Evolution of Wheat Gliadins Conformation during Film Formation: A Fourier Transform Infrared Study

Cécile Mangavel,* Jacky Barbot, Yves Popineau, and Jacques Guéguen

Institut National de la Recherche Agronomique, Unité de Biochimie et Technologie des Protéines, Rue de la Géraudière, BP 71627, 44316 Nantes Cedex 03, France

The secondary structures of wheat gliadins (a major storage protein fraction from gluten) in film-forming solutions and their evolution during film formation were investigated by Fourier transform infrared spectroscopy. In the film-forming solution, wheat gliadins presented a mixture of different secondary structures, with an important contribution of β-turns induced by proline residues. The presence of plasticizer did not have any influence on protein secondary structure in the film-forming solution. The evolution of protein conformation was followed during drying; the major feature of this evolution was a clear growing of the infrared band at 1622 cm^{-1}, characteristic of intermolecular hydrogen-bonded β-sheets. This revealed the formation of protein aggregates during film drying. The influence of the drying temperature on film properties and gliadin secondary structures was also investigated. Higher drying temperatures induced an increase of both the tensile strength of the films and the amount of β-sheets aggregates. Although the appearance of heat-induced disulfide bridge cross-links has already been described, there is clear evidence that hydrogen-bonded β-sheets aggregates are also induced by thermal treatment. It was not possible, however, to determine whether there is a direct relationship between the occurrence of these aggregates and the increase of the tensile strength of the films.

Keywords: *Wheat; gliadins; protein films; secondary structure; infrared spectroscopy*

INTRODUCTION

During the past few years, numerous studies have been aimed at the preparation of materials from renewable resources. Among these substrates, plant proteins seem to present interesting potentialities to form biodegradable films (*1*−*3*) whose applications could partially overcome those of synthetic polymer films.

Some of the most important characteristics for thermoplastic materials are their mechanical properties; in the case of proteins, a lot of work has been done to improve these properties and particularly to increase the tensile strength of the films. Several approaches have been used to induce cross-linking in the films, either by chemical (*4*), enzymatic (*5* and *6*), or radiative treatments (*7*−*10*). However, the most promising results seem to arise from the thermal posttreatment of the films (*11* and *12*).

Very few data are available at this moment about the microstructure of the films and the organization of the protein network. A previous work (*13*) concerning the conformation of glycinin (a storage protein from soybean) in aqueous solution and in films has brought to light the occurrence of conformational changes in the secondary structure of proteins upon the film-forming process; these modifications lead to intermolecular hydrogen-bonded β-sheets structures. However, very few data are available concerning the structure of wheat gluten proteins in film-forming solutions and their evolution during drying to generate films. The role of disulfide bridges seems nevertheless to be also very important (*11* and *14*) in the network organization.

On the contrary, a lot of studies have been aimed at the determination of wheat gluten protein conformation, and more precisely of gliadin structure in solution (*15* and *16*) or in the solid state (*17*). Most results demonstrated the occurrence of α-helices for the nonrepetitive domain and of β-turns for the repetitive domains of the gliadins. The authors emphasized the importance of both the hydrophobic interactions and the hydrogen bonds to stabilize the secondary structures of the gliadins. Intermolecular β-sheets appeared in the functional (doughy) state (*18* and *19*) as well as in the solid state (*17*). The authors suggested that these structures arose from the close contact between neighboring molecules.

Our objective was to follow the conformational changes of the wheat gliadins during the film formation by Fourier transform infrared spectroscopy and to determine whether there is a relationship between mechanical properties of the films and particular secondary structures of the proteins.

EXPERIMENTAL PROCEDURES

Materials. A gliadin-rich fraction (86.4% proteins, N × 5.7) was obtained by fractionation of industrial vital gluten (Roquette, Lestrem, France) at the pilot scale, as described previously (*20*). It has been shown that these process conditions did not affect the solubility, nor the technological properties, of the gliadin-rich fraction (*20* and *21*). Furthermore, drying in dilute acetic acid was shown to maintain functionality of gluten fractions (*22*).

