Gluten Viscoelasticity Is Not Lipid-Mediated. A Rheological and Molecular Flexibility Study on Lipid and Non-Prolamin Protein Depleted Glutens

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Wheat flours differing in their lipid and non-prolamin protein (NPP) contents were obtained by chloroform or Lubrol PX extraction. Starch was hand-washed out of the flour, and the resulting glutens were studied by dynamic rheology and electron spin resonance spectroscopy (ESR) spin probing and spin labeling. Compared to control gluten, the storage modulus of NPP—including lipoprotein—depleted gluten is unchanged, and the loss modulus is not significantly different. The mobility of spin probes differing in size in the gluten aqueous phase suggests the existence of at least two compartments: a network with a mesh size of about 0.8 nm and larger water pockets. These compartments are present in all glutens. The spectra resulting from ESR spin labeling experiments are composite, reflecting the contribution of at least two types of polypeptides differing in flexibility. The population of mobile spin labels linked to the lysine residues is much larger in control gluten compared to NPP depleted gluten, indicating the lysine residues of these proteins are in a less flexible environment. On the contrary, only a slight difference is observed between the various samples when cysteine residues are labeled. These results suggest that NPP and lipids behave only as fillers in the prolamin network.

Keywords: *Wheat proteins; lipid-binding proteins; viscoelastic properties; ESR; spin probing; spin labeling*

INTRODUCTION

Wheat prolamins—glutenin and gliadins—comprise the major wheat flour proteins, but the other proteins, accounting for 10–20% of the total protein contents, are important enough to be worth considering. These non-prolamin proteins (NPP) are composed of soluble and insoluble macromolecules whose main biological function is not nitrogen and carbon storage. For example, enzymes and enzyme inhibitors are the main components of the soluble proteins, also known as albumins and globulins, while the insoluble NPP are part of supramolecular structures (membrane proteins, nucleoproteins, cell wall proteins, etc.). Proteins presenting a high affinity for lipids are found both in the insoluble (membrane proteins) and in the soluble NPP fractions (lipid transfer proteins, puroindolines, ligoline, thionins, etc.) (Carr et al., 1992; Désormeaux et al., 1992; Marion, 1992).

From a technological point of view the overall role of these proteins in flour quality is still obscure, although the examination of some specific non-prolamin proteins has revealed some probable relationships to the functionality of flour (Wrigley and Bietz, 1988; Basuki et al., 1991; Payne et al., 1985). These proteins may facilitate sulfhydryl–disulfide interchange (Prieto et al., 1993) and have been found in disulfide-linked aggregates (Gupta et al., 1991; Okada et al., 1987; Kobrehel et al., 1991). Other proteins, including lipid-binding proteins, would be involved in the stabilization of air– or oil–water interfaces in bread doughs and cake batters (Marion, 1992; Wilde et al., 1993).

It is generally assumed that soluble proteins are extracted with starch during hand kneading, although this is not actually correct. Thus, some soluble and insoluble non-prolamin proteins are found in gluten, the impact of which, on gluten viscoelasticity, is not clear. Lipid-mediated aggregates comprising storage proteins were isolated from flour and gluten, and their implication in bread-making quality was postulated (Bekes et al., 1992).

In this paper, we used an extraction procedure that has been recently developed to prepare glutens with only traces of NPP and lipids by using nonionic detergents (Blochet et al., 1991). We present results on the rheology of lipid and NPP depleted glutens to define any incidence of these components on gluten viscoelasticity. Electron spin resonance (ESR) spin probing and spin labeling enabled us to investigate the liquid phase of hydrated glutens and the contribution of NPP to the average polypeptide flexibility of hydrated gluten proteins.

EXPERIMENTAL PROCEDURES

Sample Preparation. The control flour was from a French common wheat, variety Aubaine, of good bread-making quality. Samples were submitted to different treatments.

Defatting of the flour was carried out at room temperature by two successive chloroform extractions for 2 h with a 1:5 w/v ratio. The flour samples were dried under a hood until the chloroform vapors had totally disappeared.

