

A Study by Infrared Spectroscopy of the Conformations of Gluten Proteins Differing in their Gliadin and Glutenin Compositions

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Received 23 February 1994

ABSTRACT

The conformations of gluten proteins extracted from three near-isogenic lines of the wheat cultivar Sicco, which differed in their high M_r glutenin subunit compositions, were studied by infrared spectroscopy. Whole gluten and gluten fractions prepared by sequential extraction were analysed in solution and in the doughy hydrated state by transmission and attenuated total reflectance spectroscopy, respectively. In the hydrated viscoelastic state, the β -sheet content, especially that of intermolecular β -sheet conformation, of gluten proteins was higher than that in solution. In viscoelastic gluten fractions, the proportion of intermolecular β -sheet increased as the glutenin content increased. In addition, the α -helix content was lowest and the intermolecular β -sheet content highest when disulphide bonds were reduced in the hydrated state because interactions between gluten polypeptides can be maximised under these conditions. These results suggest that the interactions between subunits in glutenin polymers and aggregates involve intermolecular β -sheets located in their repetitive domains or in the region of chain entanglements that are responsible for the viscoelastic properties of gluten proteins.

INTRODUCTION

Wheat storage proteins (prolamins) exhibit the unusual property of being viscoelastic when hydrated. This behaviour determines to a very large extent the technological values of the flours. The physicochemical basis of wheat prolamins viscoelasticity has not yet been fully elucidated. The role of the aggregative properties of gliadins and glutenins has been demonstrated¹ and it is now

well established that high M_r glutenin subunits contribute particularly to gluten elasticity^{2,3}. On the other hand, gluten viscoelasticity depends mostly on its content of protein polymers and on the size distribution of the protein polymers that form a transient network⁴. It has been postulated that gluten viscoelasticity is related to special sequence domains of high M_r glutenin subunits that are rich in β -turns⁵. Most structural studies on prolamins have been carried out in dilute solution by circular dichroism spectroscopy⁶, however. Recently, it has been shown by attenuated total reflection (ATR) infrared spectroscopy⁷ that the conformation of functional (viscoelastic) gluten proteins differs markedly from that in solution. Hydrated doughy samples of gluteins and gliadins contain less α -helix and β -turn structures and more β -sheet structure, es-

ABBREVIATIONS USED: ATR = attenuated total reflectance; DTT = dithiothreitol; EDTA = ethylenediaminetetraacetic acid; NIL = near-isogenic lines; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; SE-HPLC = size-exclusion high-performance liquid chromatography; TEA = triethylamine.

pecially intermolecular β -sheet, than in solution. It was suggested that gluten proteins may interact in the network through aligned β -sheets corresponding to their repetitive domains⁷.

In this paper, transmission and ATR infrared spectroscopy have been applied to a series of glutens and gluten fractions differing in their rheological behaviour⁸. They were prepared from three near-isogenic lines of wheat Sicco with different high M_r glutenin subunit compositions. The effect of the reduction of disulphide bonds was also investigated. The objective was to examine how the conformations of the prolamin fractions are related to their gliadin and glutenin polypeptide compositions. An attempt was made to relate their conformations to their interactions in the network formed by hydrated storage proteins.

EXPERIMENTAL

Near-isogenic lines

Analyses were carried out on three near-isogenic lines (NIL) of wheat Sicco bred by P. Payne (Plant Breeding International Ltd., Cambridge, UK). Flour samples were kindly provided by A. Tatham (AFRC Institute of Arable Crops Research, Long Ashton Research Station, Bristol, UK). Their high M_r subunit compositions are as follows: NIL 5+10: 1/7+9/5+10; NIL 2+12: 1/7+9/2+12; NIL double-null: -/7+9/- . These lines have been fully described by Payne *et al.*⁹.

