

Changes in Secondary Protein Structures During Mixing Development of High Absorption (90%) Flour and Water Mixtures

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ABSTRACT

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Wheat flour and water mixtures at 90% absorption (dry flour basis) prepared at various mixing times were examined using Fourier transform infrared (FT-IR) reflectance spectroscopy. Spectra were obtained using a horizontal attenuated total reflection (ATR) trough plate. The apparent amount of protein and starch on the surface of the dough varied with mixing time but this was likely due to the polyphasic nature of the substrate and the changing particle distributions as the batter matrix was

developed. Deconvolution of the Amide I band revealed contributions from alpha helical, β -turn, β -strand, β -sheet, and random conformations. The ratio of β -sheet to nonsheet conformations reached its greatest value about the same time that the mixture was most effectively separated by a laboratory-scale, cold-ethanol-based method but before the peak consistency measured by a microfarinograph.

Wheat flour dough or batter may appear to be uniform and well mixed but actually it is multiphasic: starch, gluten, lipids, and water representing the principal phases. Furthermore, the form of these phases changes during periods of mixing that prepare them for separation or food uses. Microscopic changes begin with the instant formation of protein fibrils at first contact of water and flour particles (Bernardin and Kasarda 1973a,b; Amend and Belitz 1991). Slow mechanical development induces these fibrils to coalesce into fibrous bands or tendons and segregates the starch into clusters. When flooded with a displacing fluid, this open, sponge-like structure readily releases the entrained starch. This unmixed or separable state is evident in dough at 56–65% absorption and in batter at 90% absorption (Tipples and Kilborn 1975; Robertson and Cao 1998; Robertson et al 2000). Additional development disbands the protein into relatively fine, uniformly distributed, and networked or webbed filaments that entrap the starch and gas bubbles formed when the dough is fermented and baked.

The physical properties of hydrated wheat proteins are the result of covalent and noncovalent interactions of wheat gluten proteins. These interactions are altered by the repeated extension, tearing, and compression during mixing or development. Specific chemical effects include 1) disulfide bond disruption, 2) chain disentanglement and rupture, 3) disulfide-sulfhydryl interchange, 4) formation of dityrosine cross-links, 5) formation of new disulphide cross-links, 6) free radical interactions, and especially 6) reorientation leading to enhanced hydrogen bonding (Golstein 1957; Dronzek and Bushuk 1968; Jones and Carnegie 1971; MacRitchie 1975; Schroeder and Hoseney 1978; Graveland et al 1980, 1984; Danno and Hoseney 1982; Singh et al 1990; Tatham et al 1990; Shrewry et al 1994; Weegels et al 1995; Skerritt et al 1999; Vera-verbeke et al 1999; Aussenac et al 2001; Tilley et al 2001).

Insight into these interactions may be derived from the secondary protein structures. Others have described regularly repeated β -turns in the repetitive domains of glutenin subunits, γ -gliadins, and ω -gliadins. The repetitive domains of glutenin subunits have many β -turns formed in regions where tyrosine is one of the four amino acids. Viscoelasticity of bread dough has been attributed to interactions between aligned β -sheets formed in the repetitive domains through a network of hydrogen bonds between hydrophilic side chains. During mixing, disulfide bonds break and the opportunity for all the gluten proteins to interact and change conformation is increased. This is reflected in loss of α -helices and β -turns, and increases in β -sheets (Pezolet et al 1992; Popineau et al 1994; Shewry and Tatham 1990, 1994).

Pertinent information about secondary molecular structures of proteins is accessible through Fourier transform infrared spectroscopy (Purcell et al 1988; Tatham et al 1990; Pezolet et al 1992; Popineau et al 1994; Belton et al 1995; Wellner et al 1996). FT-IR spectroscopy has recently been applied to assess structural factors associated with dough stickiness at 36% absorption (van Velzen et al 2003) but has not been applied to the controlled development of high-absorption (90%) wheat flour compositions of importance to wheat separation, fractionation, or refining. In the present report, attenuated total reflectance Fourier-transform infrared spectroscopy was applied to high-absorption wheat flour mixtures to identify the underlying protein secondary structure and to correlate this with gluten-starch separability and the extent of mixing.

Experimental Procedures

Unbleached flour obtained from a commercial supplier (Giusto, San Francisco, CA) and stored at -30°C was used as the source flour for experimentation. This flour is a blend of dark northern spring wheat and hard red winter wheat from Montana. Proximate analysis (Anresco, San Francisco, CA) for this flour on a dry or moisture-free basis (mfb) was 13.4% protein ($N \times 5.7$) by micro Kjeldahl method (960.52); 5.6% lipids (922.06); 0.6% ash (923.03); 69.1% carbohydrates including fiber by difference and total solids (925.09) (AOAC International 2000). Protein was assayed at 13.5% in this laboratory by nitrogen determination (model FP428, Leco, St. Joseph, MI).

