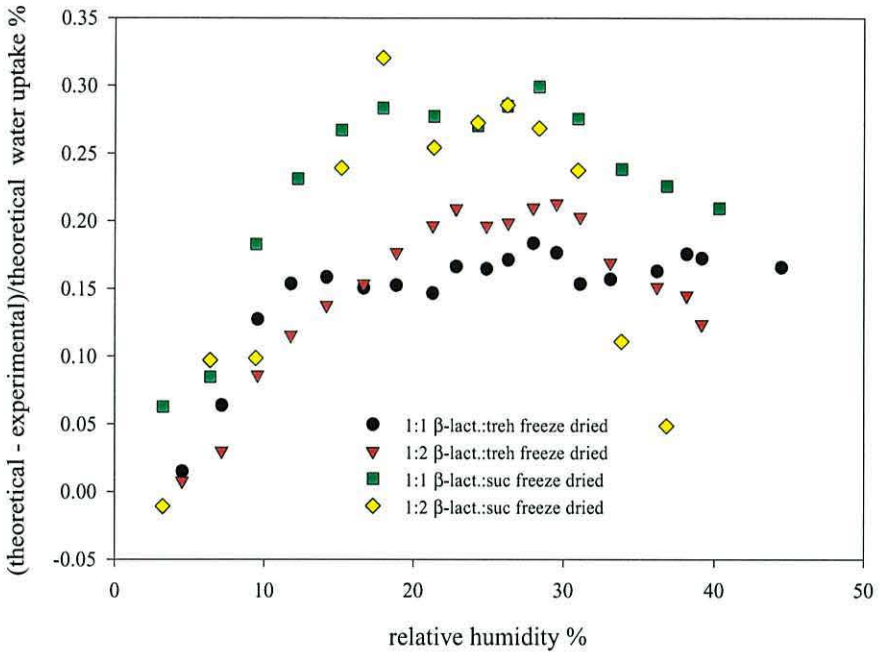


Figure VIII.13: Difference between theoretical isotherm for non-interacting protein:sugar and the experimental isotherms, (1:2 and 1:1 freeze-dried samples).

and trehalose induces structural changes in these sugars which were related to crystal formation. It is not therefore surprising that the isotherms of protein sugar mixtures showed a similar transition. It can be clearly seen, however, that the positions of the transitions are not well predicted by the theoretical isotherms. This probably indicates that the presence of protein molecules reduce the ability of sugar molecules to form intermolecular sugar-sugar hydrogen bonds, thereby retarding crystal formation. This is consistent with the study on the influence of proteins on the infrared spectra of dried carbohydrates (Carpenter and Crowe, 1989) which showed that the spectra of the saccharides which reflected that their structures were affected by the presence of protein molecules.

Following the crystallization transitions, when the hydration measurements were repeated on the resulting sample, very different characteristics were recorded. The subsequent hydration isotherms following the transition are successfully modelled using a suitable combination of isotherms of the pure protein and the evaporated sugar sample[4].

[4]A form which for both sugars has been shown to be in a crystalline state (chapter VII)

From these observations there is a clear indication that, whilst considerable interaction occurs between the protein and sugars when the sugars are in an amorphous, glassy state, there is little or no interaction between the protein and sugars when trehalose and sucrose are present in the mixture in their crystalline forms.

Although the water-binding characteristics of the protein-sugar complexes are essentially independent of preparation method, it is important to note a deviation from this which occurs for evaporated samples at low hydrations. Both β-lactoglobulin sucrose and β-lactoglobulin-trehalose samples prepared in this way appear to have the ability to retain water under conditions of extreme dehydration in marked contrast to identical samples prepared by freeze-drying and spray-drying. This might indicate that under certain conditions protein-sugar mixtures are capable of retaining water molecules when dehydrated, an ability that could have either a beneficial or deleterious effect on the protein structure, beneficial since it could mean that vital water molecules for the protein conformation are retained but deleterious since those water molecules although preserving the protein structure might allow the protein to have a certain degree of mobility that could affect the long term stability of the protein.

Recent studies (Crowe et al., 1998) have concluded that effective preservation requires both the existence of a glassy state and the formation of hydrogen bonds between sugar and protein. In addition, it has also been shown that crystallization of additives has deleterious effects on the protein stability (Terebiznik et al., 1997; Cardona et al., 1997). A finding of this investigation, that hydrogen bonding only occurs when trehalose or sucrose is present in the glassy form, is consistent with this theory.

The formation of a glassy state is, however, no guarantee that hydrogen bonding will occur as evidenced by measurements on dextran (Allison et al., 1999) which readily forms a glass but does not hydrogen bond to the protein. The effectiveness of trehalose in preservation may be thought to be due to a high glass transition temperature(Green and Angell, 1989; Franks et al., 1991), indicating a readiness to form a glassy structure over a relatively wide hydration range, combined with the ability to form significant hydrogen bonding interactions with the protein. However, this appears not to be consistent with measurements on raffinose, a trisaccharide, which is relatively ineffective in preserving protein structures (Terebiznik et al., 1997; Schebor et al., 1999) but which possesses a

glass transition temperature comparable with trehalose (Saleki-Gerhardtand et al., 1995; Wolkers et al., 1998) together with a hydrogen bonding potential reported to be greater than that exhibited by trehalose (Gaffney et al., 1988).

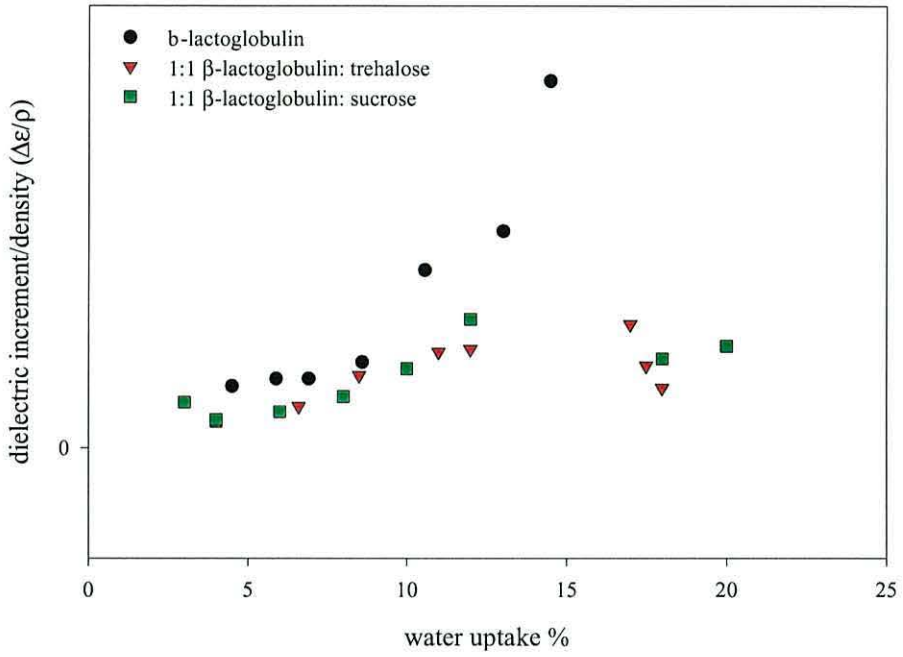


Figure VIII.14: The dielectric increment of the lower frequency dispersion plotted against protein water uptake, 1:1 β-lactoglobulin trehalose and sucrose mixtures

Unfortunately dielectric measurement on protein sugars mixtures did not show any remarkable difference between sucrose and trehalose and could not discriminate between methods of preparation. It appears that the transition seen in the hydration isotherms attributed to crystallization of the sugar component of the mixtures has a dramatic effect on the dielectric response, i.e at the transition humidity level the dielectric increment shows a marked discontinuity. Furthermore, following this transition the dielectric data may not be reliable since sugar crystallization was observed to affect the physical integrity of the samples and its distribution around the inner electrode of the sample cell. Together these facts indicate that the crystallization tendency hampers the measurement of the dielectric response of the protein. Therefore, no definite conclusion could be extracted from the hydration dependent of the dielectric data of protein-sugar

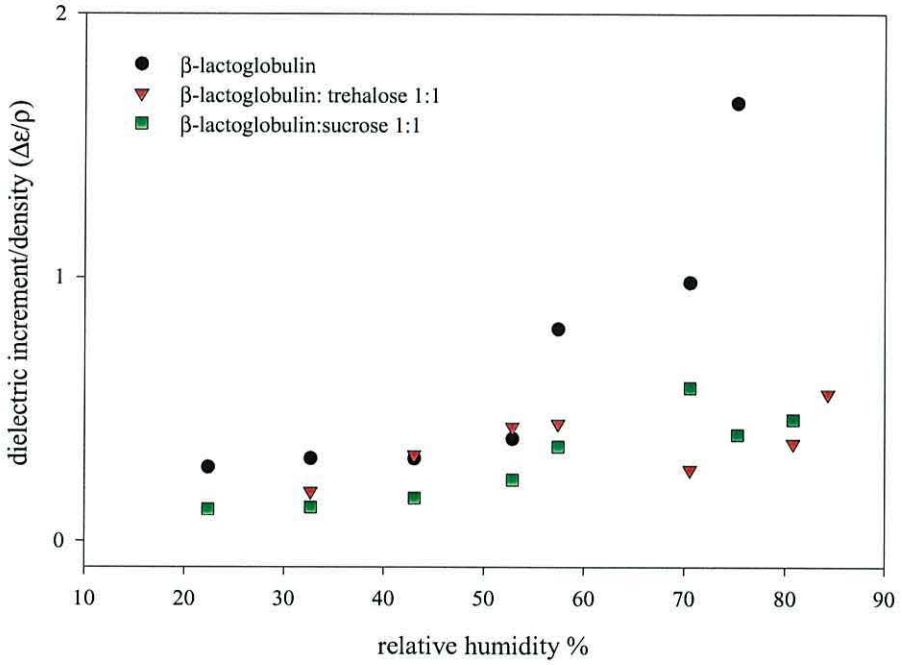


Figure VIII.15: The dielectric increment of the lower frequency dispersion plotted as a function of the relative humidity, 1:1 β-lactoglobulin trehalose and sucrose mixtures

1:2 samples. In spite of these difficulties, since crystallization of sucrose in the 1:1 freeze-dried sample was inhibited and trehalose crystallization was slightly delayed, these samples were also tested. The dielectric increment for these samples also presented a discontinuity (see figures VIII.14 and VIII.15) although at higher relative humidities than in the case of the 1:2 samples. This might indicate that a lower proportion of sugar in the protein-sugar formulation may allow the study of the effect of sugars on the dielectric properties of proteins. It is important to note that, in the case of the sucrose mixture which did not show a transition in the isotherm, a transition is reflected in the behaviour of the dielectric increment, possibly indicating that some structural changes are taking place which are not seen by gravimetric measurements.

5 Conclusion

β-lactoglobulin sugar mixtures have been studied with the aid of gravimetric and high frequency dielectric techniques. It has been found that,

- There is evidence of interaction between the sugar and protein specifically in the hydration region where single hydrogen-bonded water is expected to be bound. The magnitude of the interaction appears to be slightly more extensive for sucrose.
- Following crystallisation of the trehalose and sucrose components of protein-sugar mixtures, phase separation of the sugar and protein takes place into separate non-interacting species.
- For the β-lactoglobulin:sucrose/trehalose samples prepared by evaporation from solution, a small amount (3% by weight) of water is trapped in the complex even under extreme conditions of dehydration. On rehydration, the trapped water is released following the characteristic structural transition.

IX

Trypsin stability

1 Introduction

Detailed knowledge of deactivation kinetics is a prerequisite for accurate interpretations of preservation phenomena. The quantitative description of deactivation dependency upon additive kind and content may allow the prediction of the usefulness of an additive. Furthermore, if correspondence between the deactivation mechanism and the observed kinetics can be established, then kinetics may be used to identify an appropriate stabilization technique.

A study on trypsin stability is presented in the following section. Long term stability was simulated by incubating the samples at 77°C. This was done under the assumption that incubation at moderately high temperature during a relatively short period of time (over 20 days) will have a similar effect that storage at room temperature for a much longer period of time. This assumption is based in two considerations: (1) molecular mobility and thus protein fluctuations are the main factor responsible for protein denaturation and (2) storage at moderately high temperature will induced the same mobility and induced the same structural rearrangements in the protein:additive formulations as storage at room temperature.

The protein degradation during incubation at relatively high temperature (77°) has been characterized and compared to the protein-additive mixtures' activity decay. This study has not only considered trehalose and sucrose, dissacharides whose preservation properties have been extensively studied, but also 2-hydroxypropyl-β-cyclodextrin, an oligosacchride whose preservation potential has recently been considered (Cooper, 1992; Branchu et al., 1999a; Branchu et al., 1999b; Li et al., 2001).

2 Materials and methods

a Sample preparation

Trypsin (from Porcine Pancreas, crystallized and lyophilized), sucrose (99.5%) and trehalose (dihydrate, from Saccharomyces cerevisiae) were purchased from Sigma Chemical Company and used without further purification. 2-hydroxypropyl-β-cyclodextrin was obtained from Fluka Chemicals and was also used with no further purification.

Protein and various protein:additive samples were freeze-dried, incubated at 77°C and then dissolved in 100 mM phosphate buffer for enzyme activity measurements.

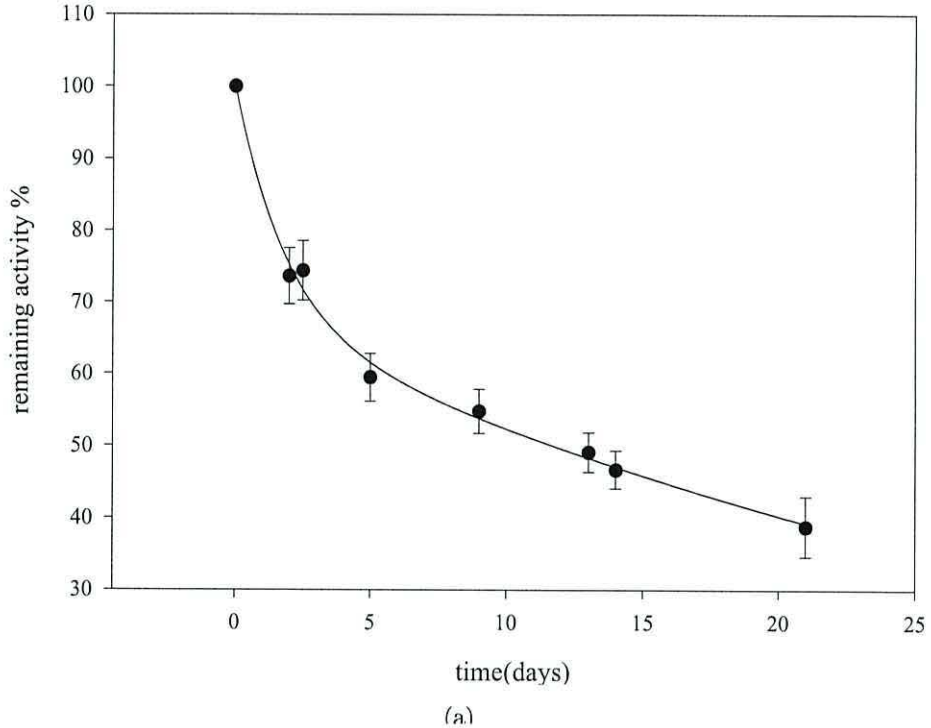
b Experimental techniques. Enzymatic assay

Activity, defined as the increment of absorbance at 253 nm per minute (Δ Abs/min), was measured using a double beam spectrophotometer (Hitachi, model U-2000)[1]. The reaction[2] used in the activity assays was Nα-Benzoyl-L-Arginine Ethyl (BAEE) hydrolysis in phosphate buffer 100 mM, pH 7.0 at 25^oC. The reaction was started by injecting a given volume of enzyme solution into a BAEE solution preheated at the reaction temperature. Each sample solution was tested three times and the mean value was considered.

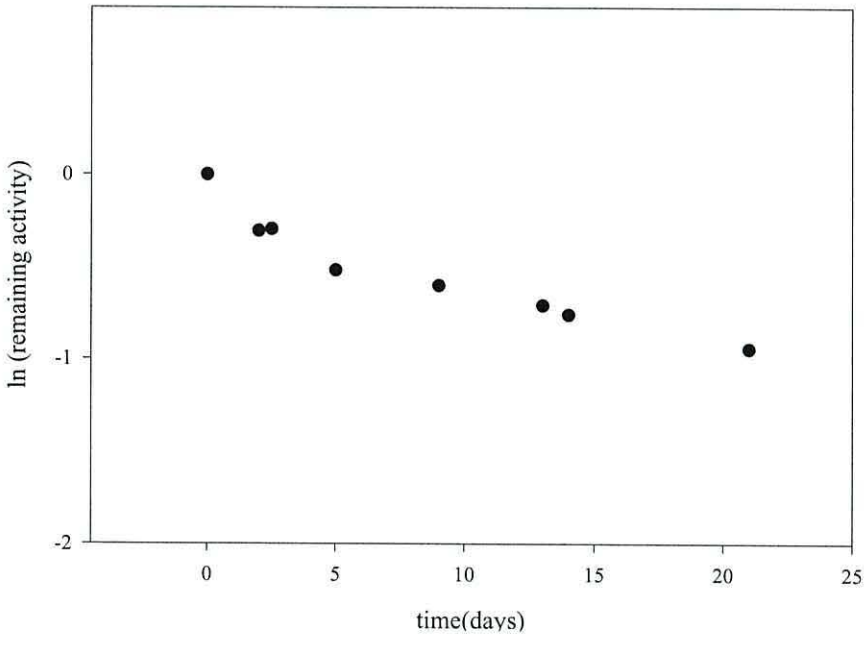
The ΔAbs/min of freeze-dried trypsin that has not been heat treated, A_o, was considered to correspond to 100% trypsin activity. ΔAbs/min at time t of heat treatment, A_t was referenced to A_o to determine remaining activity (r.a.), so that r.a.= $A_t/A_o \times 100\%$.

Error assessment

The reproducibility of the activity determination was estimated as follows; 10 replicates of trypsin solutions were prepared and the activity was measured as described previously. The standard deviation of the mean values is represented graphically as error bars in figure IX.1.