Extraction of gluten and gluten fractions

Glutens were extracted from defatted flours as described previously⁸. Gluten fractions were prepared by extraction with dilute HCl solutions¹⁰, using the procedure described previously⁸. Sequential extraction, comprising 10 steps, was performed as follows: fractions F1–F3, three extractions with 0.3 mM HCl (100 ml); fractions F4–F6, three extractions with 0.625 mM HCl (50 ml); fractions F7–F10, four extractions with 1.5 mM HCl (50 ml); fraction F11, residual material.

This extraction procedure yields gluten proteins retaining their functionality.

Reduced and alkylated glutens

Gluten was extracted from flour of the wheat cultivar Aubaine as described above. Gluten was

dispersed in 0.05 M Tris buffer, pH 8, containing 0.01 M EDTA and 0.5% (v/v) triethylamine (TEA). Various amounts of 2-mercaptoethanol were added to obtain final concentrations of 6, 9, 24, 60 and 150 mM. The reaction was continued for 30 min at room temperature under a stream of nitrogen. Sulphydryl groups were alkylated with 4-vinylpyridine for 30 min in the dark. The reaction was terminated by adjusting the pH to 3 by the addition of concentrated HCl. The samples were dialysed exhaustively against water, then against 0.01 M acetic acid, and were then freeze dried. Reduction of intermolecular disulphide bonds was followed by size-exclusion high-performance liquid chromatography (SE-HPLC), using the procedure described recently⁴. This showed that the size of the glutenin polymers decreased progressively as the 2-mercaptoethanol concentration was increased. With 150 mM 2-mercaptoethanol, glutenin subunits and gliadin monomers were observed mostly, indicating that reduction of intermolecular disulphide bridges was complete.

Deamidated ω -glutens

Partly deamidated ω -glutens were prepared by mild acid hydrolysis¹². Two samples were prepared, the extents of deamidation of which were 23 and 41%.

Infrared spectroscopy

For the infrared spectroscopy transmission measurements, proteins, glutens or fractions were stirred for 2 h in 0.1 M acetic acid, pH 3.5. Solutions containing about 7% (w/w) protein were allowed to stand overnight at 4 °C, centrifuged, and the supernatants were used to record the spectra. Doughy samples used for attenuated total reflectance (ATR) measurements were prepared by adding excess water (10 μ l) at pH 7.0 to the dry solid material (4 mg). To study the reduced proteins, a solution of 0.6 M dithiothreitol (DTT) was used to hydrate the samples instead of water.

Fourier transform infrared spectra (1000 scans) were recorded at a resolution of 2 cm^{-1} on a Bomem DA3-02 spectrophotometer equipped with a narrow-band mercury-cadmium-telluride detector. All spectra were corrected for the spectral contribution of water using the method of Dousseau *et al.*¹³ and normalised to a total inte-

grated intensity of 1 to allow easier comparison of spectra obtained at different concentrations and pathlengths. Fourier deconvolution of the amide I region was achieved by the method of Griffiths and Pariente¹⁴ using a γ factor of 7.5 and a filter of 0.14.

Transmission spectra were obtained at 20.0 ± 0.1 °C using a cell composed of two CaF₂ windows separated by a 6- μ m spacer¹³. Spectra of doughy samples were obtained by ATR at room temperature using a single reflectance cell (Harriick Scientific, U.S.A.) fitted with a zinc selenide prism. Hydrated samples were pressed against the prism and covered with an excess of water.

RESULTS AND DISCUSSION

Biochemical and rheological characteristics of glutens and gluten fractions

The prolamin compositions of glutens and gluten fractions, as determined by SDS-PAGE and SE-HPLC, and their viscoelastic properties were reported previously⁸ and are summarised below.

The differences in high M_r glutenin subunit compositions among the Sicco NIL result in variation in the size distributions of the glutenin polymers and in different extractibility and rheological properties of the glutens. The absence of three high M_r subunits (double-null line) increased the extractibility of glutenin because the number and the size of glutenin polymers was diminished. Glutenin viscoelasticity was drastically decreased compared with that of the standard 5+10 line. The substitution of subunits 2+12 for subunits 5+10 also caused a decrease in gluten viscoelasticity. Subunits 5+10 promote the formation of less-extractable glutenin polymers.