Samples of wheat flour (10 g) and distilled water (9 g) were mixed in a Brabender microfarinograph for 5–45 min as described previously (Robertson and Cao 1998; Robertson et al 2000). Samples were removed from the microfarinograph, transferred to the FT-IR spectrometer, and spectra were collected within 7 ± 1 min.

FT-IR spectra of each sample were obtained using the Perkin Elmer System 2000 FT-IR spectrometer equipped with a DTGS

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a color version of Fig. 6 not included in the print edition.

(deuterated triglycine sulfate) detector and a horizontal ATR accessory (out-of-compartment contact sampler, Spectra-Tech, Shelton, CT). A trough sampling plate (7 × 1 cm) with a ZnSe crystal (45° angle of incidence, RI = 2.4) was used. Spectra were collected over the range of 4000–650 cm⁻¹ with a resolution of 4 cm⁻¹. A background spectrum of the empty trough sampling plate was collected before each sample. Freshly mixed dough was transferred to the trough using a soft plastic spatula and covered with a plastic wrap to prevent moisture loss. The dough was pressed firmly onto the crystal to eliminate air and to achieve better contact. The first spectrum (25 scans) of each sample was obtained immediately and subsequent spectra were collected (25 scans) every 3 min up to 45 or 60 min. After each sample, dough was removed from the plate with a soft spatula under running warm water. The residue left on the crystal was rubbed off with tissues using a back and forth motion under flowing warm water and then under flowing distilled water. Any water left on the sampling plate, excluding the crystal, was wiped off with tissues and then evaporated with warm air. The sample compartment, detector and ATR accessory were not purged with dried nitrogen gas. Experiments were performed in an air-conditioned room but neither temperature nor humidity was constant. Data from duplicate or triplicate dough samples were averaged. A water spectrum was obtained. Spectra were converted from transmittance to absorbance, and the water contribution was subtracted. The difference spectrum was smoothed twice.

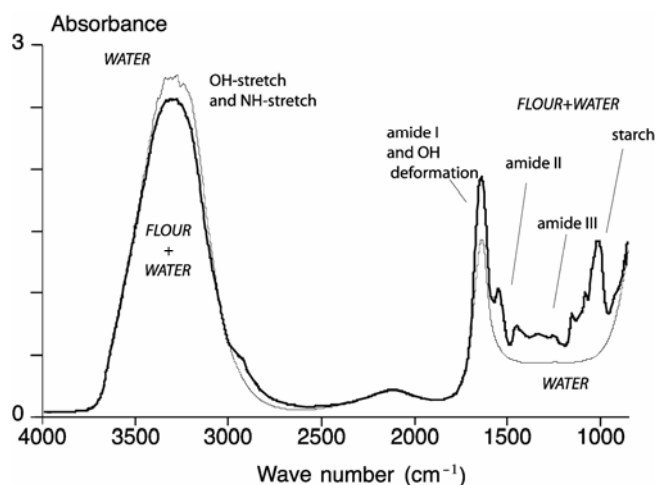


Fig. 1. FTIR/ATR spectrum for water and for 90% absorption flour and water mixture developed in a microfarinograph for 24 min.

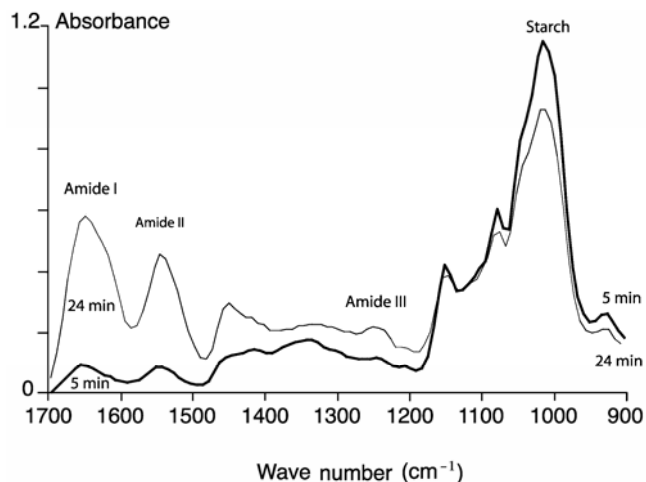


Fig. 2. FTIR/ATR spectrum of 90% absorption batters (5 and 24 min of mixing) after correction by subtraction of water spectrum.