Preparation of the Solutions. For spectroscopic measurements of protein solutions, gliadins were mechanically stirred for 2 h in 0.1 M NaOH, pH 11. Solutions containing about 7% proteins by weight were allowed to stand overnight at 4 °C and centrifuged before recording the spectra.

* To whom correspondence should be adressed; email: mangavel@nantes.inra.fr.

10.1021/jf0009899 CCC: $20.00
Published on Web 01/09/2001

Preparation of the Protein Films. Gliadin films plasticized with diethylene glycol were prepared as described previously (*2*). The film-forming solution (pH 11) presented a 16% w/w protein content and a 0.37 plasticizer/protein ratio. The solution was spread on a glass plate covered with a polyester sheet, and then dried in a ventilated oven until the weight of the film reached a constant mass (typically 1 h at 70 °C). Other drying conditions were also investigated (from 16 h at 25 °C to 30 min at 90 °C). The gliadin film was peeled from the plate and equilibrated during 72 h in a cabinet under standard conditions of temperature (20 °C) and relative humidity (60% RH, produced by a saturated solution of sodium bromide).

Plasticizer Content. The residual amount of plasticizer in the films was determined by high-performance liquid chromatography (*23*). Film samples (500 mg) were extracted twice with water (25 mL) for 60 and 30 min and the total volume of the extract was adjusted to 250 mL. After filtration, the solution (100 μL) was injected on an ion-exchange column (Sucrex-CIL Ca^{2+}) heated at 60 °C. The plasticizer was eluted by water (0.8 mL/min) in isocratic conditions and detected by a differential refractometer (Jobin-Yvon). The concentration was calculated by reference to a calibration curve of the same plasticizer.

Protein Contents. Total protein content (N × 5.7) was determined after digestion of the gliadin films in concentrated sulfuric acid by an automated ammonia/salycilate reaction (*24*). The method was slightly modified: selenium was used as a catalyst and the digestion was realized at 300 °C during 2 h. Protein solubility in water was determined by extracting 500 mg of film with 250 mL H_2O for 90 min; the solution containing the water extractable proteins was recovered by filtration. The final water extract presented a pH of 9.5, due to solubilization of residual sodium hydroxyde from the film into the water medium. The total nitrogen content of the extract was assayed according to the previous method.

Residual Water. The water content in films was determined from weight difference of the samples (500 mg) after 24 h drying in a desiccator containing P_2O_5 under reduced pressure (*2*).

Mechanical Properties. Mechanical properties were evaluated on 5A-type specimens according to the ISO-527-2 standard ($l_0 = 20$ mm). Film thickness was evaluated at five points with a micrometer. Elongation at break and tensile strength were measured at 20 °C and 60% RH with a DY34 Adamel-Lhomargy device, equipped with a 10 N load cell. The initial grip separation was set at 50 mm, and the cross-head speed was 20 mm·min^{-1}. Each measurement was replicated five times. The statistical analysis of the temperature effect on film mechanical properties was done by a simple randomized ANOVA technique using Statgraphics software. A Duncan's multiple range test was used for the multiple comparison of the means.

FTIR Measurements. Fourier transform infrared spectra (250 scans) were recorded at a resolution of 2 cm^{-1} on a Nicolet Magna IR 550 spectrometer equipped with a liquid nitrogen cooled Mercury−Cadmium−Telluride detector. The instrument was continuously purged with dry air. Transmission spectra were obtained at 20 °C using a cell composed of two CaF_2 windows separated by a 6 μm spacer. All transmission spectra were corrected for the spectral contribution of the aqueous solvent, according to the method of Dousseau et al. (*25*). Spectra of films and film-forming solutions were obtained by attenuated total reflection (ATR) using a single reflection accessory fitted with a thermostated diamond crystal. Correction for the spectral contribution of water was done by applying a subtraction factor to the water spectrum to ensure a constant value (0.7) for the Amide I/Amide II ratio. It was not necessary to subtract any water contribution for the film spectra; the sharp band near 3400 cm^{-1} indicated indeed a negligible contribution of the O−H stretching with regard to the N−H stretching (*26*).

