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THE ROLE OF MOISTURE IN PROTEIN STABILITY

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ABSTRACT

The importance of water sorption on solid state stability of proteins can be addressed through an understanding of properties of the sorbed water and its impact on the properties of the protein. Most decomposition reactions are minimal at or below the monolayer level of hydration. Sorption of water beyond that of the monolayer generally results in increased rates of decomposition due to the increased conformational flexibility of the protein and the ability of the less tightly bound water to mobilize reactants. In many cases the rates of decomposition can be influenced by the addition of excipients. An understanding of how such excipients can influence water sorption and stability will allow for improved development strategies to minimize the decomposition of proteins in the solid state.

INTRODUCTION

The importance of moisture on solid-state stability of drugs and drug-containing formulations is well documented [1,2]. However, few cases are reported in which the solid drug is a protein or polypeptide [3-5]. Despite such voids in the pharmaceutical literature, a significant amount of information on protein hydration and stability in the solid state is available from the food industry [6-9] Solid state stability of proteins is a concern for bulk solid storage and processing, lyophilized formulations, and controlled release formulations. In most cases, administration of controlled release systems expose the protein to elevated temperature and high water activity. These unfavorable environmental conditions cannot easily be controlled and formulation strategies are crucial to protect the protein over extended periods. Most controlled delivery systems result in the protein either being in solution or a highly hydrated state prior to its release; the possible exception being

surface-eroding polymers. Therefore, effective formulation strategies will require an understanding of protein hydration and its impact on stability in the solid state.

HYDRATION OF PROTEINS

Due to the importance of proteins in biological systems, food sources and textiles, their hydration has been the subject of extensive investigation [6-12]. The most common measure for water uptake by protein powders is equilibrium moisture content (EMC). The water content is determined following equilibration at a given relative humidity. A sorption isotherm is generated by assessing EMC at varying relative humidities (Figure 1). The use of partial pressures or water activities in place of relative humidities is common. Numerous efforts have been made to develop mathematical models with relevant and measurable parameters to describe the sigmoidal shaped isotherms [13]. The classic physical adsorption model or the BET equation is typically used to determine the amount of water (EMC value) necessary to form a "monolayer" [14]. In the case of proteins this "monolayer" is more correctly defined as the amount of water necessary to cover the highly active sorption sites, and does not correspond to monolayer coverage of the particles.

The sorption isotherm for proteins can be roughly separated into three regions (Figure 1). The first region is binding of water to highly active sites such as charged and highly polar groups. The second region is a transition region from monolayer to multilayer coverage. It occurs with the binding of water to weaker sorption sites such as the peptide backbone and polar surface groups. Additional water binding occurs via clustering at or near charged and highly polar groups and through filling of voids created by swelling of the polymer. The last region, or multilayer region, occurs with condensation of water at very weak binding sites and layering of loosely held water. This multilayer region is more effectively described by solution sorption models than physical sorption models [13].

The binding of water to proteins is a result of numerous molecular and intermolecular interactions (Table 1). Efforts to establish stoichiometric relationships between the types of functional groups for water binding and the sorption isotherm have been pursued with limited success [10,11,15]. Examples of water binding capacity of various groups as a function of relative humidity are listed in Table 2. Using these binding capacities, Leeder and Watt calculated isotherms based on the primary sequences of several proteins. These isotherms compared favorably with experimental isotherms up to about 40-50% relative humidity [11]. Our experience with recombinantly-derived bovine somatotropin (rbSt) resulted in reasonable to

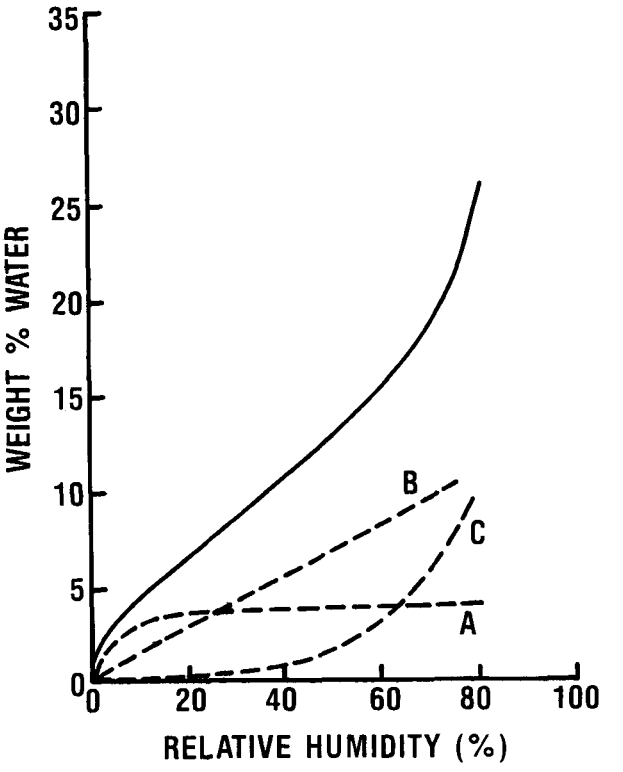


FIGURE 1
Sample sorption isotherm for the hydration of Lysozyme broken down into component parts using D'Arcy-Watt analysis [11]. (A) Sorption onto strong sorption sites, (B) Sorption onto weak sorption sites and (C) multilayer sorption.
(Reproduced with permission of Academic Press, [11])

TABLE 1
Factors Involved in Water Binding to Proteins

Molecular Level Interactions

1. Coulombic forces (ionized groups, ions)
2. Hydrogen bonds (polar groups)
3. Van der Waals forces (dipole interactions)
4. Steric effects (hydrophobic groups)

Intermolecular Level Interactions

5. Mobility changes in polymer segments (plasticizing effect of water)
6. Capillary condensation (hydrodynamic curvature effects)

TABLE 2
Water Associated with Various Groups in Proteins [11].

Sorption site	Moles H_2O per mole of sorption site at R.H. of:						
	5%	10%	20%	35%	50%	65%	80%
Carboxyl (-COOH)	0.7	0.92	1.2	1.63	2.0	2.3	2.5
Aliphatic hydroxyl (OH)	0.05	0.09	0.17	0.27	0.34	0.46	0.60
Phenolic hydroxyl (-OH)	0.16	0.25	0.5	0.75	1.0	1.3	1.8
Peptide (-CO•NH-) Amide (-CO•NH_2) Heterocyclic imino (-NH-)	0.04	0.006	0.11	0.17	0.25	0.36	0.56
Amino (-NH_2) Guanidino (-NH•C(=NH)NH_2)	0.6	0.83	1.2	1.63	2.1	2.4	2.7

poor predictions of isotherms depending on the salt of rbSt used (Figure 2) [16]. Overestimation of EMC values when calculating isotherms from various stoichiometric analysis is common [10].