NPP were extracted from control and chloroform defatted flours by three successive 2-h gentle mixings in phosphate

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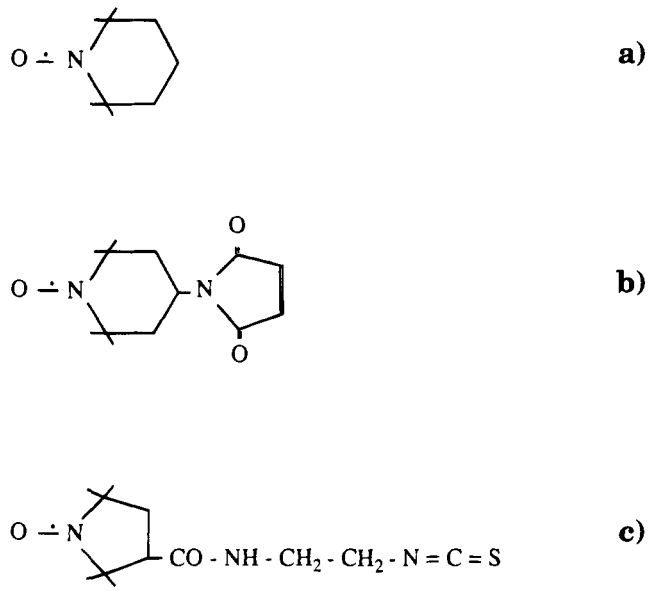

Figure 1. Chemical structures of the nitroxide radicals employed: (a) TEMPO; (b) 4-maleimido-TEMPO; (c) 3-(2-isothiocyanatoethylcarbamoyl)-PROXYL.

buffer (0.1 M, pH 7.8, 0.1 M KCl, 5 mM EDTA) and centrifugations (30 min, 5000*g*). The albumins and globulins are the main components extracted by the first treatment, carried out at 4 °C. Lipid-binding proteins and lipids are extracted at room temperature in the last two extractions by adding 2% of Lubrol PX. This detergent consists of a polyoxyethylene polar head and a hydrophobic aliphatic chain and exists as a micellar solution in water above its critical micellar concentration (cmc). This nonionic detergent is used to extract amphiphilic transmembrane proteins (Hjelmeland, 1990).

Gluten was extracted from the flours after the different treatments by extensive hand-kneading with deionized water to remove the detergent. The glutens were then freeze-dried and ground to a powder. The resulting samples are named by code: gluten from chloroform extracted flour (CG); gluten from Lubrol extracted flour (LG); and gluten from chloroform and Lubrol extracted flour (CLG).

Biochemical Characterization. The protein composition of the glutens was analyzed by SDS–PAGE on a 10–20% gradient polyacrylamide gel (Popineau and Pineau, 1985). The relative content of low molecular weight NPP was determined on the densitograms of the gels, by determining the absorbance at 600 nm of the 14 000–16 500 molecular weight bands relative to the sum of the protein absorbance. These measurements yield approximate values and enable us to give a quantitative estimate of the differences observed visually on the electrophoresis gel (Fullington et al., 1980). No repeats were made.

The fatty acid content of the flour was determined by transmethylation of lipids in dry gluten with methanol–sulfuric acid (98:2 v/v) followed by hexane extraction (Welch, 1977). Quantification of the fatty acid methyl esters was carried out by gas chromatography with margaric acid as an internal standard.

Rheological Measurements. Dynamic measurements in shearing were performed as in Cornec et al. (1994), on a Carri-Med CSL100 constant stress cone-plate rheometer. Gluten powder (300 mg) was rehydrated with deionized water (10 mL) for an hour and then centrifuged (500*g*, 15 min). Samples were rested for an hour and then placed in the rheometer and covered with water to avoid evaporation. After an hour of rest, the measurement was started. A 3% strain amplitude was applied, with a frequency sweep from 10^{-3} to 36 Hz at a temperature of 20 °C. Four repeats were performed on samples from the CLG batch to control the repeatability of the procedure.