All data manipulations were performed with the Grams/32 software (Galactic Industries Corporation, Salem, NH). The parameters of Fourier deconvolution were chosen after several trials in order to obtain enough band narrowing to see the major components of the amide I band but without introducing significant side-lobes in the 1690−1720 cm^{-1} region where there is no protein band. Transmission spectra of protein solutions have been deconvoluted with a deconvolution filter constant, γ, of 7 and a smoothing filter of 74%. For the infrared ATR spectra of films and film-forming solutions, these parameters have been set to 13 and 90%, respectively.

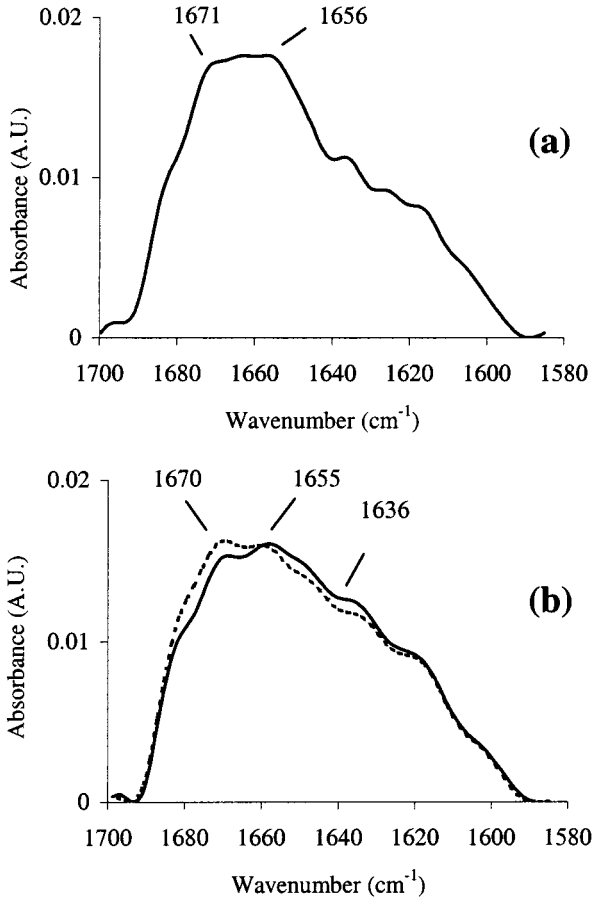

Figure 1. Fourier deconvoluted infrared spectrum of (a), a gliadins solutions (7% w/w) at pH 11 (transmission); (b), gliadins film-forming solutions (16% w/w) at pH 11, without (—) and with (---) diethyleneglycol as a plasticizer (ATR).

RESULTS AND DISCUSSION

Protein Conformation in Film-Forming Solutions. Figure 1a displays the deconvoluted infrared spectra of a gliadins solution (7% w/w) at alkaline pH in the amide I band region (1585−1700 cm^{-1}). This band is mainly due to the stretching vibrations of the carbonyl groups and is representative of the secondary structure of the proteins (*27*). It is composed of several overlapping components, with two prominent features at 1671 and 1656 cm^{-1}. The 1671 cm^{-1} band can be assigned to the β-turn conformation (*28*): the repetitive domains of gliadins, rich in proline residues (about 30%), are indeed supposed to contribute to these turns (*29*). Moreover, the gliadins are very rich in glutamine residues (30−50%), and some authors (*30*) attributed the band at 1671 cm^{-1} to the contribution of glutamine side-chain residues. However, in the case of ω-gliadins, Popineau et al. (*19*) showed that the spectral contribution of glutamine side-chain residues remained fairly weak. The band at 1656 cm^{-1} is assigned to α-helical conformation (*18* and *19*), though some contribution of random coil is also possible (*31*). The presence of some α-helical conformation is generally attributed to the nonrepetitive

domain of the gliadins (*16*). The minor features at 1682, 1636, 1625, and 1619 cm^{-1} are highly characteristic of β-components presenting differences in hydrogen bonding between β-strands (*13* and *32*). This spectrum is in good agreement with previous infrared data concerning acetic acid solutions of gluten or purified gliadins (*18* and *19*).