The differences is sorption isotherms for various salts, as noted in Figure 2, result from changes in the ionization of amino acid side chains and the presence of highly polar counterions which affect the nature of the binding sites. Comparisons of sodium and hydrochloride salts of casein show a minimum degree of hydration when the protein is prepared at a pH near its isoelectric point of 4.6 [17]. The effect of ionization state has also been demonstrated by the sorption isotherms (Figure 3) and monolayers (Table 3) obtained for the synthetic polypeptides poly-l-lysine and poly-l-glutamic acid and their salts [18]. Also of note in Table 3 are the similar monolayer values obtained for the same protein in different physical states.

Monolayer data for water or gas sorption, obtained by the BET equation, are commonly used to determine surface areas of solid particles [20]. Although the areas determined by nitrogen sorption are highly dependent on the particle size or

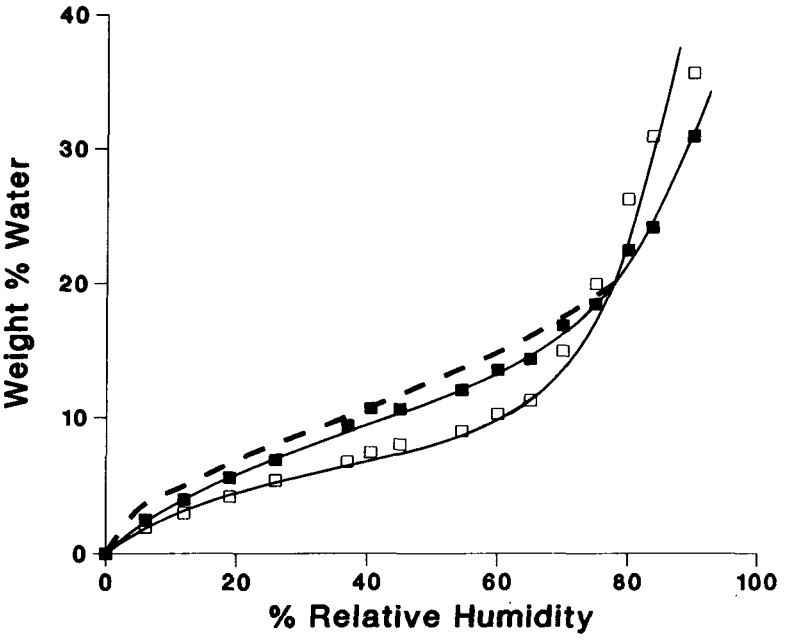


FIGURE 2
Comparison of predicted (--) and experimental isotherms for two different salts of rbSt [16].

physical state of the solid, surface areas of protein powders from water sorption data are unaffected by such factors (Table 4) [21]. The very large H_2O/N_2 surface area ratios are consistent with penetration of moisture into the solid and have been noted with other noncrystalline polymeric materials such as starch, cellulose and hydrogels [22,23]. Penetration of water results in macroscopic polymer deformation and swelling. This is one proposed explanation for the sorption-desorption hysteresis commonly observed with most proteins (Figure 4) [23-25]. These macroscopic intermolecular interactions were previously described in Table 1. Other explanations involving molecular interactions, such as conformational changes or phase changes, have also been proposed to account for hysteresis [26].

Increasing temperature, decreasing solid packing density and attaining desorption in a single step, as opposed to several steps, have been found to decrease the size of the hysteresis loop [25]. Likewise, long-term storage under set conditions may have a significant impact on subsequent hysteresis loops [23]. Consequently, the

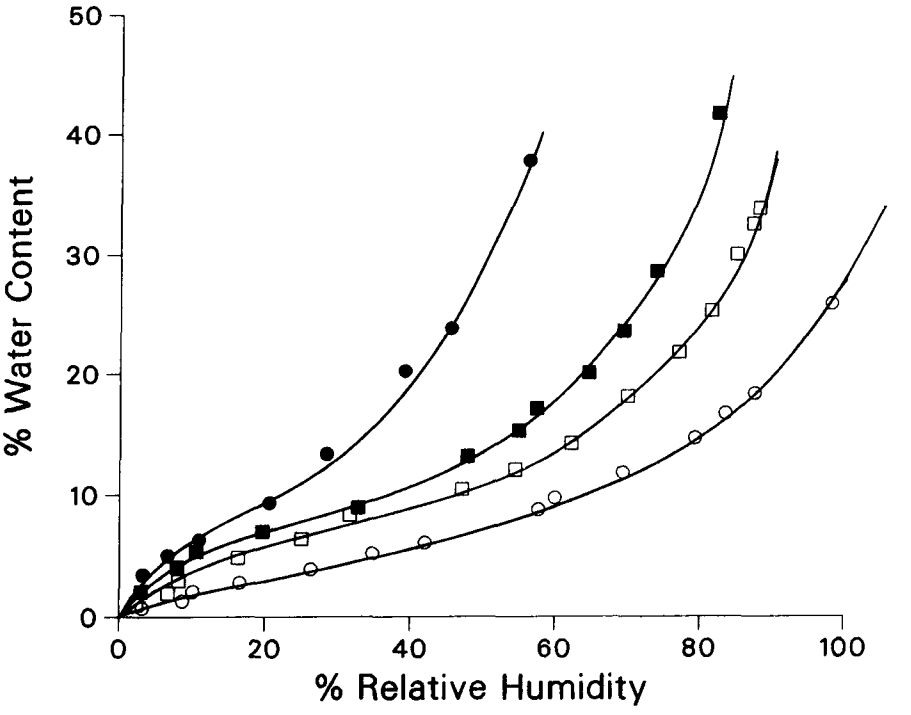


FIGURE 3
Sorption isotherms for poly-L-lysine (□), poly-L-lysine hydrobromide (■), poly-L-glutamic acid (○) and sodium poly-L-glutamate (●) at 25°C [18].

water content of a protein sample at a given relative humidity can be influenced by its prior process and storage conditions.