The high M_r glutenin subunit contents of the fractions prepared by sequential extraction in dilute HCl increased with their rank number (F1 to F11), i.e. as their extractibilities decreased. The content of the largest glutenin polymers (excluded peak in SE-HPLC, $M_r > 0.5$ million) was higher for fraction F11. The excluded peak accounted for less than 2% of the protein in F1 but for 47% in F11 of Sicco NIL 5+10. In the same fractions, gliadin monomers accounted for 50% and 18%, respectively. Fractions with the same size distribution (as estimated from SE-HPLC patterns) displayed similar viscoelastic be-

haviours independently of their high M_r subunit allele composition. This demonstrated that the viscoelasticity of gluten proteins is related to the polymeric and aggregative nature of the system⁸.

Amino acid side-chain spectral contribution in the amide I region

Gluten proteins contain between 33% and 50% of glutamine side chains¹⁵, which may contribute to the absorption in the amide I region. Methods to correct the absorption spectra of protein solutions in either H₂O or D₂O for the spectral contribution of amino acid side chains have been proposed by Chigardze *et al.*¹⁶ and Venyaminov and Kalnin¹⁷. In order to perform this correction, the infrared spectra must be converted to molar absorbances from sample concentrations obtained by ultraviolet absorption measurements, and the proportion of each amino in the sample must be known accurately. This procedure has been used by Purcell *et al.* to correct their infrared spectra of α - and ω -gliadins in solution for the glutamine absorption¹⁸. In the case of gluten proteins, which are protein mixtures, it is very difficult to meet these conditions and to perform an accurate correction for the contribution of the amino acid side chains. In addition, it has been shown recently by Fabian *et al.*¹⁹ that, even for a pure protein like streptokinase, the correction for side-chain contributions is difficult to realise since the molar absorbances of free amino acids in solution may differ from that of the side chains in specific protein microenvironments.

Nevertheless, in order to estimate the extent of the glutamine side-chain contribution in the amide I region of the spectra of gluten proteins, the infrared spectra of native and partly deamidated (23 and 41%) ω -gliadins were recorded and are presented in Fig. 1. ω -Gliadins are the prolamins that contain the highest proportions of glutamine residues (40–50%). As can be seen from Figure 1, the spectra of 23 and 41% deamidated ω -gliadins were very similar, showing clearly that the spectral contribution of the glutamine side chains was fairly weak and was not responsible for the spectral changes presented in this paper. The difference between the spectrum of native ω -gliadins and those of the deamidated proteins was most likely due to a conformational change induced by the deamidation process. On the basis of these results, and without a reliable correction criterion, we have preferred not to

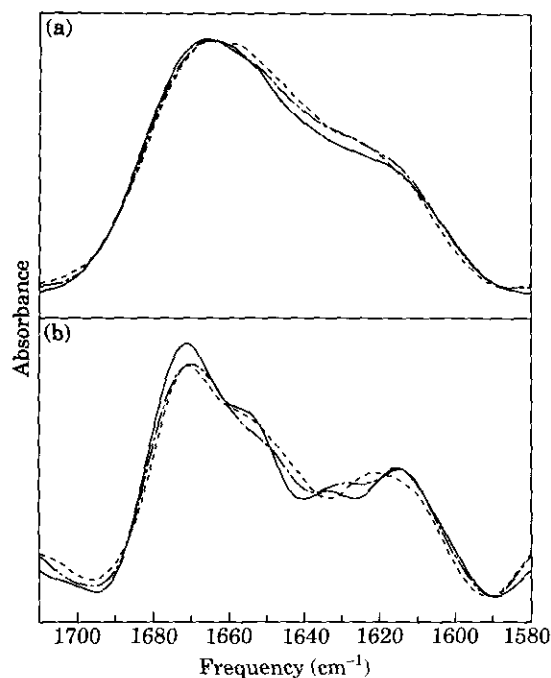


Figure 1 (a) Original and (b) Fourier deconvoluted infrared spectra in the amide I region of native and partly deamidated ω -gliadins in solution. Deamidation: 0% (—); 23% (---); 41% (— — —).