In film-forming conditions, i.e., at higher protein concentration (16% w/w) (Figure 1b), the major features remained at the same wavenumbers (1670 and 1655 cm^{-1}). The significant difference is a growing contribution of the 1636 cm^{-1} band, representative of β-sheets (*27*).

The influence of the plasticizer on protein conformation was investigated. In protein films, plasticizers (usually of polyol type) are required to reduce the brittleness of the material and are then incorporated in the film-forming solution (*2* and *11*). In this case, diethylene glycol was chosen as a plasticizer (Figure 1b). It is clear that both spectra present the same features, the slight shift observed being smaller than the spectral resolution. Therefore, the plasticizer does not play any role on protein secondary structure in the film-forming solution. Because water also acts as a plasticizer by creating hydrogen bonds with the proteins, these results could be expected. However, they differ from those of a previous work (*13*), in which an increase of the α-helical content of glycinin in the presence of ethylene glycol was noticed. The authors attributed this effect to both the high dipole moment of ethylene glycol and its low dielectric constant, supposing that the interaction of the plasticizing agent with glycinin would lead to some rearrangement of the protein toward a more stable conformation. In the case of gluten proteins, no structural changes were observed in the α-helical domain. These helices had been attributed to the nonrepetitive domain of the gliadins; their stability was previously described (*16*), as circular dichroism studies enlightened a low sensitivity to temperature changes. In the case of wheat gliadins, the interactions of proteins with the plasticizing agent, therefore, did not involve structural rearrangements.

Evolution of Protein Conformation during Drying. The evolution of protein conformation during the drying, at the 70 °C temperature used in a previous work (*2*), of film-forming solution was followed by infrared spectroscopy (Figure 2). The resolution of the amide I band is clearly improving with time; the main feature of this evolution is the replacement of the 1626 cm^{-1} shoulder by a well-defined band at 1622 cm^{-1}; this peak features the appearance of β-sheets presenting a high level of hydrogen-bonding. From the appearance of a shoulder at 1693 cm^{-1}, a splitting of the β-sheet band could be suggested, indicating an antiparallel orientation of these sheets (*33*). β-turns are present during the whole drying kinetics, as revealed by the presence of the shoulder at 1670 cm^{-1}. The band at 1653 cm^{-1} featuring α-helices and random coil structures was not affected by the drying kinetics. Spectra corresponding to intermediate steps of drying (more particularly after 30 min of drying) present a better resolution, revealing the appearance of a band near 1636 cm^{-1}, attributed to the presence of β-sheets. The drying step from the film-forming solution to the film consequently induces major changes of the protein conformation: the departure of water leads to some rearrangement in the hydrogen-bonding pattern, influencing the secondary structure of the proteins.

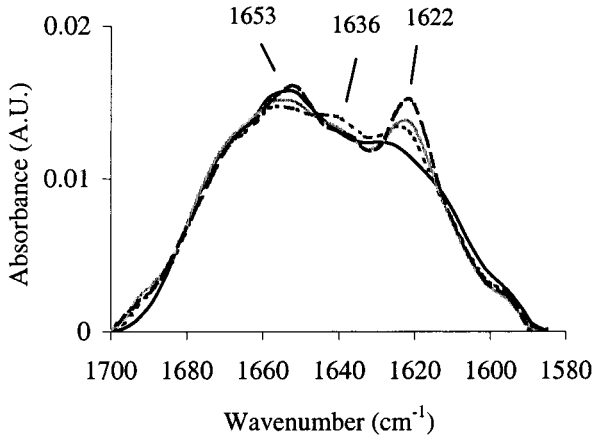

Figure 2. Fourier deconvoluted infrared spectra (ATR) of gliadins film-forming solutions (16% w/w) at pH 11 during drying at 70 °C: — (solid line), initial time; ····· (dotted line), after 30 min; (shaded line), after 45 min; – – (dashed line), after 60 min (film state).