The block average smoothing algorithm was used to smooth all spectra. By this method, spectra were smoothed using nine points in each block. The method averages points in a block around the target point to eliminate sharp noise peaks. The method was applied twice. The peak areas and height intensities of amide I, amide II, amide III, and starch bands of each sample were calculated. For protein, secondary structure determination the smoothed spectrum was then deconvoluted (Spectrum, v. 2.00, Perkin Elmer, Norwalk, CT). The variables selected were Bessel filter, 2.4 gamma, 24 length. The method used for deconvolution was the Fourier self-deconvolution method that operates on the Fourier transform of the spectrum (Kauppinen et al 1981). The deconvoluted spectra were curve-fitted (Grams/32 v. 5.03, Thermo Galactic/Thermo Electron, Salem, NH). The parameters used for the calculation suggested by C.-Y. Ma (macy@hkucc.hku.hk, *personal communication*) were peak-fitting function, baseline function select (none), Gaussian function, sensitivity (low), and maximum iteration (50). We modified this by increasing the iterations to 100. The trace limits selected for the present calculation were 1690–1592 cm⁻¹ with a FWHH set at 2.5. The area of each curve-fitted peak was taken as the integrated intensity of that band.

The data of summary data plots 3, 4, 8–10 were fitted using a locally weighted least squares error method (Chambers et al 1983) employing a 95% smoothing factor as implemented in Synergy software (Kaleidagraph 3.6.4). Data in data plot were fitted with a polynomial regression.

Methods and instrumentation for measurement of consistency and separability and for visualization by microscopy have been described previously (Robertson et al 2000).

RESULTS

FTIR/ATR Spectroscopy

A characteristic FTIR/ATR spectrum of a 90% absorption mixture (batter) mixed in a microfarinograph for 24 min is shown in Fig. 1. IR bands of note are the OH and NH stretches at 3300

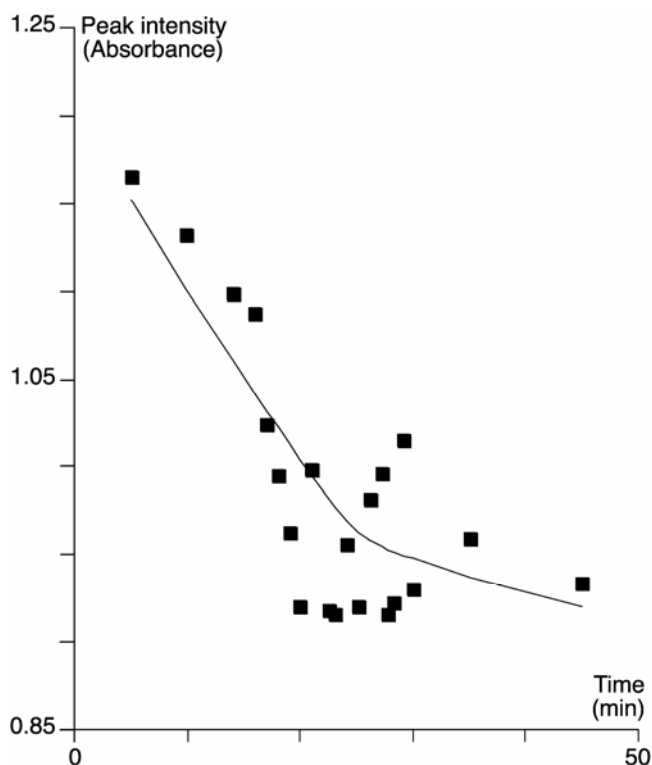


Fig. 3. Mixing-effected changes to intensity of starch-absorption band for 90% absorption flour and water mixtures. Coefficient of variation was 5.5%.

cm⁻¹, OH deformation and amide I (80% C=O stretch, 10% C-N stretch, and 10% N-H bend) at 1650 cm⁻¹, amide II (60% N-H bend, 40% C-N stretch) at 1546 cm⁻¹, amide III (mixture of more than four components involving the amide group of the peptide chain) at 1245 cm⁻¹ (Stuart 1997), and starch (C-O, C-C stretches, and C-OH bending) at 1017 cm⁻¹ (Kacurakova and Mathlouthi 1996). Water makes large spectral contributions to the intensities of the broad band at 3300 cm⁻¹ and the amide I at 1650 cm⁻¹ as shown in the water spectrum in Fig. 1. However, when the water spectrum is subtracted, the flour-related spectral features become

more prominent (Fig. 2). Amide bands arise from interactions between amino acid residues within a polypeptide chain or protein. The amide I band is almost entirely due to C=O stretch vibration and is sensitive to protein conformation and has been used to study protein secondary structure.