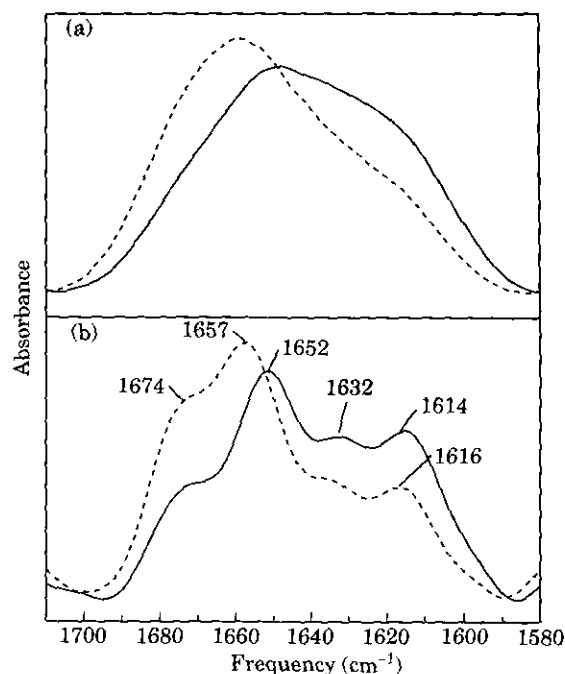


Figure 2 (a) Original and (b) Fourier deconvoluted infrared spectra in the amide I region of fraction F6 of Sicco NL 5 + 10 in 0.1M acetic acid solution (---) and in the hydrated doughy state (—).

correct the spectra presented in this paper. For most proteins, the side-chain contribution has been estimated to be approximately 10% of the intensity of the amide band I¹⁹.

Comparison of the conformations of gluten proteins in solution and in the hydrated doughy state

Infrared spectra in the amide I region of the fraction F6 of Sicco NL 5 + 10 in solution and in the hydrated doughy state are presented in Fig. 2. This fraction contains about 43% of glutenin polymers⁸. After deconvolution, the spectrum of the dissolved protein showed a prominent band at 1657 cm^{-1} that is associated with the α -helical conformation²⁰. The band at 1674 cm^{-1} is associated with the presence of β -turns^{20,21}, a structure predicted in the long repetitive domains of prolamins⁶. Accordingly, it is the major band in the spectrum of ω -gliadins in solution⁷. The two weaker bands at 1616 and 1632 cm^{-1} were assigned to intermolecular and extended β -sheet structures, respectively^{7,20,21}. As expected, the

spectra shown in Fig. 2 were similar to those observed for gluten and γ -gliadins under the same experimental conditions⁷, which were also found to be in quantitative agreement with structure contents estimated by circular dichroism⁷.

In the doughy viscoelastic state, the spectra were quite different from those of the proteins in solution. The α -helix band was shifted from 1657 to 1652 cm^{-1} , indicating that the α -helices were probably more stable in functional proteins than in solution. This may be due to stronger hydrogen and hydrophobic bonding in prolamin aggregates. Simultaneously, the intensity of the β -turn band decreased, whilst that of the bands at 1614 and 1632 cm^{-1} increased, showing that β -sheet structures were favoured in the doughy state. Amide I bands around 1615 cm^{-1} have been reported for cholera toxin²², cytochrome c²³ and parvalbumin at high temperature²⁴. Such a low frequency for the amide I band has been associated with the presence of intermolecular β -strands in aggregated structures²⁵⁻²⁷ and is believed to be a general phenomenon in thermally denatured proteins²⁴. It seems likely, therefore, that β -sheets are involved in the intermolecular

association of prolamins. These results are in very good agreement with those obtained previously on whole gluten and purified γ - and ω -gliadins⁷.