(a)



(b)

Figure IX.1: Thermal deactivation of freeze-dried trypsin incubated at 77°C (a), remaining activity vs. time (b) ln(remaining activity) vs. time

3 RESULTS

a Trypsin

Figure IX.1 shows the deactivation curve for trypsin. Protein denaturation is usually regarded as a two state process, i.e a transition from a native, folded state to a denatured, unfolded state. If this were so, one would expect the deactivation process to follow first order kinetics (see chapter III). By contrast, however, it can clearly be seen that trypsin deviates from this behaviour as shown by the convex ln(remaining activity) vs. time curve (fig. III.1). In literature, (for references see (Toscano et al., 1994)) departure from first order kinetics has been explained as being due to different mechanisms, for instance, aggregation and/or enzyme hetereogeneity, and consequently modelled by the appropriate equations.

It has been pointed out however, that several of those mechanisms can be described by a three parameter bi-exponential equation (Aymard and Belarbi, 2000), of the form

$$\frac{A_t}{A_o} = Ae^{-\alpha t} + (1 - A)e^{-\beta t} \; . \tag{IX.1}$$

This equation has been traditionally associated with the existence of two isoenzymes deactivating at different rates (α, β), but, as previously stated, it can also describe different deactivation mechanisms, such as the series model where the deactivated state is achieved by a sequence of first order reactions, or the competitive model where the enzyme is inactivated via two independent reactions, one of them reversible or by enzyme hetereogeneity (Aymard and Belarbi, 2000; Toscano et al., 1994).

Aymard and Belarbi (Aymard and Belarbi, 2000), suggested the use of equation IX.1 as a mechanism free model, that is, one which does not consider any particular deactivation process. The use of the bi-exponential, three parameter equation is then justified since it facilitates the analysis of the data and reduces the risk of cross-correlation, (i.e reduces the possibility of finding several sets of parameters with equivalent goodness of the fit) in comparison with an equation with a higher number of parameters.

[1] A schematic diagram of a light absorption measurement is shown in figure III.7 to be found in chapter III

[2] details of the reaction to be found in chapter III, section 8

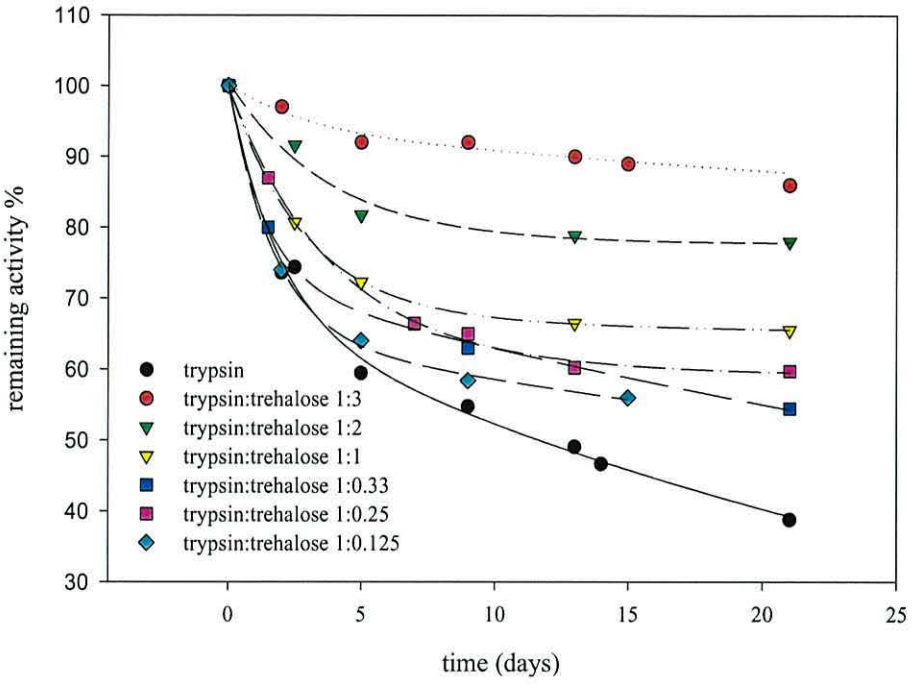


Figure IX.2: Thermal deactivation of freeze-dried trypsin:trehalose mixtures incubated at 77°C

Trypsin data has been successfully modelled ($R^2 > 0.99$) by equation IX.1, giving the values 0.3252 (32.52%), 0.54 and 0.026 days^{-1} for A, α and β respectively, which compare very well with previously published results (Aymard and Belarbi, 2000).

b Trypsin:trehalose/sucrose mixtures

The effect of trehalose molecules on trypsin deactivation can be seen in figure IX.2 which shows, together with the loss of trypsin activity, the deactivation curves for trypsin-trehalose mixtures during incubation at 77 ± 1°C. The protein-sugar ratio was varied from 1:0.25 to 1:3. It can be seen that identical activities were recorded for all mixtures at the beginning of the experiment ($t = 0$) indicating that sugar concentration had no effect on the enzyme activity just after freeze-drying. It is evident from figure IX.2 that for samples with increasing amounts of trehalose, the loss of enzymatic activity decreases.

The stabilization effect of sucrose on trypsin is shown in figure IX.3. It can be seen that sucrose mixtures follow the same trend as trehalose mixtures; initially addition of

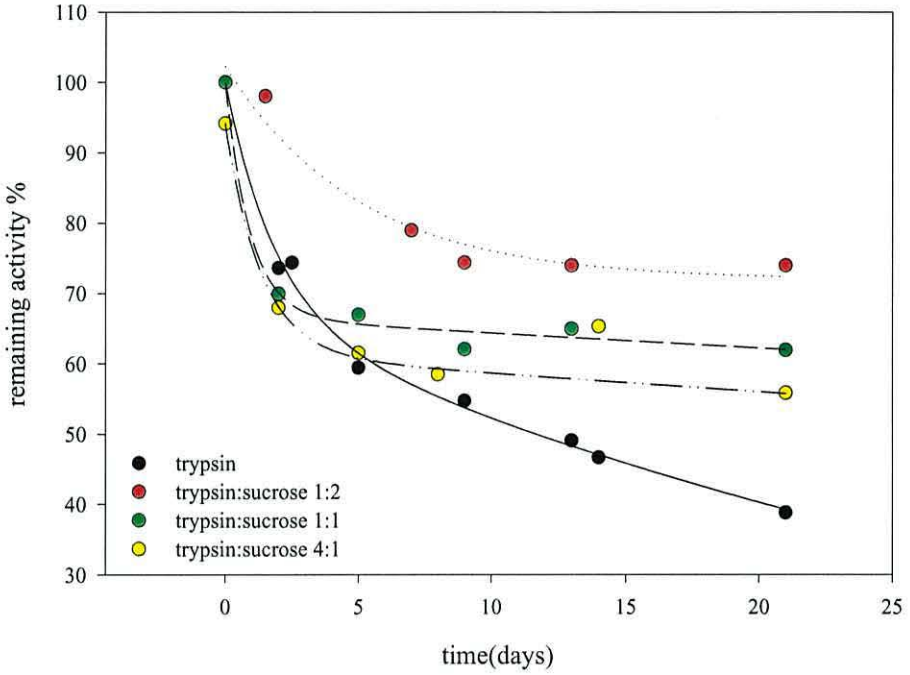


Figure IX.3: Thermal deactivation of freeze-dried trypsin:sucrose mixtures incubated at 77°C

sucrose to freeze-dried trypsin samples does not affect the enzyme activity, i.e remaining activity at $t = 0$ days is 100% and, throughout the storage period, with increasing fraction of sucrose, the loss of enzymatic activity decreases. Furthermore, for both sugars, mixture deactivation curves show the same trend as the protein on its own, that is, mixtures deactivate according to a non-first order kinetics. However the mixture characteristics showed an important feature; after a few days of storage the degradation curves saturate and reach an almost constant value.

The complex behaviour of protein-sugar degradation kinetics was observed previously (Pikal and Rigsbee, 1997; Davidson and Sun, 2001). Pikal and Rigsbee interpreted it as due to the presence of a degradation intermediate and their data was analyzed using "the square root of time kinetics". In contrast, Davidson and Sun did not find this "square root of time kinetics" behaviour and used first order kinetics and, after a certain time of storage, zero order kinetics for data analysis.

In the present work, the bi-exponential, three parameter equation IX.1 (Aymard and Belarbi, 2000), used to model trypsin degradation, has been used to model the mixtures'

trypsin:additive	A	α	β	R^2
1:0	32.52	0.54	0.026	0.99
trehalose				
1:3	4.9	0.45	0.004	0.98
1:2	22.30	0.26	$2.28 \cdot 10^{-10}$	0.97
1:1	33.09	0.34	0.001	0.99
1:0.33	28.13	0.72	0.013	0.97
1:0.25	30.05	0.99	0.009	0.99
1:0.125	35.70	0.58	0.01	0.99
sucrose				
1:2	28.41	0.18	0.026	0.95
1:1	33.43	0.16	0.0034	?
1:0.25	38.35	0.84	0.0048	0.95

Table IX.1: Bi-exponential parameters

deactivation curves. Goodness of the fit can be evaluated by the close match between experimental data and the modelled curved, as well as by the R^2 values which vary from 0.99 to 0.95.

If the bi-exponential equation is considered to represent simultaneous deactivation isoenzymes with the same catalytic properties the parameters A, $(1-A)$ α and β would be, respectively, the initial proportions of each form of enzyme and the inactivation rate constants. Dependency of these parameters with content of sugar seems to indicate, as expected, that the initial proportion of each isoenzyme is not affected by the presence of sugar. However, the rates of deactivation α and β, although they do not appear to follow a definite trend somewhat decrease with the amount of sugar, that is the rate at which the enzyme deactivates decreases with increasing amount of sugar.

It can be clearly seen that, although the equation fits the data reasonably well ($R^2 > 0.95$) the observed trend of the estimated parameters with the content of either sugar in the mixture, does not seem to clarify the mechanism responsible for the denaturation of the enzyme in the presence of sugars.

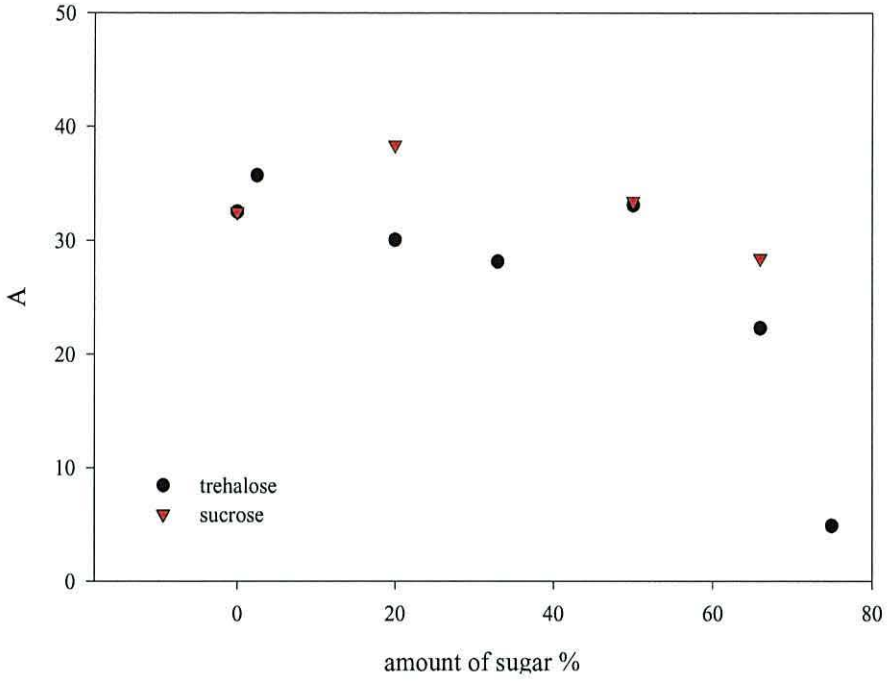
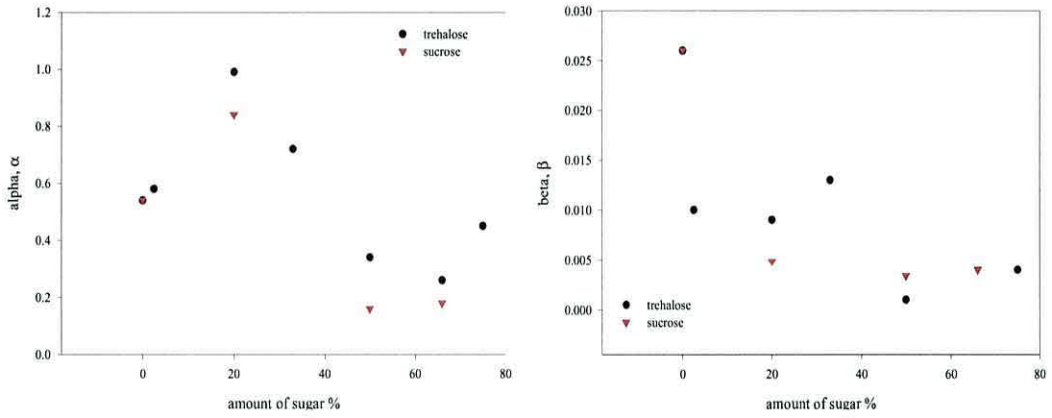


Figure IX.4: Variation of the constant A with amount of sugar.



(a) (b)

Figure IX.5: Variation of the rate constant (a) α and (b) β with amount of sugar.

c *Trypsin:2-hydroxypropyl-β-cyclodextrin mixtures*

2-hydroxypropyl-β-cyclodextrin (hp-β-CD) was also tested for comparison. hp-β-CD has been reported (Branchu et al., 1999a; Branchu et al., 1999b) to protect proteins under certain circumstances (e.g. spray dried induced damage) and has also been shown that it can favour denaturation in other cases (thermal stability in solution) (Cooper, 1992).

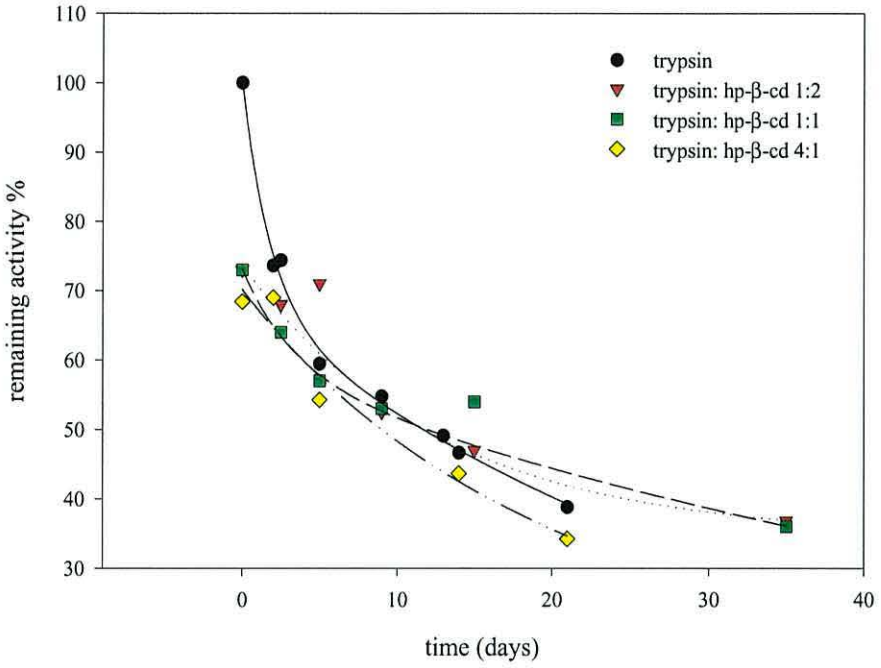


Figure IX.6: Thermal deactivation of freeze-dried trypsin:2-hydroxypropyl-β-cyclodextrin mixtures incubated at 77^{o}C

Under the circumstances considered in this study hp-β-CD is clearly not protecting the enzyme. After freeze-drying, all samples studied showed less activity than the actual protein on its own, and during storage hp-β-CD does not show any apparent effect on the protein, if anything it seems it slightly destabilizes it (figure IX.6). The hp-β-CD containing mixtures could not be successfully modelled by the bi-exponential equation, curves in the graph are only drawn as a guide.

4 Discussion

Enzyme activity measurements performed in this study have characterized the deactivation kinetics of trypsin and related mixtures throughout the incubation period. Addition of trehalose and sucrose resulted in stability enhancement whereas the presence of hp-β-CD does not appear to have a beneficial effect on trypsin deactivation.

In order to quantify the extent to which the sugars protected trypsin against heat stress when in the solid state, the data has been analyzed as follows; the initial rapid activity decay during the first five days of incubation has been characterized by applying

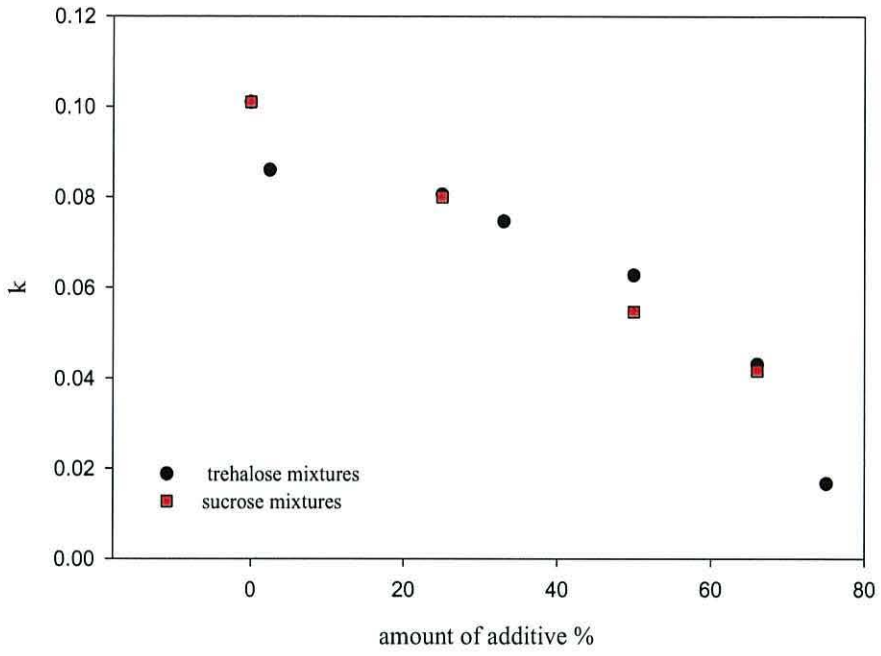


Figure IX.7: Variation of the first order rate constant k with amount of trehalose and sucrose%

first order kinetics, while the quasi-steady state part of the mixtures' deactivation curves (6 to 22 days) has been described with a parameter or preservation factor calculated using the following expression,

$$P(reservation) = \frac{ra^{av}_{mix} - ra^{13}_{tryp}}{ra^{13}_{tryp}} 100 \qquad \text{(IX.2)}$$

where ra^{av}_{mix} is the average of the remaining activity for each mixture and ra^{13}_{tryp} the remaining activity of trypsin at 13 days of incubation (49.10%)[3]. If denaturation is considered as a continuous conformational change of the enzyme (Toscano et al., 1994) in which the activity is gradually lost and if, during the process the number of catalytic sites is not reduced, the activity of each site is decreased because of the time-progressive loss of the original conformation. In this case the preservation parameter may be understood to be a percentage of structure protected by the additive.