Repeated scans of the same sample for up to 1 hr of resting on the sampling plate were collected to assess stability. We observed peak-specific effects of slowly increasing amide intensities and slowly decreasing starch band intensities. The amide band intensity changes were linear with a rate of change of ≈ 0.001 AU min⁻¹ for a sample mixed for 5 min and 0.0009 AU min⁻¹ for a sample mixed for 21 min. Starch band intensity changes were nonlinear for short mixing times (5 min) with a -0.0027 AU min⁻¹ for the first 15 min of rest, but -0.001 AU min⁻¹ for the following 15–45 min resting period. Starch band intensity changes were essentially linear at -0.0002 AU min⁻¹ for the 21-min mixing times. Hence, we expected only very small differences in the spectra due to differences in the time from the end of the mixing to the spectra collection, even though this period varied by as much as 2 min. Three possible explanations for this instability are 1) relaxation and flow of the mixture, 2) instrumental drift, and 3) water evapor-

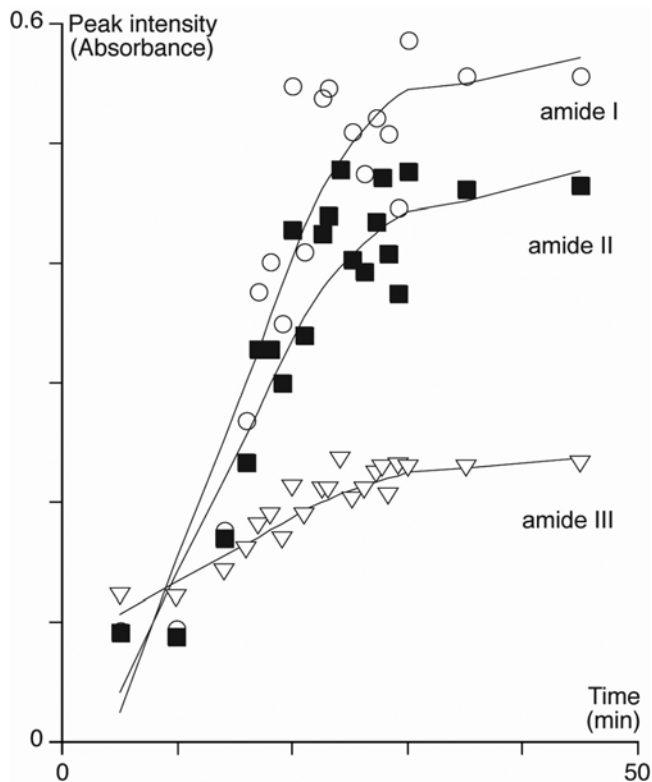


Fig. 4. Mixing-effected changes to intensity of amide-absorption bands for 90% absorption flour and water mixtures. Coefficient of variation was 7.8% Amide I; 5.2% Amide II; and 3.6% Amide III.

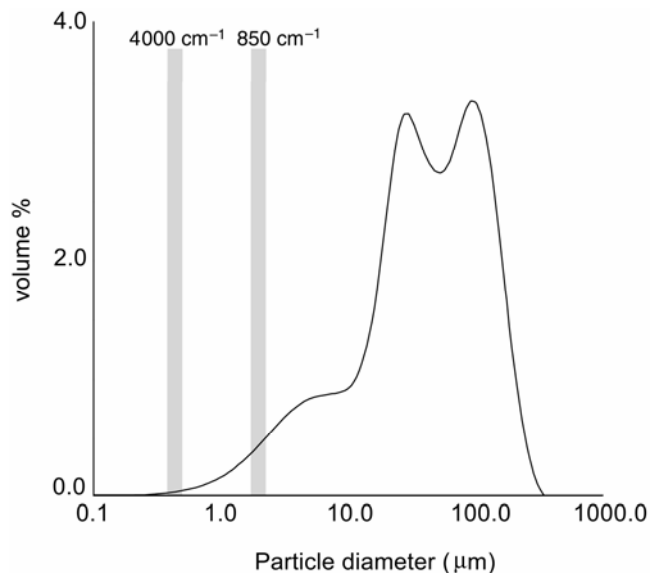


Fig. 5. Particle-size description for flour in water at high dilution. Vertical bands represent computed depth of penetration of IR beam at 4000 and 800 cm using refractive index of water, hydrated protein, and starch.

