Effects of Temperature and Water Content on the Secondary Structure of Wheat Gluten Studied by FTIR Spectroscopy

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The effect of temperature on gluten conditioned at the following water contents, 0%, 13%, and 47% (wet weight basis), was studied by FTIR spectroscopy over the temperature range of 25−85 °C. A detailed discussion of the assignment of the amide I band is given. At 0% hydration no changes in the secondary structure with temperature could be detected; spectra were consistent with a tight disordered structure with many protein−protein interactions. At 13% hydration, distinctive changes occurred in the low-frequency region of the amide I band (1630−1613 cm^{-1}). This was attributed to changes in the β-sheet structure. On cooling to 25 °C, these changes were mainly reversed. It was noted that most of the changes observed occurred above the glass transition temperature. At 47% hydration, more complex changes took place: as the temperature was raised distinct bands at 1630 and 1613 cm^{-1} merged. However, this process was partially reversed, with recovery of both bands, on cooling. The significance of these results in relation to other changes in gluten proteins in flour and dough with temperature and water content is discussed.

Introduction

During bread making, the ability of dough to leaven, hence to confer on bread its final characteristics, originates largely from wheat gluten. The latter is constituted predominantly by the storage proteins called prolamins[1−3] and comprises two major fractions: glutenins, which contribute to elasticity,[4] and gliadins, which play a role in the viscous flow behavior of the dough. For over a decade, spectroscopic techniques such as FTIR (Fourier transform infrared spectroscopy)[5] have helped to elucidate the nature of the gluten structure and dynamics. FTIR has proved to be a very useful tool in determining the secondary structure of gluten proteins. For example, in gluten, the β-sheet content was reported to change as hydration increased.[6] In hydrated gluten containing increasing ratios of glutenins to gliadins, the proportion of intermolecular β sheets to α helices increased, suggesting that interactions between glutenin subunits occurred via intermolecular β sheets.[7] In ω gliadins it was found that the amount of β sheet increased and then decreased with increasing hydration level.[8] At high hydration, β sheets were replaced with β turns. In a hydrated, purified high molecular weight subunit of wheat glutenins, α helices are present in the N and C terminal domains with β turns and intermolecular β sheets in the central repeat domain.[9] It was suggested that the terminal domains promote the formation of β sheets. A "loop and train" model,[10,11] which is consistent with the spectroscopic findings, has been proposed. In this model very low-moisture conditions result in unordered structures in the glutenins which are tightly packed and stabilized by hydrogen bonds. At intermediate moisture contents, competition between glutamine side chains and water for hydrogen bonding create the "loop" segments which have a β-turn structure, whereas the interchain interactions form β-sheet regions called "trains". As water content increases, the equilibrium between β turns and β sheets shifts toward turns. Elasticity is proposed to be the result of the loop/train equilibrium.

During the making of bread, water content varies from 13% to about 50% and temperature ranges from 25 to 200 °C. Currently there has been no consistent study of the role of water and heat on gluten conformation. Previous work gave details of irreversible changes in gluten rheology at temperatures greater than 40 °C. This study also showed that, when using size exclusion HPLC, upon heating at 70 °C, some aggregation was occurring producing large glutenin polymers and unextractable protein residue.[12] It seems likely that this is accompanied by changes in the secondary structure of gluten. There has been one report concerning the changes in the secondary structure of gluten in relation to cereal-based products manufacture.[13] In this study, it appeared that kneading and stretching resulted in a decrease in α helices accompanied by an increase in extended β sheets, but no heating effect was described. Overall the effects of heat on the structure of variously hydrated gluten have not been reported.

In this paper we describe experiments conducted on extracted gluten, which has been conditioned at various moisture levels (0%, 13%, and 47%) and submitted to heating and cooling. These values represent a control, dry value of moisture content, a value close to that in flour, and a value close to that used in dough. Spectra were obtained both during the heating process and after cooling to determine reversible and irreversible changes.

Materials and Methods

Gluten Samples. Fresh gluten samples were obtained from milled wheat flour (cv. Hereward), kindly provided by the Institute of Food Research, Norwich, U.K. Flour (30 g) and deionized water (18 g) were mixed manually for approximately 15 min. The finished dough was washed thoroughly with deionized water until the water ran clear; the sample was then freeze-dried. The yield of dry gluten based on the initial wheat flour was approximately 6.9%. The freeze-dried gluten was ground with mortar and pestle and stored in a sealed jar until further use. Typical protein contents of such material are between 90% and 95% dry weight.