Figure IX.7 shows the dependence of the first order kinetics rate constant, k, on

[3]the 13th day is the middle one in the 'stable' range of the mixtures' curves after the first rapid decay

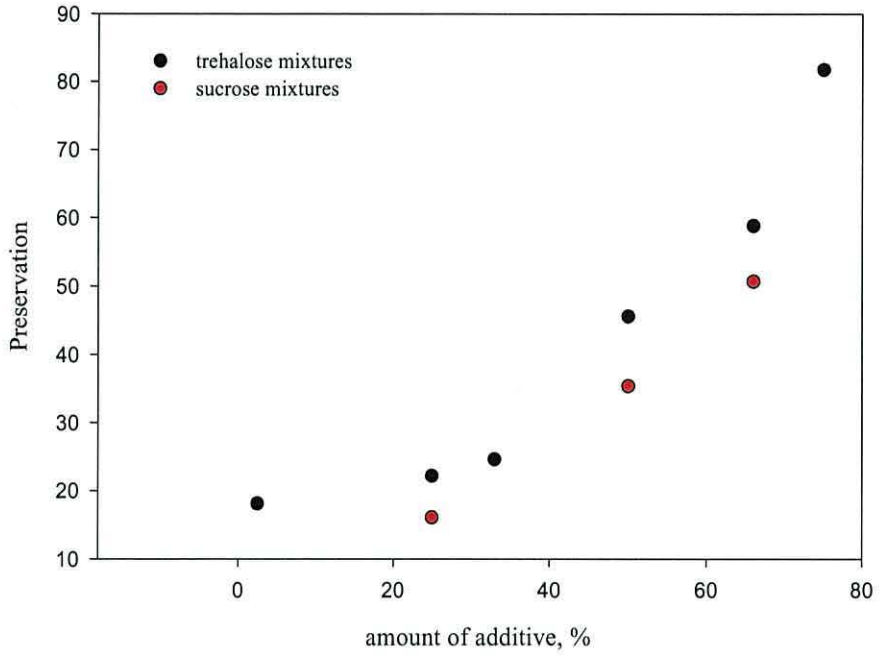


Figure IX.8: Preservation parameter dependency on amount of trehalose and sucrose%

amount of sugar present in the studied formulations. It can be seen that k decreases as the amount of sugar is increased indicating that the rapid decay of enzyme activity is inhibited by the presence of additive. It can also be seen that both trehalose and sucrose are equally effective in retarding the initial activity decay, i.e the mechanism responsible for this initial decay, is only affected by the proportion of additive and not by the particular sugar. However, equivalent formulations of protein:sucrose and protein trehalose, although losing activity at the same rate during the first few days of incubation, reach different steady values (see figure IX.8) between five and twenty two days. The preservation factor, P, therefore not only reflects the fact that increasing amounts of additive enhance the stability of trypsin but also that trehalose is slightly more effective that sucrose. On average, over the range of protein:sugar ratios studied, trehalose seems to improve the protection of the enzyme by approximately 8% compared to sucrose.

Summarizing, results presented here indicate that trehalose appears to be more effective than sucrose in preserving the enzyme which, in general terms, is in agreement

with the work of several others (Sarciaux and Hageman, 1997; Cardona et al., 1997; Mazzobre et al., 1997; Terebiznik et al., 1997; Costantino et al., 1998). However, comparison with other studies should be made with caution since sample preparation and experimental conditions have been found to vary greatly. For example, some authors (Cardona et al., 1997; Mazzobre et al., 1997; Terebiznik et al., 1997) have investigated the stability of several proteins in disaccharide matrices in which the amount of protein is relatively very small, varying temperature of incubation and ambient relative humidity. Other authors have investigated preservation by preparing protein-sugar mixtures with samples having the same glass transition temperatures (Sun and Davison, 1998; Sun et al., 1998) and studied the deactivation at different incubation temperatures. Others have varied the amount of additive in protein-sugar formulation (Sarciaux and Hageman, 1997; Costantino et al., 1998), and some have tried different combinations of additives (Allison et al., 1999; Davidson and Sun, 2001).

Despite sample and experimental condition disparity, the general conclusion that can be obtained from these studies is that trehalose is more effective at protecting proteins than any other saccharide and that the glass transition temperature of samples seems to be closely related to protection, although this relationship does not seem to be a simple one.

One of the current hypotheses which attempts to explain bio-protection by sugars considers the formation of glassy matrices which restrict molecular motion thus reducing, if not completely inhibiting, degradation (Green and Angell, 1989; Franks et al., 1991; Levine and Slade, 1992) (for details see chapter II). Consequently, it has been suggested that the parameter which could determine the stability of a given formulation at a certain temperature is its glass transition temperature, T_g. Since at temperatures below T_g the material is in its glassy state and is considered to be physically stable, degradation should be significantly inhibited. By contrast, at temperatures above T_g, the glass rapidly loses its low molecular mobility e.g. its high viscosity, hence the glassy matrix recovers molecular motion and the formulation is more likely to suffer degradation. However, it is widely known (Crowe et al., 1998) that a pure protein alone will form an amorphous phase in the final dried solid having a relatively high glass transition

temperature[4] (e.g. see (Sochava, 1997)). Yet simply keeping an unprotected protein sample below its T_g is not sufficient to ensure preservation over an extended period. It has been suggested that in addition to storage below T_g, long term stability could be dependent on retaining the native structure in the dried solid (Prestrelski et al., 1993b; Prestrelski et al., 1995); Studies on interleukin-2 showed that the protein, when stored unfolded[5], was less stable than when stored in its native form. All the same, degradation of the native form was still significant. Consistent with this, it has been observed that protein degradation, quantified by the extent of deamination and aggregation, was greatly accelerated at temperatures above T_g whilst rates of degradation were greatly reduced at temperatures below T_g. However, in several cases storage below T_g did not ensure stability (Prestrelski et al., 1995). In addition, it was noted following IR spectroscopic studies, that structural changes arising during lyophilization led to further deactivation during subsequent storage even if the storage temperature was less than the T_g of the system (Chang et al., 1996).

As for protein:sugar mixtures, it has also been shown that stability is significantly enhanced when the storage temperature is below T_g, although in contradiction to the 'glass transition' theory, degradation at temperatures below the T_g was still observed (Cardona et al., 1997; Sarciaux and Hageman, 1997; Mazzobre et al., 1997; Terebiznik et al., 1997; Sun and Davison, 1998; Sun et al., 1998; Davidson and Sun, 2001).

In the light of these results, several authors (Cardona et al., 1997; Mazzobre et al., 1997; Terebiznik et al., 1997) have indicated that T_g might not be the best parameter for predicting stability of protein:additives, since, although molecular mobility may be significantly decreased in the glassy state, the protein molecule is still mobile enough to lead to denaturation, and loss of enzymatic activity. They proposed, based on experimental data, that, instead of using T_g as the stability threshold, the difference between storage temperature and the T_g of the sample, $T - T_g$ should be used; the greater the difference the more efficient the protection (Cardona et al., 1997; Mazzobre

[4]According to Slade and Levine (Slade and Levine, 1995) the glass transition temperature of any dry biopolymer is about $200 \pm 50°C$ and at low moisture ($\leq 10\%$) an increase of 1% in its water content reduces the glass transition temperature by $10 \pm 5°C$

[5]note some proteins although unfolded in the dried state, after being freeze-dried, can regain the native conformation when rehydrated (Prestrelski et al., 1993a)

et al., 1997). In addition, it has been observed that crystallization of additives had a deleterious effect on enzyme activity, i.e. when trehalose was allowed to crystallise either by exposing the samples to high relative humidities or high temperatures the protective effect was lost as trehalose formed a separate phase no longer associated with the protein (Cardona et al., 1997; Terebiznik et al., 1997).

Unfortunately, the present study did not provide any information about the physical state of the matrices during storage or at the end of heat treatment. However, the glass transition temperature of the protein:sugar mixtures may be estimated using the Gordon-Taylor equation (Schneider, 1997; Khalloufi et al., 2000; Matveev et al., 2000) which appears to be 'the simplest and most reliable approach for use with amorphous pharmaceutical materials' (Hancock and Zografi, 1997) and has the form,

$$T_{gmix} = \frac{\alpha_s T_{gs} + K\alpha_p T_{gp}}{\alpha_s + K\alpha_p} \tag{IX.3}$$

where $\alpha_{s,p}$ are the fraction of each component present in the system, sugar and protein and K is a constant given by $\Delta C_{pp}/\Delta C_{ps}$ where ΔC_p is the heat capacity increment at the glass transition.

trypsin:additive	T_g (°C)	$T - T_g$
trehalose		
1:0	115	−38.0000
1:3	106.07	−29.0714
1:2	106.52	−29.5254
1:1	107.64	−30.6471
1:0.25	110.96	−33.9016
sucrose		
1:2	83.51	−6.5106
1:1	87.56	−10.5926
1:0.25	99.58	−22.5833

Table IX.2: Estimated glass transition temperatures for trypsin:trehalose/sucrose mixtures

Consequently, the T_g values of mixtures used can be calculated using equation IX.3, and considering 115°C, 0.2 J/Kmol , 105°C, 0.55 J/Kmol, 78°C, 0.56 J/Kmol as the $T_g's$ and heat capacity increments at the glass transition of anhydrous trypsin[6](for reference

[6]Note that although the glass transition temperature of a dried pure protein is expected to be about $150 - 200$°C, values ranging from 110 to 200°C have been reported (Morales and Kokini, 1997; Sochava,

see (Angell, 1995a)), anhydrous trehalose (Shamblin et al., 1999) and anhydrous sucrose (Shamblin et al., 1999) respectively. Estimated T_g values, shown in table IX.2 are in good agreement with those measured by other authors (Sarciaux and Hageman, 1997; Costantino et al., 1998). Estimated values for the studied mixtures indicate that the incubation temperature is below the glass transition temperature, confirming that degradation occurs even when samples are in the glassy state. Moreover, estimated $T_g's$ reveal that the protection effectiveness does not seem to be related solely to the T_g of the sample, since as the amount of sugar increases, the preservation increases but the T_g's of the samples decrease.

If, instead of using T_g as the stability threshold, $T - T_g$ is used, the trend of those values again does not correlate with protection (see table IX.2). Even though this seems to be opposite to what some authors have observed, it may just be due to the large proportion of additive used in their experiments (protein proportion was very small) whereas work carried out here employed formulations in which the proportion of protein is significantly higher.

Summarizing, the protection conferred by the additives to trypsin does not appear to correlate with the parameters, T_g and $T - T_g$, related to glass transition theory. This may not be surprising since formulations studied here have a high protein content and therefore the glass forming properties of the additives may not determine the stability of the studied formulations. Of course, it is necessary to bear in mind that the glass transition temperatures have been estimated from an equation that could be considered rather simplistic.

Nevertheless it is important to note that although in glassy material molecular motion is restricted, it can still be significant below T_g (Hancock et al., 1995; Hancock and Zografi, 1997; Hatley and Blair, 1999; Shamblin et al., 1999; Hancock and Shamblin, 2001). It has been shown that at temperatures as much as 50^o below T_g there can be

1997); this discrepancy may be due to different experimental conditions or just simply because different proteins have different T_g values (Morales and Kokini, 1997) but it may also be due to the difficulty involved in determining the glass transition temperature of proteins in the solid state. This is so because of their intrinsic characteristics, i.e a protein can exist in a large number of sub-states of similar but slightly different free energy and are 'trapped' as such upon dehydration. Consequently, it is reasonable to envision the existence of a wide distribution of relaxation times leading to the lack of an easily observable cooperative transition by thermal analysis. The value chosen here may seem rather low but was taken from the one publication which also gave the heat capacity change at T_g.

significant molecular mobility, which can promote degradative reactions (Hancock et al., 1995). The following question related to protein stability and integrity of biological systems then arises, *"at what temperature do the molecular motions responsible for physical and chemical instabilities cease to become likely over the lifetime of that particular system?"* (Hancock and Zografi, 1997). It has been suggested that this lower temperature limit might correspond to the Kauzmann temperature, T_K[7] (Hancock et al., 1995). Although this might be the case for some systems, the reported the temperature of negligible molecular mobility appears to depend on the method used to assess the molecular motions and also it depends on the particular glass former used.

As yet there are no reliable means of predicting the temperature of negligible molecular mobility in amorphous solids. A conservative approach is usually recommended when defining product conditions of storage (Hancock and Zografi, 1997). Therefore to achieve good stability, samples should be store below their T_K (Hancock and Shamblin, 2001). However, this temperature regime (temperatures below T_g) is particulary difficult to characterize because of the long time-scales required for molecular motions in the glassy state. To overcome this difficulty it has been proposed that the T_K values can be roughly approximated as being two thirds of the glass transition temperature T_K (Hancock and Shamblin, 2001).

It is clear from the previous discussion that a freeze-dried protein can be easily degraded at moderate temperatures probably due to the high mobility and flexibility of the protein structure/side chains. From the deactivation curves (figures IX.2 and IX.3) it can be seen that, (1) sugars prevent the initial enzyme activity decay, an effect that seems to be related to the amount of sugar rather than to the particular sugar and (2) that after a certain period of time (approximately 5 days) the activity decay of the sugar mixtures saturates to almost constant values, trehalose mixtures doing so at higher activity values than the sucrose ones.

The preservation effect observed here may be explained in terms of molecular mobility; initially mixtures are almost as mobile as the protein on its own so they degrade at a similar rate as the protein. It seems possible that during this initial period

[7]In theory, this temperature corresponds to the point where structural and configurational mobility is reduced to a level equivalent to that of the crystalline state.

of incubation both protein and sugar are mobile and therefore can arrange themselves in a new configuration in which mobility responsible for degradation is totally or partially inhibited. As a result degradation seems to stop or at least is greatly retarded.

5 Conclusion

In this study trypsin activity assays were performed in an attempt to characterize the deactivation kinetics of the enzyme in the presence of additives. The obtained results seem to indicate that

- Trypsin on its own denatures at moderate temperatures following non-first order kinetics.
- Trypsin-sugar also denatures during incubation although to a lesser extent than the protein.
- Initial activity decay in the presence of sugars is affected by the amount of sugar in the sample and not by the sugar type. However, the overall effect of the sugars on the enzyme deactivation is that (1) sugar reduces the extent of the deactivation, (2) trehalose is more effective than sucrose.

X

TRYPSIN:SUGAR MIXTURES

1 INTRODUCTION

Protein preservation throughout a particular storage regime has been characterized in the previous chapter. However, activity measurements on their own can not explain the mechanism/s by which such preservation is achieved. This chapter attempts to gain an insight into the protein:additive interactions which might be relevant to protein stabilization, through the study of the hydration properties of trypsin:additive mixtures.

2 MATERIALS AND METHODS

a Sample preparation.

A commercial preparation of trypsin (from Porcine Pancreas, crystallised and lyophilized) was used for all the experiments run in this work, together with sucrose (> 99.5%) and trehalose (dihydrate, from Saccharomyces cerevisiae) was purchased from Sigma Chemical Company and used without further purification. 2Hydroxypropil-β-cyclodextrin (HBCD) was obtained from Fluka Chemicals and was also used without further purification.

Samples of different trypsin:additives ratios by weight were freeze-dried and subsequently incubated at 77°C.

b Experimental techniques.

Enzymatic assay Activity, defined as the increment of absorbance at 253 nm per minute (ΔAbs/min), was measured using a double beam spectrophotometer (Hitachi, model U-2000) (for further details on the spectrophotometer see chapter III). The reaction[1] used in the activity assays was Nα-Benzoyl-L-Arginine Ethyl (BAEE)

[1] details on the reaction to be found in chapter III, section 8

hydrolysis in 100 mM phosphate buffer , pH 7.0 at 25°C. The reaction was started by injecting a given volume of enzyme solution into a preheated BAEE solution at the reaction temperature. Each sample solution was tested three times and the mean value was considered.

The ΔAbs/min of freeze-dried trypsin that has not been heat treated, A_o, was considered to correspond to 100% trypsin activity. ΔAbs/min at time t of heat treatment, A_t was referenced to A_o to determine remaining activity (r.a.), so that r.a.= $A_t/A_o \times 100\%$.

The reproducibility of the activity determination was estimated as follows; 10 replicates of trypsin solution were prepared and the activity was measured as described previously. The standard deviation of the mean values was 6%.

Hydration isotherms Measurement of the hydration isotherms for trypsin:additive structures were performed using a computer controlled Sartorius vacuum microbalance (type 4332). Prior to each hydration isotherm experiment, samples were dried in vacuum ($1 \cdot 10^{-2}$Torr) for at least 24 hours. The water uptake of the protein samples was measured for step increases in water partial pressure of approximately 2.5% over the hydration range 0 to 90%. The relative humidity level was controlled to within 0.1% and the temperature was maintained at 25 ± 0.1°C. The partial pressure measurements were made by manometer ($0 - 15\%$ relative humidity) and a calibrated Humilab HL24D vacuum humidity probe (10-90% RH)[2].

Usually at least two hydration runs were performed on each sample before and after incubation. Measurements on each sample were repeated to ensure reproducibility.

Dielectric measurements The hydration dependent dielectric response of the protein-additive samples was investigated using Time Domain Reflectometry (TDR) which covers the frequency range 1 MHz to 1 GHz [3].

As in previous studies (chapter VII and VIII), care was taken to ensure that the choice of time window allowed an adequate recording of the observed waveforms.