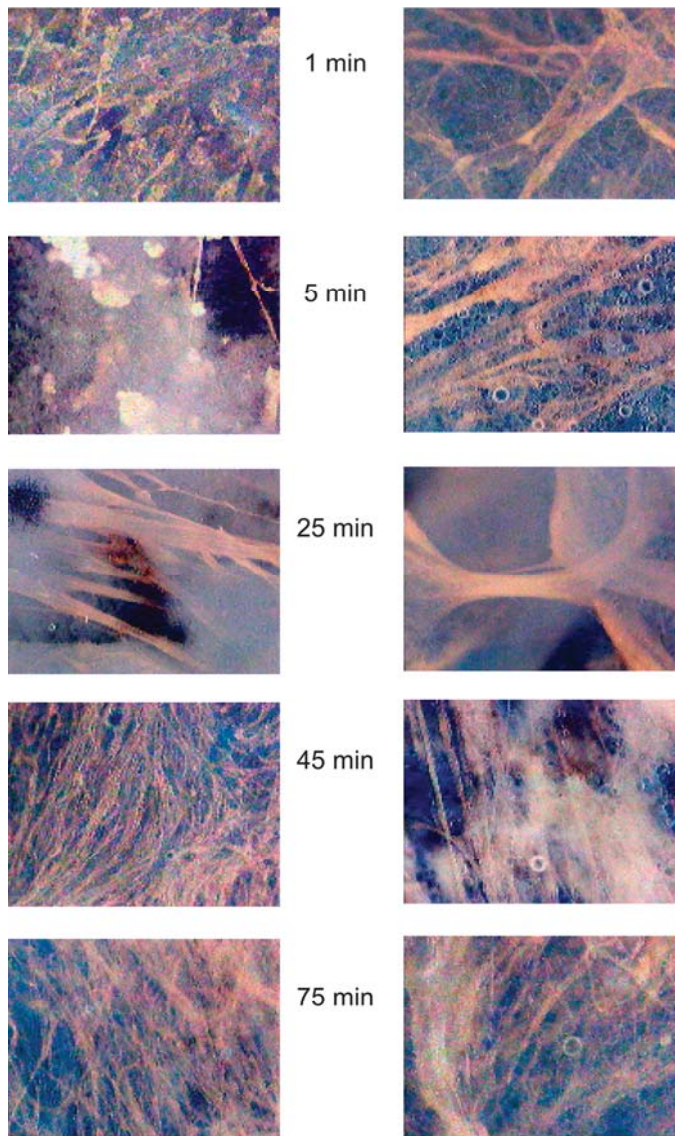


Fig. 6. Images of wheat-flour mixtures at 90% absorption, developed by mixing in a microfaringraph using a blue-staining protein dye and digital inversion (Adobe Photoshop). Field of view: horizontal width of left column images is 1.54 mm and right column is 0.6 mm.

ation. Flour and water mixtures at high absorption are fluids that typically relax and exhibit slow flow after the stresses of mixing are relieved. This flow may lead to displacement of starch from the measuring region. The second possibility, instrumental drift, would have been revealed as uniformly increasing or decreasing peak heights. The third possibility, evaporation from the top dough surface, was excluded because of the extremely low water vapor transmission rate (WVTR) of the plastic covering. We expected a fractional loss of water from the sample to be $\approx 10^{-5}$ g/g based on a WVTR for polyvinylidenechloride (Saran wrap) of $2 \text{ g/m}^2 \cdot 24 \text{ hr}$ (Food Science & Technology, University College Cork) (Available at <http://www.ucc.ie/fcis/PKplastics.htm>).

FTIR/ATR spectra for 90% absorption mixtures that were mixed or developed for up to 45 min had increased amide and lowered starch band intensities in proportion to the time of mixing up to optimum mixing for starch-protein separation (Figs. 3 and 4). Bulk concentration does not change as a result of mixing or while spectra were collected so that these differences in the FTIR/ATR spectra report actual concentration changes within the depth of penetration by the IR beam.

Depth of penetration (D_p) at which the beam is reduced by a factor of e^{-1} may be estimated by

$$D_p = \frac{1/\omega}{2\pi \cdot \eta_1 \sqrt{\sin^2 \theta - \left(\frac{\eta_2}{\eta_1}\right)^2}}$$

where the factors (and instrument or estimated values) are ω , the wave number of the incident light ($4000\text{--}850 \text{ cm}^{-1}$); θ , the incident angle (45°); η_1 , the refractive index of the ATR crystal (2.4); and η_2 , the refractive index of the sample (starch 1.5, hydrated protein between that for water [1.33] and that for dry protein [1.5]) (van Veltzen et al 2003; Vörös 2004). The calculated depth of penetration of the IR beam was $0.38\text{--}0.5 \mu\text{m}$ at 4000 cm^{-1} and up to $1.8\text{--}2.3 \mu\text{m}$ at 850 cm^{-1} . Hence, FTIR/ATR will best represent particles that are wholly within this depth window (van Veltzen et al 2003). Initially the particle dimensions in this mixture are up to $25 \mu\text{m}$ for starch and up to $50 \mu\text{m}$ for protein, as shown in dilute aqueous suspensions obtained by laser light scattering (Fig. 5). The distribution includes the expected bimodal distribution for wheat starch as well as a third modality for protein and protein-starch aggregates. Microscopy (Fig. 6) further illustrates the heterogeneity of the mixture. Although the starch granule dimensions do not change with mixing, the protein characteristic dimensions (fibril diameter) change progressively to smaller and smaller values (Fig. 6). We believe that the finer protein fibrils increas-

