Molecular flexibility in wheat gluten proteins submitted to heating

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Abstract Prolamin proteins are responsible for the network that gives wheat dough its viscoelastic properties. Non-prolamin depleted gluten was prepared under conditions that preserve its functionality. Electron Spin Resonance (ESR) was used to provide information about the dynamics of the protein at temperatures between 5 and 90°C by specific spin labelling of its cysteine residues. The spectra were of a composite type, resulting from at least two populations of spin labels largely differing in molecular mobility. The correlation time of the less mobile nitroxide radicals was determined by saturation transfer ESR. Upon heating there was a transfer from the slow to the fast moving population of radicals, and an increase of mobility of this last catagory that followed the Arrhenius law. The effect of temperature on molecular flexibility was reversible. This was not the case for purified, polymerised glutenin subunits extracted from gluten. Urea created similar modifications on gluten as heat.

Key words: Prolamin; Gluten; ESR; ST-ESR; Temperature; Urea

1. Introduction

Gluten is a protein part of dough that gives it unique technological characteristics, making wheat the most wide-spread cereal. The protein network of gluten is mainly composed of monomeric (gliadins) and polymeric (glutenins) water-insoluble storage proteins, associated into aggregates that interact by weak bonds [1]. This viscoelastic mass is what remains when starch and and water-soluble components are washed out of wet flour. Although it is accepted that gliadins contribute to flow and glutenins to elasticity [2], the molecular basis of the functionality of the protein network of gluten in unclear. The secondary structure of wheat proteins has generally been studied after solubilisation, sometimes involving the modification of the polypeptides [3,4]. However, recent attenuated total reflection infra-red (ATR-IR) studies on functional gluten indicated a decrease of intra- and mainly inter-molecular β-sheet conformation when proteins in a doughy state are solubilised [5,6]. An IR study on alkylated and unalkylated high molecular weight glutenin subunits showed an increase in β-sheet type structure upon hydration, and an enhancement of low energy intermolecular interactions in the presence of disulfide bonds [7]. No difference was found by ^{1}H and ^{13}C NMR spectroscopy between hydrated gluten, glutenin enriched fraction and gliadin enriched fractions at 30°C. The relaxation times of the proteins were not modified after heating to 80°C [8]. The degree of segmental mobility in the proteins in D_2O was high (~65%) and increased with temperature.

Electron spin resonance (ESR) spectroscopy can yield information about polymer networks by the use of a stable paramagnetic compound [9]. Spin probing informs about the solvent environment, whilst covalent spin labelling reflects the segmental mobility of labelled molecules. Previous work has proven the usefulness of ESR spectroscopy in understanding the properties of functional gluten. Spin probing showed a compartimentation of the liquid phase of hydrated gluten, namely, the existence of a lipid and of an aqueous phase [10,11], and of two different microenvironments in the water phase [12,13]. The polypeptide flexibility was found to depend on the gliadin/ glutenin ratio and on the organisation of glutens [10,11].

To improve our understanding of the organisation of gluten and of the interactions in the protein network, we investigated, in this paper, the effect of temperature and chemical denaturation on the molecular flexibility of gluten and glutenin subunits by ESR and saturation transfer (ST)-ESR.

2. Materials and methods

2.1. Sample preparation

Gluten was hand extracted from a good bread-making flour of the French wheat cultivar (cv) Aubaine. The flour was extracted with chloroform so all the gluten was defatted, and when specified, the flour was also extracted with a non-ionic detergent (Lubrol, 2% v/v), to prepare non-prolamin protein (NPP) depleted gluten [13,14]. The gluten was then freeze-dried and ground to a powder.

