Hydration of Gluten: A Dielectric, Calorimetric, and Fourier Transform Infrared Study

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The nature of the hydration of proteins and the subsequent implications for functionality is a matter of importance in both pharmaceutical and food applications. Most published studies rely on the use of one technique and attempt to characterize the system. Few studies have used combinations of techniques. In this paper we report on the use of infrared, dielectric, and calorimetric methods to examine the hydration process of wheat gluten. This has been the subject of considerable study by other techniques and has been well characterized by our group. Results show that in both the infrared and dielectric measurements there is a change in behavior at about 35% water content. This is also the water content below which lowering the temperature of the sample does not result in ice formation. We suggest that at this water content the protein amide groups are fully hydrated, and beyond this point addition of water results in protein dilution rather than further hydration.

Introduction

Many proteins that are exploited for their material properties do not exhibit their functionality under conditions of high water content. Instead water content is controlled to optimize particular material properties. In the case of therapeutic proteins and peptides, it is often desirable to reduce water content to very low levels to preserve activity during storage. Thus the behavior of proteins under low water content is an important area of study and requires the development of suitable techniques to do so. While infrared and low-frequency dielectric methods have been used separately to examine protein behavior and have been shown to yield useful information, the techniques have not been used together. The nature of the data obtained by each technique is, however, complementary. The infrared data contain information that is essentially structural in nature. The changes in vibrational frequency represent changes in the electron distribution and density in the bonds involved in vibration. The time scale of the measurement is such that all states with lifetimes greater than about 10^{-13} s contribute to the spectrum and are manifested in terms of the distribution of absorption frequencies observed. In contrast, the dielectric measurements are sensitive to processes that take place between 7 and 15 orders of magnitude more slowly than the vibrational processes. Changes in dielectric behavior will therefore reflect dynamical processes that are the result of changes in structure as observed by infrared methods. The combined methods therefore offer a route to understanding the structure/function relationships arising from hydration. Thus it is hoped that the combination of data will yield a more complete picture of hydration process than when the methods are used separately.

The system chosen for the first investigation is wheat gluten. Gluten is the water-insoluble proteinaceous component of wheat flour. When hydrated, it forms a viscoelastic mass that is mainly responsible for the mechanical properties of dough and pastry. The behavior of dough and pastry is very dependent on water content, and precise control of water content is vital for maintaining the required properties of the material;[1] hence the interaction of water and gluten is in itself of considerable technical interest. Gluten hydration has also been studied previously by NMR[2] and Fourier transform infrared (FTIR)[3] spectroscopy, so comparative results are available. In the previous FTIR paper,[3] interpretation centered on the changes in the amide I band, but earlier work[4] suggests that the amide II band may be particularly sensitive to hydration, and it is on this that we concentrate in this paper.

There do not appear to have been any dielectric studies of gluten. However, there have been a number of studies on the dielectric response of proteins at low water content, which have shown the considerable sensitivity of the method to the effects of water content. Early work by Rosen[5] on three proteins in the frequency range 10^4-10^8 Hz indicated that generally the magnitude of the real and imaginary parts of the permittivity increase with water content, with a break in the curve at about 33% water content. Later work has been reviewed by Pethig.[6] However, no work has covered the range of both frequency and hydration considered here.

Materials and Methods

Gluten Preparation. Gluten was extracted from flour from the single cultivar Hereward: 180 g of flour and 108 g of deionized water were mixed in a beaker for 15 min until the mixture became a homogenized dough. The dough was washed thoroughly with deionized water until the wash became clear from starch. The resultant gluten was housed in a 500 mL round-bottom flask and freeze-dried for 2 days at 253 K, following which the dry gluten was milled to fine powder with a pestle and mortar. The fine powder of gluten was stored in a refrigerator before use.