ESR Measurements. The nitroxide radicals used are shown in Figure 1; they were purchased from Aldrich Chemicals (Strasbourg, France). The samples were prepared by saturating (300%) gluten powder with a nitroxide spin radical aqueous solution (0.1–0.5 mg/mL) and gently hand-mixing. In spin probing, the samples were left to rest 30–60 min and then placed in an ESR quartz tissue cell, and the spectra were recorded. In spin labeling, the samples were left to incubate for about 2 h and then extensively dialyzed (48–60 h) against distilled water with gentle mechanical agitation. The ESR spectra were recorded after the sample had rested for about 30 min. The samples were inserted in an ESR quartz tissue cell. Measurements were carried out on a Varian E9 spectrometer linked to a tracer. The scan width was 100 G. The spectra were often composite, resulting from at least two populations of nitroxide radicals, one fast moving ($\tau_c < 10^{-8}$ s) and one less mobile ($\tau_c > 10^{-8}$ s). The rotational correlation time (τ_c) was calculated from features of the fast moving nitroxide radicals according to the theory developed by Freed and Fraenkel (1963), as described in Hargreaves et al. (1994a). The relative importance of less mobile spin labeled residues was estimated by the ratio $R = i/m$, where i and m are the amplitudes of the low field peaks of the slow and fast moving radicals, respectively.

ESR measurements were performed at room temperature in three replicates. The results presented are the averages and standard deviations of τ_c and R.

Table 1. Biochemical Characteristics of the Gluten Samples

	gluten from			
	control flour	defatted flour	Lubrol extracted flour	defatted Lubrol extracted flour
protein content[a]	85.3 ± 1.1	86.9 ± 0.5	84.4 ± 1.5	84.1 ± 2.0
% NPP[b]	100	85	23	19
lipid content	6.6 ± 0.3	0.49 ± 0.01	0.145 ± 0.003	0.062 ± 0.004

[a] Determined by Kjeldahl method with a factor 5.7. [b] NPP, nonprolamin proteins determined by densitometer on SDS–PAGE in the 14 000–16 500 molecular weight band; no replicas. The control gluten was used as reference.

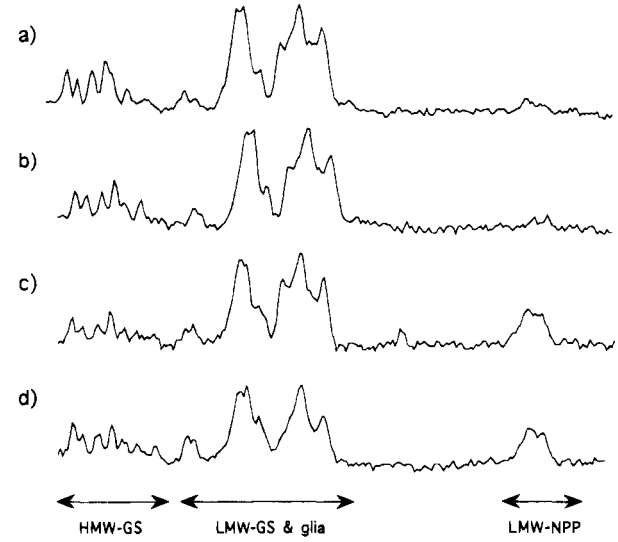

Figure 2. Densitograms obtained from 10–20% gradient SDS–PAGE in reducing conditions, stained with Coomassie blue: (a) gluten from chloroform and Lubrol extracted flour; (b) gluten from Lubrol extracted flour; (c) gluten from chloroform extracted flour; (d) control gluten. HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; glia, gliadins; LMW-NPP, low molecular weight non-prolamin proteins (about 14 000–16 500).

RESULTS AND DISCUSSION

Compositions of the Glutens. Table 1 presents the compositions of the glutens. The protein contents of the different samples are not significantly different, with