Low molecular weight (LMW) and high molecular weight (HMW) glutenin subunits were purified from bread-making flour (cv Etoile de Choisy) after NPP-extraction, based on a method developed by Marchylo et al. [15]. The gluten was extracted 4 h by 70% ethanol and centrifuged (8,000 × *g*, 15 min). The insoluble fraction was freeze-dried and ground to a powder. A 1 g aliquot of this powder was extracted 30 min with 50 ml of 50% 1-propanol and centrifuged (20,000 × *g*, 10 min). The insoluble fraction was further extracted for one hour at 60°C with addition of 0.3% dithiotreitol (DTT) and centrifuged as previously. This operation was repeated twice and the supernatants were pooled. The propanol content was adjusted to 60%, and the solution was left to stand overnight at 4°C, then centrifuged at this temperature as previously, the supernatant and insoluble being respectively LMW and HMW glutenin subunits. The proteins were dialysed against dilute acetic acid and freeze-dried. The purity was assessed by electrophoresis under reducing conditions as indicated elsewhere [16]. Under non-reducing conditions the glutenin did not penetrate the gel, showing that the glutenin subunits had polymerised.

2.2. ESR and ST-ESR measurements

Spin probing and spin labelling were performed as described previously [11]. Gluten powder was saturated (300%) with an nitroxide spin radical aqueous solution (0.1 to 0.8 mg/ml) and left to hydrate 30–60 min. In spin probing, the spectra were then recorded, whilst the samples were left to incubate (~1 h) and were then dialysed before the ESR measurements in spin labelling. TEMPO and TEMPOL were used in spin probing experiments. The spin labels 3-maleimido-PROXYL

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(3MP) and 4-maleimido-TEMPO (4MT) were used to covalently bind sulfhydryl groups (cysteine residues), the former label being slightly more rigid. The spin label 3-(2-isothiocyanatoethyl-carbamoyl)-PROXYL (32IP) labels amino groups (lysine and possibly N-terminal amino-acid residues). The paramagnetic compounds were purchased from Aldrich and Sigma.

Unless otherwise indicated, the fully hydrated samples were inserted into a glass capillary, the extremity was sealed with a two component glue, and the samples covered with water and paraffin oil to avoid drying. ESR measurements were carried out on a Bruker ESP 300E spectrometer equipped with a computer. A microwave power of 2 to 10 mW was used for conventional ESR, avoiding saturation phenomena. In ST-ESR experiments, the second harmonic of the absorption was detected at a modulation 90° out of phase, with a power of 100 mW. The temperature was controlled by a stream of gas nitrogen in a quartz dewar. The ESR measurements were performed between 20°C and 90°C with 10° increments. Some measurements were carried out at 5°C. After heating, the samples were cooled down to about 20°C and ESR spectra recorded to check the reversibility of the heating.

In a few cases, experiments were carried out at room temperature on a Varian E9 spectrometer, the samples being placed in a quartz tissue cell in excess water.

2.3. Analysis of ESR and ST-ESR spectra

The ESR spectra were often of a composite type, reflecting the existence of at least two populations of nitroxide radicals differing in their mobility, as described previously [11]. A value R reflecting the proportion of slow moving to fast moving population of spin labels was calculated for composite spectra:

$$R = i/m$$

Fig. 1 shows the definition of i and m, the heights of the low field peaks corresponding respectively to slow moving ('immobile') labels ($\tau_c > 10^{-7}$ s) and to fast moving ('mobile') labels ($\tau_c < 10^{-8}$ s). The rotational correlation time (τ_c) of the mobile population of spin labels was calculated from the spectra according to Freed and Fraenkel [17]:

$$\tau_c = 6.65 \times 10^{-10} \times H_{+1} \times [(I_{+1}/I_{-1})^{1/2} - 1]$$

with H_{+1} the peak to peak width of the low field line in Gauss, I_{+1} and I_{-1} the heights of the low and high field lines respectively (see Fig. 1).

ST-ESR spectroscopy enables to measure slower motions (10^{-3} s $< \tau_c < 10^{-7}$ s) as compared to conventional ESR (10^{-8} s $< \tau_c < 10^{-11}$ s). Only the high field features (H" and H) of the ST-ESR spectra were taken into account, as the low (L and L") and central (C and C") field features showed an overlap with the spectrum of the fast moving nitroxide radicals (Fig. 2). τ_c was determined graphically, on a H"/H vs. τ_c reference curve (Fig. 3), established with spin-labelled haemoglobin, as described elsewhere [18].