[2]for further details see chapter VII

[3]for further details see chapter VII and chapter VI, section VII.2

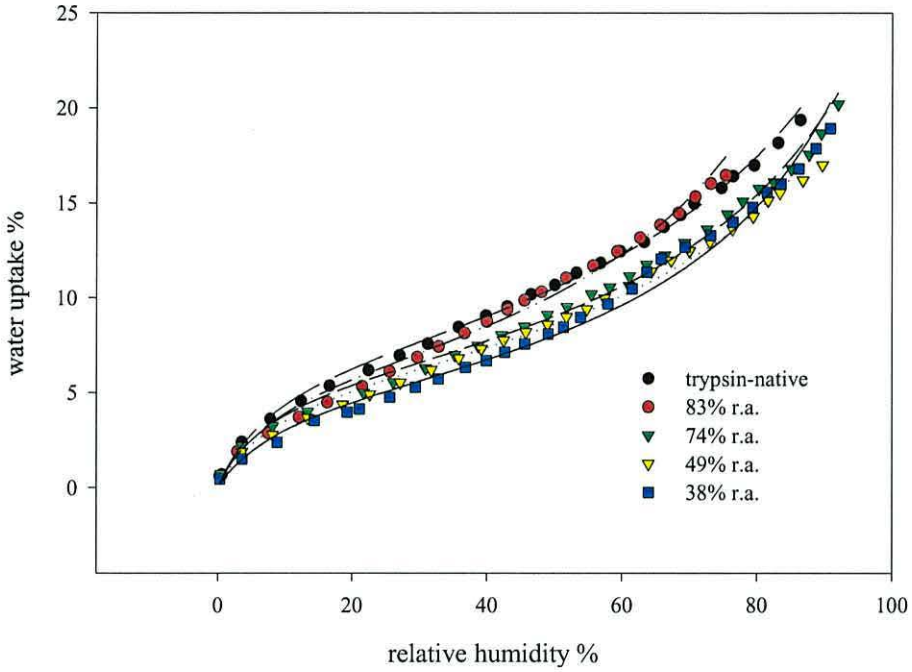


Figure X.1: Trypsin hydration isotherms

Investigating the dispersion centered about $\sim$ 3 MHz a 250 ns time window was used.The inner electrode length was 20 mm.

Packing of samples into the sample cell resulted in measured density values varying between 0.50 and $0.70 \cdot 10^6$ g m^{-3}. Hydration of the samples was achieved as previously described, chapter VII.

3 Results

a *Hydration isotherms*

Denatured trypsin The hydration isotherm of native trypsin at 25 ± 1°C is shown in figure X.1. Also shown are the isotherms for different, incubated trypsin samples at 77°C, which show a decrease in water absorption after incubation. Activity recovery of these samples after incubation was assessed as previously described, see chapter IX; it was found that incubation resulted in loss of enzymatic activity thus in partial denaturation. Activity recovery values are given in table X.1.

The different trypsin isotherms were fitted to a modified GAB equation (Gascoyne and Pethig, 1977) (eq. VII.1) and values for the hydration parameters were obtained

(shown on table X.1). Goodness of the fit may be evaluated by the close match between modelled and experimentally determined isotherms (see figure X.1) and also by the R^2 which varies from 0.97 to 0.99). The value of v_m, corresponding to a monolayer coverage of the primary, highly active binding sites, of native trypsin is 7.65% and it decreases as the protein is denatured. The value of b, a measure of how protein interact with sorbed water molecules, appears to be relatively constant with denaturation but values for denatured trypsin structures are somewhat higher than for the native protein. The closer the b value is to unity the weaker the water-protein interaction is, furthermore, when $b = 1$, the water molecules attached to the protein have the same properties as bulk water.

	a	b	v_m
native	14.27	0.71	7.65%
denatured A[a]	13.42	0.86	6.35%
denatured B[b]	15.96	0.77	6.06%
denatured C[c]	14.29	0.76	5.91%
denatured D[d]	12.02	0.82	5.29%

[a] 83%remaining activity
[b] 75%remaining activity
[c] 49%remaining activity
[d] 38%remaining activity

Table X.1: Hydration parameters for native and denatured structures of trypsin

It seems that, throughout thermal denaturation in the solid state, trypsin undergoes conformational changes which decrease the affinity of the protein for water; denatured trypsin clearly binds less water that the native form.

Decreased water uptake upon denaturation has been previously reported (Rüegg et al., 1975). Rüegg and coworkers observed that thermal, irreversible denaturation of β-lactoglobulin[4] was accompanied by a decrease in the water uptake. The difference between the protein isotherms before and after denaturation being significant after 30% RH, and more pronounced at very high RH. This apparent decrease in water binding observed in sorption measurements after heat denaturation was explained as being due to changes other than deconformation, since unfolding of proteins should result in water uptake.

[4]denaturation was achieved by heating an aqueous solution of the protein for 1 h at 90°C

However Bone (Bone, 1994) found that the denatured form of β-Lactamase produced by chemical stress sorbed more water than the native structure, indicating that denaturation had induced the protein structure to unfold thus making available more binding sites. v_m value was found therefore to increase with denaturation whilst b values of these structures seem to decrease with unfolding.

Together these results seem to indicate that different denaturation pathways can produce changes in a protein isotherm in both directions, i.e. denaturation can cause a protein to sorb more water molecules or to apparently lose water affinity.

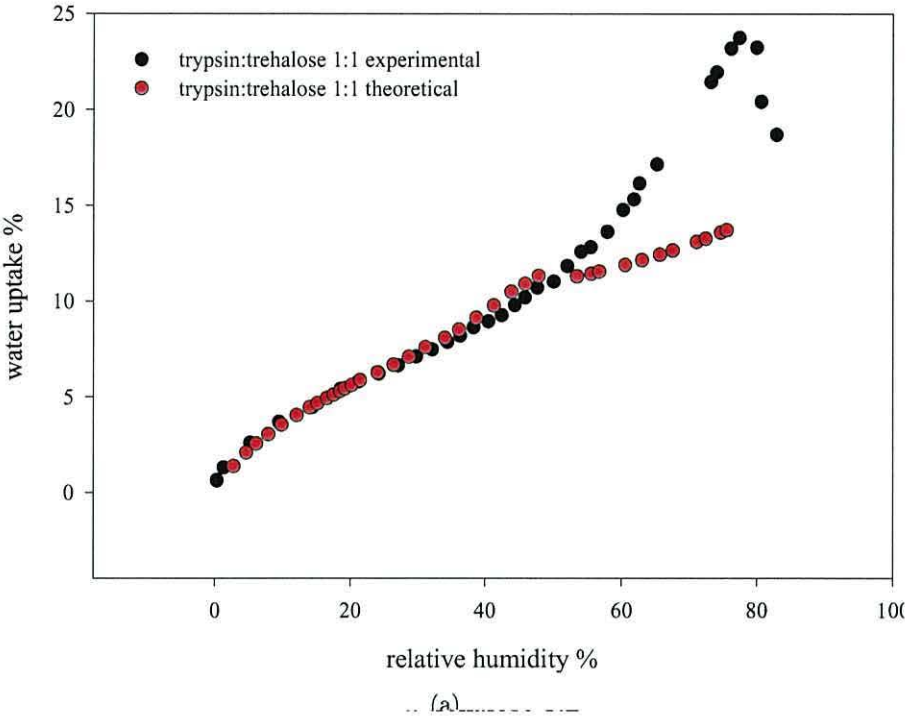
Trypsin-trehalose/sucrose mixtures prior to incubation The hydration characteristics of freeze-dried protein:additive samples (ratios from 1:0.25, to 1:3) were studied prior to and after incubation in an oven at 77°C.

A representative number of the typical isotherms are shown in figures X.2and X.3. These show initial (prior to incubation) water adsorption isotherms for trypsin:trehalose and trypsin:sucrose formulations together with the theoretical isotherms. These have been constructed from the experimental isotherms of the constituents on their own and assuming no interaction between protein and sugar molecules (see chapter VIII).

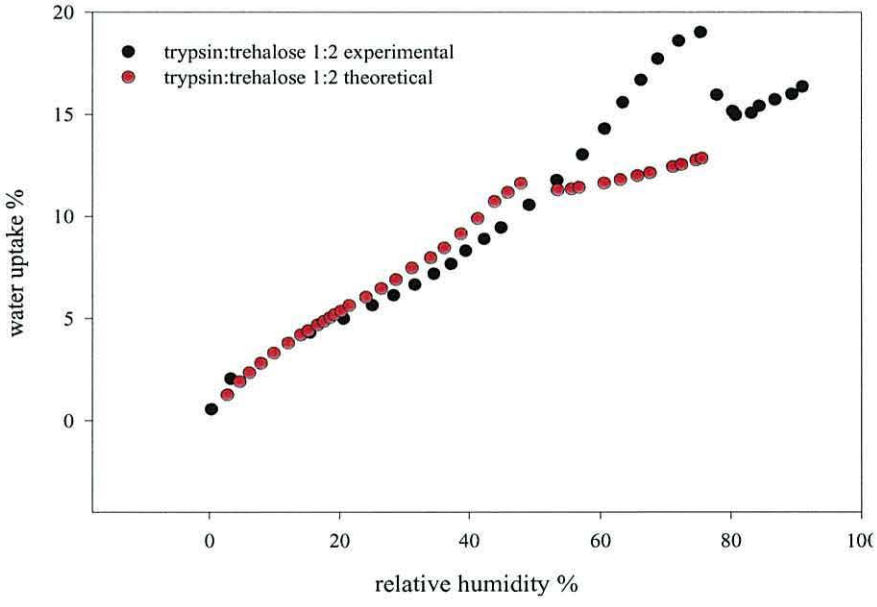
Comparison of the experimental and theoretical isotherms shows that the amount of water adsorbed in the mid-hydration range is less than that predicted by the theoretical isotherm. As previously discussed in the chapter on β-lactoglobulin mixtures, this indicates the likelihood that there is interaction between the protein and the sugars and that a complex is formed between the protein and sugar molecules involving sites on the protein and/or on the sugar which would normally be involved in binding water molecules (Lopez-Diez and Bone, 2000).

In agreement with the results obtained with the β-lactoglobulin mixtures, the interaction parameter previously defined (see chapter VIII) indicates that sucrose shows a more extensive interaction with trypsin than trehalose (see figure X.4 and table X.2).

It is also interesting to note that, on reaching a sufficiently high water content, protein-trehalose samples (all but the 4:1 ratio) experience a transition which was previously attributed to crystallization (see chapter VIII). By contrast, none of the sucrose mixtures show evidence of any transition. Furthermore, for all the samples, the

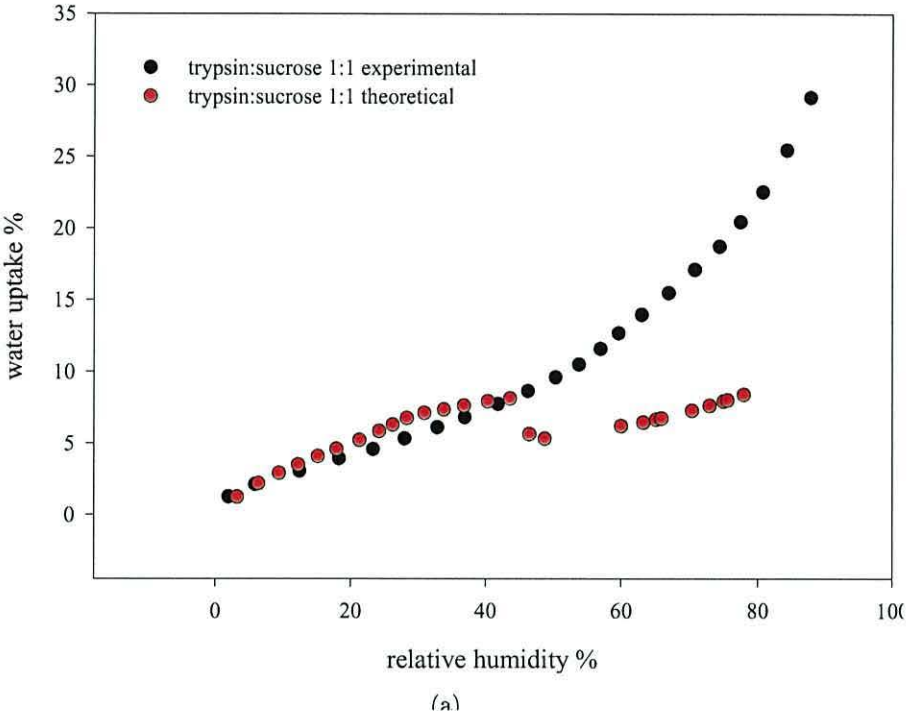


(a)

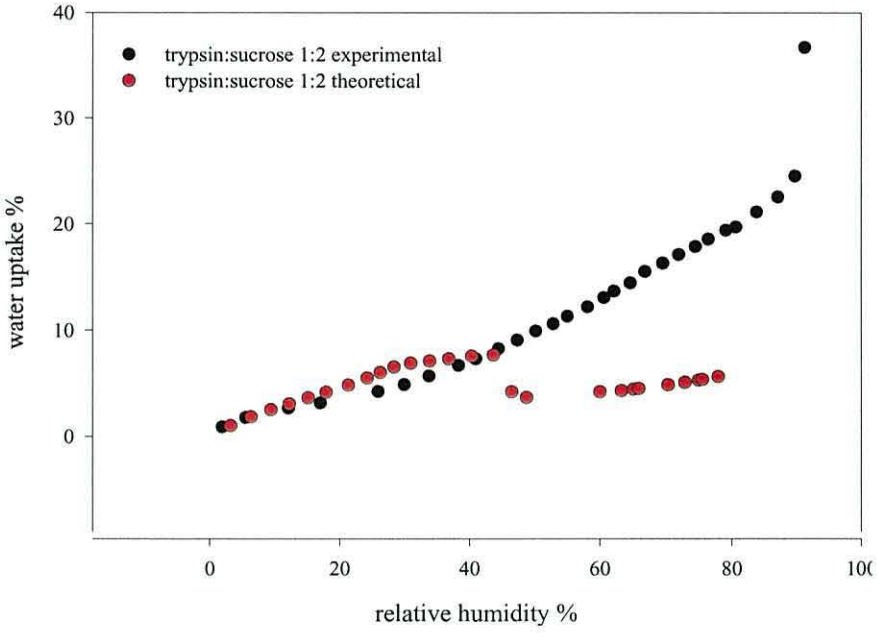


(b)

Figure X.2: Hydration isotherms of trypsin-trehalose samples **before** incubation. Ratios (a) 1:1, (b) 1:2



(a)



(b)

Figure X.3: Hydration isotherms of trypsin-sucrose samples **before** incubation. Ratios (a) 1:1, (b) 1:2

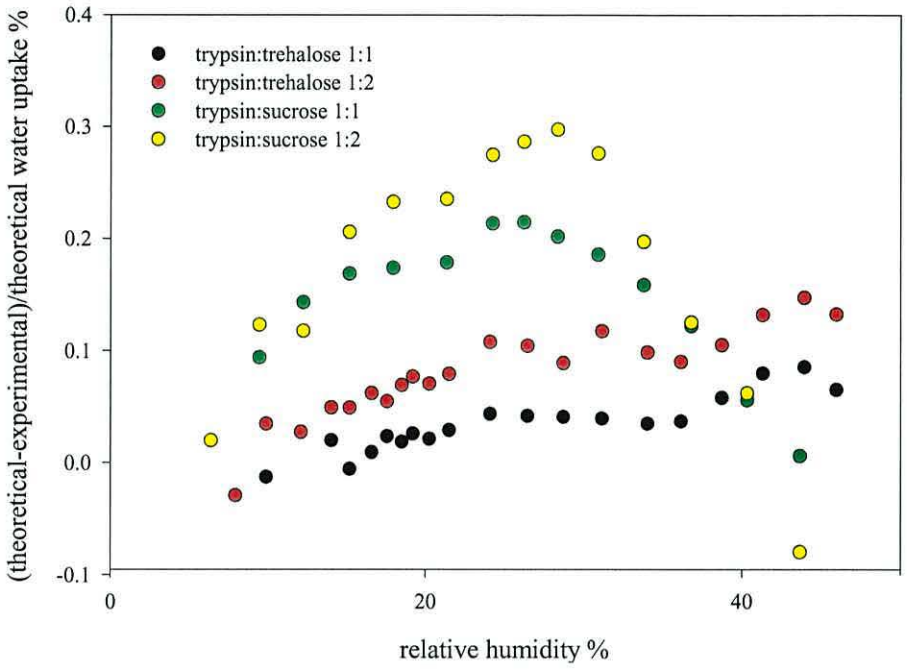


Figure X.4: Difference between theoretical isotherm for non interacting trypsin-sugar and the experimental isotherm

positions of the discontinuity in the hydration isotherm are not successfully predicted by the theoretical model, which probably indicates that the presence of protein molecules reduces the ability of sugar molecules to form intermolecular sugar-sugar hydrogen bonds, thereby retarding crystal formation.

Trehalose & sucrose mixtures after incubation After incubation of the samples at 77 ± 1^{o}C for 13 days, the hydration isotherms were recorded which produced considerably altered characteristics compared with the hydration runs collected before incubation. This was not totally unexpected since incubation of samples resulted in loss of activity (see table X.3 and also chapter IX), and denatured trypsin was found to produce different isotherms to that of the native protein, i.e as denaturation increased the water uptake decreased (see chapter VII). Activity was assayed as described in chapter IX, enzyme activity values are listed in table X.3.