The evaluation of protein stability is more complex in heterogeneous systems such as a protein formulation. In most cases, the hydration and equilibration of various components are dictated by the individual isotherms and total available water in the formulation [27-28]. Additives such as glycerol, propylene glycol or other polyhydric alcohols are commonly used in protein formulations as cryoprotectants during freezing and lyophilization [29,30]. The presence of these additives can increase water content at a constant relative humidity or water activity. However, if the water content of the formulation is constant, the presence of these additives will decrease the water activity in the formulation. The presence of soluble salts will also

TABLE 3
Water Monolayers Determined Using the BET Equation [16, 18, 19]

Protein	gm H_2O/ 100 gm Protein
rbSt	
Lyophilized (pH 9-10)	7.6
Lyophilized (pH 6-7)	5.2
Ovalbumin	
Air-dried	11.6
Lyophilized	11.6
Denatured	13.6
β-Lactoglobulin	
Crystallized	8.6
Lyophilized	9.4
Poly-L-lysine	
Lyophilized (pH 5.6)	7.1
Lyophilized (pH 11)	6.2
Poly-L-glutamic acid	
Lyophilized (pH 8.7)	7.0
Lyophilized (pH 2.0)	4.1

TABLE 4
Surface Areas Calculated From Water and Nitrogen Sorption Data Using the BET Equation [16, 21, 22]

	m^2/Gm		
	N_2 area	H_2O area	H_2O/N_2
Ovalbumin			
Air-dried	20.4	218	10.7
Lyophilized	5.8	200	34.5
rbSt			
Lyophilized	1.3	188	145
Microcrystalline Cellulose	-	-	85
p-HEMA	-	-	1.2×10^5

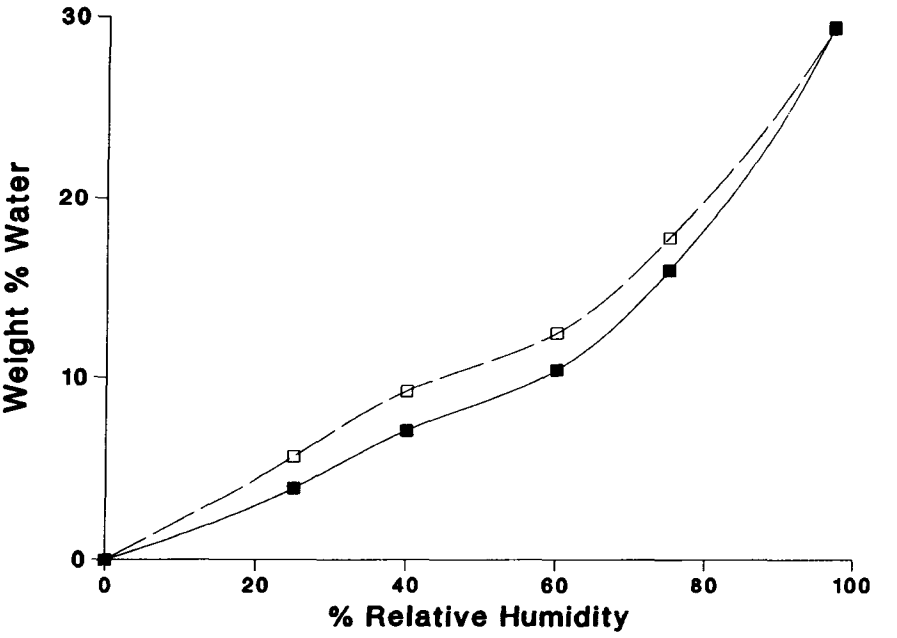


FIGURE 4
Sorption (■)/Desorption (□) hysteresis loop for rbSt at 25°C [16].

increase water uptake at high relative humidities by dissolving in the loosely bound water and decreasing the vapor pressure, causing increased condensation. At relative humidities less than 50%, such electrolytes may actually decrease the water uptake by occupying protein binding sites for water [31]. Therefore, the activity of water in a heterogeneous system can be varied through the appropriate use of additives.

The effect of water content and/or water activity on the solid state stability of proteins results from one of the following factors:

1) changes in dynamic activity of the protein,
2) changes in conformational stability of the protein,
3) participation of water as a reactant or inhibitor,
4) participation of water as a medium for mobilization of reactants.

Extensive studies in the hydration of lysozyme [32-34] and other proteins [35-36] have identified several critical levels of hydration at which significant changes in properties of the protein and the bound water occur. Initial hydration up to a level

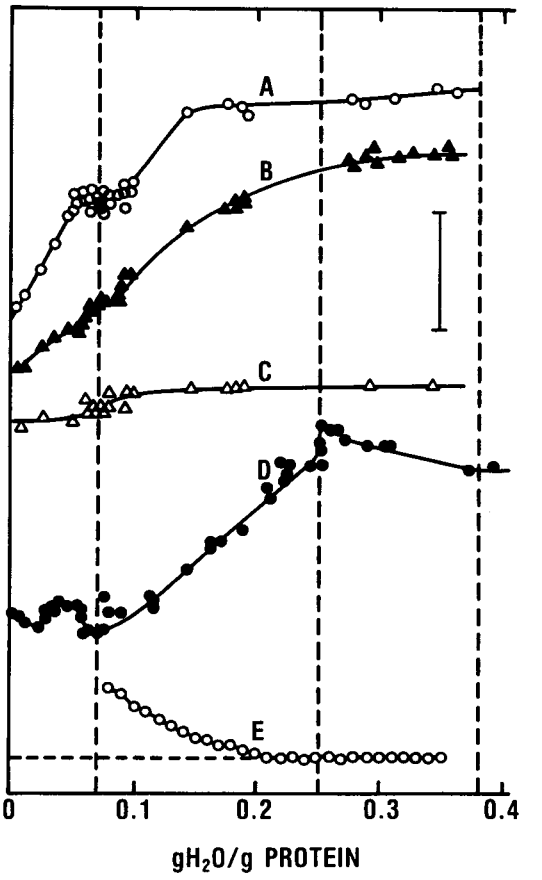


FIGURE 5
Properties of lysozyme measured as a function of the degree of hydration [32]. (A) Carboxylate absorbance at 1580 cm^{-1}, (B) Amide-1 shift 1660 cm^{-1}, (C) OD stretching frequency at 2570 cm^{-1}, (D) Apparent specific heat capacity and (E) Diamagnetic susceptibility.

of 6-8% water content (monolayer levels) results in very little change in the properties of the tightly bound water (Figure 5); water mobility is approximately 1/100th that of bulk water. Significant changes in the carboxylate and amide regions of the protein occur with increasing hydration above the monolayer (Figure 5). Consult references 32-34 for a more extensive discussion. From 6-25% water content, the properties of the bound water change significantly. Above 25% water content, the properties of the bound water are similar to those of bulk water. The dynamic