Hydration of Samples. Eleven different saturated aqueous solutions of salts were used to cover as wide a range of relative humidity (RH) or water activity (a_w) as possible at 298 K. Values of the relative humidities were taken from Meites.[7] Phosphorus pentoxide and deionized water were used in addition to the saturated solutions to give the minimum and maximum water activity at 298 K, respectively.[8] A 50 mL portion of each solution was kept in a wide short-neck 100 mL beaker, which was placed in a sealed glass jar. Two duplicate samples of about 0.5 g of gluten were accurately weighed in small plastic dishes and placed over a plastic net inside the jars. The jars were housed in

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a controlled temperature incubator at 298 K. Mold growth in the high water activity jars (relative humidity >80%) was prevented by use of a small open dish with about 1 g of thymol. The samples were weighed by microbalance every day until constant weight was obtained. Equilibrium was assumed after two identical readings, with the process taking about 6−7 weeks. The initial water content of the samples was calculated from the weight loss of the sample exposed to zero humidity. The water content of the samples is expressed as a percentage calculated as $(W_w \times 100)/W_g$, where W_w is the weight of water and W_g is the weight of dry gluten. Three replicate experiments were carried out.

FTIR Measurements. Spectra were obtained on a Bio-Rad FTS165 FTIR spectrometer fitted with a single-reflection diamond attenuated total reflection (ATR) accessory (SPECAC). A mercury−cadmium−telluride (MCT) detector was used. The background spectrum of the ATR cell was recorded at 200 scans and 2 cm^{-1} resolution. The samples were placed on the ATR crystal, sufficient pressure was applied to the sample to enable good contact with the ATR crystal, and the spectra were recorded under the same conditions as the background. Two subsamples were taken and scanned separately. Experiments were carried out at room temperature on each of the replicates from the hydration procedure.

The infrared spectra were analyzed by use of the Omnic software package (version 6.1a, Thermo Nicolet Corp.). The spectra of two replicate measurements for each sample were averaged and a reference water vapor spectrum was subtracted if required; this was then followed by subtraction of the water signal from the protein region as described previously.[3] The resultant spectrum was Fourier self-deconvoluted, by use of the parameters Bandwidth = 30 and Enhancement = 1.3, and automatically baseline-corrected. The relative heights of the peaks at 1630 and 1645 cm^{-1} in the amide I region and at 1548, 1533, and 1515 cm^{-1} in the amide II region were measured.

Dielectric Spectroscopy. Low-frequency dielectric measurements were carried out on a BDC-N broadband dielectric converter (Novocontrol GmbH, Germany) linked to a frequency response analyzer (Solarton-Schlumburger, Germany). A parallel plate stainless steel electrode system with electrodes of area 254.5 mm^2 and separation distance 1.5 mm was used for all runs, with a frequency range of 10^6−10^{-2} Hz and an applied voltage of 0.5 Vrms. Isothermal dielectric spectra were obtained at a range of specific temperatures from 298 to 243 K as the temperature was decreased. The sample temperature was reduced at a rate of 2 K/min between measurement temperatures, by use of a Quatro temperature control system (Novocontrol GmbH, Germany).

Differential Scanning Calorimetry. DSC measurements were taken on a DSC Q1000 (TA Instruments) with nitrogen as the carrier gas. Full calibration was performed before the acquisition of experimental data. Samples of weight 6−17 mg were placed in matched hermetically sealed aluminum pans (TA Instruments). The sample and reference pans were loaded at ambient temperature, held at 298 K for 10 min, cooled to 238 K at 10 K/min, and then held at this temperature for 10 min. The sample temperature was then increased at 10 K/min to 358 K.

Results and Discussion

Absorption Isotherms. A plot of the absorption isotherm at 298 K is shown in Figure 1. The data were fitted to the Guggenheim−Andersen−de Boer (GAB) isotherm[9] in the form

$$\frac{w}{w_m} = \frac{Cka_w}{(1 - ka_w)(1 - ka_w + Cka_w)} \quad (1)$$

where w is the water content as a percentage calculated as described above, w_m is the water content corresponding to monolayer formation, C is the Guggenheim constant, k is a constant compensating for multilayer formation, and a_w is the water activity expressed as relative humidity. The values of w_m, C, and k were found to be 0.050, 20.0, and 0.0092, respectively. The fit parameters are of the same order of magnitude as those reported previously for gluten,[9] and although the actual values are somewhat lower for w_m and higher for C and k, the observed and predicted values of amounts of water absorbed are close to the previously published values.