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Water Conditioning and Water Content Determination. The procedure followed closely that employed earlier.[14] Two compounds, P_2O_5 and KCl, were used to give very dry and semidry gluten samples, respectively. Glass jars (0.5 L), tightly sealed with a rubber ring, were used. A crystallizing dish containing a saturated salt solution of KCl or dry P_2O_5 was placed at the bottom of the container. The samples were left over the salt solutions or P_2O_5 with the help of a plastic grid until equilibrium was reached. The jars were maintained at 25 °C. The weight change was monitored until no further variation could be detected. The final moisture content on a wet weight basis (w.w.b.) was determined by drying in an oven at 130 °C for 4 h, after which the weight was constant. The determinations were duplicated.

The material was fully hydrated at 46.7% moisture content (w.w.b.) by manually mixing freeze-dried gluten proteins together with deionized water to reproduce as much as possible the hydration conditions encountered in dough processing. This corresponds to 60−70% added water usually used in bread dough mixes (based on the initial flour content).

FTIR Spectroscopy. Spectra were recorded on a BioRad FTS 165 FTIR (Varian Limited, 6 Mead Road, Oxford Industrial Park, Yarnton, Oxford, OX5 1QU) spectrometer with a mercury/cadmium/telluride detector. The 0% and 13% hydrated gluten samples were taken from the sealed jars, whereas the 47% hydrated material was freshly produced each time. Then they were placed on a single-reflection diamond ATR (attenuated total reflectance) accessory (SPECAC, Orpington, U.K.) and carefully pressed down to ensure a good contact with the ATR crystal. A rubber O-ring was placed around the sample and was compressed by the action of the lever pressing the sample to the ATR crystal. The seal thus created ensured that there was no water loss during the experiments. For each sample, 200 spectra at 2 cm^{-1} resolution were averaged, and three replicates were taken. Each scan lasted approximately less than 3 min. The empty ATR crystal served as a reference. With the help of the heating Golden Gate unit (SPECAC, Orpington, U.K.), the temperature of the sample could be adjusted. For the dry, relatively wet, and fully hydrated samples (0%, 13%, and 47%), a temperature ramp of 30 °C/min was used to heat the sample to the experimental temperature. After the temperature of the experiment was reached, the sample was left on the ATR to stabilize for 15 min prior to scanning. It was then allowed to cool until the ATR heating plate was at 25 °C, at which point another scan was performed. Unfortunately, cooling could not be controlled based on the design of the heating system. For each temperature in the range of 25−85 °C a new sample was used. Spectra for deionized water and water vapor were measured and subtracted from the sample spectra[8] using Omnic v6.1A software (Thermo Nicolet Cooperation, Madison, WI). Each spectrum was baseline corrected following the method of Wellner et al.[15] by ensuring that the spectrum was zeroed at 1800 cm^{-1} where the baseline was relatively flat. Fourier self-deconvolution (FSD) was also carried out with an enhancement factor of 1.3 and bandwidth of 30. Positions of the absorbance peaks located in the amide I region were determined using the second derivative.[16] Intensities at six different wavenumbers (Table 1) were measured and normalized to the glutamine absorbance peak taken at 1600 cm^{-1}. Ratios were calculated. Differences between the values obtained at different temperature treatments (heating or heating/cooling) and those from spectra measured at 25 °C were also determined to establish any relative changes in the secondary structure of the proteins.

Results and Discussion

FTIR Spectra and Assignment. A typical FTIR spectrum for a 0% hydrated gluten sample is presented in Figure 1. For higher moisture systems, the water and water vapor spectra were subtracted. There are several typical absorbance peaks that may be discerned. A peak occurring at 1748 cm^{-1} is characteristic of the C═O stretch from lipids. This is expected, as the gluten was not defatted. Around 1650 cm^{-1}, the amide I band originates from the C═O stretching with some contribution of the N−H vibration. Between 1480 and 1575 cm^{-1}, another broad peak, the amide II, appears and is due to NH bending combined with CN stretching. Our spectra are similar in general appearance to those of a previous study[17] which described the FTIR of extracted gluten.

Figure 2 shows an example of a Fourier self-deconvoluted spectrum with a second-derivative spectrum showing clearly the valleys arising as individual components. In an attempt to

Table 1. Assignment for Secondary Structure Analysis of the Amide I Bands in the FTIR Spectra (Based on Second-Derivative Spectra) at 25 °C

position (cm^{-1})	assignment
1699−1698	turns or β hairpins
1684−1681	β sheets
1670−1665	β turns
1650−1649	random coils and α helices
1632−1629	antiparallel β sheets, more weakly hydrogen-bonded β sheets
1614−1613	strongly hydrogen-bonded β sheets, β edges, extended hydrated chains,[a] some possible contribution from glutamine side chains, intermolecular β sheets
1598−1594	glutamine side chain (NH_2)

[a] These are only likely to occur in hydrated materials.