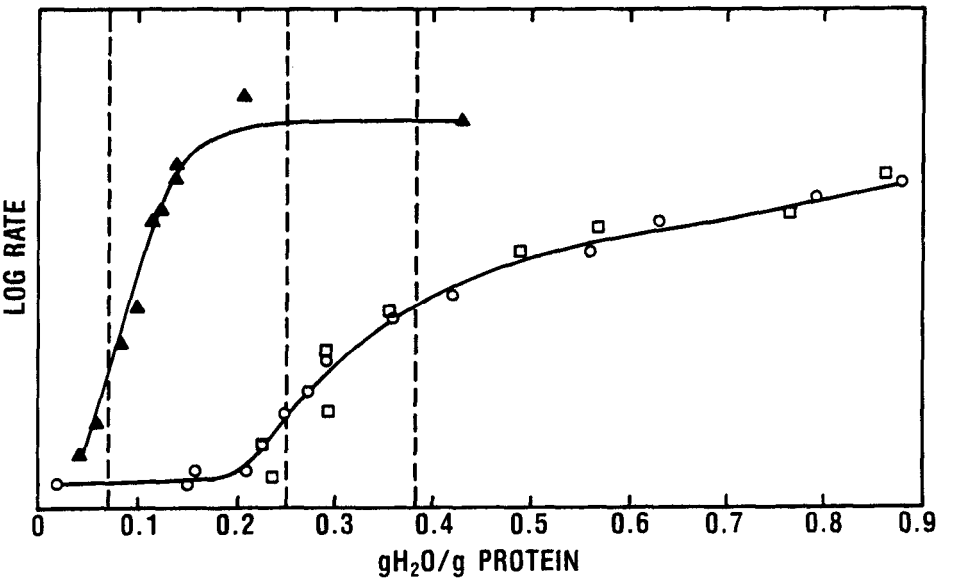


FIGURE 6
Dynamic properties of lysozyme as a function of water content [32]. Log rate of peptide hydrogen exchange (▲), rotational relaxation time of an ESR probe (○), and enzymatic activity as log V_o (□).

activity of the protein is detected at water levels just below the monolayer as evidenced by increased internal motion leading to hydrogen exchange (Figure 6). A significant increase in flexibility and subsequent enzymatic activity at approximately 20% water content is noted (Figure 6). The sequential hydration of a protein as described above has been proposed by Rupley et. al. [34].

DECOMPOSITION OF PROTEINS

The structural heirarchy that exists in proteins results in a multiplicity of decomposition pathways which may or may not be inter-dependent (Table 5). Alterations in tertiary and secondary structure can occur without any chemical changes or changes in the primary structure. Such alterations are regarded as conformational changes or denaturation and may be reversible or irreversible [29,37-38]. Irreversibility generally results from the exposure of hydrophobic surfaces which then interact inter-molecularly, leading to aggregation. Chemical modification generally results in changes to the primary sequence and may or may not have a

TABLE 5
Pathways For Solid State Decomposition of Proteins

Cleavage	Denaturation
Oxidation	Transpeptidation
Crosslinking	Photodegradation
Deamidation	Interaction with Additives

subsequent effect on conformational structure. Chemical modifications can result from intra-molecular reactions, inter-molecular reactions or involve reactions with other components of a heterogeneous system. The effect of moisture on solid state stability will be discussed regarding conformational integrity, inter-molecular protein-protein interactions and interactions between the protein and the other reactants. Unfortunately, there is not any good information available on intra-molecular reactions such as deamidation or disulfide shuffling in the solid state.

The temperature required for thermal denaturation of proteins (TD) in the solid state decreases with increasing protein hydration until the a water content reaches 50%. At this point TD of the solid state is approximately equal to that in solution (Figure 7) [39-41]. A mathematical relationship between the volume fraction of water in the polymer and the melting or denaturation temperature of the polymer-water system has been indicated. Thermal denaturation temperatures at very low water content are difficult to obtain due to other predominating decomposition pathways.

The effect of elevated temperatures, which may be encountered during processing, has been evaluated for proteins such as rbSt [16] and bovine serum albumin (BSA) [42] at hydration levels below that of the monolayer. The major decomposition pathway for rbSt results in the formation of covalently bound aggregates. The kinetics are modeled as an apparent second-order reaction over the first half-life (Figure 8). It is unlikely that a second-order model will be acceptable beyond a half-life, due to subsequent reactions between monomers and formed dimers to yield trimers and higher oligomers. While the nature of the crosslinking in rbSt is not clear, studies with BSA have shown similar covalent aggregation [42]. The involvement of free lysine groups in crosslinking reactions of BSA have been noted. The effect of temperature on the apparent second-order rate constants for rbSt is linear with an

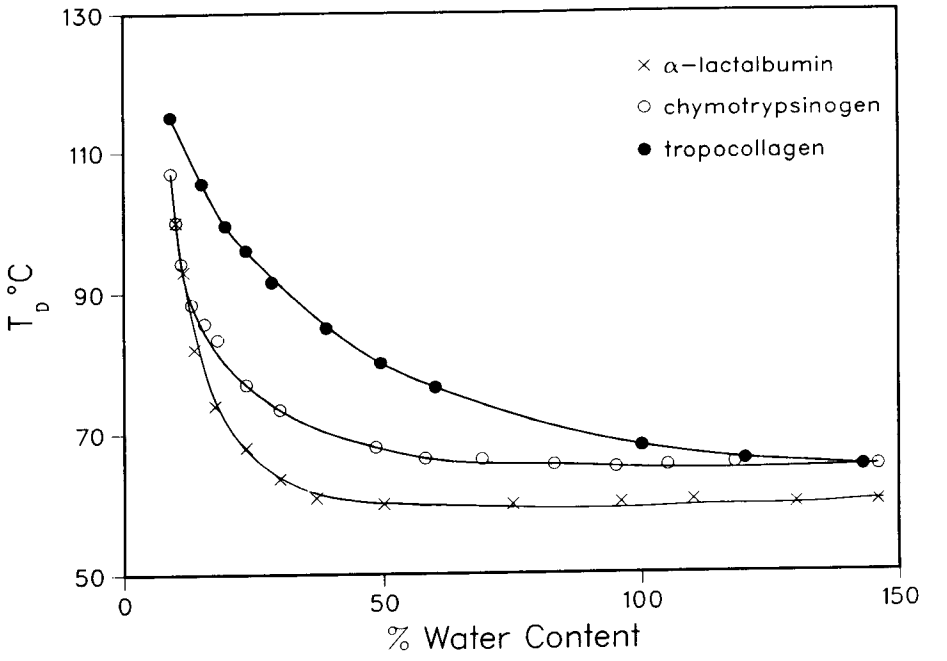


FIGURE 7
Denaturation temperatures as a function of water content for three proteins [39-41].