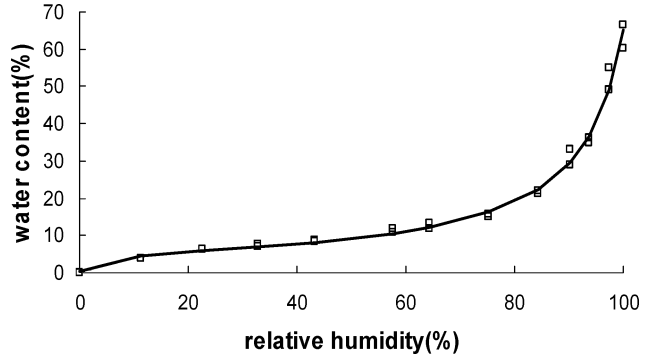

Figure 1. Absorption isotherm of Hereward gluten. Data combined from replicate measurements are shown. The continuous line is a fit of the GAB isotherm to the combined data.

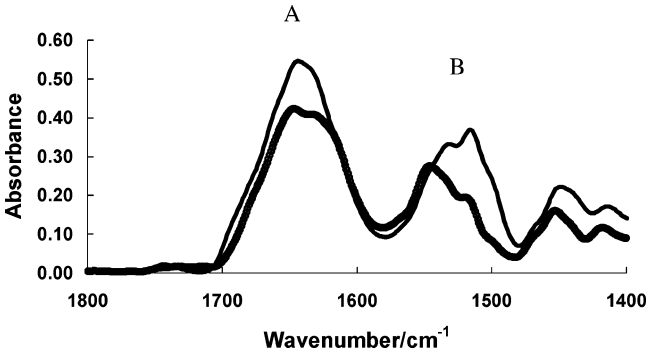

Figure 2. FTIR spectra of dry (light line) and hydrated (60.3%) (heavy line) gluten showing (A) the amide I and (B) the amide II bands.

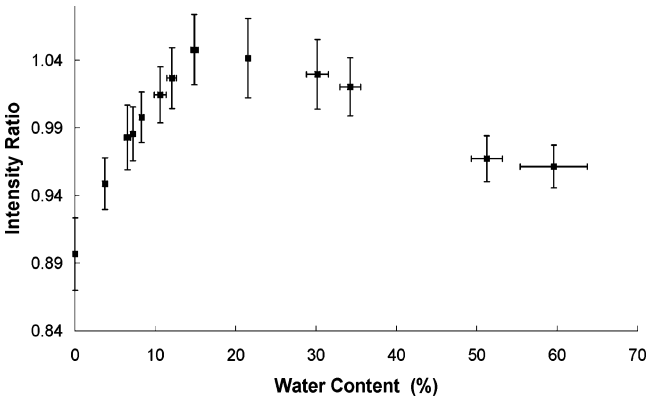

Figure 3. Ratios of the intensities at 1630 cm^{-1}/1645 cm^{-1} plotted against water content. Error bars indicate the deviations of the replicates.

Infrared Spectra. The infrared spectra obtained from the gluten samples with 0% and 63% water content are shown in Figure 2. On hydration, the single main amide I band maximum at about 1645 cm^{-1} splits into two main bands centered at 1650 and 1630 cm^{-1}. The effect of hydration status on this splitting is mapped in Figure 3 by plotting the ratio of intensities at 1630 cm^{-1}/1645 cm^{-1} as a function of water content.

The data given are for three samples subjected separately to the hydration regime. Although there are some differences in the absolute values, the trends are very similar for all samples. There is a maximum in the 1630 cm^{-1}/1645 cm^{-1} intensity ratio

at about 15% water content, indicating that there is a maximum in the β-sheet component at this level of hydration, and a slow decline thereafter, indicating further change in the protein behavior as the water levels increase.