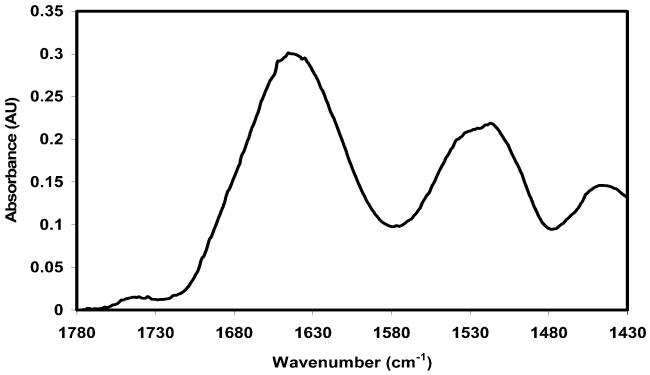

Figure 1. Typical FTIR scan of 0% hydrated gluten (over P_2O_5) extracted from Hereward wheat flour showing the C═O lipid vibration at 1748 cm^{-1}, the amide I vibration around 1650 cm^{-1}, and the amide II vibration around 1530 cm^{-1}.

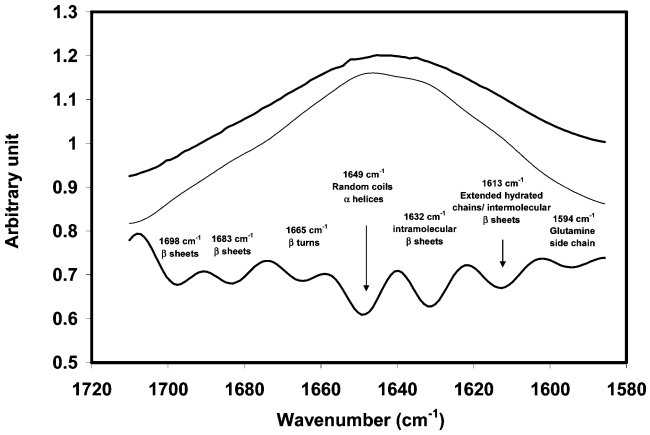

Figure 2. FTIR deconvolution and second-derivative spectra of the amide I region for dry gluten: top line, original spectrum; middle line, deconvoluted spectrum (FSD); bottom line, second derivative showing the valleys associated with secondary structures. The assignments indicated are only partial; a full discussion of the assignment is given in the text.

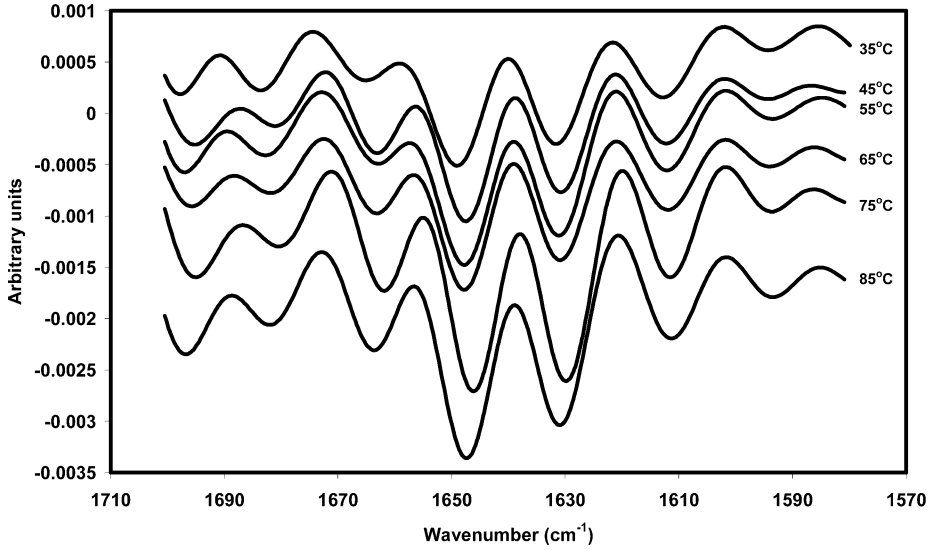

Figure 3. FTIR second-derivative spectra for 0% hydrated gluten, curves placed in descending order with temperature.