Comparison of the conformation of the proteins of gluten fractions

Fractions F2, F6 and F10 of gluten extracted from Sicco NIL 5 + 10 were chosen as examples in this section. These fractions differed largely in their prolamins compositions and size distributions and are representative of all fractions of the three NIL that were studied. The glutenin/gliadin ratios, determined by SE-HPLC, were 0.76 for F2, 1.8 for F6 and 3.6 for F10⁸. The largest glutenin polymers (SE-HPLC excluded peak) accounted for 4.5, 18.7 and 40.4% of the protein in these fractions, respectively.

The original and deconvoluted infrared spectra of fractions F2, F6 and F10 are presented in Fig. 3. The shape of the amide I band changed notably from one fraction to another, the maximum near 1652 cm^{-1} being stronger in the F2 fraction. These differences are highlighted in the deconvoluted spectra [Fig. 3(b)]. It is clear from Fig. 3(b) that the 1652 and 1672 cm^{-1} bands, associated with the α -helix and β -turn structures, respectively, decreased in intensity from F2 to F10, while the intensity of the lower-frequency bands due to the presence of β -sheet structure increased. Therefore, the proportion of α -helix and β -turn structures became less important when the glutenin content of the fractions and their content of the largest glutenin polymers increased. On the other hand, the β -sheet content increased in going from fractions F2 to F10. Similar patterns were observed for the fractions extracted from each near-isogenic line.

To facilitate comparison of the different fractions, the peak height intensity ratio of the 1650 (α -helix) and 1615 cm^{-1} (β -sheet) bands, h_{1650}/h_{1615} , was calculated from the infrared spectrum of each fraction of the Sicco 5 + 10 NIL. As shown in Fig. 4, the ratio of α -helix to β -sheet decreased as the fraction number increased, i.e. as the content of glutenin polymers and aggregates increased⁸. In addition, Fig. 5 also shows that there was a good correlation (correlation coefficient of 0.94) between the h_{1650}/h_{1615} ratio and the glutenin polymer content of the fractions, as determined by SE-HPLC. This relationship appeared to be determined essentially by glutenin polymer content and not by the high M_r glutenin

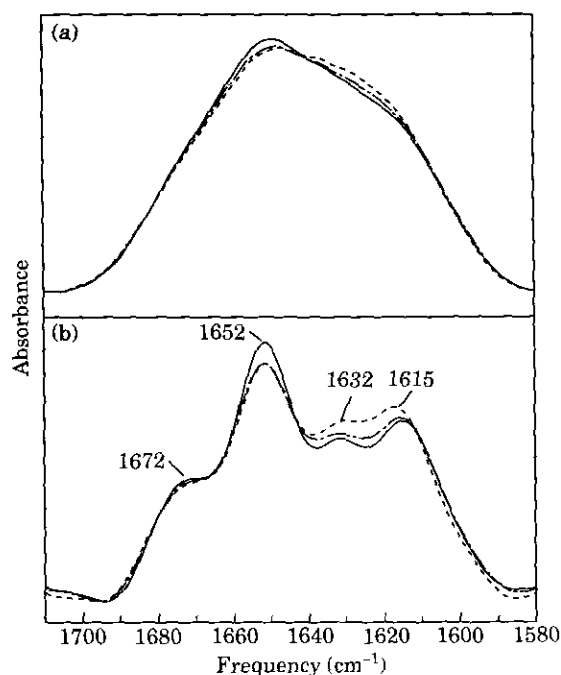


Figure 3 (a) Original and (b) Fourier deconvoluted infrared spectra in the amide I region of fractions F2 (—), F6 (— — —) and F10 (— · — · —) of Sicco NIL 5 + 10 in the hydrated doughy state.

subunit alleles represented in these polymers, since the three near-isogenic lines gave almost indistinguishable results. It should be noted that whole glutes and fractions F6 and F7, which had almost the same prolamins composition, had the same h_{1650}/h_{1615} ratio also.