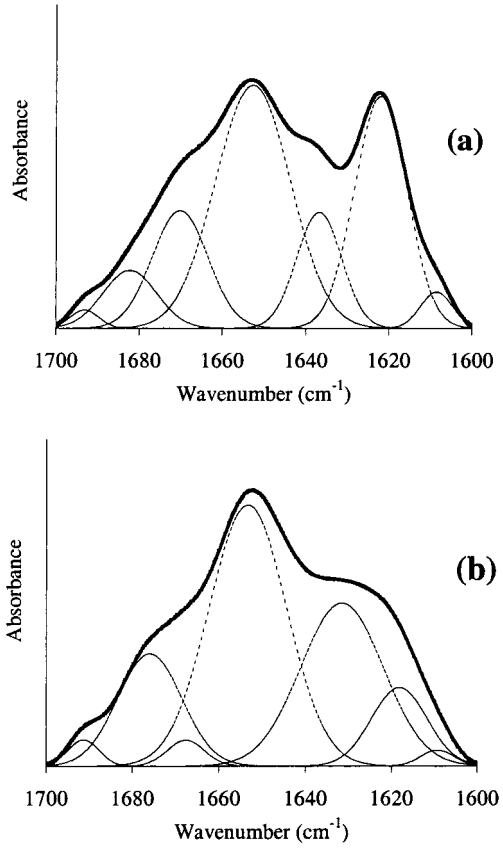

Figure 3. Fourier deconvoluted amide I band of gliadin films fitted with sums of Gaussian bands: (a), film dried at 70 °C, (reduced $\chi^2 = 6.74$, $r = 0.99750$, standard error = 0.00003725); (b), film dried at 25 °C, (reduced $\chi^2 = 30.4$, $r = 0.99627$, standard error = 0.00004713).

From the last spectrum of the kinetics, protein conformation in the film state is characterized by two major bands at 1653 and 1622 cm^{-1}. Band fitting with Gaussian band-shapes was performed on this spectrum to estimate the contents of various protein conformations (Figure 3a and Table 1). Though the curve fitting is subject to some errors, it remains very useful to compare the evolution of single components of the amide I band. As in the film-forming solution, the major band

Table 1. Positions, Widths, and Relative Areas of the Bands Fitted to the Fourier-Deconvoluted Spectra of Gliadins Films Dried at Different Temperatures

Drying Temperature (°C)																	
25			40			50			60			70			90		
pos (cm^{-1})	width (cm^{-1})	rel. area (%)	pos (cm^{-1})	width (cm^{-1})	rel. area (%)	pos (cm^{-1})	width (cm^{-1})	rel. area (%)	pos (cm^{-1})	width (cm^{-1})	rel. area (%)	pos (cm^{-1})	width (cm^{-1})	rel. area (%)	pos (cm^{-1})	width (cm^{-1})	rel. area (%)
1691	8.4	1	1691	8.5	2	1692	8.6	1	1694	7.2	1	1693	8.0	1	1693	8.0	1
1676	17.5	13	1681	14.8	7	1682	15.2	6	1682	14.1	7	1682	15.2	7	1682	15.2	8
1668	10.1	17	1667	18.9	19	1667	21.3	24	1668	15.6	17	1670	15.7	14	1669	16.2	15
1653	21.1	36	1652	14.0	28	1652	14.4	27	1652	15.8	33	1653	21.1	39	1653	21.1	39
1631	22.5	24	1637	18.8	23	1639	17.4	19	1637	12.2	14	1636	12.7	11	1637	12.0	10
1618	15.2	8	1622	16.6	20	1624	16.8	20	1622	15.4	27	1622	14.8	26	1623	14.1	24
1609	9.0	1	1610	10.4	1	1611	10.3	3	1609	9.1	2	1609	9.1	2	1609	9.5	3