The overall trends in the FTIR data shown here for gluten are similar to those previously reported for the model protein ω-gliadin.[4] However, in that case, the spectral changes were interpreted in terms of an equilibrium between β sheets and β turns. In the case of whole gluten, the situation is somewhat more complicated. Gluten proteins may conveniently be categorized into two classes of prolamin proteins: sulfur-rich and sulfur-poor. ω-Gliadin and the high molecular weight subunits are examples of sulfur-poor prolamins. There is more sulfur-rich prolamin than sulfur-poor prolamin in gluten. The sulfur-rich species tend to have a higher α-helical content than the sulfur-poor species, although some also contain repeat regions similar to those in ω-gliadin and the high molecular weight subunits. The repeat regions have the potential to transform from disordered structures when dry to β-sheets at low water contents, further transforming to β-turns at high water contents[4] (for a more detailed discussion of the gluten proteins, see Mills et al.[10]).

Thus the changes observed are likely to arise from a number of different contributions that, because of the protein heterogeneity in the system, will occur at different rates. Overall, the process starts with the dry protein being conformationally disordered and giving rise to the main band at 1645 cm^{-1} (assignments given here are based on the detailed assignments of Wellner et al.[4] and Georget and Belton[3]). Addition of water allows conformational relaxation and the formation of α-helices (1650 cm^{-1}) and β-sheets (1630 cm^{-1}), with further hydration favoring the formation of extended hydrated chains and β-turns from the β-sheets.[3,4]

The changes in the amide II band are more dramatic than in the amide I region. There is a complete change in band shape with a reversal of relative intensities between the low- and high-frequency ends of the band. This behavior is consistent with that observed in ω-gliadin.[4] The amide II band arises mainly from a combination of C−N and N−H vibrations and is much more sensitive to the environment of the N−H group than the amide I band; there is also a contribution from tyrosine side chain vibrations at 1515 cm^{-1}. Exchange of H for D on the amide shifts the amide II band much more than the amide I band. In contrast, the amide II band is much less conformationally sensitive than the amide I band. Thus, we use the changes in the amide II band simply as an index of changes to the environment of the N−H group and do not attempt to deduce conformational information. The 1515 cm^{-1}band, which should be relatively insensitive to conformational and other changes, is used as reference frequency.

A plot of the ratios of the intensities at 1548 cm^{-1}/1515 cm^{-1} and at 1533 cm^{-1}/1515 cm^{-1} is shown in Figure 4.

Two distinct regions are apparent: the first region, up to about 30% water content, shows a highly linear variation of intensity ratio with water content. After this point, the rate of change declines sharply and a relatively flat curve is observed. These results suggest that between 30% and 40% water content there is a significant change in the environment of the N−H groups. This is compatible with the results of Belton et al.,[11] who noted that a large change in the shape of the amide II band occurred at about 30−37% water in the analogous high molecular weight subunits of gluten. The results obtained here are also similar to a calibration curve reported[12] for the water content of gluten from the ratios of intensities at 1512 cm^{-1}/1547 cm^{-1}.

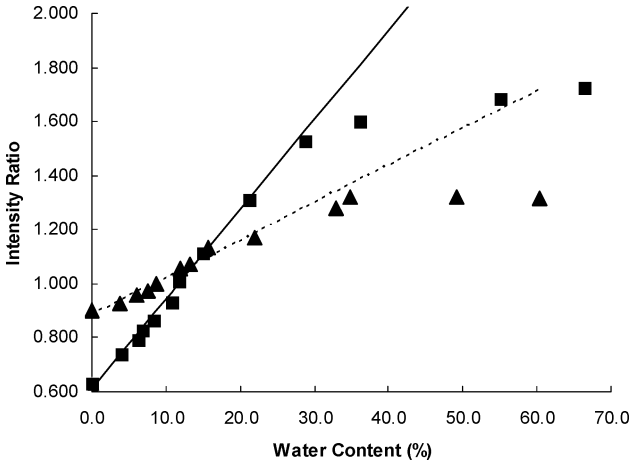

Figure 4. Changes in ratios of band intensities with water content: (■) ratio of intensities at 1548 cm^{-1}/1515 cm^{-1}; (▲) ratio of intensities 1533 cm^{-1}/1515 cm^{-1}. Straight line fits to the two sets of low water content data are shown.