These results suggest that functional glutenin polymers are richer in β -sheet structure than gliadins. The origin of the conformational differences between the fractions is unclear. It may arise from their different contents of gliadins, low M_r glutenin subunits and high M_r glutenin subunits. Sequence studies and circular dichroism measurements on α - and γ -type gliadins and low M_r glutenin subunits have shown that the proportion of α -helix is as high in low M_r glutenin subunits as it is in gliadins⁶. High M_r glutenin subunits contain a very long repetitive domain, which has been shown to be rich in β -turn conformation in solution²⁸. Previous results obtained for prolamins by infrared spectroscopy have shown that the β -sheet conformation is favoured in the doughy state rather than the β -turn conformation, as in the case of ω -gliadins, which also have a highly repetitive central domain⁷. The high M_r

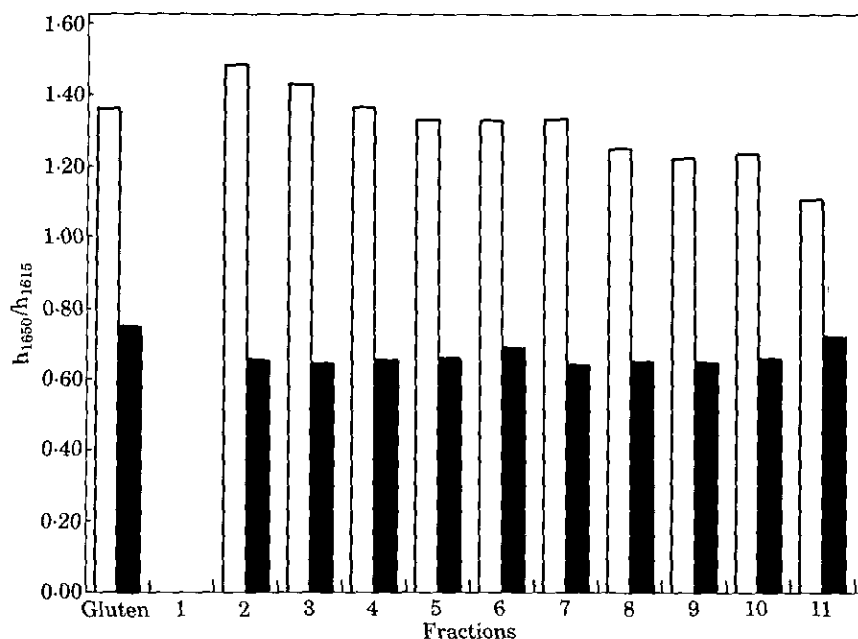


Figure 4 Effect of the fraction number on the h_{1650}/h_{1615} intensity ratio in the deconvoluted spectrum of each fraction of Sicco NIL 5 + 10 in the hydrated doughy state. Unreduced proteins: clear bars. Proteins reduced with 0.6M dithiothreitol: black bars.

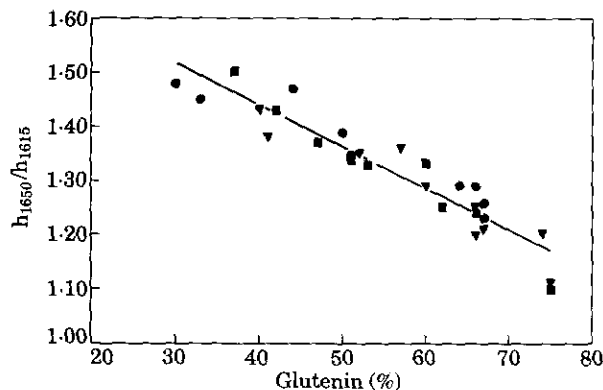


Figure 5 Effect of the glutenin content of the different fractions on the h_{1650}/h_{1615} intensity ratio in the deconvoluted spectra of Sicco NIL 5 + 10 (■), 2 + 2 (▼) and double null (●) in the hydrated doughy state.