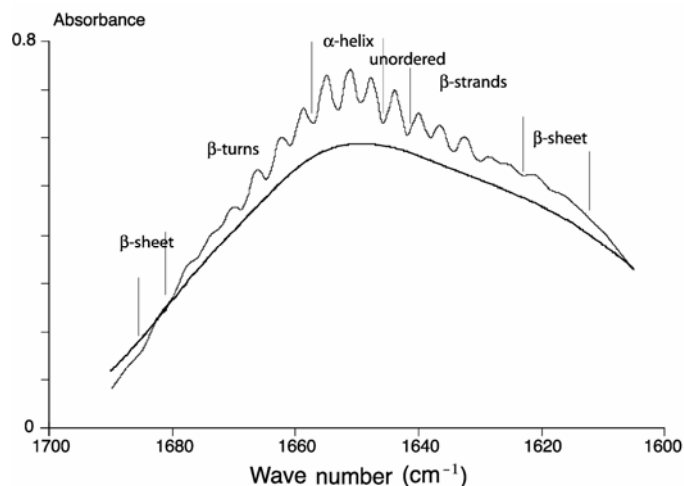


Fig. 7. Normal and deconvoluted, water-corrected spectrum of 90% absorption, flour and water mixture after 24 min of mixing using a micro-farinograph. Structural annotation based on assignments of Table I.

ingly fall within the measurement window as they flow into the space between the starch granules. Only a fraction of most starch granules in this zone are perceived by the beam. Hence, the changes in intensities reflect increasing bulk homogeneity, dispersion of protein, and flow of protein to the measurement window.

FTIR/ATR Monitoring of Secondary Structure

When the amide I band was deconvoluted, peaks representing known secondary structural features of proteins were revealed. The peak assignments include β -strands, β -turns, β -sheets, α -helices, and unordered structures (Table I). An example of one such deconvolution for 24 min of mixing development is shown in Fig. 7.

To monitor homogeneity during mixing, the ratios of the individual amide I components to the total area of the amide I band were calculated from the deconvoluted spectra. These include con-

TABLE I
Assignment of Deconvoluted Amide I Band of Wheat Flour Dough

Wave number (cm^{-1})	Structural Assignments	References ^a
1686–1680	β sheet, antiparallel	1, 2, 5–9, 11, 12, 16
1677–1666	β turns	1–5, 7, 8, 10–17
1662–1659	β turns and irregular structure	1, 4, 7, 8, 11, 15
1655	α helix	2–10, 12–14, 16
1651–1648	α helix H-bonded with water	1, 3–5, 7, 9, 11–17
1644	Unordered structures	1, 2, 4–8, 11, 16
1640–1637	β strands, weakly H-bonded	2, 4, 7–12, 15, 16
1633–1625	β strands, strongly H-bonded	1–10, 12–17
1622–1613	β sheets, extended and intermolecular bonding	1–7, 9–17

^a (1) Allain et al 1999, (2) Arrondo et al 1994, (3) Belton et al 1995, (4) Byler and Susi 1986, (5) Dornberger et al 1996, (6) Fernandez-Ballester et al 1992, (7) Goormaghtigh et al 1990, (8) Hadden et al. 1993, (9) Jackson et al 1991, (10) Mangavel et al 2001, (11) Meng and Ma 2001, (12) Naumann et al 1993, (13) Pezolet et al 1992, (14) Popineau et al 1994, (15) Serina et al 1996, (16) Surewicz et al 1988, and (17) van Velzen et al 2003.