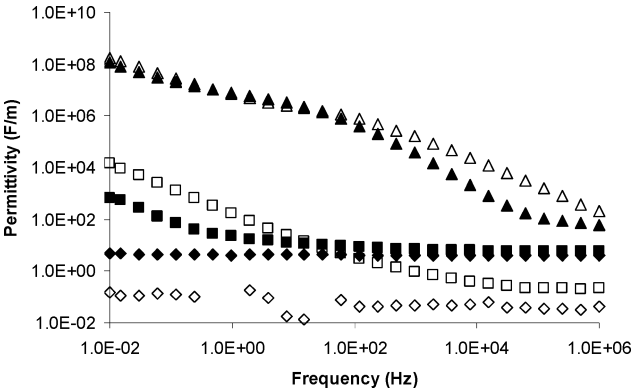

Figure 5. Low-frequency dielectric response of hydrated gluten at 288 K. Diamonds, 0% water; squares, 22% water; triangles, 60.3% water; solid symbols, real permittivity; open symbols, imaginary permittivity.

The changes reported here are not due to a conformational change. The changes in the amide I band show a maximum at about 15% water and continue to change slowly thereafter. A possible explanation is that at around 30−40% water content the N−H groups become fully hydrated and their hydrogen-bonding environment changes only slowly from that point. These changes are due to a decrease in protein−protein interactions with increased water content and other small conformational changes not related to amide group hydration. Thus, after 30% water content the rate of change of the N−H environment is much slower.

Dielectric Measurements. The dielectric response of the hydrated gluten samples fell into three distinct categories, as represented in Figure 5 for gluten samples with 0%, 22%, and 60.3% water content at a representative temperature of 288 K.

The spectral shape may be used as an indicator of the process of charge transport through the material. At the lowest water contents, the dielectric response is essentially flat, indicating virtually no charge movement in the sample and suggesting that there are no charge displacements in this frequency range at these low hydration levels. At intermediate levels of hydration, shown here for the 22% sample, the shape is typical of a system in which charge moves through the sample via a process of charge-hopping between discrete sites.[13] At the highest levels of hydration, the spectral shape is that of a Maxwell−Wagner dispersion,[14] with a bulk and an electrode barrier region. In this case, there is continuous movement of charge through the bulk

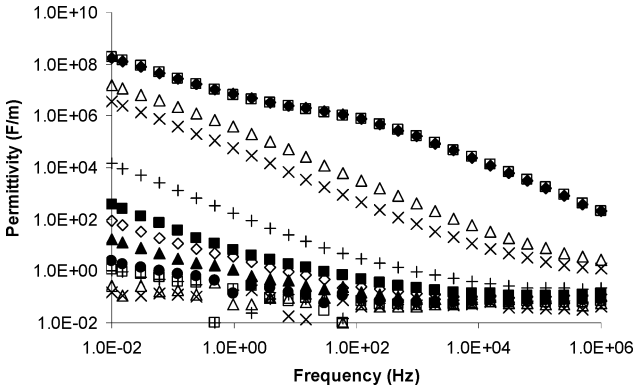

Figure 6. Variation of imaginary permittivity with water content at 288 K. (◆) 60.3%; (□) 49.2%; (△) 34.9%; (×) 33.0%; (+) 22.0%; (■) 15.6%; (◇) 13.2%; (▲) 11.9%; (●) 8.6%; (□, lower range) 7.5%; (+, lower range) 6.1%; (△, lower range) 3.8%; (×, lower range): 0%.