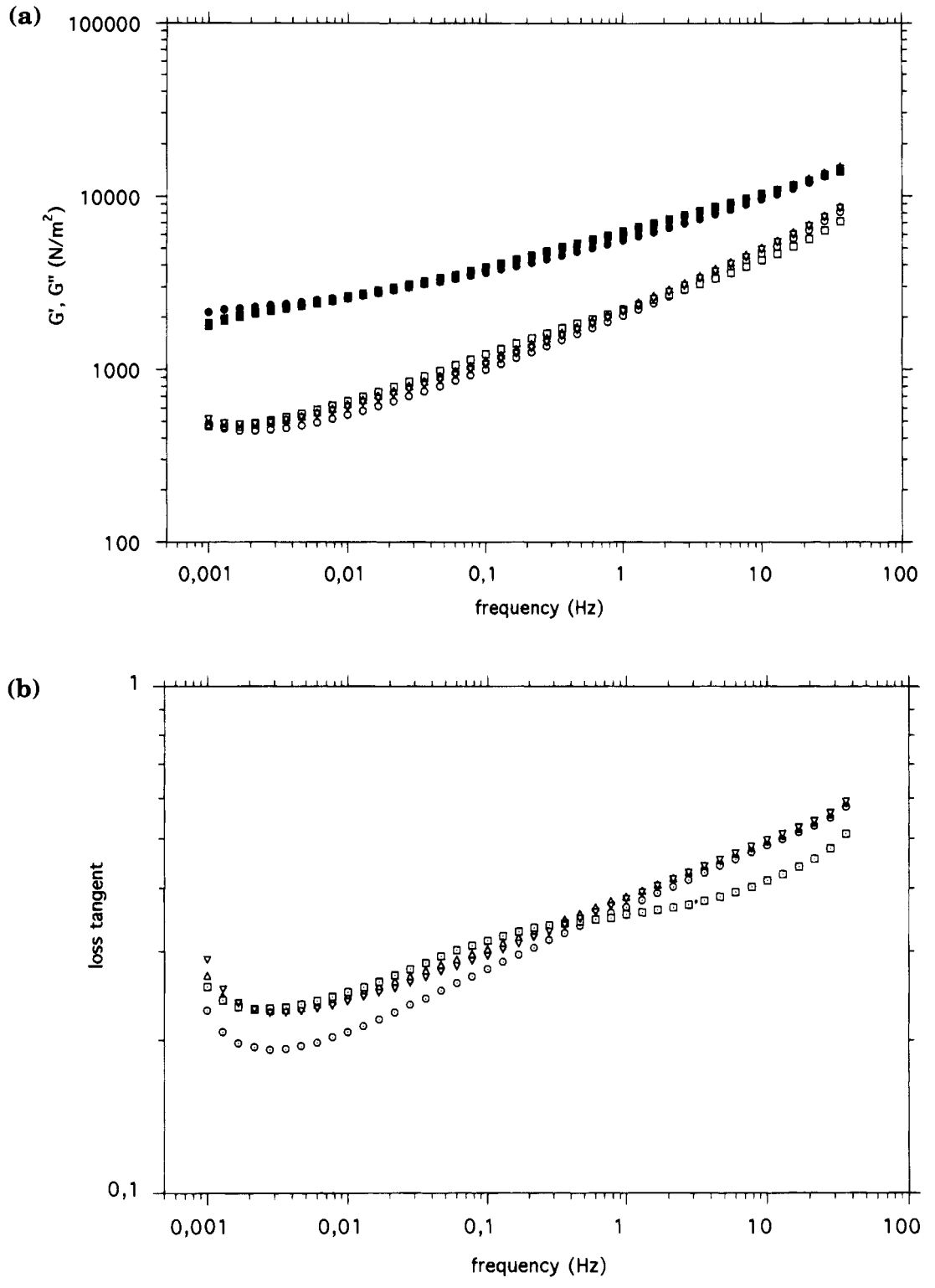

Figure 3. Mechanical spectra of the totally hydrated glutens obtained by frequency sweep in a cone-plate rheometer: □, control gluten; ○, gluten from chloroform extracted flour; △, gluten from Lubrol extracted flour; ▽, gluten from chloroform and Lubrol extracted flour. (a) G' and G'' are the storage and loss moduli, respectively. Solid symbols represent G' and open ones G''. (b) Loss tangent, representing the ratio G''/G'.

an average protein content of 85%, which is a high value due to the thorough hand extraction. The chloroform defatted samples (CG) contained less than 10% of the amount of lipids found in control gluten, and the chloroform and Lubrol extracted samples (CLG) contained only 1% of this amount. The non-prolamin proteins (NPP) are only very partially extracted by the chloroform treatment of flour. On the other hand, they are extracted very efficiently by the nonionic detergent, as can be seen on the densitometric profiles (Figure 2).