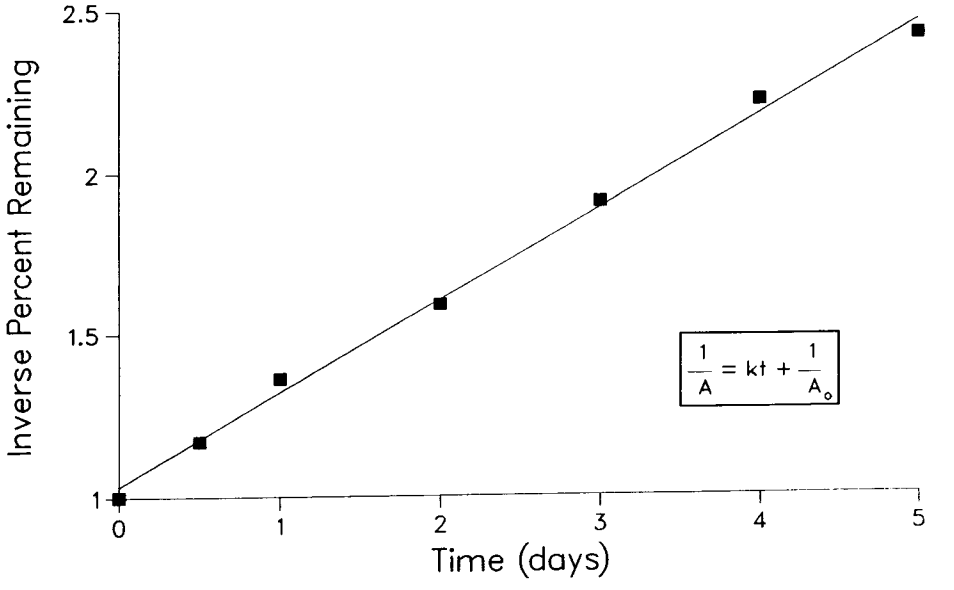


FIGURE 8
Representative plot for a 40-60% loss of rbSt as measured by RPHPLC, assuming a second order decomposition after exposure to elevated temperatures [16].

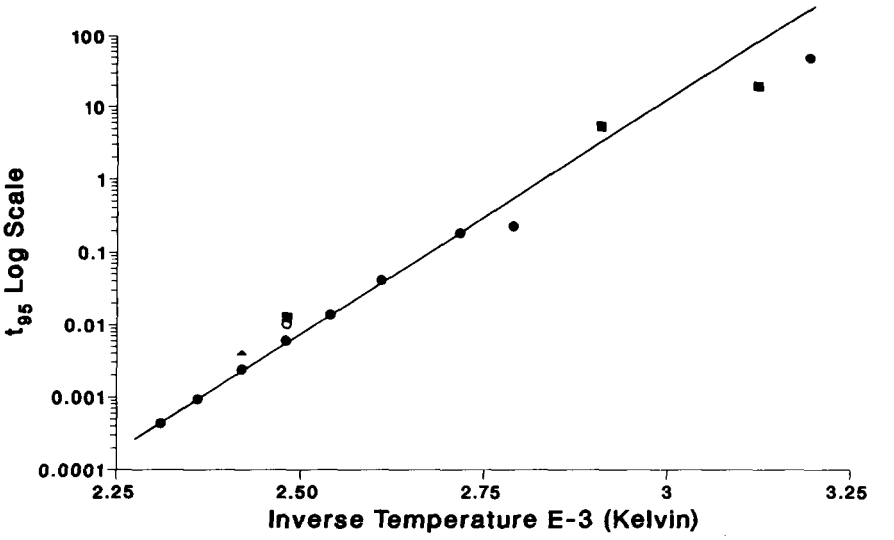


FIGURE 9
An Arrhenius plot of apparent t_{95} values calculated from second order rate equations for solid state decomposition of rbSt. Different symbols represent different salts or preparations [16].

apparent E_{act} of 121 kJ mol^{-1} (Figure 9). This compares well with an E_{act} of 130 kJ mol^{-1} for the initial rates of loss of lysine groups in BSA. Several observations of note with the BSA system, are that BSA decomposes faster in the case of pure protein as compared to heterogeneous systems containing the protein and the rate of loss of ε-amino groups of lysine decreases drastically as the reaction proceeds. Both observations are consistent with the bimolecular nature of the proposed crosslinking.

Studies of protein cakes which undergo similar decomposition to BSA show increasing rates of loss of lysine groups with increasing relative humidity up to the expected monolayer coverage area of 8-10% moisture [42]. Beyond the monolayer, increasing humidity only had a slight effect on the rate of loss of free lysine groups. However, changes in other amino acids and/or conformational structure are likely to occur as the water content increases. The impact of relative humidity on solid state decomposition of proteins can be easily detected at lower temperatures and is dependent on the particular salt of the protein studied (Figure 10). In many cases, more than one decomposition pathway will occur as demonstrated by the different stability profiles for rbSt obtained by the use of different assays (Figures 10 and 11).

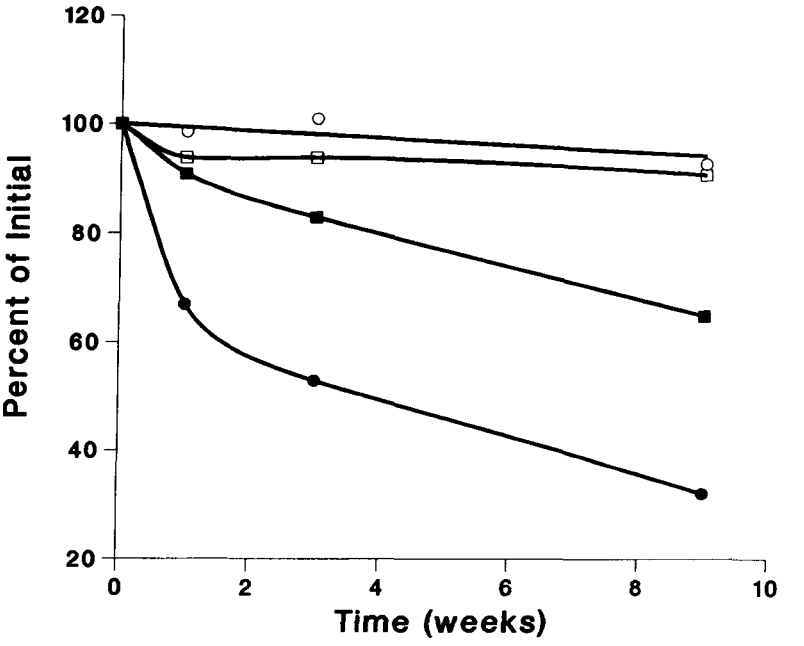


FIGURE 10
Effect of humidity on the decomposition of two different rbSt salts at (○ , □) 10-15% R.H.and (● , ■) 75% R.H. Loss was monitored by RP-HPLC [16].