Figures X.5and X.6 show the experimental isotherms together with the theoretical hydration isotherms for the incubated trypsin-sugar mixtures. The theoretical isotherms

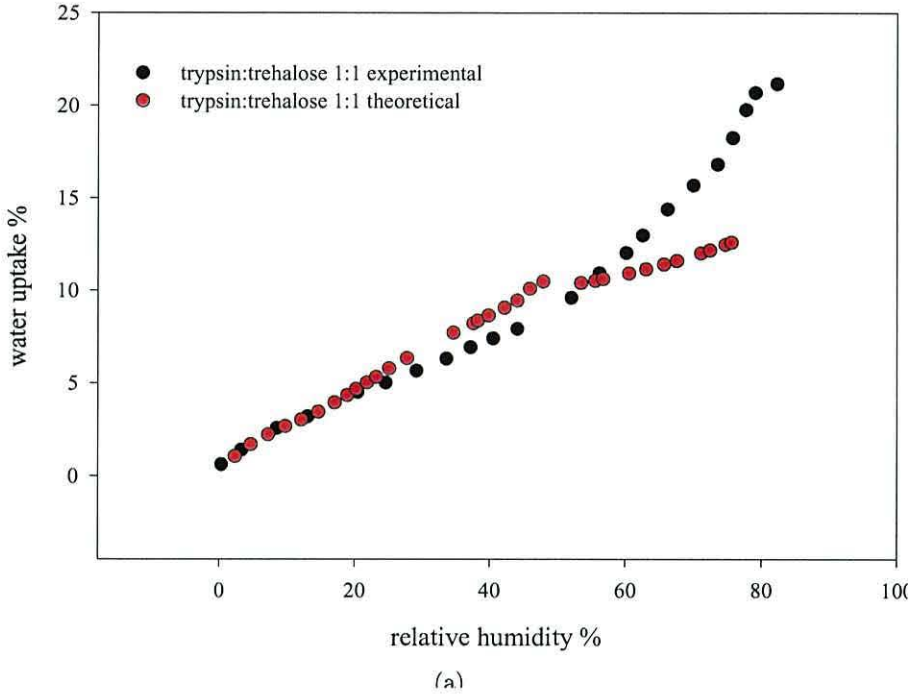
	prior to	*after*
trypsin:trehalose (1:3)	0.1	0.3
trypsin:trehalose (1:2)	0.1	0.20
trypsin:trehalose (1:1)	0.05	0.18
trypsin:trehalose (1:0.25)	0.01?	0.01
trypsin:sucrose (1:2)	0.27	0.14
trypsin:sucrose (1:1)	0.2	0.125
trypsin:sucrose (1:0.25)	0.15	0.02

Table X.2: Estimated interaction parameter for trypsin:trehalose/sucrose mixtures, prior to and after incubation

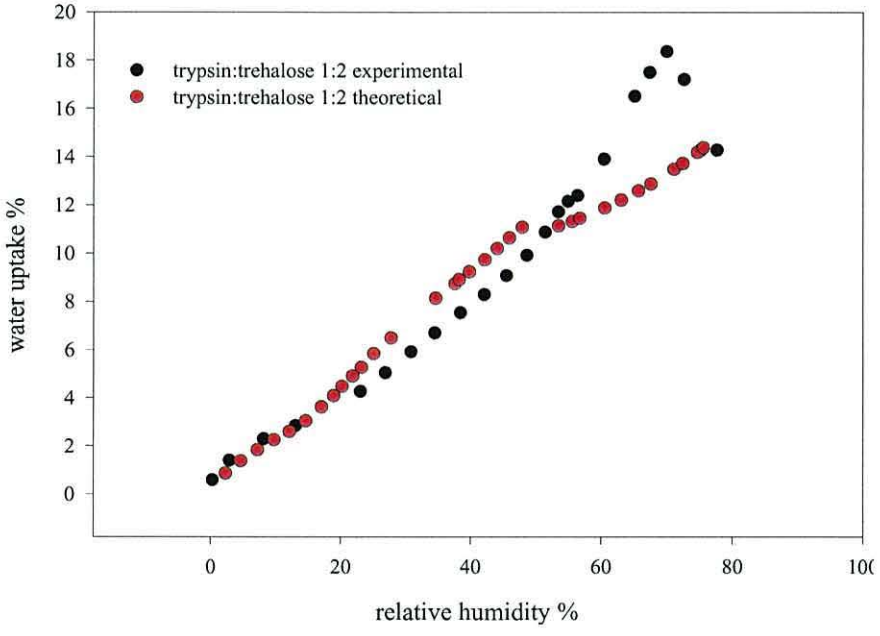
were calculated as previously, that is assuming that species do not interact, but since incubation has an effect on both protein and additive care must be taken when selecting/choosing the appropriate isotherms to construct the theoretical characteristics. When modelling mixtures containing trehalose, the heated form[5] of the sugar was considered whereas for samples prepared with sucrose the freeze-dried form was used. For the calculation of the theoretical isotherms containing sucrose, it is clear that the most appropriate isotherm should have been the heated form of sucrose but the following was observed: the heated form of sucrose showed little water uptake indicating a crystalline form. A theoretical isotherm based on such an isotherm will predict low water uptake for the trypsin:sucrose samples, however sucrose mixtures show much more water uptake than that predicted. Such discrepancy between experimental and theoretical would seem to indicate that the protein is greatly unfolded which does not agree with the measured remaining activity. Furthermore, the heated 1:2 protein:sucrose mixture showed the transition associated with crystallization, which clearly indicates the sugar was not in the crystal state prior to incubation. Therefore, as previously stated, the freeze-dried amorphous form of sucrose was used. Note that for trehalose the heated form could be used since it did not appear to go to any major structural transition through heating.

The selection of the trypsin isotherm to model a given formulation is dependent on each mixture since all of them presented different activities (see table X.3). For a given mixture/activity a trypsin isotherm was estimated with the help of the previously collected isotherms of denatured forms of trypsin whose remaining activity varied from

[5]see chapter VII

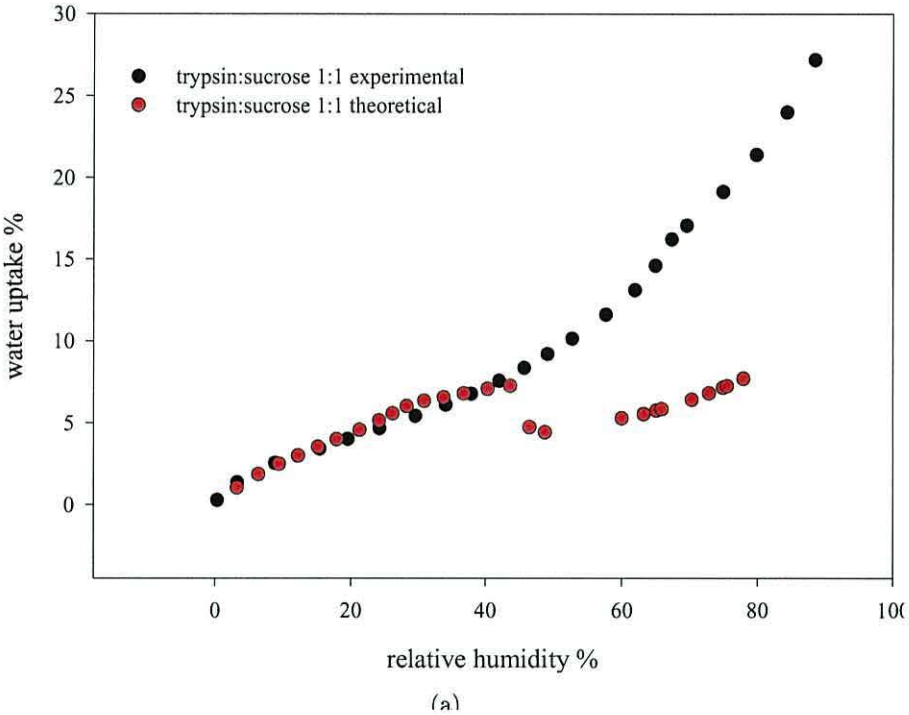


(a)

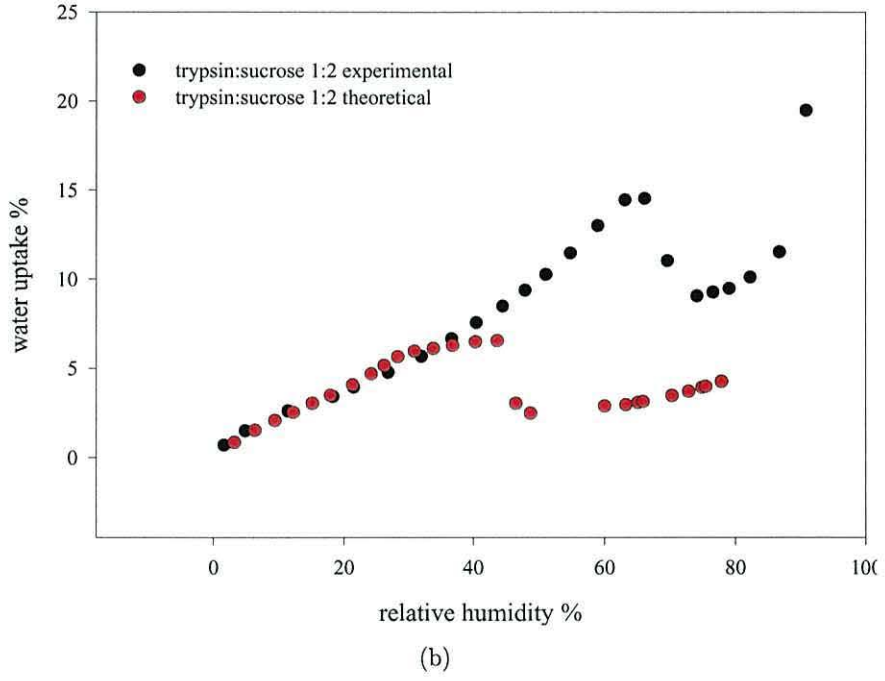


(b)

Figure X.5: Hydration isotherms of trypsin-trehalose samples **after** incubation. Ratios (a) 1:1, (b) 1:2



(a)



(b)

Figure X.6: Hydration isotherms of trypsin-sucrose samples **after** incubation. Ratios (a) 1:1, (b) 1:2

	remaining activity
trypsin:trehalose (1:3)	90
trypsin:trehalose (1:2)	83
trypsin:trehalose (1:1)	75
trypsin:trehalose (1:0.25)	71
trypsin:sucrose (1:2)	74
trypsin:sucrose (1:1)	67
trypsin:sucrose (1:0.25)	65
trypsin:hp-β-CD (1:2)	52.1
trypsin:hp-β-CD (1:1)	42.7
trypsin:hp-β-CD (1:0.25)	43

Table X.3: Remaining activity % of incubated trypsin:additive mixtures.

38 to 100%. A Matlab routine[6] was written so that a hydration isotherm could be predicted for any given enzyme activity within the activity range 38 to 100%. In this analysis, it was assumed that trypsin should adopt the same conformation or at least to show the same isotherm for a given activity, i.e the same degree of denaturation would always lead to the same conformation, regardless of the presence of the additive. In fact, this assumption was tested with a trypsin trehalose 1:1 formulation. The sample was incubated in the oven at 77°C for five days, activity was measured (remaining activity $\sim$ 74%), then trehalose molecules were dialysed out and subsequently the protein solution was freeze-dried. Activity at this point was checked to ensure dialysis had not further damaged the protein, and finally an isotherm was obtained and compared to that of a denatured form of trypsin with remaining activity of 75%. As it can be seen in figure X.7, both isotherms coincide indicating the initial assumption is valid.

As can be seen in figures X.5 and X.6, in the case of trehalose, the difference in the mid-hydration range between the experimental and the theoretical sorption isotherms appears to have increased compared with unheated samples whereas in the sucrose formulations the difference decreases. Consequently the difference between the theoretical and experimental isotherms and the estimated interaction parameters vary according to this trend (see figure X.8 table X.2). This effect would seem to indicate that through heating, the interaction between protein and sugar constituents changes, moreover trehalose and sucrose seem to show opposite trends.ways/fashion.

[6]see appendix D

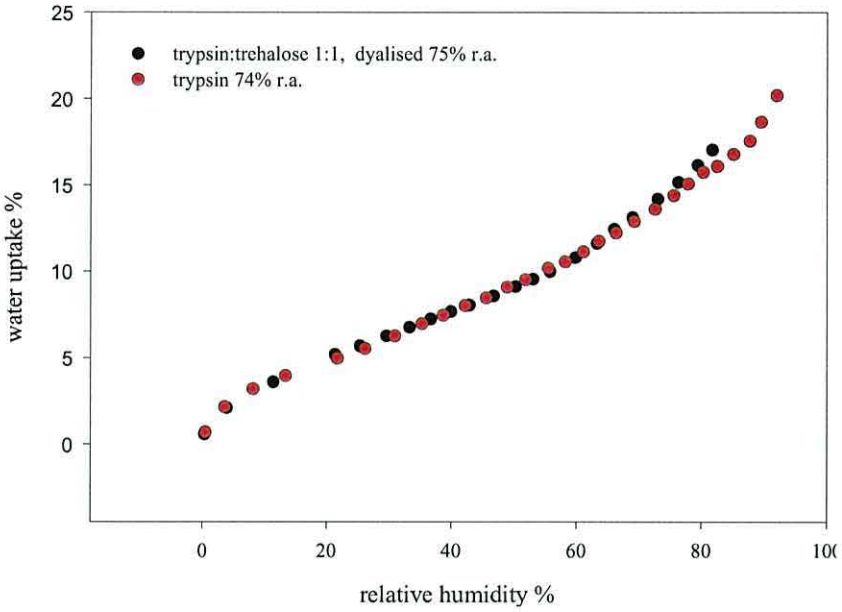


Figure X.7: Hydration isotherm of a dialysed trypsin:trehalose sample

Regarding the crystallization transition, the isotherms of the incubated samples show different features compared with the non-incubated ones. It can be seen that the trypsin:sucrose 1:2 sample, now shows a transition after heating, indicating that throughout incubation at 77°C crystallization of sucrose molecules has been promoted. In addition, the transition observed in the trypsin:trehalose mixtures seems to occur at slightly higher relative humidities than in the non-incubated samples.

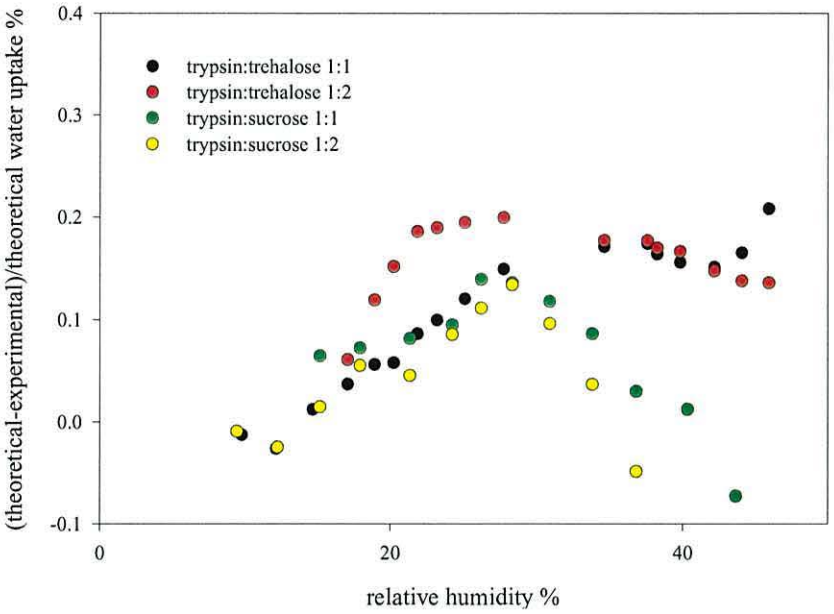
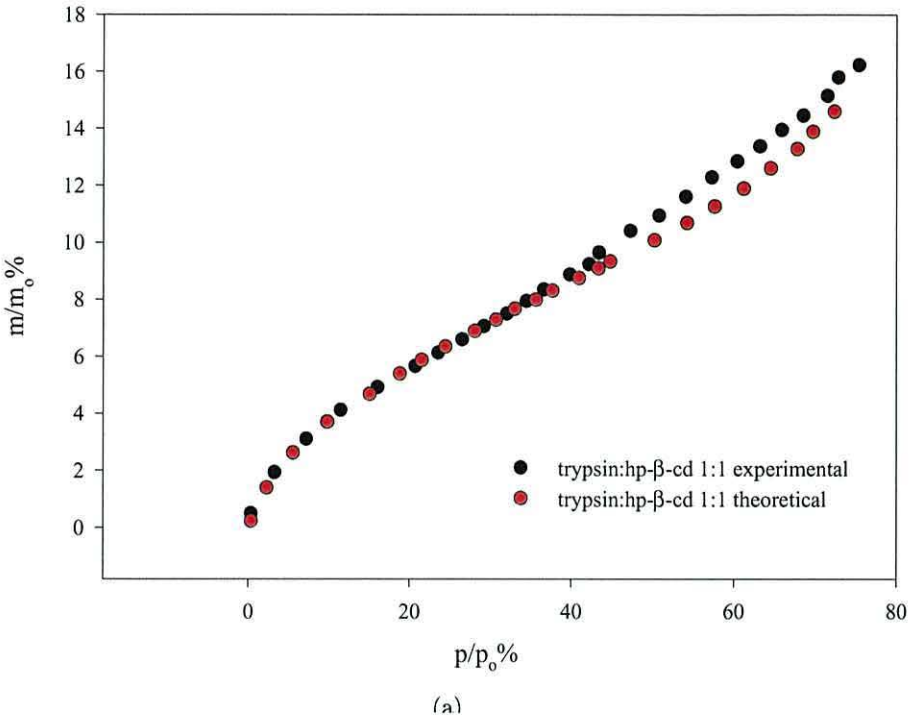
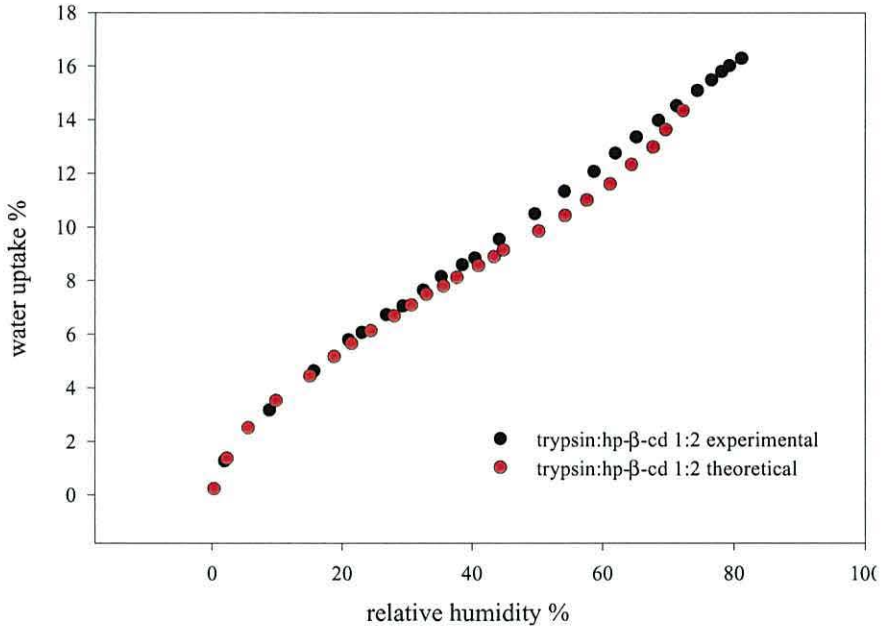


Figure X.8: Difference between theoretical isotherm for non interacting trypsin-sugar and the experimental isotherm after heating

2-hydroxypropyl-β-cyclodextrin mixtures, before and after incubation Moisture sorption isotherms of the 2-hydroxypropyl-β-cyclodextrin (hp-β-CD) formulations show very different features to those of the disaccharides (see figures X.9 and X.10). Theoretical isotherms show a very close match with the experimental hydration characteristics up to 50% relative humidity. However at higher hydrations the theoretical isotherm lies slightly below the theoretical which could indicate the existence of a denaturing interaction between the additive and the protein. hp-β-CD is reported to interact with aromatic groups of the protein (Branchu et al., 1999a) which, due to their hydrophobic character, are expected to be in the interior of the protein molecule. Therefore an interaction between protein and hp-β-CD could result in exposure of these groups thus induce general unfolding which results in increasing the number of sites available to sorb water molecules.