3. Results

3.1. Influence of temperature on the flexibility of the mobile population

Typical ESR spectra recorded between 20 and 90°C on 4MT spin-labelled gluten are presented in Fig. 1. Similar spectra were also obtained with 4MT spin-labelled NPP depleted gluten, HMW and LMW glutenin subunits and with 32IP spin-labelled LMW subunits. Increasing temperature, there is a progressive transfer of radicals with a slow motion to a more mobile population of labels. Finally, at 90°C, all the spin labels are in the mobile state. Concomitantly, the rotational correlation time (τ_c) of the mobile fraction decreases with increasing temperature. When the logarithmic of the R-values and τ_c are plotted vs. the reciprocal of the temperature, a linear relation is found (Figs. 4 and 5). From the τ_c (Fig. 5) it is possible to determine an activation energy (E_a) according to the Arrhenius equation:

$$\tau_c = \tau_{c0} \times \exp(E_a/RT)$$

with τ_{c0} the pre-exponential factor, T the absolute temperature

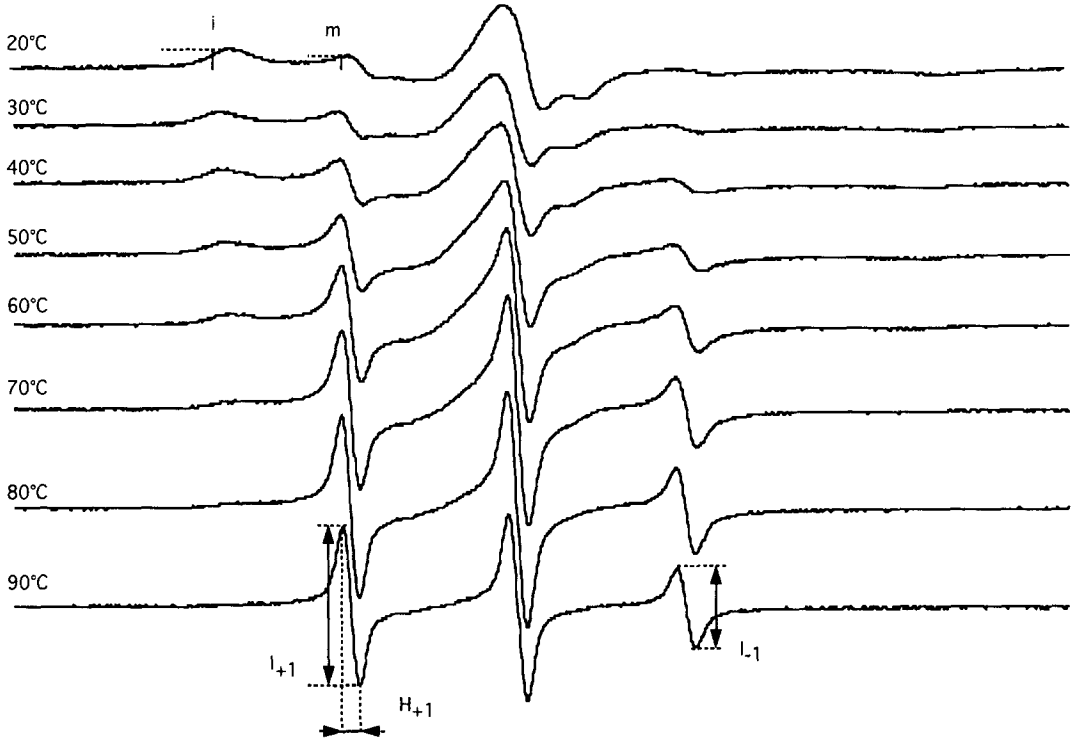

Fig. 1. Conventional ESR spectra of 4MT spin-labelled gluten recorded at different temperatures. The spectral features used to calculate the R-value and the rotational correlation time (τ_c) are indicated.