glutenin subunit contents of the fractions from near-isogenic lines 5 + 10 and 2 + 12 vary from 1.6 to 12% of the total protein⁸, which may explain the differences in the infrared spectra of fractions F2 to F11. The value of the h_{1650}/h_{1615} ratio cannot be determined solely by the content of high M_r glutenin subunits of the fractions, however. The contents of high M_r subunits in all fractions of the double-null line were only half those in corres-

ponding fractions (same rank number) of lines 5 + 10 and 2 + 12, but they had identical infrared spectra. Similarly, high M_r glutenin subunits account for only about 5% of gluten proteins in the double-null line, but for about 10% in the 5 + 10 and 2 + 12 NIL, yet the infrared spectra of whole gluteins from these three lines were again similar.

These results led to the hypothesis that the association of the subunits in polymers and aggregates is the cause of the variation of the relative contents of α -helix and β -sheet of gluten fractions, rather than the subunit composition of glutenin polymers. Intermolecular β -sheet structures may be involved in protein-protein interactions⁷. To test this hypothesis the influence of disulphide bond integrity on the structure of prolamins was also studied.

Effect of the reduction of the disulphide bonds

To determine the effect of the disulphide bonds on the conformation of gluten proteins, gluten (cv. Aubaine) was reduced and alkylated at different concentrations of reducing agent so as to vary the proportion of intact and reduced disulphide bonds. The spectra of five gluten samples in

the doughy state reduced with 6, 9, 24, 60 and 150 mM of 2-mercaptoethanol are presented in Fig. 6. This figure shows clearly that the reduction of disulphide bridges has a substantial effect on the infrared spectrum of gluten. The intensity of the band due to α -helix structure (1651 cm^{-1}) decreased markedly with the reduction of disulphide bonds, whilst that of the band associated with the intermolecular β -sheet structure (1617 cm^{-1}) increased considerably. The concomitant increase in the high-frequency component at 1693 cm^{-1} indicated that the β -sheet structure was antiparallel since the splitting of the amide I mode caused by interstrand interactions occurs only for the antiparallel structure²⁹. The ratio h_{1651}/h_{1617} decreased from 1.28 to 0.73. Furthermore, isosbestic points were clearly seen at approximately 1626 and 1662 cm^{-1} , revealing a two-state equilibrium between the oxidised and reduced forms of gluten. At a 2-mercaptoethanol

concentration of 150 mM, the reduction of intermolecular disulphide bonds, at least, was almost complete according to SE-HPLC patterns (not shown). Cysteine residues are located in the unreplicative regions of wheat prolamins¹⁵. They form either intramolecular bonds (gliadins) or intra- and intermolecular bonds (glutenin)¹⁵. Reduction of disulphide bonds can alter the conformational stability of proteins and their intermolecular interactions, resulting in changes in their infrared spectra.

After reduction with 0.6 M DTT, the spectra of all gluten fractions from Sicco NIL were identical and resembled that of Aubaine gluten reduced with 150 mM 2-mercaptoethanol. They exhibited the same h_{1650}/h_{1615} ratios (Fig. 4). This was unexpected because they differed considerably in their prolamins compositions. After reduction, the prominent structure in the hydrated doughy prolamins was the intermolecular β -sheet ($h_{1650}/h_{1615}=0.7$) as in reduced and alkylated cv. Aubaine gluten. The conformations observed in the reduced fractions were not influenced by their composition (glutenin/gliadin ratio, low M_r /high M_r glutenin subunit ratio). They were also completely different from their conformations in solution in the absence of reduction (Fig. 2), for which the most prominent bands in the infrared spectrum of sulphur-rich and sulphur-poor prolamins are located at 1655 and 1671 cm^{-1} , respectively⁷. It is important to stress that, in the doughy state, the protein concentration was about 40% (w/w) and protein-protein interactions are favoured in concentrated systems such as this. The formation of intermolecular β -sheet structure may be facilitated in highly concentrated reduced prolamins because disulphide bonds do not limit the unfolding of the proteins.