Table 2. Mechanical Properties and Compositions of Gliadins Films Dried at Different Temperatures

drying temperature (°C)	maximal stress (MPa)	elongation at break (%)	residual plasticizer (%)	residual water (%)	protein content (%)	soluble protein/total protein
25	0.61 ± 0.06[a] a[b]	1156 ± 86 a	21	11.8	58.7	0.57
40	0.64 ± 0.05 a	1070 ± 89 ab	21	11.1	59.3	0.55
60	0.95 ± 0.10 b	1044 ± 80 b	21	10.5	58.7	0.40
70	1.14 ± 0.13 c	873 ± 73 c	21	11.1	59.1	0.35
80	2.49 ± 0.25 d	429 ± 43 d	21	11.1	59.5	0.21
90	2.29 ± 0.21 d	460 ± 77 d	20	11.4	59.5	0.19

[a] Values represent the mean of five replicates. [b] The mean values having the same letters do not differ statistically ($p < 0.05$) according to Duncan's multiple-range test.

at 1653 cm^{-1} (39% of the total protein secondary structures) is attributed to α-helix and random coil (*17*). In accordance with previous results, the combination of bands at 1622 and 1693 cm^{-1} is assigned to antiparallel intermolecular hydrogen-bonded *β*-sheets and features the presence of protein aggregates (*13, 31, 34*). These aggregates are found to contribute to about 26% of the signal. The 1670 cm^{-1} band, representing 14% of the total amount of structures, was assigned to *β*-turns, whose importance can be related to the large number of proline residues in the repetitive domain of gliadins (*29*). The band at 1609 cm^{-1} is related to NH_2 scissoring from the glutamine side-chains (*30*). Other bands in the spectrum are assigned to *β*-sheets, i.e., 1636 and 1682 cm^{-1} (respectively 11% and 7% of the total secondary structures).

It is interesting to notice that the general features of the gliadins film spectrum are very close to those of wheat gluten or purified gliadins in their hydrated state (*18* and *19*). In this study, the same intermolecular associations giving rise to a *β*-sheet band at low wavenumbers were observed.

Furthermore, it is clear that this particular band at 1622 cm^{-1} is more generally observed in protein networks, as it also appeared in glycinin (*35*), *β*-conglycinin (*36*), and *β*-lactoglobulin (*37*) gels, as well as in glycinin films (*13*). This suggested a special role of intermolecular hydrogen bonds as stabilizing interactions for the network. As glycinin proteins are globular in the native state and do not present any repetitive domain, it is assumed that repetitive sequences of wheat gliadins are not necessary to generate intermolecular *β*-sheets aggregates, which are mainly induced by protein−protein interactions during the filming process. Concerning high molecular weight glutenins, it has been previously shown (*38*) that their nonrepetitive domains (which are analogous to those of gliadins) may also present *β*-sheets in the hydrated state.

Influence of the Drying Temperature on Protein Conformation in Film. The influence of high-temperature posttreatments on the mechanical properties of proteins films has already been described (*11* and *12*). However, in most of these studies, the films were previously dried at ambient temperature; the influence of drying temperature on film properties and on protein conformation was never investigated. In our case, the drying temperatures of gliadins film-forming solutions were set to various values (from 25 to 90 °C) and the corresponding infrared spectra of the films were recorded and band-fitted (Table 1).