extract more information from the spectra a principal components analysis was carried out on the 13% and 47% water content samples during heating and cooling. The PCA clearly discriminated between samples and sample histories, but the loadings for the main components indicated the main wavelengths as being the same as those found by the second-derivative method. Thus, as no new information was forthcoming no further analysis was carried out. Table 1 gives the positions and the assignments of the amide bands based on previous work.[5,8,9,18−21] The amide I region has been chosen for detailed investigation as it is the most informative part of the spectrum with regard to the secondary structure of proteins. Several bands in the amide I region between 1613 and 1632 cm^{-1} could be identified and attributed to β-sheet conformations. The band at 1613 cm^{-1} is at quite low frequency but is consistent with bands observed at 1619[19] and 1616[20] cm^{-1} and assigned to β structures. The full assignment of these bands is not straightforward; low-frequency bands can represent conformers with more strongly hydrogen-bonded peptide groups[22,23] in β sheets or may arise from intermolecular β sheets or extended hydrated structures. They may also originate from "β edges",[21] that is the outer strands of β sheets. Higher frequency bands are thought to derive from antiparallel intramolecular β sheets; however, in prolamins, where there are many repeated sequences, the distinction between inter- and intramolecular β sheets is less clear, and antiparallel sheet formation would be possible between proteins. In this system it is better in general to discriminate on the basis of hydrogen-bond strength and extended hydrated turns rather than attempt to distinguish between inter- and intramolecular interactions.

An exception to this may be interactions arising from denatured α-helical structures, since these regions generally do not involve repeat sequences. The band at 1669 cm^{-1} is assigned to β turns, which form the β-spiral structures in the repetitive domain of the HMW (high molecular weight) glutenins[24,25] but are also found in gliadins.[19] Random coils and α helices absorb around 1650 cm^{-1}, and separate peaks from each of these contributions could not be observed. The band around 1680 cm^{-1} is associated with the β-sheet absorbance at around 1630 cm^{-1}.[9]

The glutamine side group has an absorption band at 1595 cm^{-1}.[9] It is arguable that glutamine has two contributions at 1658 and 1605 cm^{-1},[20] and this intensity may contribute to the intensity of the broad band at 1650 cm^{-1} and to the band around 1610 cm^{-1}. There are some small absorptions from amino acids in this region, but their intensity is low and constant,[26,27] and the principal contributing amino acids all occur at lower levels in gluten proteins than in many other proteins. Thus we, in common with other workers [7,13,16,19,22,24,25] in the field, have ignored them in assessment of changes to intensity

Experiments with replicate samples showed that the spectral region from 1680 to 1655 cm^{-1} was very variable. This region usually has strong contributions from β turns. The β-turn content is highly dependent on the mechanical history of the sample[15] and thus is sensitive to handling. We attribute the variability of samples in this region to differences in handling during extraction and make attempt to examine changes in this region during treatment.

Dry Gluten. The second-derivative spectra are useful in illustrating changes occurring in the secondary structures. Figure 3 shows the second-derivative spectra for the 0% hydrated gluten extract undergoing heating from 25 to 85 °C. For clarity, the spectra have been represented on an offset scale. Each scan originates from a different sample heated from room temperature to the temperature stated. In each case we have observed a higher level of irreproducibility in the 1665−1690 cm^{-1} region of the spectrum than in the rest of the spectrum. This probably is due to the effects of mechanical work on the protein conformation during extraction and handling[15] so that the interpretation of changes in this region needs to be circumspect. In the 0% hydrated sample (Figure 3), heating the material does not lead to any significant conformational changes. This is consistent with previous work[28] which reported that heating dry radish seeds up to 145 °C did not cause any protein structural changes that could be observed by FTIR. Analysis of the 0% hydrated gluten showed no structural changes were observed upon recooling the samples to room temperature (data not shown).

13% Hydrated Gluten. *Heating.* When the 13% hydrated material is tested (Figure 4), there is a clear change in the 1610−1630 cm^{-1} region. The two bands at 1629 and 1613 cm^{-1} are assigned to the β sheets (Table 1). It seems unlikely that at this level of hydration extended hydrated chains exist. It is arguable that the 1613 cm^{-1} valley may have contributions from chain side group glutamine (gluten proteins contain 30−50 mol % glutamine[29]).