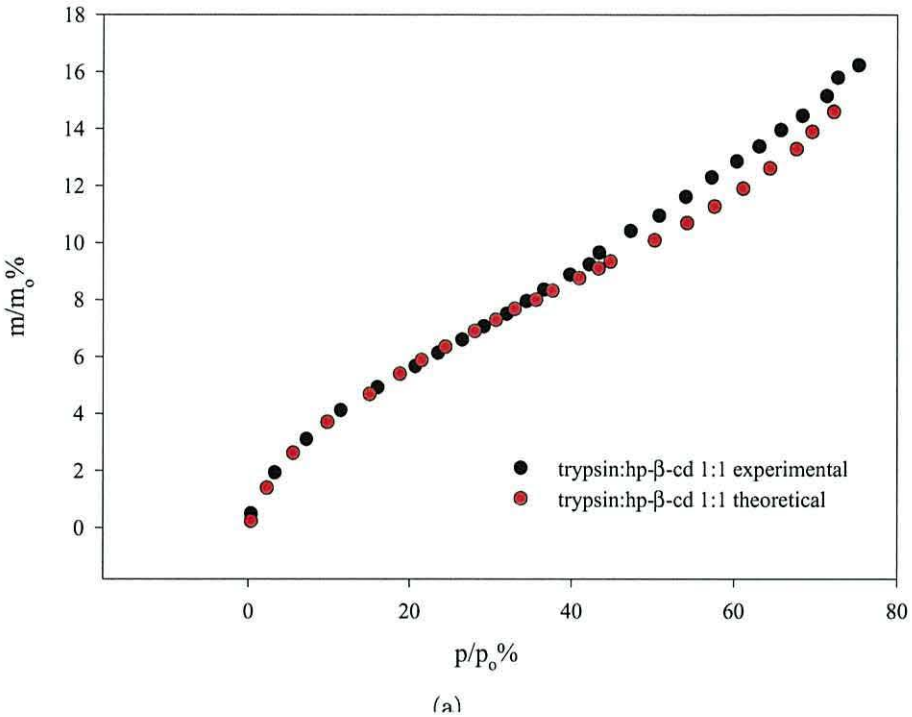


(a)

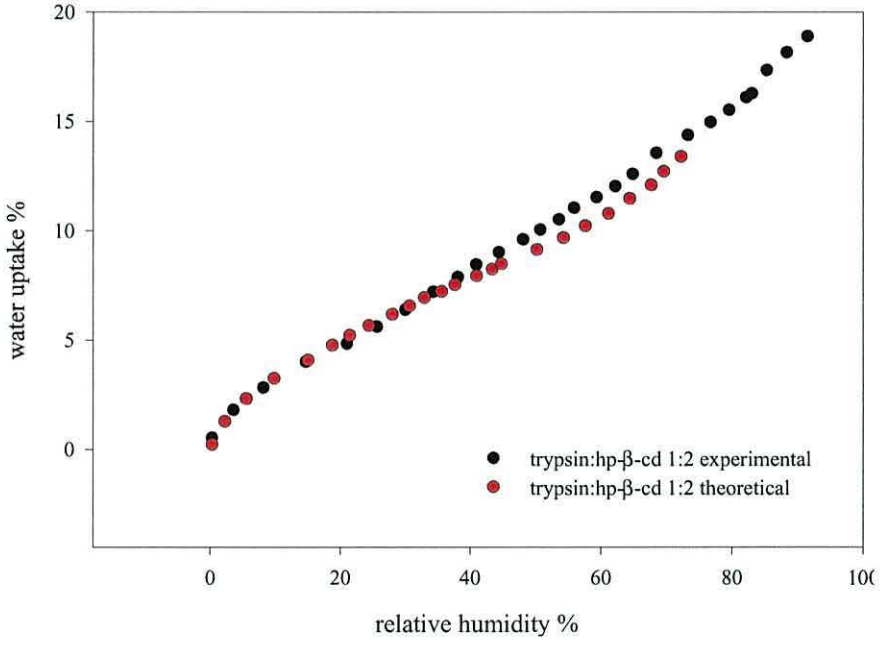


(b)

Figure X.9: Hydration isotherms of trypsin:2-hydroxypropyl-β-cyclodextrin samples **before** incubation. Ratios (a) 1:1, (b) 1:2



(a)



(b)

Figure X.10: Hydration isotherms of trypsin:2-hydroxypropyl-β-cyclodextrin samples **after** incubation. Ratios (a) 1:1, (b) 1:2

b Dielectric Measurements

The dielectric response of the hydrated trypsin-sugar mixtures were studied in a similar way to the β-lactoglubulin mixtures described in chapter VIII, over the frequency range 1 MHz to 1 GHz. Because the results obtained for the β-lactoglobulin mixtures indicated that the sugar content masked the protein response and no definite conclusion regarding the effect of the sugar on protein flexibility and mobility could be inferred, not all the trypsin samples studied gravimetrically were characterized using dielectric measurements. Nevertheless, trypsin:trehalose and trypsin:hp-β-CD 1:2 were investigated to confirm previously obtained trends and incubated trypsin-trehalose, sucrose, and hp-β-CD 4:1 were studied in an attempt to relate trypsin preservation (see chapter IX) to dielectric parameters.

For all samples studied, in the frequency range considered (1 MHz to 1 GHz), a dielectric dispersion centered at approximately 3 MHz was observed, which was consistent with the previously observed dispersion of the protein on its own. The study of the dielectric properties of the trypsin mixtures was restricted to this dispersion. The hydration dependence of this dispersion has been previously (see chapter VII) related to the glass transition, therefore given the relevance of the physical state on the stability of the samples and the effect that sugars could have on this transition, only this dispersion was characterized.

As with β-lactoglobulin mixtures, it seems that the presence of additives does not have an effect on the relaxation frequency. The effect of increasing water content on the dielectric response of the hydrated protein can be monitored by looking at the dependence of the dielectric increment, $\Delta\epsilon$, on the relative humidity (figure X.11).

For the sample with the higher content of trehalose, the previously observed trend (see chapter VIII) was confirmed. i.e at a certain relative humidity (water content) the dielectric increment showed a discontinuity, which as previously stated greatly complicated the comparison with the dielectric response of the protein. It was found, however, that the trypsin:hp-β-CD sample did not show any significant difference to the protein response. This seems to indicate that, in this case, the presence of the additive does not have an effect on the protein dielectric properties.

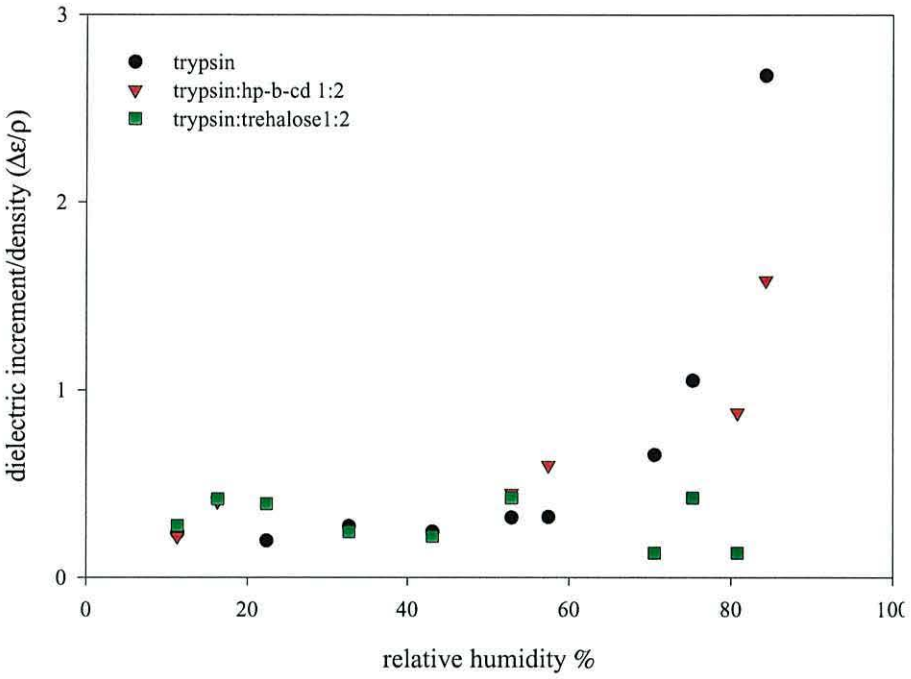


Figure X.11: The dielectric increment of the lower frequency dispersion plotted against protein water uptake of 1:2 trypsin mixtures

Figure X.12 shows the dielectric increment as a function of relative humidity for the incubated 4:1 trypsin additive samples. It can be seen that the characteristic for both trehalose and hp-β-cd mixtures shows the same pattern as for the protein on its own. This indicates that the protein does not seem to be affected by the presence of trehalose or hp-β-cd. By contrast the sucrose mixture shows a discontinuity at approximately 40-50% RH. Although the hydration isotherm of the trypsin:sucrose 4:1 sample did not show any transition indicative of sugar crystallization, it seems that dielectric techniques may identify structural rearrangements which may be the precursor of a later crystallization.

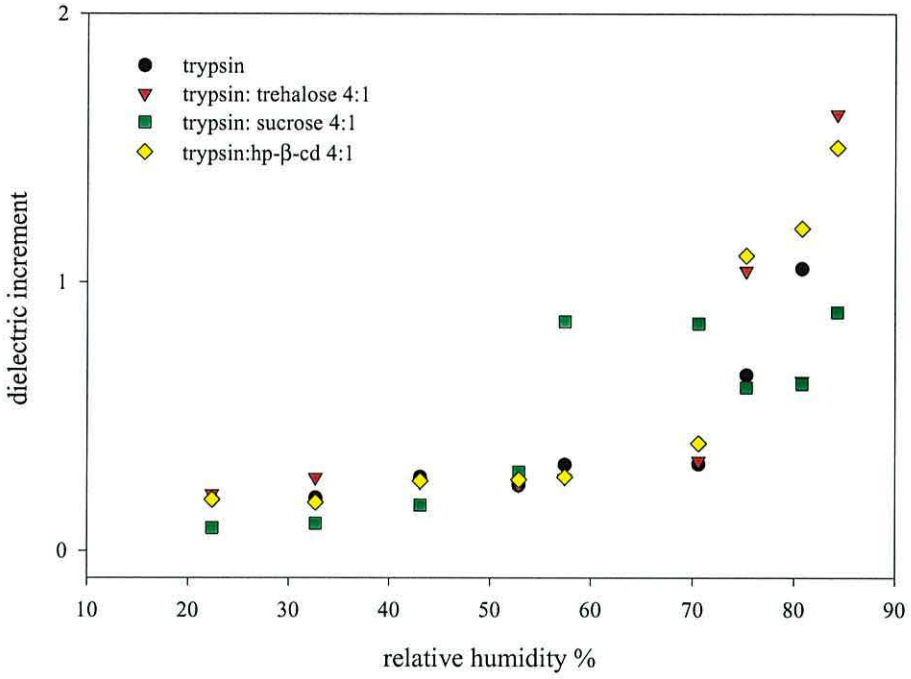


Figure X.12: The dielectric increment of the lower frequency dispersion plotted against protein water uptake of incubated 1:4 trypsin mixtures

4 Discussion

This study has attempt to characterise the water-binding properties of freeze-dried trypsin:trehalose, trypsin:sucrose and trypsin:hp-β-CD samples before and after incubation over the hydration range 0 to 85% relative humidity.

Isothermal hydration of the trypsin:sugar mixtures was found to corroborate previous results on β-lactoglobulin, that is it has shown the existence of interaction between protein and disaccharides which results in less water being taken up than predicted. Furthermore, this interaction has been found to be affected by incubation of samples at 77^{o}C. It has been shown that, for trehalose mixtures, interaction seems to increase, (e.g. for the 1:1 samples the interaction parameter increases from 0.05 to 0.18), whereas for sucrose formulations the interaction actually decreases, (e.g. the interaction parameter for the 1:1 sample goes from 0.2 to 0.125). By contrast, hp-β-CD samples did not present any evidence of interaction, either before or after incubation.

The interaction in unheated freeze-dried samples appears to be more extensive in sucrose formulations compared to trehalose as previously discussed (see chapter VIII)

and this has been attributed to preferential exclusion of trehalose molecules in frozen solutions (Izutsu et al., 1996), an effect which appears to continue in the solid state.

The incubation seems to affect not only the extent of the interaction between protein and sugar but also the crystallization transitions observed in most of the samples, the more extreme effect being observed for trypsin:sucrose 1:2. The hydration isotherm of this sample measured prior to heating did not show the characteristic discontinuity of samples which crystallize, while the isotherm recorded after incubation did show this transition. This observation appears to be correlated with the fact that the interaction between sucrose and protein decreases after heating; the first isotherm (prior to incubation), indicates that protein-sucrose interaction, perhaps together with steric effects, prevents the sugar from crystallising even when the water content is high. However, the second hydration isotherm (after incubation) seems to indicate that throughout incubation the protein-sucrose hydrogen bonds are being disrupted, resulting in a decreasing interaction with the protein. Therefore, pre-incubation 'barrier' inhibiting sucrose crystallization is reduced, thereby allowing sucrose molecules to become sufficiently mobile to form a crystalline structure when hydrated. In addition, looking at the trypsin:trehalose isotherms, the opposite effect can be seen, that is, the increase in the interaction correlates with a 'delay' in the crystallization transition.

In the light of these observations the following questions clearly arise,

- why is the extent of interaction between sugar and protein affected by heating?
- why is this effect different for sucrose and trehalose when both sugars have similar properties?
- how is this phenomena related to preservation?
- is the extent of the interaction related to protection?
- what is the nature of the interaction that determines the preservation?

That the extent of the interaction varies upon heating is consistent with the fact that molecular mobility in a glass can be significant even at temperatures below the glass transition temperature, T_g (Hancock et al., 1995). Therefore, it seems likely that

molecular motion during heat treatment may have induced the disruption of the initial hydrogen bond configuration to form a new, and probably more energetically favourable, configuration.

In order to explain the apparently different effect that incubation has on trypsin-sucrose mixtures' compared to that on the trehalose-protein formulations, the coupling of the molecular motions occurring under the conditions considered here with the thermodynamic driving force (of the sugars) toward a (more) stable crystalline state needs to be considered.

The most common form of sucrose is an anhydrous crystal in which the crystal structure is stabilized by intramolecular hydrogen bonds. In contrast, amorphous sucrose does not present any spatial order and it is possible that many hydrogen bonds are unsatisfied. Therefore as amorphous sucrose is incubated at high temperature (77^{o}C) and in the absence of water, crystalline sucrose forms spontaneously as evidenced by the isotherm of heated sucrose (see figure VII.14 in chapter VII).

In the case of protein-sucrose formulations, the decrease in the number of hydrogen bonds may then be explained by the natural tendency of sucrose molecules to form anhydrous crystals. This is clearly evidenced by the hydration characteristics of the 1:2 samples described above: in the freeze-dried form, before heat treatment, the level of interaction with the protein is relatively high and, over the hydration range investigated, there is no discontinuity which might indicate a tendency for the sugar to separate and form crystals. However, after heat treatment, there is a considerable decrease in the calculated interaction between the sucrose and protein and, in addition, phase separation and the formation of sucrose crystals is indicated in the hydration isotherms at approximately 40%. These observations seem to indicate that, as the temperature is raised, the tendency is to form a greater number of sucrose-sucrose hydrogen bonds since this ultimately (in the crystal form) allows complete hydrogen bonding compensation and therefore the lowest free energy. This necessarily means fewer sucrose-protein bonds remain.

By contrast, trehalose naturally forms dihydrate crystals in which the two glucopyranosyl moieties are linked by the two water molecules of hydration. In addition to this crystal form, trehalose can adopt different anhydrate crystal structures which have

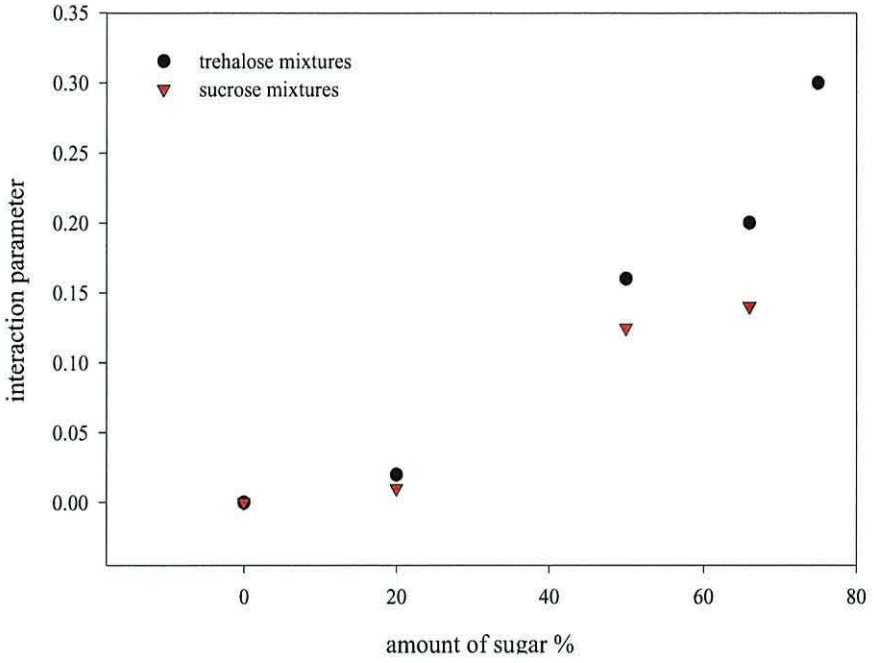


Figure X.13: Interaction parameter measured after incubation against sugar concentration

been reported to have a similar configuration to that of the solution and amorphous states (Taylor et al., 1998). Furthermore, it has been demonstrated that the dihydrate solid state appears to be almost completely hydrated indicating that two water molecules in the dihydrate solid-state structure are largely capable of satisfying the hydration requirement of the solute (Engelsen and Pérez, 2000). Together these results may indicated that incubation of amorphous anhydrous trehalose is likely to result in a trehalose form in which the structure of the dihydrate crystal is conserved and where trehalose is forced to support uncompensated hydrogen bonds. As a result, the free energy of this structure is likely to be relatively high, so a rearrangement of the trehalose structure to lower the free energy, is likely to occur by the formation of intramolecular hydrogen bonds albeit at the cost of a loss of configurational entropy. In the presence of trypsin, an alternative interaction to trehalose internal hydrogen bonding is available, that is direct hydrogen bonding to the protein, which both maintains the high entropy of the system and at the same time allows considerable hydrogen bond interaction.