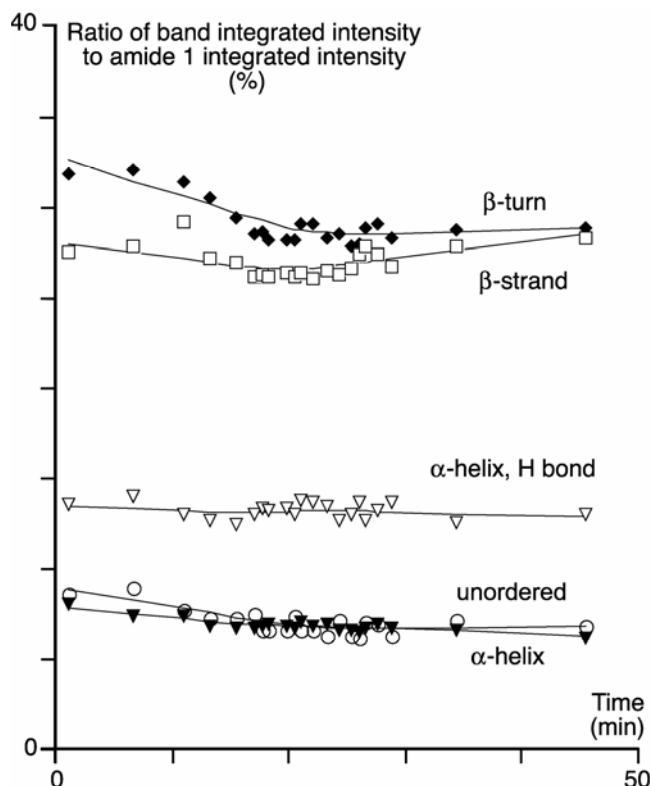


Fig. 8. Nonsheet form protein band intensities as fraction of total protein for 90% absorption flour and water mixtures after mixing using a micro-farinograph.

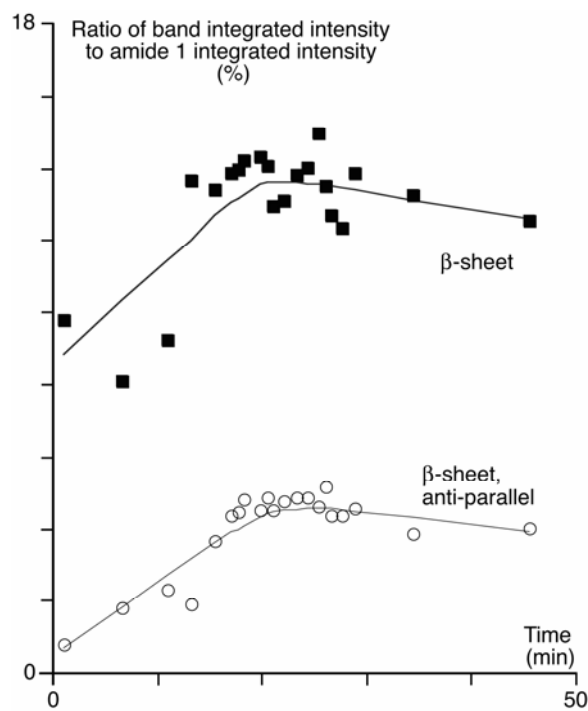


Fig. 9. Sheet-form protein band intensities as fraction of total protein for 90% absorption flour and water mixtures after mixing using a micro-farinograph.

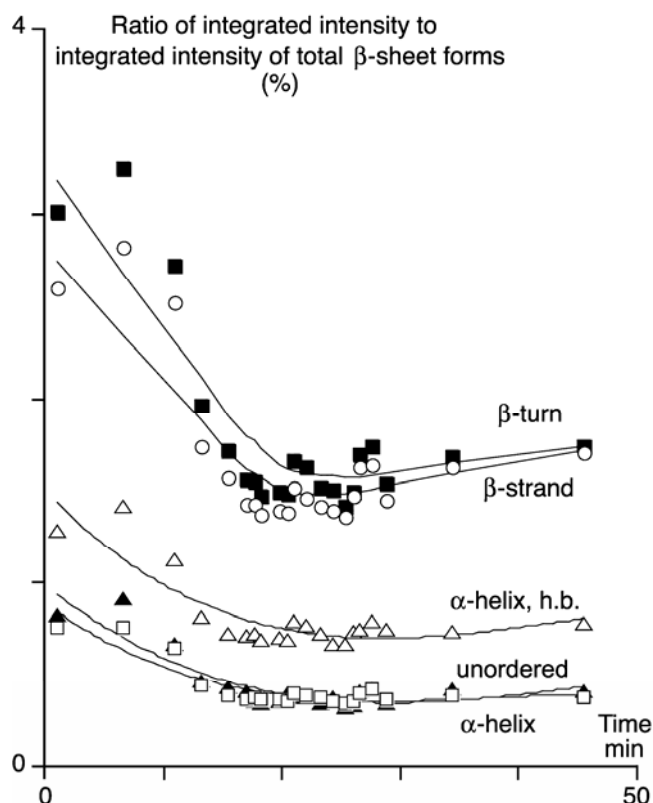


Fig. 10. Ratio of integrated intensities of nonsheet secondary structures to the total β -sheet structures.