In a previous work, the same treatments were applied to glutens instead of flour. The extraction of the NPP by the nonionic detergent was then much less efficient, and the content in residual lipid was higher (Hargreaves et al., 1994a). This underlines the greater difficulty in extracting components from gluten rather than from flour. This could be due to the reorganization of the prolamin proteins upon hydration and hand-kneading of flour, forming a network that traps different components and limits the diffusion of extraction solvent. This

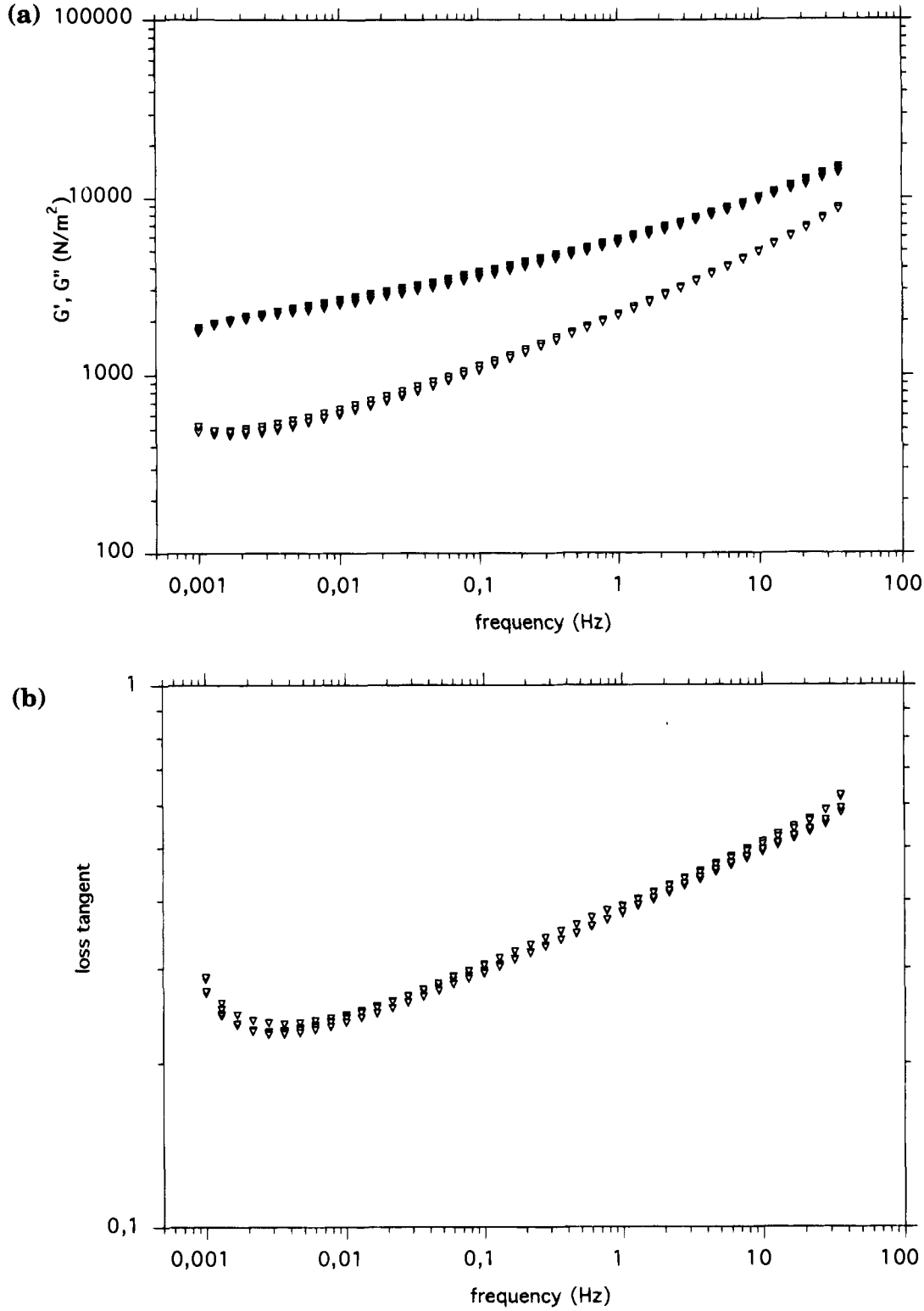

Figure 4. Repeatability of the rheological test. Mechanical analysis was carried out on four gluten samples from chloroform and Lubrol extracted flour: (a) G' (solid symbols) is the storage modulus and G'' (open symbols) the loss modulus; (b) loss tangent, representing the ratio G''/G'.

effect is amplified as the concentration of the proteins increases from about 10% of the dry matter in flour to 80% in gluten.

Rheological Behavior of the Glutens. The mechanical spectra of the four glutens are shown in Figure 3, while Figure 4 shows the repeatability of the determination, being four measurements on the same type of gluten. All of the glutens exhibit the same type of viscoelastic behavior, whatever the conditions of preparation. This behavior in which the storage (G') and loss (G'') moduli present a minimum is typical of a transient network (Cornec et al., 1994).