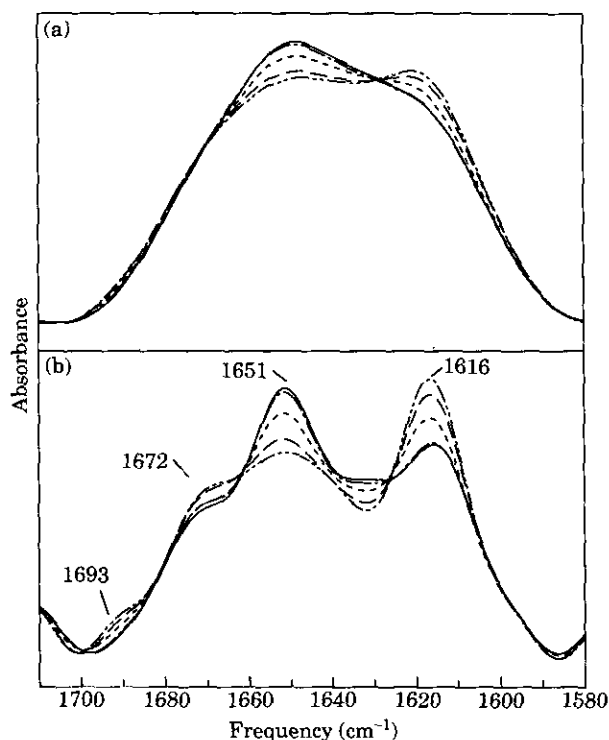


Figure 6 (a) Original and (b) Fourier deconvoluted infrared spectra in the amide I region of cv. Aubaine gluten reduced with various concentrations of 2-mercaptoethanol and then alkylated. Measurements were performed in the hydrated doughy state. 2-Mercaptoethanol concentrations: 6 mM (—); 9 mM (---); 24 mM (----); 60 mM (— · —); 150 mM (— · · —).

CONCLUSION

The results obtained for reduced and unreduced fractions indicate that the conformation of glutenin polymers depends not only on the subunit composition and intramolecular interactions, but also very strongly on their polymer organisation and intermolecular interactions. The more abundant and the longer the glutenin polymers, the higher their relative contents of intermolecular β -sheet structure. Furthermore, the cleavage of disulphide bonds maximises interactions between all prolamins, gliadins as well as glutenin subunits, and result in the highest relative content

of intermolecular β -sheet structure. Viscoelasticity is lost after reduction, however, because of glutenin depolymerisation.

In a recently proposed model for glutenin, it was envisaged that low M_r and high M_r subunits interact in glutenin polymers through their repetitive domains³⁰. The contribution of amide groups to the physical properties of hydrated gluten has been shown³⁰⁻³³. The results reported here are consistent with our previous hypothesis that the polypeptide chains in glutenin polymers interact in viscoelastic gluten most probably through β -sheet structures, formed by interactions among their repetitive domains⁷. These domains are very rich in glutamine⁶ and can form an extended network of hydrogen bonds. One might expect therefore that these regions are involved in gluten viscoelasticity, which appears to be related to transient interactions of large gluten concatenations⁴.

Acknowledgements

This research was supported by grants from the Fonds FCAR of the Province of Québec, the Natural Sciences and Engineering Research Council of Canada, the France-Québec exchange programme and the Institut National de la Recherche Agronomique, France. The authors thank P. Payne and A. Tatham for providing Sicco NIL flour samples.

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