For reactions which are bimolecular, the progress of the reaction depends on the mobility of one or both reactants. Hydration levels at or below the monolayer level have very little water available for solubilization of reactants. As the water content exceeds the monolayer, sufficient loosely bound water is available to mobilize reactants, especially if they are of small molecular weight and highly polar. The "effective viscosity" of bound water decreases linearly with increasing water content, allowing for more facile diffusion of reactants [43]. Therefore, at water levels greater than the monolayer, the reaction rate will increase with increasing water content (Case I, Figure 12). This increase may continue until a point is reached where all the reactant has been solubilized and dilution of reactant(s) offsets decreasing viscosity (Case II, Figure 12). Finally, if the dilution factor is predominant or water is a product inhibitor of the decomposition at higher water levels, a decrease in the rate as shown in Case III can occur (Figure 12).

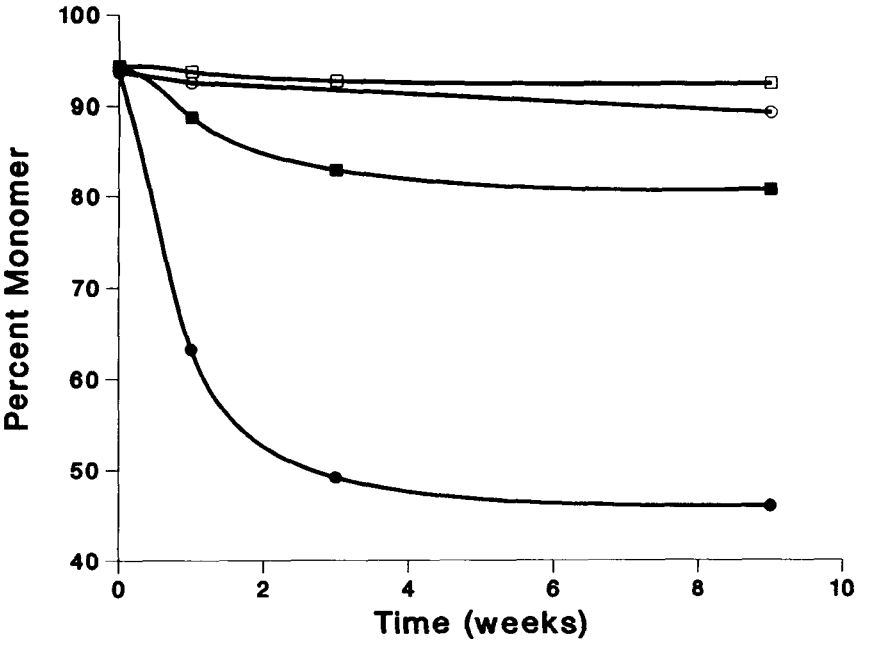


FIGURE 11
Effect of humidity on the decomposition of rbSt salts as described in Figure 10. Loss was monitored by size exclusion chromatography [16].

The mobilization of hydrogen-containing reactants can be monitored by wide-line NMR [31]. An increase in the specified NMR resonance occurs upon dissolution or mobilization of the reactant. This mobilization point for additives can vary from system to system, but generally, the same rank order of mobilization points for additives will be observed in each system. These mobilization points are likely to be related to the critical relative humidities of the water soluble solutes (i.e. the relative humidities at which the solutes will undergo deliquescence). The interaction of various solutes can affect each others mobilization in several ways [44]. Added electrolytes with ions of small radius can result in significant decreases in the percent water content required for reactant mobilization [31]. These increases are generally attributed to exclusion of water at the protein surface by preferential binding of the electrolytes, thus increasing available water. The addition of liquids such as glycerols and glycols, which act as humectants, greatly decrease the amount of water necessary for reactant mobilization even though they act to decrease water activity

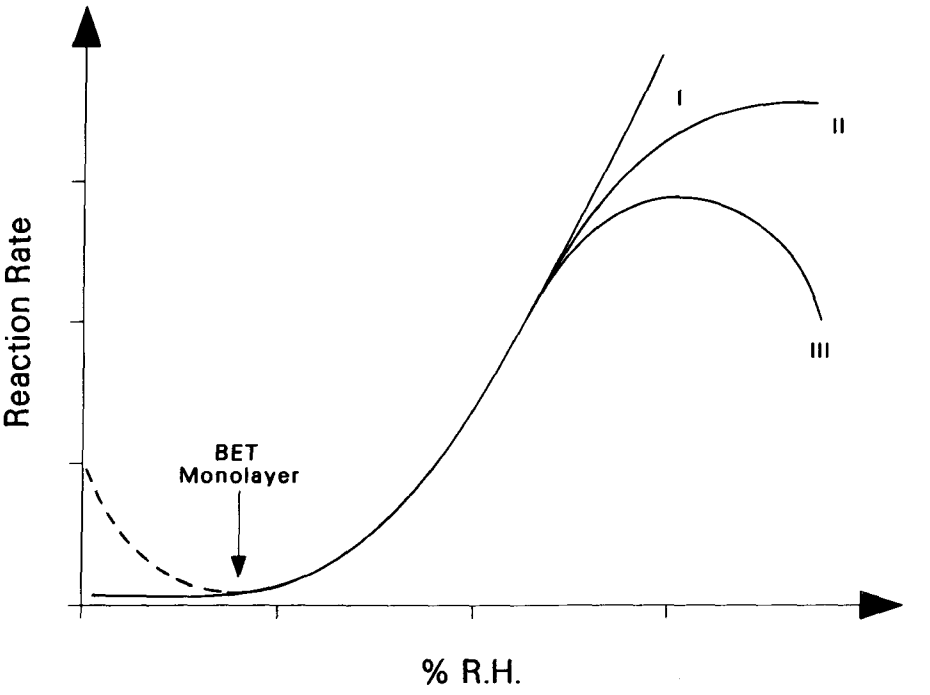
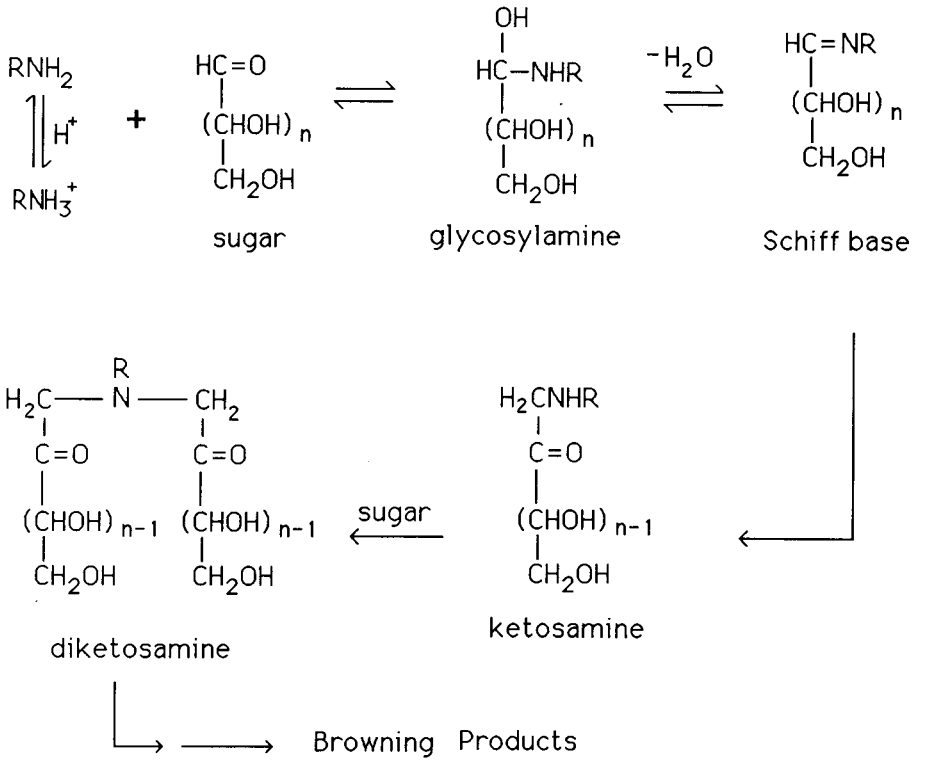