The NPP depleted glutens (LG and CLG) have slightly higher G'' and lower G' values at low frequencies compared to the chloroform defatted sample (CG). This is more easily observed when one considers the tangent of the loss angle ($\tan \delta = G''/G'$). The control gluten—that is not defatted—also presents a slight difference in the features of the $\tan \delta$ curve, which has a sigmoid shape, resulting mainly from a variation in

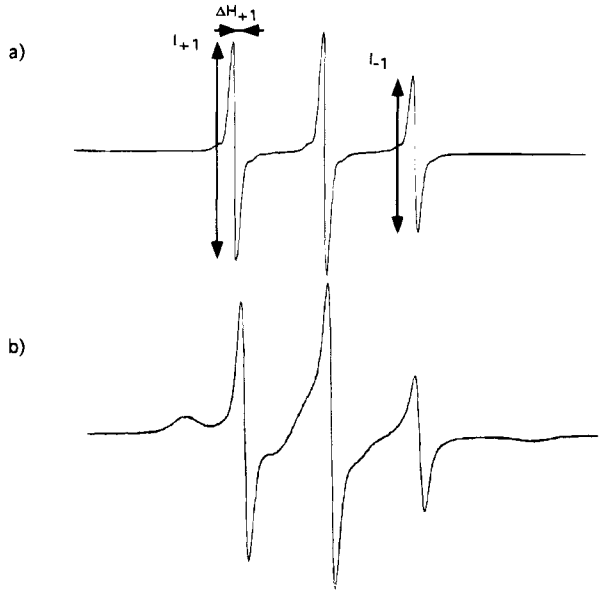

Figure 5. ESR spin probing spectra of fully hydrated defatted and non-prolamin protein depleted gluten: (a) with TEMPO; (b) with 4-maleimido-TEMPO. The spectral features shown are used for calculating the rotational correlation time.

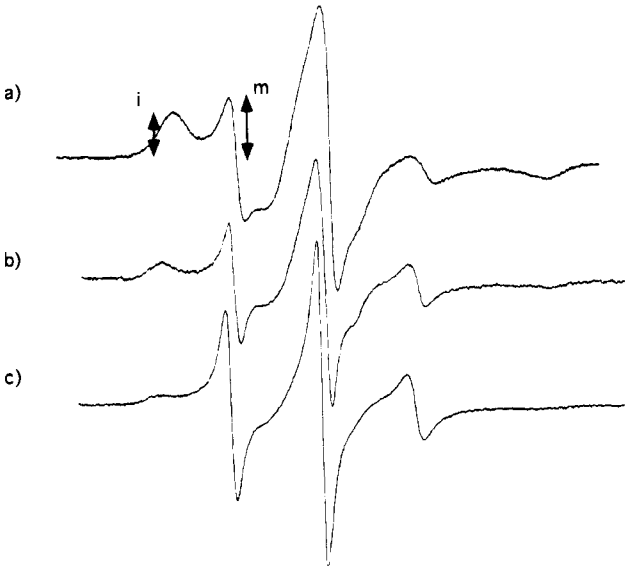

Figure 6. ESR spin labeling spectra: (a) labeling of cysteine residues in fully hydrated defatted and non-prolamin protein depleted gluten; (b) labeling of lysine residues in fully hydrated defatted gluten; (c) labeling of lysine residues in fully hydrated defatted and non-prolamin protein depleted gluten. The lines marked "i" and "m" are the low field lines corresponding to the immobile and mobile spin radicals, respectively.