All spectra recorded between 40 and 90 °C presented bands at the same positions, indicating that the secondary structures of the proteins were similar in type. The relative areas of these bands were, however, quite different, depending on the thermal treatment. Among the main features, it is interesting to notice that an increase of the drying temperature from 40 °C to 90 °C induced a small rise (from 20 to 24%) of the 1622−1624 cm^{-1} band relative to aggregates of hydrogen-bonded *β*-sheets, concomitant with a decrease of the *β*-sheet band around 1637 cm^{-1} (from 23% to 10%). The 1652 cm^{-1} band underwent an increase from 60 °C to 90 °C (from 33 to 39%); though this band was first assigned to α-helices, the contribution of random coil structures cannot be ruled out, more particularly at higher temperatures (above 70 °C), when the band became broader (up to 21.1 cm^{-1}). The *β*-turns feature (near 1670 cm^{-1}) drastically decreased after having reached a maximum at 50 °C (from 24% at 50 °C to 15% at 90 °C). From these results, it seems that the higher-temperature treatments favor α-helices (and/or random coil structures) as well as hydrogen-bonded *β*-sheets at the expense of other sheets and turns.

Concerning the film dried at 25 °C (Figure 3b), the distribution of the various types of *β*-sheets was slightly modified: two strong features were noticed at 1676 cm^{-1} (13% of the structures) and 1631 cm^{-1} (24%), instead of at 1682 and 1637 cm^{-1} for higher temperatures; the shifts of both bands to lower wavelengths reveal a different distribution of the hydrogen-bonding pattern.

The major feature (36% of the secondary structures) remained the band at 1653 cm^{-1}, attributed to α-helix; some spectral contribution of β-turns (17% at 1668 cm^{-1}) was also noticed. Concerning the strongly hydrogen-bonded β-sheets, a shift to lower wavelengths (1618 cm^{-1} instead of 1624 cm^{-1}) was observed, but the important feature is the small relative area of this peak (8%). This indicates a very low contribution of the β-sheets aggregates in the protein structure when the films were dried under mild conditions.

It is clear that the proportion of the aggregates increases (from 8% to 24−26%) when the drying temperature increases; higher drying temperatures may then favor the rearrangements of the proteins into aggregates during film formation.

The mechanical properties of films dried at different temperatures were also tested. All films presented the same global composition, i.e., similar protein, water, and plasticizer contents (Table 2), but their mechanical properties were clearly influenced by the drying temperature: as the temperature increased, there was a significant rise of the tensile strength of the films (from 0.61 to 2.29 MPa), together with a decrease of their elongation at break (from 1156 to 460%). Moreover, there was a clear decrease of protein water solubility (from 0.57 at 25 °C to 0.19 at 90 °C) when the drying temperature increased. As these differences could not be attributed to changes in film composition, it appears then quite clearly that some network strengthening was induced by the thermal treatment. Similar changes in mechanical properties have been previously observed, when heating was used as a posttreatment after film drying at ambient temperature (*11* and *12*); in some cases, a decrease of protein solubility was also noticed (*39*). These modifications were attributed to some heat-induced aggregates formation; the authors suggested that the aggregates could involve hydrogen bonds as well as disulfide bonds. The occurrence of these disulfide bridges was also clearly demonstrated when gluten film-forming solutions were pretreated at 75 or 95 °C (14). In the case of wheat gliadins, we clearly underlined the role of hydrogen bonds in the appearance of protein aggregates after a heat treatment; nevertheless, the occurrence of disulfide bridges cross-linking cannot be ruled out.

This study clearly enlightened our understanding of the occurrence of intermolecular hydrogen-bonded β-sheets aggregates in wheat gliadins during the drying step of film formation. In previous papers, the respective influence of disulfide bridges and hydrogen bonds on the mechanical properties of films was not clearly elucidated. From our study, it could be claimed that a higher density of hydrogen bonding, induced by thermal treatment, might play a major role in network formation and on film properties.

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Received for review August 8, 2000. Revised manuscript received November 6, 2000. Accepted November 6, 2000. This research was funded by the European Community in the frame of "Gluten Biopolymer" (FAIR CT 96 1979).

JF0009899