FIGURE 12
Possible effects of water content on reaction rates of bimolecular reactions in the solid state (see text for description of different cases). Dotted line refers to special case typical of many oxidation reactions.

43,45. Solid humectants such as sorbitol, mannitol and other polyhydric alcohols which dissolve in the bound water can also decrease the water activity at a given water content (consistent with Raoults Law). This leads to a decrease in available water for reactant mobilization.

An excellent example of the effects of reactant mobilization on reaction rate are observed in studies of the Malliard Reaction, Scheme I. The reaction is initiated by the attack of amino nucleophiles such as lysine on reducing sugars and the formation of glycosylamines. The glycosylamine undergoes further rearrangement with degradation to unsaturated carbonyls and is commonly termed the browning reaction. The effect of relative humidity on the Malliard Reaction in various glucose-containing systems all show the characteristic maxima of the Case III situation (Figure 12). The location of the maxima may vary from 40-80% relative humidity depending



SCHEME 1
The Maillard Reaction

on reactants and presence of additives (Figure 13) [5,46-48]. The addition of liquid humectants such as glycerol or proplylene glycol causes a lowering of the mobilization point and has a significant effect on the location of the maxima (Figure 14) [45]. The addition of a solid humectant such as sorbitol will tend to decrease the rate of browning significantly by decreasing the free water for mobilization of reactants (Figure 14). The mobilization point and onset of decomposition involving the Maillard Reaction correlate exceptionally well (Figure 15) [44]. The importance of pH in both the initial phases of amino group reaction with the sugars and the subsequent browning reactions has been shown [46]. The increasing rate with increasing pH is as expected based on the degree of ionization of the nucleophilic amino group.

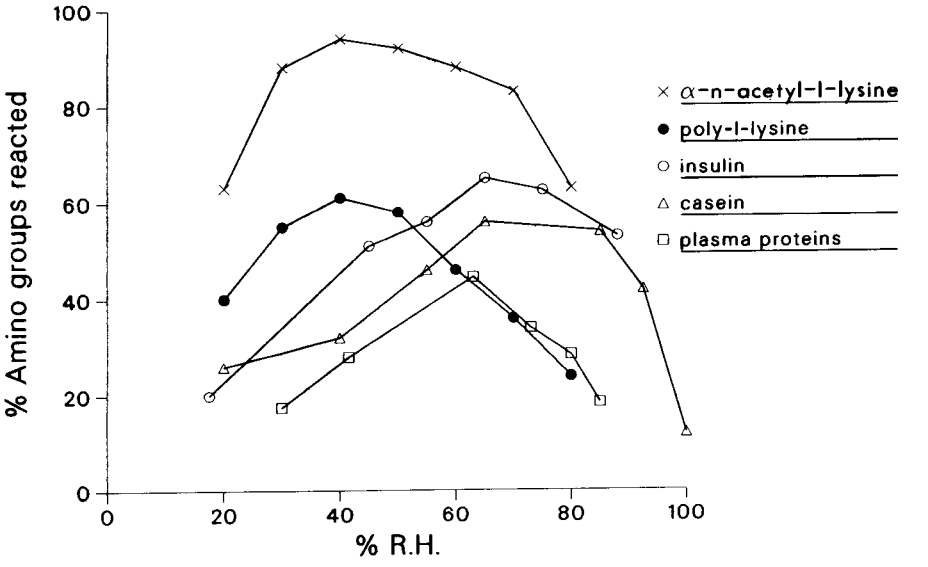


FIGURE 13
The effect of moisture on the progress of the Maillard Reaction [5, 46-48].

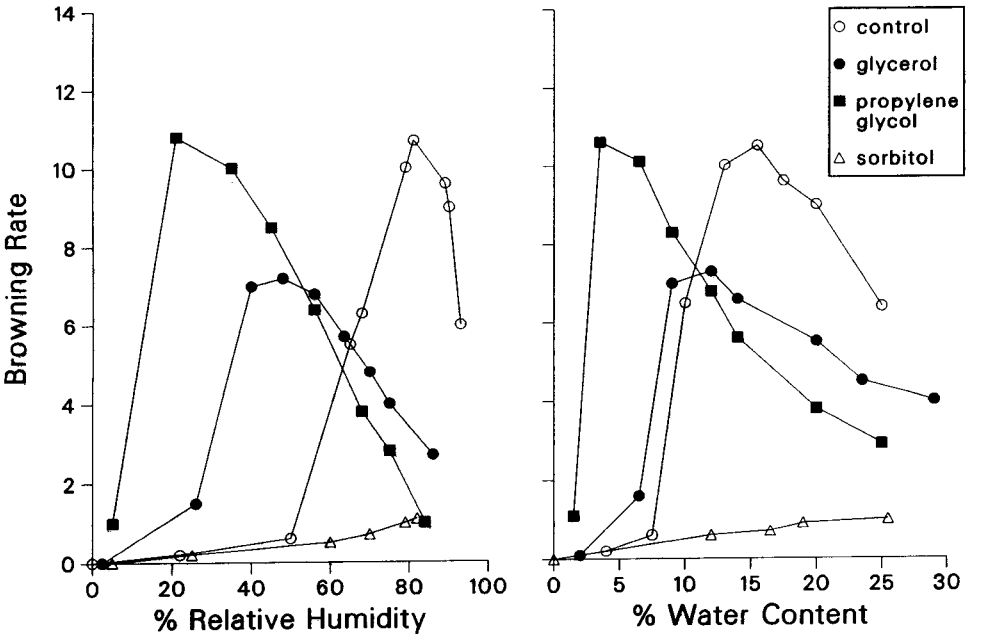


FIGURE 14
The effect of humectants on the progress of the Maillard Reaction as indicated by browning rate [45].