the viscous component G'' (Figure 3). These features were confirmed by a replicate and definitely attributed to lipids as they were observed on gluten with a reduced NPP content, but still containing lipids, obtained by a buffer extraction with no nonionic detergent (results not presented). This shape may be due to a filling phenomenon (van Vliet, 1988). Indeed, the lipids form vesicles in the gluten network (Marion et al., 1987; Carr et al., 1992) that could act as a filler. The variation in the viscoelasticity of the system would only be slight because of the low content in lipid and the deformability of the lipid vesicles.

However, the variation of the G' and G'' values between samples never exceeded 10%. This is negligible when compared to that between samples differing in gliadin/glutenin ratio or in glutenin polymer composition (Cornec et al., 1994; Popineau et al., 1994). These authors worked in conditions similar to ours, with gluten subfractions differing in solubility and gluten from near-isogenic wheat lines differing in their composition in high molecular weight glutenin subunits. They found that the values of G' and G'', at low frequencies, vary by a factor 10. This suggests that the NPP may contribute to the system in a passive way but do not interfere in the organization of the glutenin polymers responsible for the rheological properties of gluten.

These results show that the viscoelasticity of fully hydrated gluten at 20 °C is determined mainly by the gliadin/glutenin ratio and the composition of glutenin. The chloroform and Lubrol extraction procedures used do not modify the rheological functionality of the prolamin proteins in a noticeable way. Furthermore, lipids do not play the role of plasticizer in the water-saturating conditions of our gluten (about 62%), confirming the idea that lipids form vesicles that are embedded in the gluten (Marion et al., 1987).

Characterization of the Liquid Phase of Gluten Systems. ESR spin probing consists in adding a compound with a paramagnetic moiety to the liquid phase of the system. The recorded spectrum reflects the mobility of this compound. The signal of the fraction of compound interacting covalently with components in the gluten system is considered to be negligible compared to the signal of the main population of spin probe. If the spin probe can reach several environments, the ESR spectrum will be of a composite type, resulting from the superimposition of the signals from the different populations of the spin probe. This is the case for the spectra obtained with TEMPO spin probing of gluten from nondefatted flour, which presents a splitting in the high field line (figure not presented), indicating there are two populations of probes differing in the polarity of their environment. This result has been discussed previously (Pearce et al., 1988; Hargreaves et al., 1994a). These authors concluded that the splitting corresponded to populations of TEMPO in an aqueous phase and in the intrinsic lipid phase of the gluten. In all of the defatted glutens (CG, LG, and CLG) the high field splitting is not present (Figure 5), confirming previous results.

Table 2. ESR Spin Probing of Gluten Samples

		gluten from			
		control flour	defatted flour	Lubrol extracted flour	defatted Lubrol extracted flour
TEMPO	τ_c (10^{-10} s)	1.34 ± 0.10	1.45 ± 0.10	1.46 ± 0.13	1.34 ± 0.10
4-maleimido-TEMPO	τ_c (10^{-10} s)	5.20 ± 0.50	4.85 ± 0.60	5.03 ± 0.30	4.94 ± 0.20
	R	0.10 ± 0.05	0.11 ± 0.02	0.06 ± 0.01	0.082 ± 0.01
3-(2-isothiocyanatoethylcarbamoyl)-PROXYL	τ_c (10^{-10} s)	nd[a]	3.23 ± 0.15	3.38 ± 0.10	3.38 ± 0.18

[a] Not determined.