However, no corresponding valley could be detected at 1658 cm^{-1} (as was found in ω gliadins[8]), and this suggests that at least most of the low-frequency band is correctly attributed to

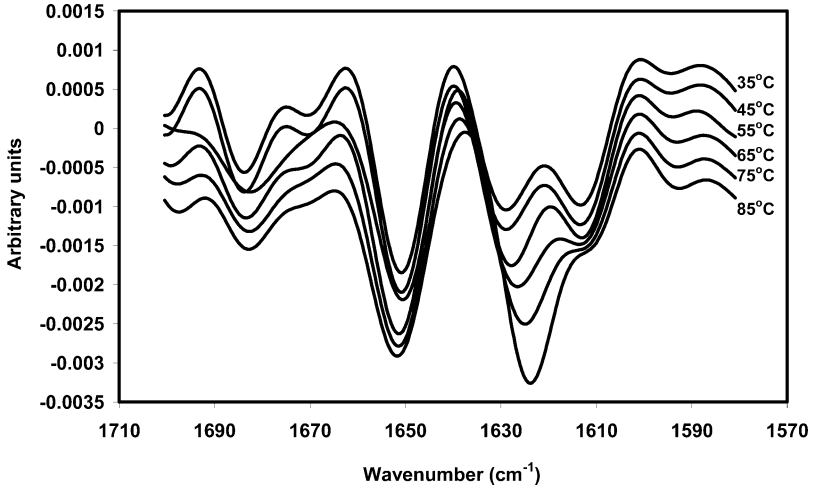

Figure 4. FTIR second-derivative spectra for 13% hydrated gluten, curves placed in descending order with temperature.

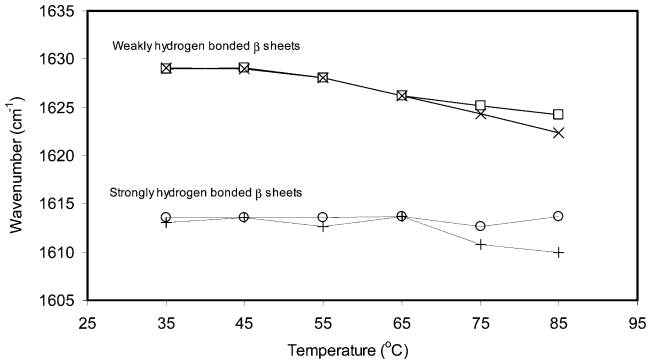

Figure 5. Band shifts in the β-sheet region from gluten at 13% hydration undergoing various temperature treatments: crosses, heated; empty symbol, heated and cooled to 25 °C. The lines are a guide to the eye.

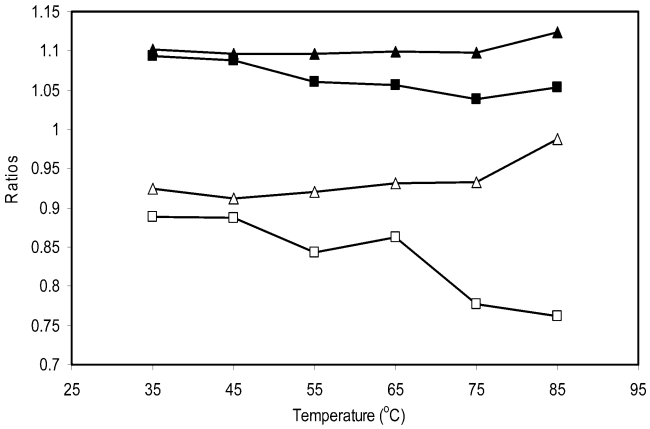

Figure 6. Intensity ratios 1613/1650 cm^{-1} and 1629/1650 cm^{-1} for 13% hydrated gluten proteins: ■ 1629/1650 cm^{-1} heated; ▲ 1629/1650 cm^{-1} heated and cooled; □ 1614/1650 cm^{-1} heated; △ 1614/1650 cm^{-1} heated and cooled.

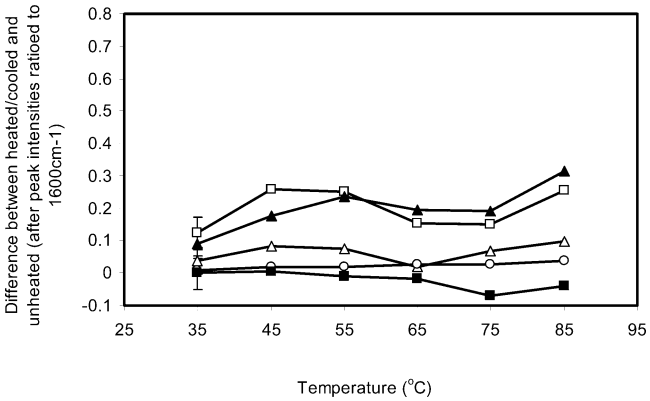

Figure 7. Differences in relative intensities for heated and cooled samples at 13% hydration. Error bars represent the standard deviation values of three replicates. ▲ 1613 cm^{-1}; □ 1629 cm^{-1}; ■ 1650 cm^{-1}; △ 1684 cm^{-1}; ○ 1699 cm^{-1}.