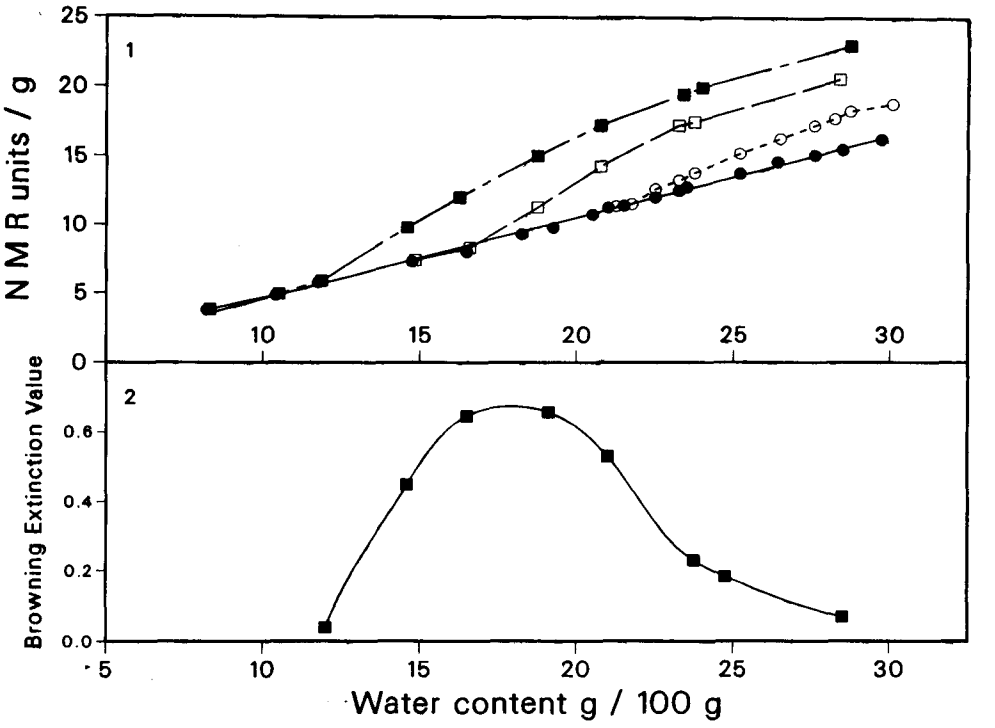


FIGURE 15
Comparison of the observed point of reactant mobilization and the degree of browning observed from the Maillard Reaction in a model starch system. (1) Mobilization of glucose hydrate (○), γ-aminobutyric acid (□), mixture of glucose hydrate and γ-aminobutyric acid (■) and the control starch (●). (2) Browning values after seven days at 25°C [44].

Most enzymatic reactions are minimal below relative humidities of 80% [49]. This is consistent with the 20-25% water content that is required for significant conformational flexibility (Figure 6). However, in some cases, significant activity can be observed at relative humidities of 20-30% or lower. This activity is consistent with the appearance of internal protein motions at water contents greater than 5-10%, or relative humidities of 10-30% (Figure 6) [49]. Water can also act as a media for transport of small molecular weight substrates which are easily mobilized. Consequently, enzymatic reactions even at the monolayer level of water are possible. If the substrates are volatile or in liquid form, their mobility may be such that enzymatic activity can be detected at monolayer or lower levels. Indoxylacetate is hydrolyzed by esterases at relative humidities below 10% [50].

TABLE 6
Effects of Water on Free Radical Oxidation Reactions in the Solid State [51,52]

Pro-oxidant Effects

1. Mobilization of catalysts
2. Swelling exposes new reaction sites
3. Decrease viscosity of sorbed phase

Antioxidant Effects

1. Retards oxygen diffusion
2. Promotes radical recombination
3. Decreases catalytic effectiveness of metals
4. Dilution of catalysts

Self-proteolysis may be a particular concern when formulating protease type drugs. However, due to the low mobility of the protein, such intermolecular reactions as self-proteolysis should be minimal below about 80% relative humidity. The addition of easily mobilized substrates which are non-cleavable should even further minimize any self-proteolysis, especially below 80% relative humidity where the substrate may be mobile but not the protein.

Finally, the last reaction to be addressed is that of oxidation. Much of the information available in this area is derived from mixed lipid-protein systems [51]. However, the observations may be useful in general for free radical propagation reactions in the solid state. Free radical reactions of proteins can be important with photocatalyzed decompositions, irradiation sterilization, exposure to high temperature, and production of radicals by physical processes such as freezing, drying and grinding [51]. The effect of free radicals on proteins is generally either scission of disulfides, scissions of the peptide backbone or inter- and intra-molecular crosslinking.

The effect of water on oxidation reactions is varied, it can have both antioxidant and proxidant effects depending on the system (Table 6). Generally, free radicals are stable at water levels below the monolayer, but decay rapidly through recombination as the water content increases [51,53]. A larger proportion of sulfur centered versus α-carbon centered free radicals occur as the water content increases, thus leading to different types of products. Because of the antioxidant effects of

water, many oxidation reactions actually show increased rates at water levels below that of the monolayer (Figure 12). At water levels exceeding the monolayer the prooxidant effects of water become important. At high water content the facilitated recombination of radicals and the dilution of trace catalysts can cause a decrease in rates similar to Case III in Figure 12.

SUMMARY

Most decomposition reactions are minimal at or below the monolayer level of hydration due to low availability of the water and limited dynamic activity of the protein. Sorption of water beyond that of the monolayer generally results in increased rates of decomposition. These increased rates are primarily due to the increased conformational flexibility of the protein and the ability of the less tightly bound water to mobilize reactants. In most cases the rates of decomposition are influenced by the presence of additives. Therefore, an understanding of how these additives can influence water sorption and stability will allow for formulation strategies to minimize the decomposition of proteins in the solid state.

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