As a result of the described phenomena, in the presence of heat and absence of

water (denaturation conditions), trehalose appears to interact more strongly with the protein than sucrose. This can be seen more clearly in figure X.13 where the interaction parameter (after heating) is plotted against sugar concentration. Over the range of protein-sugar ratios studied, there appears to be a steady rise in the interaction with increasing sugar, with trehalose showing greater interaction for the same proportion of sugar.

Comparing this characteristic with that describing the greater preserving ability of trehalose (see figure IX.8, chapter IX), it is tempting to conclude that the preservation is a direct consequence of interaction between protein and sugars.

This conclusion may be confirmed from the plot of preservation against interaction for both sugars. The study carried on trypsin stability (see previous chapter) allowed the estimation of a preservation parameter, P, which measured the extent to which trehalose and sucrose inhibit enzyme deactivation. In an attempt to relate the interaction parameter obtained from gravimetric studies to preservation, both parameters have been represented graphically as preservation vs. interaction (after incubation), figure X.14. This representation reveals that a direct relationship between these parameters is clearly evident with data points from both sugars lying on the same line. It appears from this data that the preservative properties of the sugars in terms of their ability to prevent protein denaturation is directly linked to their ability to take part in hydrogen bond interactions with the protein. Furthermore, given the results presented here it is tempting to attribute the preservation effect to the resulting interaction after heating.

According to the previous discussion, it seems that trehalose better preservative efficiency compared to that of sucrose is due to its particular structure which allows maximum hydrogen bonding to the protein. Therefore, it appears that trehalose is more effective than sucrose, simply, because it hydrogen bonds to the protein more effectively.

In such a picture raffinose, a trisaccharide which forms pentahydrate crystals, has a similar T_g to trehalose (Wolkers et al., 1998) and a higher hydrogen potential (Gaffney et al., 1988). Furthermore, it forms penta-hydrate crystal so it might be expected to interact with the protein even more strongly than trehalose when water of hydration is completely removed, and therefore be more effective than trehalose in protecting proteins against long term storage-induced damage. However, reported results indicate

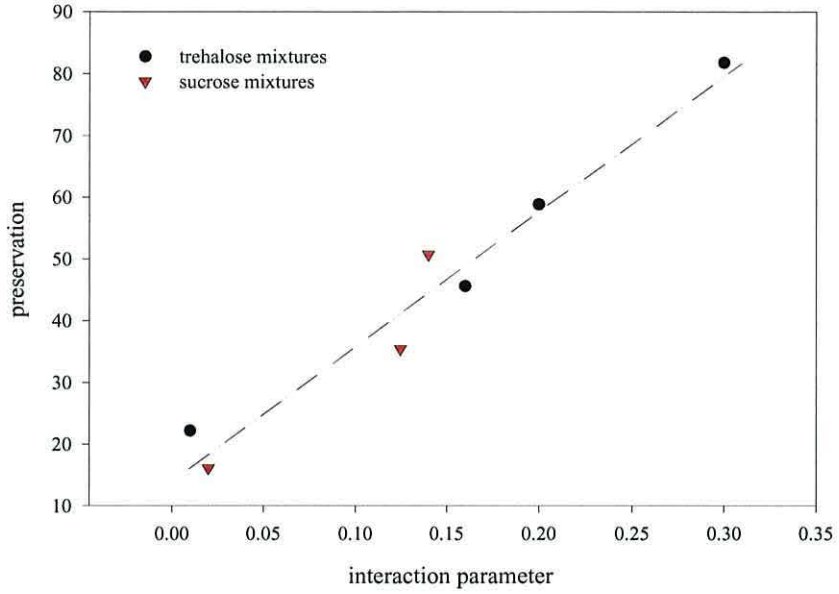


Figure X.14: Preservation factor represented as a function of the interaction parameter for incubated mixtures

that raffinose is considerably less effective than trehalose (Cardona et al., 1997). This may be explained by to structural collapse of the raffinose molecule upon dehydration which is likely to result in considerable intramolecular hydrogen bond compensation. As previously mentioned, the dehydrated trehalose seems to remain in the same configuration as in the solution state leading to uncompensated H-bonds, therefore increasing its ability to hydrogen bond to the protein.

It is interesting to note that the representation of the preservation factor vs. interaction (prior to incubation) (figure X.15) also indicates that the extent of the interaction is directly proportional to protection, that is as the interaction increases, protection increases. However the interaction prior to incubation, in contrast to the interaction between trehalose/sucrose and trypsin after incubation does not appear to be correlated. It has been suggested (Prestrelski et al., 1995; Chang et al., 1996) that, to ensure storage stability, damage during freeze-drying has to be minimised, that is, a freeze-dried formulation in which a protein structure resembles its folded state is more stable during storage than one in which the protein is almost unfolded. Additives which

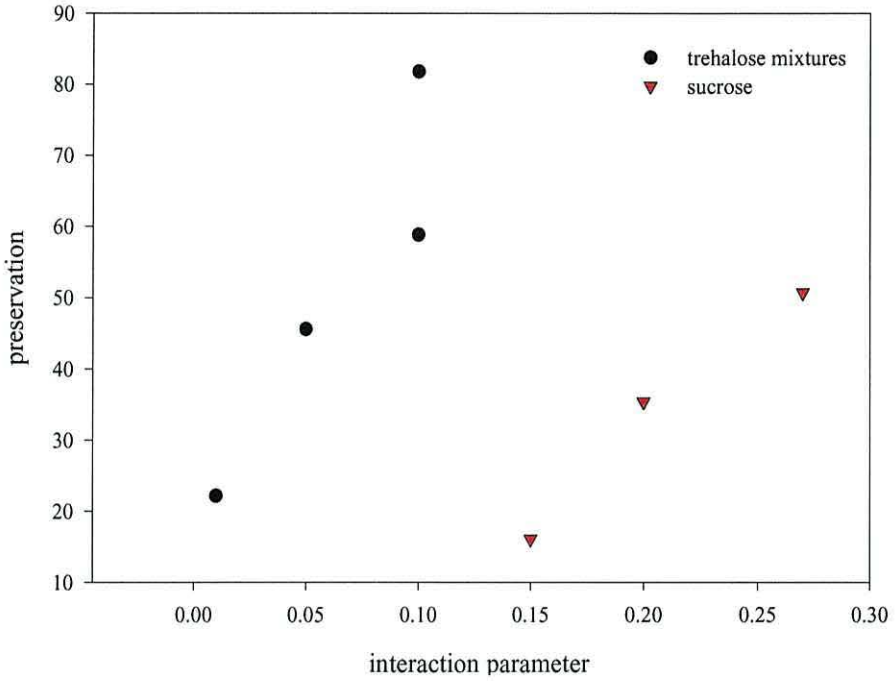


Figure X.15: Preservation factor represented as a function of the interaction parameter for non-incubated mixtures

hydrogen bond to the protein have been shown to prevent, to some extent, protein deconformations during freeze drying (Prestrelski et al., 1993b; Prestrelski et al., 1995; Chang et al., 1996; Allison et al., 1999; Allison et al., 2000) and also these additives have been shown to enhance protein stability during storage (Prestrelski et al., 1993b; Prestrelski et al., 1995; Chang et al., 1996). In contrast additives which do not hydrogen bond with the protein, such as dextran, have been shown not to be effective protectants against heating stress (Crowe et al., 1994; Allison et al., 2000). Therefore, initial hydrogen bonding seems to determine whether an additive will preserve the protein structure during storage.

Under the conditions studied here both trehalose and sucrose have been shown to form hydrogen bonds during freeze drying, and as expected, they have also been shown to protect the protein during incubation. However, although hydrogen bonding seems to be an initial requirement for protein stabilization, the preservation vs. interaction plot (figure X.14) show that the extent of the hydrogen bond interaction after heating is the crucial factor which determines the efficiency of a given additive.

5 Conclusion

The study carried out on trypsin-additive mixtures' hydration properties has revealed important features which can be summarize:

- According to previous studies (see chapter VIII and also (Prestrelski et al., 1993b; Allison et al., 1999; Allison et al., 2000)) trehalose and sucrose freeze-dried formulations show evidence of hydrogen bond interaction with trypsin, with sucrose interacting to a greater extent than trehalose.

- Interaction between protein and sugar seems to be disrupted as a result of incubation at elevated temperatures. As a consequence, in the case of sucrose the interaction diminishes whereas trehalose exhibits an increase in interaction with the protein.

- Hydrogen bond interaction appears to be related to the proportion of remaining trypsin activity and hence to the preservation of the protein structure.

- The apparent better efficiency of trehalose protecting against trypsin deactivation is determined to be due to a high hydrogen bond potential together with the special characteristics of its anhydrous structure.

XI

Conclusion

The objective of this work was to investigate protein sugar interactions which may be relevant to biological preservation and to study the relationship between protection, direct interaction through hydrogen bonding and glass formation. In order to achieve this, the water binding properties of various protein-additives formulations which have been prepared using β-lactoglobulin or trypsin and either trehalose, sucrose or 2-β-hydroxypropyl-cyclodextrin as carbohydrate additive have been characterised.

The study of the individual components of the mixtures revealed that, in the frequency range considered, the proteins showed two dispersion centered approximately at 3MHz and 100MHz in agreement with previously published results. Furthermore, their hydration dependency seemed to indicate that the nature of these dispersions may be related to different events in the hydration process. The higher frequency dispersion has been shown to be closely related to the relaxation of loosely-bound water molecules and the lower frequency dispersion connected to plasticization of the protein conformation by water possibly resulting in a physical state transition from glass to rubber.

The hydration isotherms characteristics of the dissacharides, trehalose and sucrose showed different features to those of the protein, in that both appear to undergo marked structural transitions during hydration and that the initial physical state of the sugars (e.g. amorphous or crystalline) largely affected their hydration characteristics. By contrast, the oligosaccharide 2-hydroxypropyl-β-cyclodextrin showed dielectric and hydration characteristics similar to the proteins that is sigmoidal shape isotherms and dispersions centered at 3 MHz and 100 MHz.

The study of protein:additive mixtures was initiated by investigating the characteristics of β-lactoglobulin:trehalose and β-lactoglobulin:sucrose mixtures prepared by two commercially used preparation methods (freeze drying and spray drying) and by

evaporation.

Importantly, although the sugars (trehalose and sucrose) take on different structures with different hydration characteristics depending on preparation method, β-lactoglobulin mixtures produced by the three methods, exhibited common hydration trends: at low and mid hydrations all samples exhibit properties consistent with the sugar component existing in the amorphous, glassy form, whereas at relatively high hydrations, following a marked transition, the trehalose and sucrose components appeared to adopt crystalline structures.

Evidence was found of interaction between the sugar and protein, specifically in the hydration region where single hydrogen-bonded water is expected to be associated with the protein. The magnitude of the interaction appeared to be slightly more extensive for sucrose. Furthermore, following crystallisation of the trehalose and sucrose components of protein-sugar mixtures, phase separation of the sugar and protein took place into separate non-interacting species.

Interestingly, it was observed that for the β-lactoglobulin:sucrose/trehalose samples prepared by evaporation from solution, a small amount ($\sim$ 3% by weight) of water was trapped in the complex even under extreme conditions of dehydration. Nevertheless, on rehydration, the trapped water is released following the characteristic structural transition.

Long term stability was simulated by incubating the trypsin:additive samples at 77^{o}C. The activity assays of trypsin and trypsin formulations indicated that trypsin on its own denatures following non-first order kinetics. Lyophilization of trypsin in the presence of sugars studied appeared not to affect the activity of the enzyme. Furthermore, mixtures denatured during incubation although to a lesser extent than the protein and in general agreement with the available literature. It was observed that trehalose is more effective than sucrose in protecting the protein showing approximately 8% higher preservation on average than sucrose. By contrast, hp-β-CD containing mixtures show loss of activity after freeze drying and denatured at the same rate as the protein.

Gravimetric measurements on trypsin mixtures indicated that trehalose and sucrose freeze dried formulations show evidence of hydrogen bonds interaction with trypsin, with sucrose interacting to a greater extent than trehalose. Importantly, incubation resulted

in the disruption of the interaction. In the case of sucrose, the interaction diminished whereas trehalose exhibited an increase in the interaction with the protein following incubation.

Good correlation between the hydrogen bond interaction and the recovery of activity after incubation indicated that the preservation effect was directly related to the ability of a given additive to interact with the protein. Furthermore, the apparent better efficiency of trehalose protection against trypsin deactivation was determined to be due to a more extensive hydrogen bonding potential together with the special characteristics of its structure.

Further work

There are several lines of research which could extent the work presented here, some examples are presented here:

- Regarding the sample preparation method studied, it would be interesting to investigate the evaporated samples and their reluctancy to lose few water molecules when dehydrated under vacuum. A complete study of their characteristics, activity recovery and reaction to incubation may determine in more detail what mechanism/s are responsible for preservation of proteins and cell components of anhydrobiotic organisms.

- The stability study focused on deactivation at a certain temperature. However, stability assessment at different temperatures may be carried out to achieve a more comprehensive characterization of the enzyme deactivation and confirm the good correlation between the preservation results and the water binding properties of the mixtures.

- Further characterization of protection and its relation with hydrogen bond interaction between the protein and additives may include the study of more of protein:additive ratios; this study may indicate whether preservation saturates at high additive content.

- Also, in relation to the deactivation studies, the glass transition, T_g, of the mixtures may be accurately determined. In this work, the T_g of the samples was estimated from the Gordon Taylor equation. Calorimetric techniques (e.g differential scanning calorimetry) could not only provide a measure of T_g but also could aid the understanding of the glass forming properties of the samples.

- Dielectric studies performed in this work have shown that the physical state (glass or crystalline) of the additives can greatly affect the dielectric response of the samples and, in fact, hamper the study of the dielectric properties of proteins embedded in the additive matrix. It may be possible to overcome this difficulty by investigating the dielectric response of the protein additive mixtures at a certain hydration, lower than 40% relative humidity, to avoid sugar crystallization, and monitor the response at different temperatures.

Appendices

A

The glass state

"Glass, in the popular and basically correct conception, is a liquid that has lost its ability to flow." C.A. Angell

Throughout this work, the glassy amorphous state has been often mentioned and its relation to protein stability has been discussed, therefore in the present chapter the basic characteristics of the glassy state are reviewed.

1 Description and definition of the glass state

A glass is usually defined as an amorphous solid. In fact a glass is actually an undercooled liquid of such a high viscosity ($\eta > 10^{10}$-10^{14} Pa·s) that it exists in a metastable, mechanical solid state. A glass forms when a typical liquid, with a disordered molecular structure, is cooled to a temperature generally about 100°C (for many pure liquids) below its equilibrium crystalline melting temperature (T_m) or freezing point, at a cooling rate sufficiently high to avoid crystallization of the liquid. This solidification process, known as vitrification results in 'freezing in' or immobilization of the disordered structure of the liquid state, such that the resulting glassy solid is spatially homogeneous, but without any long-term lattice order, and is incapable of exhibiting any long-range, cooperative relaxation behaviour (e.g translational mobility) on a practical time scale.

The glassy state can be obtained by many different routes and yet appear to be experimentally the same structure.

The four most common means of obtaining an amorphous solid are condensation from the vapour state, supercooling of the melt, mechanical activation of a crystalline mass and rapid precipitation from solution (i.e. freeze drying or spray drying which are some of the most popular formulation preparation methods in the pharmaceutical and food industries).

The three-dimensional long-range order that normally exists in a crystalline material does not exist in the amorphous state, and the position of molecules relative to one another is more random, as in the liquid state. Structurally a glass is barely distinguishable from the liquid. For substances and mixtures of substances which can form stable glasses there are non-crystalline packing modes of the atoms and molecules into which they easily assemble. These may not be the lowest energy packing modes (as is demonstrated by the release of heat when crystallization takes place), but their energy is sufficiently low in comparison to alternative packings to make their occurrence as a state of matter very probable when the time available for finding a condensed state is a factor. For the so-called "good glass-formers", the probability of germinating a crystal rather than forming a glassy solid during cooling at normal rates is so small that crystals simply do not form.

A glass transition in amorphous systems is a temperature-, time- and composition dependent, material-specific change in physical state, from a glassy mechanical solid to rubbery viscous liquid (capable of flowing in real time). In terms of thermodynamics, the glass transition is defined as a second-order transition (as opposed to a first-order transition such as crystalline melting), and denoted by a change in slope of the volume expansion (a first derivative of the free energy), a discontinuity in the thermal expansion coefficient and a discontinuity in the heat capacity (a second derivative of the free energy). Figure A.1 shows an schematic graph of the variation of enthalpy (or volume) with temperature.