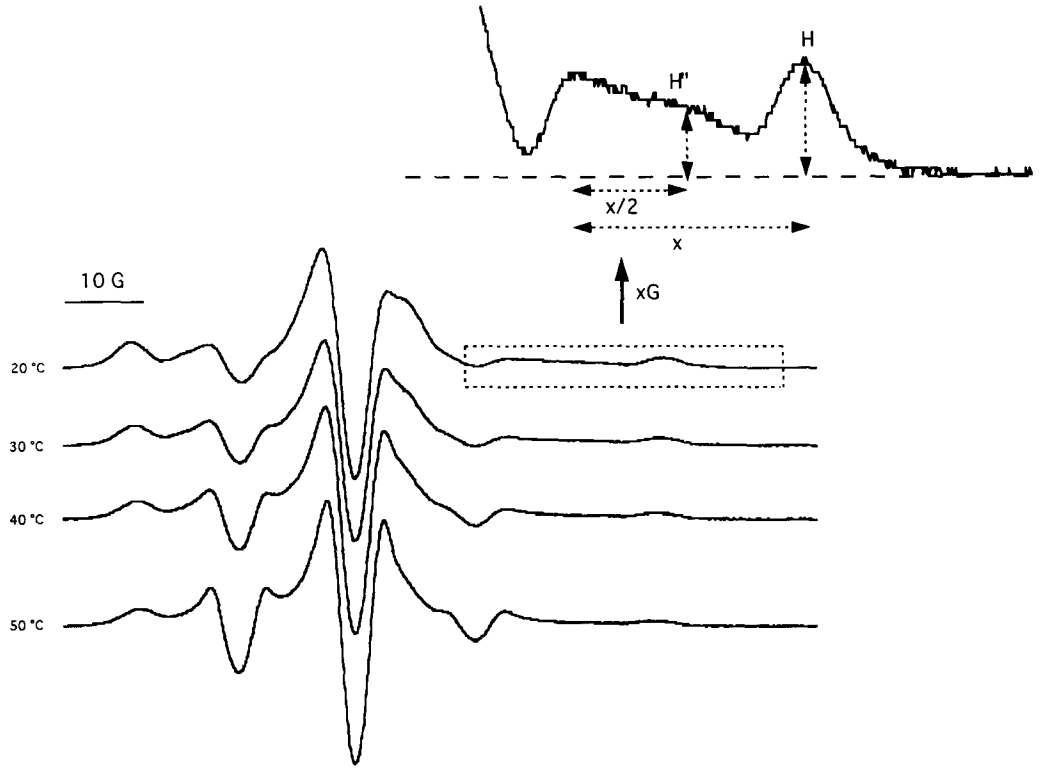

Fig. 2. Saturation transfer (ST) ESR spectra of spin-labelled gluten recorded at different temperatures. The spectral features used in estimating the correlation time (τ_c) are indicated.

and R the ideal gas constant. For the NPP-depleted gluten the value of E_a was found to be 13.5 kJ/mol. Gluten and NPP-depleted gluten samples spin-labelled with 3MP gave similar E_a values as 4MT labelling. This value of E_a is in agreement with the value found for gliadin or glutenin enriched fractions [19]. The E_a value determined with 4MT spin-labelled HMW glutenin subunits is slightly higher than that of the LMW subunits (21.0 and 14.5, respectively).

The change in the molecular flexibility of 3MP spin-labelled gluten kept at 80°C showed that the modification due to heat was very rapid (< 5 min), and that the sample was then quite stable, with only a slight and progressive decrease in τ_c (6.7 to 4.9 10^{-10} s) and R (~0.08 to ~0.03) over 15 h.

After heating, the samples were cooled down to room temperature, and ESR spectra were recorded. On average, the gluten samples presented similar spectra before and after heating, apart form a decrease in the intensity of the signal due to a reduction of the spin label and a concomittant loss of paramagnetism. Measurements performed on a Varian E9 spectrometer with 4MT spin-labelled gluten samples before and after undergoing 10 min heating at 100°C in excess water confirmed that no difference in flexibility of the spin-labelled cysteine residues was observed due to heat-setting in gluten. Conversely, the spectra of 4MT and 32IP spin-labelled LMW and HMW glutenin subunits after heating showed that the alterations in the molecular flexibility induced by heat were not completely reversible: the R and τ_c values remained close to those found at 50 to 60°C.