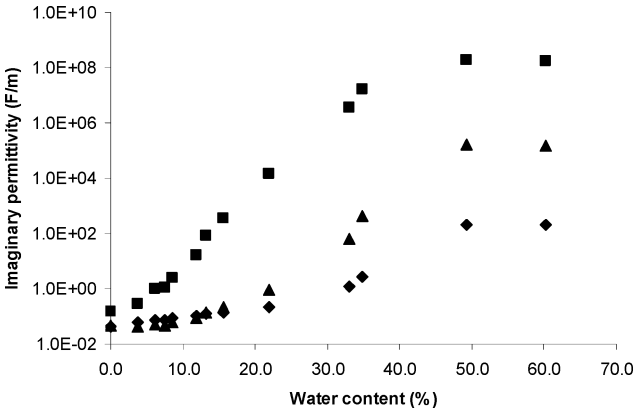

Figure 7. Variation of the imaginary permittivity at 288 K with water content: (■) 10^{-2} Hz; (▲) 10^{3} Hz; (◆) 10^{6} Hz.

region, in a manner analogous to direct current charge conduction, confirmed by inspection of the log−log gradient of the imaginary permittivity in the higher frequency region, which is close to the expected value of −1.

As the level of hydration increases, there is an increase in the extent of charge movement and a change in the mechanism of charge transport. Limited change occurs between the samples with 0% and 8.6% water content. A change in the charge transport mechanism to charge-hopping occurs with the 11.9% sample, the same mechanism being seen in samples up to and including the 34.9% sample. The 42.9% sample is the first to exhibit Maxwell−Wagner behavior, with the final sample, the 60.3% sample, showing a response superimposeable with that of the 49.2% sample. The variation in the imaginary permittivity with water content is shown in Figure 6.

The species that gives rise to the Maxwell−Wagner dispersion is most likely bulk water, by similarity to the extensive published data on aqueous solutions and suspensions, for example, ref 15. It is therefore suggested that the surface hydration of the gluten protein has already reached a maximum by a water content of 49.2%, with the true maximum occurring at some point between 34.9% and 49.2% water content, and hence "excess" water being free to exhibit essentially direct current movement between the electrodes. Thus there is little change between the two most hydrated samples.

Dielectric data on proteins such as hydrated albumin and ovalbumin[16] and polymers such as cellulose[17] have been ascribed to proton-hopping conduction mechanisms. It is entirely feasible that a proton-hopping mechanism is responsible for the dielectric response of the hydrated gluten samples. If it is accepted that this occurs, the data shown here imply that it occurs only above a water content of about 11.9%, as below this level the response is essentially flat. An increase in the hydration level therefore allows greater proton movement and hence greater dielectric response, up to the point whereby a continuous channel of water is produced and a Maxwell−Wagner response due to the excess water is predominant.

In order to compare the effects of water content more easily, values of the imaginary permittivity at 288 K for three individual frequencies are plotted against water content in Figure 7. A sigmoidal relationship is apparent between the imaginary permittivity at low frequencies and the water content, with a plateau at approximately 35% water content. This is similar to the behavior observed in the infrared response of the amide II band.

It is interesting to compare the changes in the dielectric behavior with changes reported by Cherian and Chinachoti[2] in the NMR behavior of a similar gluten system. They observed that when the system was hydrated with D_2O, all the detected intensity from the NMR signal was in the motional narrowing regime. However, below 35% water content (all data given are recalculated from the original data to correspond to the definitions given here) not all the expected signal was observed, implying a possibility of unobserved lines. The increase in observable signal corresponded to a change in the stiffness of the material measured by dynamic mechanical analysis and was related to the glass transition. However, since in the NMR measurements there was an acquisition delay of 100 μs and the pulse length was 16.5 μs, there would have been signal loss due to relaxation before acquisition. Given that the line width ranged from 6 kHz at 2% water to 200 Hz at 35%, the expected loss due to relaxation would range from about 90% at 2% water to about 11% at 35% water. The loss therefore seems to be mainly due to instrumental effects rather than intrinsic phenomena. However, the data do show that the line changes much more slowly below about 30% water.