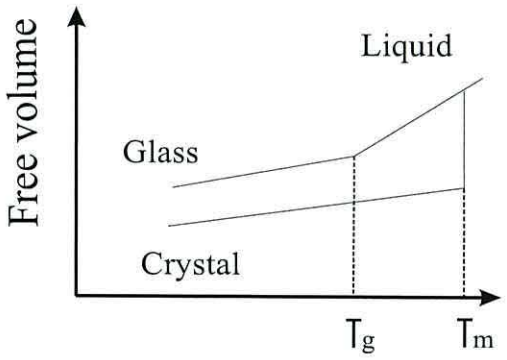


Figure A.1: Schematic graph of the variation of volume with temperature

The glass transition may also be defined, based on mechanical properties, in terms

of a mechanical relaxation process such as viscosity. Figure A.2 shows that as the temperature, T is lowered from that of the low viscosity liquid state above T_m, where Arrhenius kinetics apply, through a temperature range from T_m to T_g, a completely different, nonlinear form of kinetics, with a large temperature dependence becomes operative. Then, at a temperature where cooperative mobility becomes limiting, a state transition occurs, typically manifested as a three order of magnitude change in viscosity, modulus or mechanical relaxation rate. At this glass transition the viscosity of a liquid is $\sim 10^{12}$ Pa·s (10^{13} Poise), and the structural relaxation time (as determined by differential scanning calorimetry (DSC)) for such a liquid is about 200 sec. A 'mechanical' glass transition can be defined by combinations of temperature and deformation frequency for which, as temperature decreases, sufficiently large number of mobile units (e.g. small molecules or backbone-chain segments of a macromolecule) become cooperatively immobilized (in terms of large-scale rotational and translational motions) during a time scale comparable to the experimental period. Perhaps the most important distinction between dimensionally extended (alpha) relaxations, which give rise to the glass transition as translational motions become constrained at T_g, and the small-scale (β and γ) relaxations, for which small-scale rotational motions do *not* become constrained as T falls below T_g, is the cooperative nature of the alpha relaxations. Arrhenius kinetics become operative once again in the glassy solid at $T < T_g$, but the rates of all diffusion-limited processes are much lower in this high viscosity solid state than in the liquid state at $T > T_g$. In fact, the difference in average relaxation times between the two Arrhenius regimes ($T < T_g$ and $T > T_m$) is typically more than 14 orders of magnitude.

a *Strong and fragile liquids*

The molecular processes which contribute to the glass transition are currently the subject of intensive research and debate. Whether the changes in thermodynamic properties (i.e. specific heat, volume) that are seen during cooling are due to a real thermodynamic phase transition or are of purely kinetic origin is a controversial issue (Angell, 1995a; Angell, 1995b). However, if only kinetic aspects are considered, it is possible to determine the temperature dependence of the molecular motions responsible for the physical properties

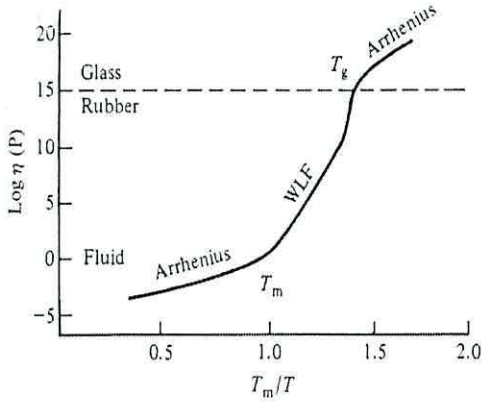


Figure A.2: Viscosity as a function of temperature for glassy polymers (Levine and Slade, 1987)

of amorphous materials. Close inspection of the broad spectrum of viscosity data available has shown that by scaling temperature in an Arrhenius plot by the glass temperature itself a pattern can be observed (see figureA.3) and the amorphous glass forming materials may be classified. Furthermore, the temperature dependence of the viscosity can be described by the following equation

$$\eta = \eta_o exp(DT_o/T - T_o) \tag{A.1}$$

where D and T_o are constants. D is the parameter which controls how closely the system obeys the Arrhenius law. As D changes, so will the value of T_o change relative to T_g.

$$\frac{T_g}{T_o} = 1 + D/(2.303 log\eta_g/\eta_o) \tag{A.2}$$

where $log(\eta_g/\eta_o)$ is $\sim$ 17 (Angell, 1995b) T_o is believed to correspond to the Kauzmann temperature (T_k) since equation A.1 yields values that agree with the Kauzmann temperature obtained from thermodynamic measurements (Kauzmann, 1948). The effect of changing D from 5 to 100 is shown in the insert to figure A.3.

Depending upon the magnitude and temperature dependence of the activation energy of molecular motions near and above T_g in supercooled liquids, it is possible to classify them as either *strong* or *fragile* amorphous systems. A *strong* liquid exhibits Arrhenius like changes in its molecular mobility with temperature and a relatively small change in

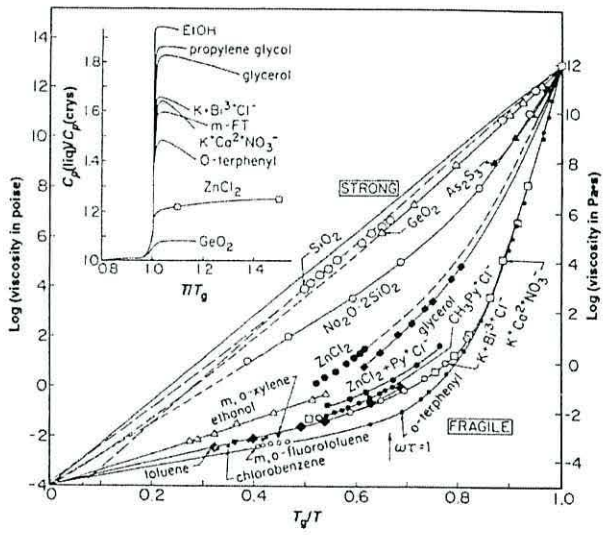


Figure A.3: T_g-scales Arrhenius plots of viscosity data showing the strong/fragile pattern of glass formers. The jump in heat capacity at T_g is generally large for the fragile liquids and small for strong liquids. (reproduced from (Angell, 1995a))

heat capacity at T_g. Strong liquids are resistant to structural changes despite changes in temperature . Proteins are an example of strong glass formers, with their changes in heat capacity at T_g often being so small that they cannot be detected using standard calorimetry techniques (Hancock and Zografi, 1997). A *fragile* supercooled liquid has a much stronger temperature dependence of molecular mobility near T_g and a relatively large change in heat capacity at T_g and will typically consist of non-directionally, non-covalently bonded molecules.

The constant D in equation A.1 is an indicator of fragility; the lower the D value, the larger the jump in the heat capacity at T_g, that is the lower the D value the more fragile is the system. Usually D values lower than 19 are characteristic of very fragile and values higher than 100 are indicative of a strong glass. As T_o is determined by D, (see equation A.2) the value of T_o is also related to the fragility of the system (Hancock and Zografi, 1997) with $(T_g - T_o) > 50$ typical of strong glass formers and $(T_g - T_o) < 50$ usual for fragile materials.

2 Crystallization of polymeric materials

Polymers in a dilute solution can be regarded as a chain whose shape is governed by short and long range inter- and intra-molecular interactions. When in the aggregate state the behaviour of the chain is influenced by the proximity of the neighbour chains. This effect determines the orientation of chains relative to to each other in the undiluted state and this is essentially an interplay between the entropy and internal energy of the system which is expressed in the usual thermodynamic form

$$G = (U + pV) - TS \tag{A.3}$$

In the melt/liquid state polymers normally attain a state of maximum entropy consistent with a stable state of minimum energy. Crystallization is a process which involves the orderly arrangement of chains and is consequently associated with a large negative entropy of activation. If a favourable free energy change is to be obtained for a crystalline formation, the entropy term has to be offset by a large negative energy contribution.

Crystal formation is a two stage process, the first one being the formation of a stable nuclei brought about by the ordering of chains in a parallel array, stimulated by intramolecular forces followed by the stabilization of long range order by the secondary valence forces which aid the packing of molecules into a three-dimensional ordered structure.

The second step in the crystallization process is the growth of the crystalline region, the size of which is governed by the rate of addition of other chains to the nucleus. As this growth is counteracted by thermal redispersion of the chains at the crystal melt interface, the temperature must be low enough to ensure this disordering is minimal.

3 Water as a plasticizer - the hydration dependence of T_g

It is well know that water, acting as a plasticizer, affects the glass transition temperature, T_g, of completely amorphous polymers and both the T_g and melting temperature, T_m of the partially crystalline polymer (Levine and Slade, 1987). Water is a 'mobility enhancer' in that its low molecular weight leads to a large increase in mobility, due to

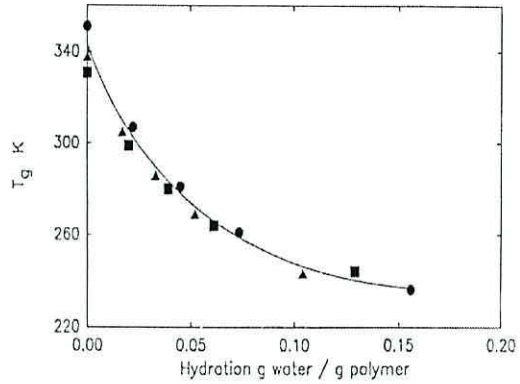


Figure A.4: The variation of the glass transition temperature with water content for polyamides (Gregory, 1995))

increased free volume and decreased local viscosity as the moisture content is increased from that of a dry solute to a solution. The direct plasticizing effect of increasing moisture content at constant temperature is equivalent to the effect of increasing the temperature at constant moisture and leads to increased segmental mobility of chains in amorphous regions of glassy and partially crystalline polymers allowing, in turn, a primary structural relaxation transition at decreased T_g.

State diagrams illustrating the extent of this T_g-depressing effect have been reported for a wide variety of synthetic and natural, water-compatible, glassy and partially crystalline polymers. In such diagrams (see for example figure A.4) the smooth 'glass curve' of T_g versus water content shows the dramatic effect of water on T_g especially at low moisture contents (that is $\leq$ 10% by dry weight). In this region T_g typically decreases by about $5 - 10^oC$ with a 1% increase in water content.

It is generally thought that at low water content the solid-water interaction is very strong, resulting in a marked plasticizing effect. As the water content increases, the strength of the solid water interaction decreases, and the plasticizing efficiency of the water is reduced. Eventually the affinity of the water for the solid decreases so much that the water preferentially associates with itself.

a Mechanism of plasticization

The mechanism of plasticization of glassy polymers by water may have two components. The first can be explained in terms of free volume and the second involves the hydrogen

bonding between water and the polar groups of the polymer. Free volume theory provides the concept that low molecular weight plasticizing components have a large free volume, so that molecular level interactions between water and a polymer lead to increased free volume in the diluted aqueous polymer rubber, which allows increased chain-segmental mobility of the backbone, which in turn results in decreased T_g. In addition, for polymers hydrogen bonding with water, it has been suggested that the free volume contribution can be augmented by a contribution due to the extent of polymer-water hydrogen bonding. The particular nature of such polymer-water interactions has been suggested to be site-specific H-bonding between water and the polar groups on the polymer, and the extent of this interaction has been said to increase with increasing hydrophilicity of the polymer. Furthermore, it has been suggested that when the glassy polymer network structure involves interpolymer H-bonds, in addition to any covalent cross-links such as are present in, for example, epoxy resins and elastin, the plasticization mechanism can be augmented further by the breakage of these interpolymer H-bonds by water, and their replacement by labile water-polymer H-bonds, which would lead to greatly increased molecular mobility, and result in even greater depression of T_g. Lastly the contribution from the dielectric properties of water has to be considered; water, due to its high relative permittivity moderates electrostatic interactions between charged and polar sites within the polymer, therefore allowing polymers to both take up different conformations and exhibit structural flexibility.

B

Raman spectra

1 Trehalose

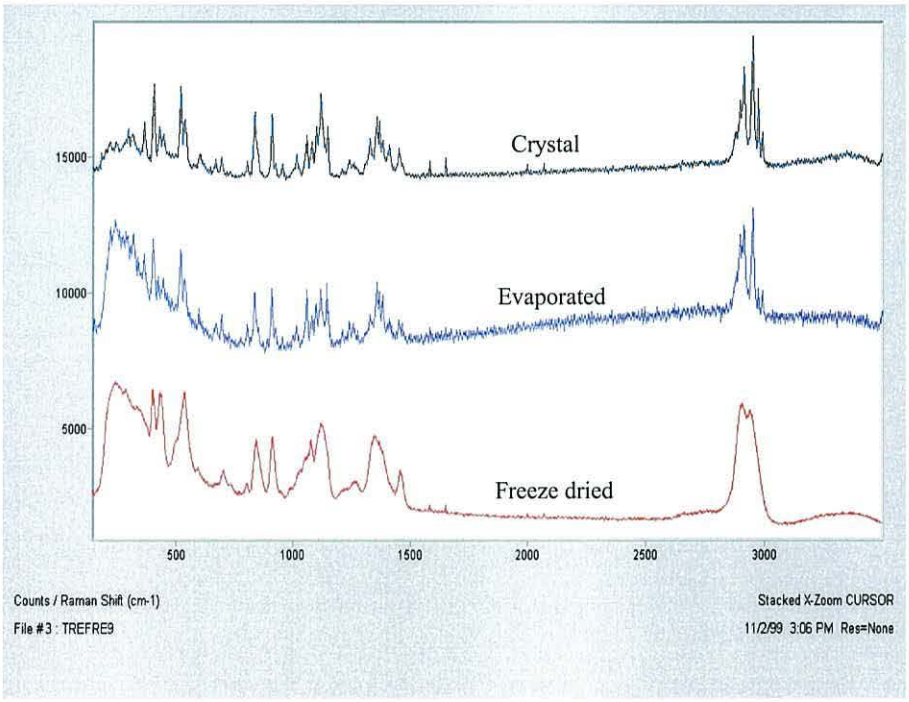


Figure B.1: Raman spectra of trehalose forms

2 SUCROSE

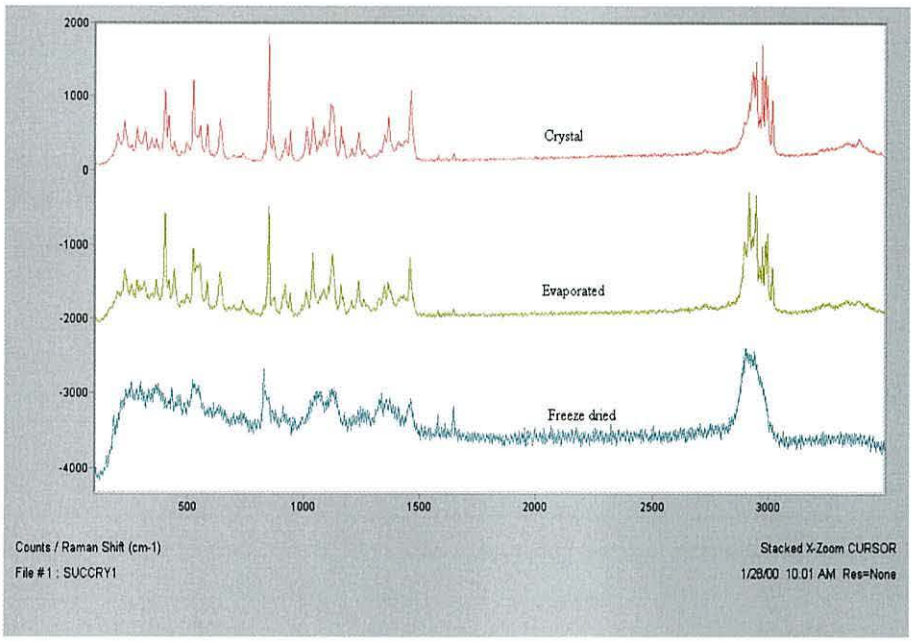


Figure B.2: Raman spectra of sucrose forms

C

A MATLAB ROUTINE

The following matlab routine has been used to predict the hydration isotherm of trypsin for any given enzyme activity within the range 100 to 38%.

clear all; **** clear all variables

hydra1 = uigetfile('*.dta','Select the RH file');

hydra2 =uigetfile('*.dta','Select the water uptake file');

hydra3 = uigetfile('*.dta','Select the remaining activity file');

rh = dlmread (hydra1,'');

m = dlmread (hydra2,'');

ra = dlmread (hydra3,'');

rhmax = max(min(rh)); rhmax = fix(rhmax);

rhmin = min(max(rh)); rhmin = ceil(rhmin);

numrh = rhmax - rhmin + 1;

ramax = min(max(ra)); ramax = fix(ramax);

ramin = min(min(ra)); ramin = ceil(ramin);

numra = ramax - ramin + 1;

rhlin=linspace(rhmin,rhmax,numrh); ralin=linspace(ramin,ramax,numra);

[RH, RA] = meshgrid(rhlin,ralin); M = griddata(rh,ra,m,RH,RA,'cubic')+ eps; figure mesh(RH,RA,M),title('hydration isotherms vs. remaining activity'); pause

RAmenu = menu('Do you what to calculate the isotherm for a given remaining activity?',... 'Yes','No');

if RAmenu == 1 $r_a = input('PleaseentertheRemainingactivity..'); elsereturnend$

minRA = min(min(RA)); minRA = ceil(minRA);

$nr_a = round(r_a); nra = nr_a - minRA + 1;$

minRH = min(min(RH)); minRH = ceil(minRH);

maxRH = max(max(RH)); maxRH = fix(maxRH);

```
newrh = (minRH:maxRH); pause
newm = M(nra,:);
figure plot(newrh,newm,'o'),title('Extrapolated Hydration Isotherm for deactivated trypsin') uimenu('label','Print figure','callback','print -v') xlabel ('relative humididty ylabel ('water uptake pause
newiso = [newrh newm];
SAVEmenupoints = menu('Save Isotherm Data?','Yes','No'); saveoptions = [1 0]; SV = saveoptions(SAVEmenupoints); if SV == 1; DIRmenupoints = menu('Choose Directory and Drive', 'A', 'C:iso'); diroptions = [1 2]; DI = diroptions(DIRmenupoints); if DI == 1; cd A: end
if DI == 2; cd C:; end
file = input('Please enter the Filename...','s');
eval(['save ' file ' newrh newm -ascii -tabs ']); cd C:11;
end
GAB
```

Publications and meetings

Scientific Publications

1 The effect of trehalose and sucrose on the long term stability of trypsin
E.C. López-Díez and S. Bone,
in preparation, (2002).

2 The effects of sugars in protecting the functional properties of dried proteins
B.S. Murray, H.-J. Liang, S. Bone and E.C. López-Díez,
Eds. E. Dickinson and R. Miller, *Royal Society of Chemistry* , pp. 272-281 (2001).

3 An investigation of the water binding properties of sugar+protein systems.
E.C. López-Díez and S. Bone,
Physics in Medicine and Biology **45**, 3577-3588 (2000).

Conferences, Workshops and Meetings

1 International School of Physics Enrico Fermi,
Protein Folding, Evolution and Design.
Varenna, Italy, 11 July – 21 July (2000).
Poster presented: Protein sugar interactions: An investigation of the water binding properties of sugar+protein systems.

2 Bioelectrostatics.
London, England, September (1999).
Poster presented:Electrostatic interactions in protein+sugar+water systems.

3 Biomolecular Folding and Self-Assembly.
Manchester, England, 12–15 April (1999).

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