backbone amide vibrations. This is consistent with previous work[30] which reported FTIR results on polypeptides modeled on the repetitive domain of wheat gliadins. The overall pattern of change is for the band at 1630 cm^{-1} to deepen with respect to the band at 1613 cm^{-1} and shift toward lower frequency (Figures 4 and 5) as temperature increases.

The shift toward lower frequency of the 1630 cm^{-1} band cannot be the result of an increase in hydrogen-bond strength as increasing temperature would weaken rather than strengthen hydrogen bonds. It probably arises from the weakening of hydrogen bonds. This causes a transfer of intensity from the low-frequency mode to a higher frequency mode; however, the strength of the bonds is still such that the absorption is still occurring at the low end of the high-frequency band. Overall, this transfer results in the appearance of a broader band which now contains a range of frequencies and is shifted toward lower frequency.

It is noticeable in Figure 6 that the ratios of the β-sheet band intensities (relative to those of the 1650 cm^{-1} absorbance peak) change significantly only after the temperature has reached 45 °C. This suggests that the critical 45−55 °C temperature might relate to the glass transition temperature (T_g). Indeed, for gluten proteins hydrated at 13% water content, a T_g value range of 40−50 °C was previously reported.[31] This value is also consistent with values found in similar gluten fractions.[32]

Heating and Cooling. It is noticeable that for the 1613 cm^{-1} absorbance band (Figure 5), up to about 65 °C the band position remains unchanged but above this temperature there is a recovery with a shift of the band back toward its original value when cooled. For the 1630 cm^{-1} peak, the shift observed after heating at 55 °C is irreversible upon cooling.

While the shifts are small the divergence in intensity ratios (Figure 6) between the heated and heated and cooled material is large. However, this arises mainly because the overall change in the ratio from its initial value at 25 °C after the cooling process is small. That is, although heating causes large changes in the spectra these are mainly reversed on cooling. The changes that do occur can be better illustrated by plotting the differences

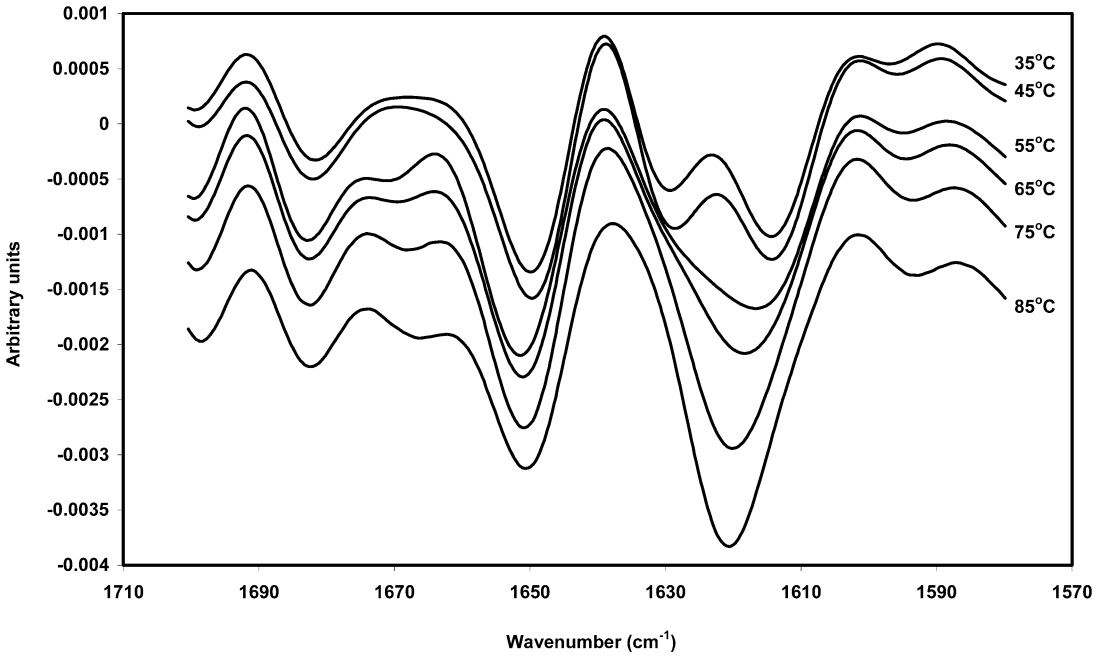

Figure 8. FTIR second-derivative spectra for gluten at 47% water, curves placed in descending order with temperature.