In a study of high molecular weight subunits of gluten,[11] it was noted that on equilibration with D_2O the proton free induction decay showed a sharp change in shape at about 30% water content, corresponding to changes in the amide II region of the infrared. Similar effects on the free induction decay have been observed in gelatin at about 30% water content.[18]

Despite, therefore, reservations about interpretation, there does seem to be a body of evidence suggesting that there is a transition point that occurs in the hydration of proteins and in particular in proteins associated with gluten. While Cherian and Chinachoti[2] associate the change with a glass transition, Belton et al.[11] suggest that there is no association and that the water content at which the glass transition occurs is lower than that at which the spectral transition occurs. Data from Kalichevsky et al.[19] suggest that for gluten the glass transition point at 35% water is 243 K, so for the data presented here in Figures 4 and 7, all the results for water contents above 35% are above the glass transition point except possibly for the data at 243 K. The effect of temperature on the relationship between water content and permittivity is illustrated in Figure 8. Clearly, the same sigmoidal relationship between imaginary permittivity and water content is maintained over a wide range of temperatures and the flattening of the curve cannot be attributed to a glass transition effect.

As the samples are cooled, ice may be formed. Calorimetric measurements indicate that in the 60.3% samples the amount of nonfreezing water is 0.45 g of water/g of protein and at 49.2% it is 0.36 g/g, calculated from the melting endotherm at ca. 273 K. Below this water content, the DSC trace was essentially flat

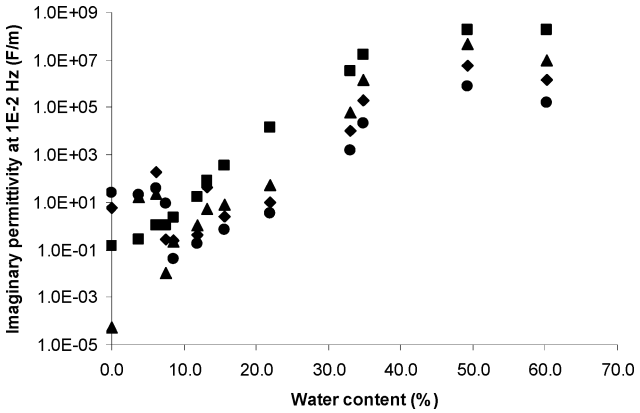

Figure 8. Variation of the imaginary permittivity at 10^{-2} Hz with water content: (■) 288 K; (▲) 263 K; (◆) 253 K; (●) 243 K.

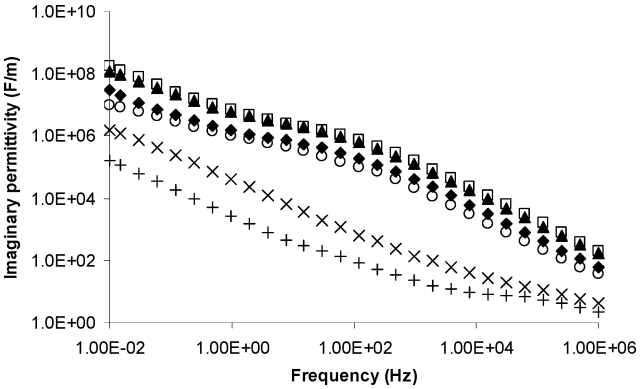

Figure 9. Variation of the imaginary permittivity of the 60.3% water content sample with temperature: (□) 288 K; (▲) 280.5 K; (◆) 273 K; (○) 263 K; (×) 253 K; (+) 243 K.

in this temperature region, with no melting endotherm being observed, implying that ice was not formed in these samples. These results are similar to those of Roman-Gutierrez et al.,[20] who observed that the water content below which no water froze in gluten/water mixtures was 38−47%

The variation of the imaginary permittivity with water content across the temperature range may therefore originate from several different causes. Below the freezing point (about 263 K) in the high water content samples, ice is present and this may contribute to the dielectric behavior. However, Barker[21] suggested that in frozen human serum albumin and polyvinylpyrrolidine solutions, nonfreezing water was a major contributor to the permittivity. However, the system under study in that case was dilute in protein, whereas the system here is very concentrated in protein and it seems likely that the protein itself contributes very significantly to the permittivity. Figure 9 shows the variation of the permittivity of the 60.3% water content sample with temperature.