Table 3. ESR Spin Labeling of Gluten Samples

		gluten from		
		defatted flour	Lubrol extracted flour	defatted Lubrol extracted flour
4-maleimido-TEMPO[a]	R	0.96 ± 0.24	0.79 ± 0.03	0.85 ± 0.10
3-(2-isothiocyanatoethylcarbamoyl)-PROXYL[b]	τ_c (10^{-10} s)	10.5 ± 0.5	10.1 ± 0.5	9.7 ± 0.5
	R	0.29 ± 0.03	0.10 ± 0.03	0.12 ± 0.01

[a] Spin labeling of cysteine residues. [b] Spin labeling of lysine residues.

The rotational correlation time (τ_c) calculated from the features of the TEMPO spectra of the samples in the aqueous environment were of the order of 1.4×10^{-10} s. There is no significant difference among the different types of samples.

The R value varied with the size of the probe (Table 2), suggesting that the aqueous phase is distributed among different types of compartments. The smallest and largest spin probes, TEMPO and 3-(2-isothiocyanatoethylcarbamoyl)-PROXYL, respectively, have a single population of mobile radicals, while the spin probe of intermediate size, 4-maleimido-TEMPO, is divided into two populations differing in mobility. Thus, it may be that there is an environment with a mesh size close to that of the 4-maleimido-TEMPO (about 0.76 nm). In this mesh the smaller spin probe remains mobile, while the larger spin probe is excluded and remains in larger water cavities, such as observed in microscopy (Hermansson and Larsson, 1986). This suggestion is confirmed by other experiments carried out on a series of gluten fractions with different compositions in prolamin proteins (Hargreaves et al., 1994c). R values and solvent viscosities (as measured by τ_c) are not significantly altered by defatting the flour or extracting the NPP, so the organization of the gluten system seems to be governed only by the prolamin proteins.

Molecular Properties of the Glutens. Cysteine residues are selectively spin labeled with the maleimide derivative and lysine residues by the isothiocyanate derivative (Figure 1). This last spin label was chosen because of its relatively long chain. Labeling with 4-isothiocyanato-TEMPO, a derivative with a shorter arm, gives rise to powder-type spectra, indicating that the lysine residues labeled are in a particularly rigid environment (Hargreaves et al., 1994a). In these experiments the ESR spectra of both labels are of a composite type, reflecting the existence of at least two populations of radicals with different mobilities (Figure 6). The R values of the labeled cysteine residues are not significantly modified by extraction of the NPP, although the values of the standard deviation are quite high (Table 3). Other results on similar samples show a slightly higher R value for control glutens compared to NPP depleted glutens (Hargreaves et al., 1994b). The removal of the NPP strongly decreases the R value of glutens when the lysine residues are labeled though the motion of the mobile population does not change significantly (Table 3). These observations could be due to one of two possibilities: either the NPP contribute to the protein network responsible for the rheological properties, and this will affect the molecular motions recorded, or the NPP make a direct contribution to the immobile component of the signal, the spin labels reacting with these small proteins being less mobile than the spin labels on the storage proteins. This second hypothesis is most likely, as we observed that their is no variation in rheological properties of the glutens upon depletion of the NPP. Furthermore, the heating behavior of cysteine spin labeled glutens is not modified by extraction of the NPP (Hargreaves et al., 1994b).

Conclusion. Two complementary methods were used to study protein–lipid–water organization in gluten. Dynamic rheology gives information about the bulk behavior of the system, while ESR spectroscopy enables us to study the molecular motions. The proportion of immobile spin labeled lysine residues decreases in non-prolamin protein (NPP) depleted glutens. The proportion of immobile spin labeled cysteine residues also seems to decrease slightly. These observations suggest that a high proportion of lysine residues of the NPP are in a very rigid environment; this proportion is even greater than in the prolamins. The cysteine residues of the NPP seem to be scarcely labeled or to present a proportion of immobile population very close to that of prolamins. On the other hand, lipid and NPP removal did not change the mobility of water soluble probes. In all samples, the aqueous phase was distributed in at least two categories of compartments differing in size. The viscoelastic properties of gluten were not significantly altered by extracting lipid and NPP. These results indicate that lipids and NPP do not participate significantly in the rheological functionality of hydrated gluten networks.

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