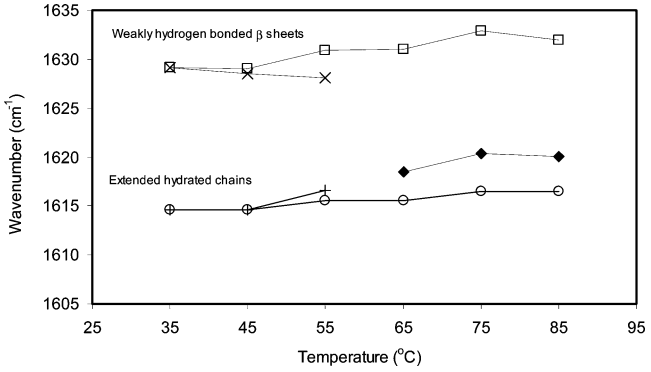

Figure 9. Band shifts in the β-sheet region from gluten at 47% hydration undergoing various temperature treatments: crosses, heated; empty symbol, heated and cooled to 25 °C. The lines are a guide to the eye. The solid diamonds represent the new merged peak that appears upon heating at high temperatures.

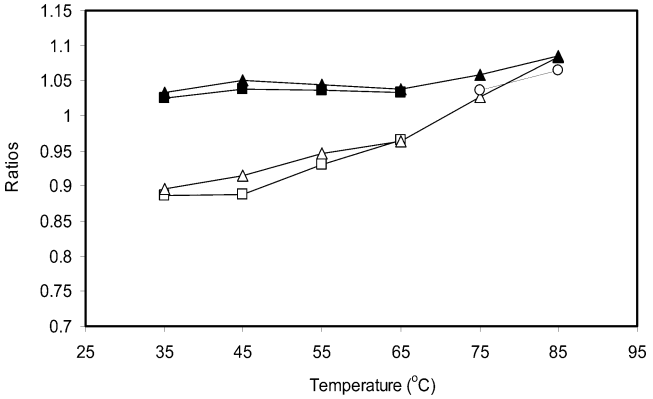

Figure 10. Ratios 1614/1649 cm^{-1} and 1630/1649 cm^{-1} for 47% hydrated gluten proteins: ■ 1630/1649 cm^{-1} heated; ▲ 1630/1649 cm^{-1} heated and cooled; □ 1614/1649 cm^{-1} heated; △ 1614/1649 cm^{-1} heated and cooled; ○ 1620/1649 cm^{-1} heated (single peak formed at high temperature).

in ratios between the heated and cooled material and unheated material (Figure 7). These plots may be regarded as quantification of the permanent changes in the spectral profile. It is apparent from the data discussed earlier (Figure 6) that heating changes the profile significantly but that the spectrum recovers to something very much like its original shape on recooling. The only significant change is that the intensities in the β-sheet region are slightly altered, but the change is small compared to that observed on heating alone.

47% Hydrated Gluten. *Heating.* When the water content is increased to 47% (Figure 8), the two peaks in the β-sheet region are evident at room temperature, but when the temperature is elevated to 55 °C these begin to merge to form a single peak which is finally centered at 1620 cm^{-1}. At this water level it is reasonable to assume that extended hydrated chains exist and that major conformational changes may occur on heating as the temperature is well above the glass transition temperature.

In Figure 9, the shift of the absorbance peaks for the weakly hydrogen-bonded β sheets and the extended hydrated chains are represented. Both bands shift toward high frequency upon heating. This probably represents a decrease in the strength of hydrogen bonds both in weakly and strongly bonded β sheets.

The relative intensity of the β region also increases at the expense of the intensity in the 1650 cm^{-1} region (Figure 10) indicating that some denaturation is taking place, possibly due to the loss of α-helical structure in gliadins and terminal regions of the high molecular weight subunits. The changes in the β region may be interpreted as the formation of a range of new β structures together with a shift to less strongly hydrogen-bonded structures. The result of this might be a range of absorbance maxima which are no longer resolvable into single peaks. An additional effect of heating is the appearance of a distinct valley at 1670 cm^{-1}. This is noticeable at 55 °C and above, shifting to lower wavenumbers upon heating. This peak may be assigned to β turns. Although this region is poorly reproducible, no peak was observed here in any sample at room temperature (Figure 8). The ready transformation of sheets to turns and back again is a feature of the behavior of glutenins and ω gliadins[5,8] and may be interpreted as the effects of heating causing the formation of turns from sheets.