The strength of the response below 263 K is related to the size of the dipole clusters in the proton-hopping mechanism[13] or, in a more straightforward conduction model,[17] to the number of conducting particles. It is true that when the high water content samples are cooled below the freezing point, their content of nonfrozen water is slightly higher than those that do not form ice, but if it were simply a water content effect, then the 49.2% sample, which has 0.36 g/g nonfreezing water, would be expected to show a weaker response than the 60.3% sample, which has 0.45 g/g. This is not the case. Since the nonfreezing water contents are at or above the level at which all the NH groups are hydrated it may be that these groups play a critical role in proton-hopping and that when they are fully hydrated the size of the clusters or the number of carriers has reached its maximum value.

Conclusions

The data presented here show that at about 35% water content there is a significant change in the behavior of hydrated gluten: below this water content there is no ice formation on cooling, and there is a change in both the dielectric and infrared spectra.

In order for ice to form, two criteria must be met. The activity and hence vapor pressure of the water must be higher than that of ice at the same temperature, and there must be regions of water sufficiently large for ice nucleation and growth to occur. In the gluten sample studied here, the failure to form ice when the water content is below 35% shows that these criteria are met only when the water content is above this level. The actual amount of ice formed will depend on the details of the sample. Both geometric and thermodynamic factors may be involved and will depend on sample history as the system is not in thermodynamic equilibrium. Hence, the actual amount of ice formed is likely to vary with the starting conditions of the sample. In addition, the melting endotherms observed on the DSC traces here and in previous studies[20] are broad, with consequent inaccuracies in the estimation of the enthalpy of the melting of ice and hence the relative contents of ice and nonfrozen water.

Thus, the nonfreezing water content is not necessarily an indication of any water that is in a special condition. It is merely an indication, in the particular sample of interest, of the hydration level at which the water activity equals that of the ice.[22] However, the inability to form ice at all must mean that at least one of the above criteria is not met. It is therefore a more significant measure than the amount of nonfreezing water, even though in a system not at equilibrium it is likely to be somewhat dependent on the system history.

The 35% water level corresponds to the concentration above which the dielectric response is consistent with freely rotating dipoles above the freezing point. This implies the existence of water whose motional constraints are not limited. As this water content is the level at which the amide II band shows a change in trend, it is reasonable to assume that it corresponds to the point at which all the amide groups are fully hydrated and water of essentially bulk character exists.

A water content of 35% corresponds to about 2.5 molecules of water per amino acid residue. It is interesting to compare this with levels of hydration that are routinely used in dough, where water control is vital for obtaining the appropriate rheology.[1] It has been calculated[23] that under typical conditions there are about 5.5 water molecules per amino acid residue in dough. The 35% level is therefore not a critical one in rheological control. This may be understood in terms of the general model of the effect of water on a dry protein. As water is added, the first stage is for its absorption into the protein at structurally important sites. This causes a shift in conformation. At the end of this process, the rate of change of conformation slows and general hydration of hydrophilic groups such as amide groups continues. When these groups are fully hydrated, dilution of the protein begins and will continue until the solubility limit of the protein is reached. At the beginning of the dilution stage there is relatively little change in the amide II band, as no further hydration of these groups occurs and bulk water begins to fill the spaces between the proteins; this results in the change in dielectric behavior. As yet more water is added, protein/protein

interactions are reduced: initially these may be weak interactions or purely mechanical collisions. This results in increased molecular mobility, and hence rheological changes, but with little change in protein structure or water behavior.

In the case of gluten, it should be recognized that not all these processes will occur in all the constituent proteins at the same rate and that individual proteins will tend to have different rates of change. However, the general trends will be true for all of them.

The combination of infrared, dielectric, and calorimetric methods shows a remarkable consistency of results. These three techniques examine different fundamental properties of the materials, that is, submolecular conformation of the protein, and dipolar movement and heat capacity changes within the sample as a whole, respectively. Hence, the consistency of the results from these three techniques is interesting in itself and lends credibility to the interpretations made.

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