tributions from nonsheet structures that decreased (Fig. 8) and those from sheet structures that increased (Fig. 9). The most intense peaks were for β -turns and strands followed by β -sheets, α -helices, and other unordered structures. Several changes in the peak ratios were significant. For instance, the relative intensity of extended and intermolecular β sheets increased from 9 to 14 with

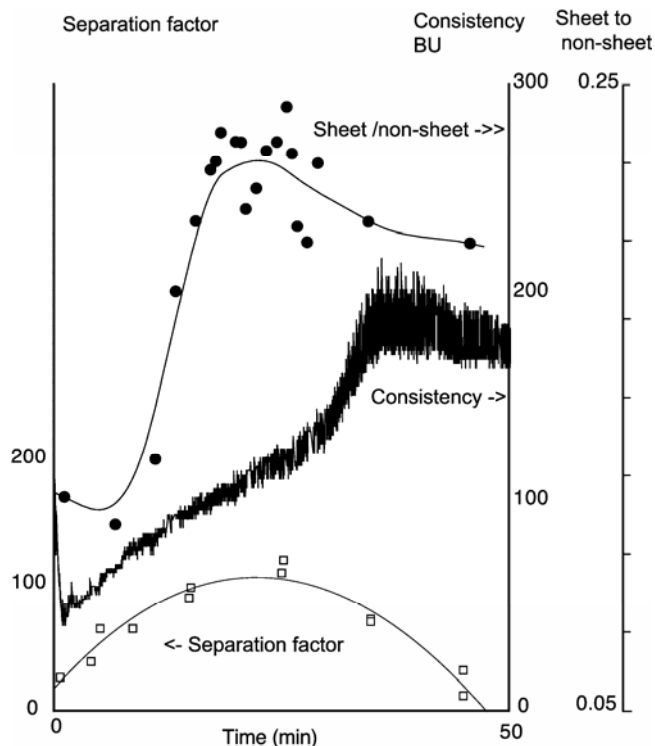


Fig. 11. Summary of molecular and macro changes during mixing of 90% absorption flour. Consistency and separation factor modified from Robertson et al (2000).

a simultaneous increase from 1 to 5 for the high frequency β -sheet, antiparallel band (Fig. 9). Most protein secondary changes occurred within the first 25 min of mixing.

When each band intensity is reported as a ratio to the sum of the β -sheet forms the conformational change from the nonsheet to sheet forms is emphasized (Fig. 10). The β -turn and β -strand structures contribute $\approx 70\%$ of the total amide band when time equals zero. At 25 min, the contribution of all nonsheet structures diminishes. Also noteworthy is a reversal of the conformational change after 25 min, although this is only minor.

All of the relative amide I band intensity changes were summarized by forming the ratio of the intensities of the principle increasing β -sheet bands to the intensity of the sum of the declining nonsheet bands. The ratio increased from 0.12 to ≈ 0.23 and then decreased to 0.2 near the highest consistency (Fig. 11). The maximum ratio of nonsheet to sheet structures coincides with the highest separation factor but precedes the peak consistency factor. Interestingly, the shape of this curve is similar to that for the farinograph or Brabender consistency. An increase in β -sheet bands has been shown previously for manually stretched and kneaded, low-moisture absorption dough (van Velzen et al 2003).

The high fluidity of the batters in the present study may diminish the intensity of structural features. For comparison, the farinograph time-to-peak and peak consistency for this flour titrated in the standard way with water to yield 500 BU (60% absorption) were $\approx 1/6$ and 2.5X, the respective values for relatively high absorption, separation batter reported here (Robertson et al 2000).

Our data showed a significant decrease of β -turns and β -strands with mixing time for optimum starch and protein separation (Fig. 9). The data suggest that β -turns in the repetitive domains of the glutenin subunits may transform to β -sheets, which can be stabilized by new covalent bonds and by hydrogen bonds between adjacent glutenin subunits. The increase in molecular sheeting noted here undoubtedly helps to set the stage for changes such as diminished separation, webbiness, and peak consistency that eventually occur.

CONCLUSIONS

This study shows that attenuated total reflectance Fourier transform infrared spectroscopy can be used to monitor relative changes in the protein secondary structures in a slowly developed, 90% absorption wheat-flour and water mixture. Changes in absolute intensities of starch and protein bands present more complex information because they are likely related to the changing macro structure of the polyphasic substrate and may index increasing extension and disbanding of the polymer protein filaments. Formation of β -sheet structures was observed to be at the expense of all other conformations during mixing. β -Sheet structures were observed to diminish after the time that the mixture is most separable.

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