Heating and Cooling. The patterns observed in the 47% hydration sample are distinct from those of the 13% sample. In the 13% sample the net change after heating is minimal; in the wetter sample, while many peaks returned to their original

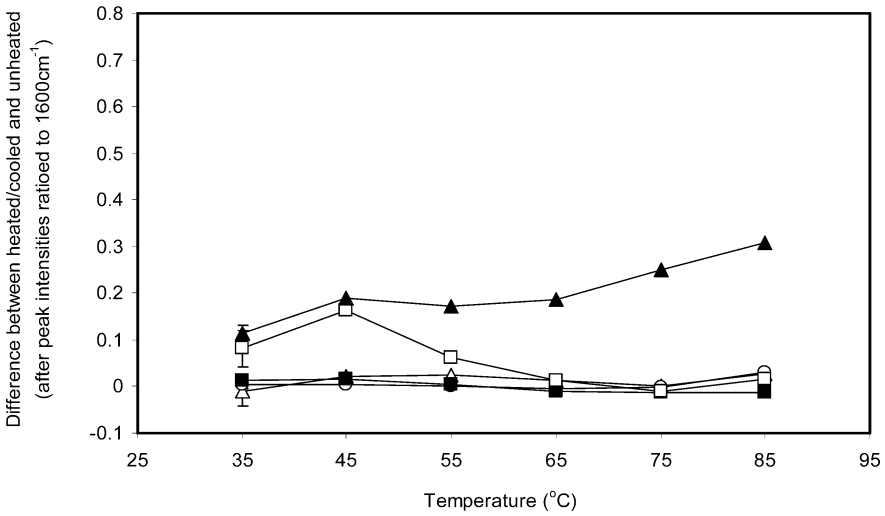

Figure 11. Differences in relative intensities for heated and cooled samples. Error bars represent the standard deviation values of three replicates. ▲ 1613 cm^{-1}; □ 1629 cm^{-1}; ■ 1650 cm^{-1}; △ 1684 cm^{-1}; ○ 1699 cm^{-1}.

intensity, the 1613 cm^{-1} peak did not (Figure 11). It is clear from this that some of the changes in the β structure appearing on heating are permanent. The recovery of the 1630 cm^{-1} peak is perhaps more remarkable—a significant conformational change has taken place in the protein, and it is apparently reversible. In many proteins this would be very unexpected, but previous work has shown that the high molecular weight subunits and ω gliadins are structurally labile.[5,8] Thus, the reversible changes observed are consistent with the large changes in structure seen previously on hydration. They are also in agreement with the loop and train model of gluten elasticity,[10,11] which requires that β-sheet formation occur on mechanical extension, and this is partially or wholly reversed on relaxation of stress. The general conclusion is that any change in the free energy of the system will result in conformational change, but in part at least the process may be reversed on returning to the original free energy conditions. This contrasts with the all or nothing event of thermal denaturation seen in proteins whose functionality depends on a fixed structure, such as enzymes.

When heated above 40 °C an irreversible effect on the rheology (dynamic measurement of the elastic and viscous moduli) of gluten has been reported.[12] As shown in Figure 11 the irreversible formation of β sheet does seem to take place between 35 and 45 °C. Two processes might be occurring: one is the formation of intermolecular β sheets by loss of α structures; the other is the formation or interchange of disulfide linkages[33] by heating. Thus, the data of the present study might explain this phenomenon by the formation of new β sheets which in turn could be locked in due to disulfide-bond formation.

Conclusions

Dry gluten proteins exposed to relatively high temperatures do not show any changes in their secondary structures. When hydrated to 13% water content, a water content typical of flour, changes in β-sheet structure occur on heating, but these changes are reversible. Thus, while the prolamins in flour are temperature sensitive no permanent changes are caused by heating to 85 °C. In the sample hydrated to 47% water content changes were greater on heating and were less reversible than those of the drier material. However, the degree of reversibility of the spectra was high compared to the degree of changes observed. These observations reinforce the notion that the gluten proteins are conformationally much more labile than many other proteins. At this level of hydration irreversible changes were only observed above 45 °C. During cooking however the flour is exposed to higher temperatures than those explored here, and there will be significant and irreversible changes to gluten structure during this process. We note that while these changes have significance for the mechanical properties of gluten there no evidence that gluten changes its bioavailability on cooking. Thus, the changes have no significance for its nutritional properties

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