3.2. *Influence of urea on gluten flexibility*

The addition of an excess solution of urea (1, 2 or 4 M) to 32IP spin-labelled gluten rendered all the nitroxide radicals mobile. The flexibility of the spin-labelled proteins increased fourfold to a τ_c of about 2×10^{-10} s, regardless of the molarity of the dissociating agent. A similar flexibility as in gluten was observed in gliadin or glutenin enriched subfractions of gluten, that differed in their mobility before addition of urea [12]. The urea solutions were found to scarcely modify the spectral features of an aqueous solution of nitroxide radicals (TEMPO, 4MT or 32IP), indicating that any variation recorded is only due to variations of protein conformations.

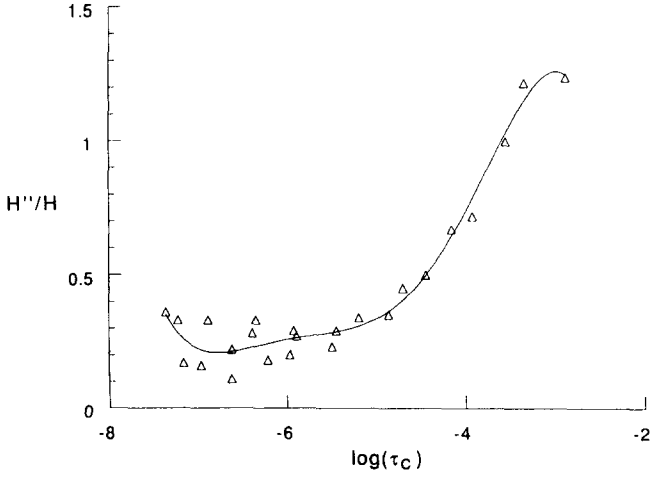

Fig. 3. Reference curve of ST-ESR spectral features H''/H versus the correlation time (τ_c), established with spin-labelled haemoglobin in environments differing in viscosity and temperature.

3.3. Mobility of the less mobile population of spin-labelled residues

ST-ESR measurements were performed on 3MP and 4MT spin-labelled gluten between 5 and 50°C (Fig. 2). Gluten and NPP-depleted gluten gave identical τ_c values, and the effect of heating up to 50°C or the choice of the spin label were negligible. The values obtained were approximate, due to a slight contribution to the spectra from the fast moving radicals, even in the high-field region of the spectrum. The values of H''/H varied between 0.6 and 0.9, indicating that τ_c is between 3×10^{-5} and 3×10^{-4} s for the slow moving population (Fig. 3). These large τ_c values, as compared to those found for the mobile fraction, stress the very slow motional properties of the immobile fraction. Thus, the spin label is rigidly bound to the gluten, with virtualy no internal flexibility.

3.4. Spin probing results

Rotational correlation times were measured for an aqueous soluble TEMPOL spin-probed gluten between 20 and 80°C. Theoretical values of τ_c were calculated for each temperature (T) from the Debye equation:

$$\tau_c = (4 \cdot \pi \cdot R_H^3 \cdot \eta)/(3 \cdot k_b \cdot T)$$

were k_b is the Boltzmann number, η the solvent viscosity, and R_H the hydrodynamic radius. The value of $(\eta \cdot R_H^3)$ determined at 20°C was used to calculate τ_c at other temperatures, after correction of the change in the viscosity due to heat by a factor corresponding to the variation in pure water viscosity for similar temperature modifications.

The measured and theoretical values of τ_c were comparable, indicating that the spin-probe reflects the behaviour of the solvent. Therefore, in the conditions of the study, the dynamic behaviour of the protein could not be observed by spin probing of the aqueous phase with TEMPOL, and the modifications induced to the network structure by the effect of temperature do not affect the mobility of small solutes.

Addition of urea to TEMPO spin-probed gluten caused a two-fold decrease in τ_c. This agrees with the suggestion that hydrogen bonds strongly participate to the gluten organisation

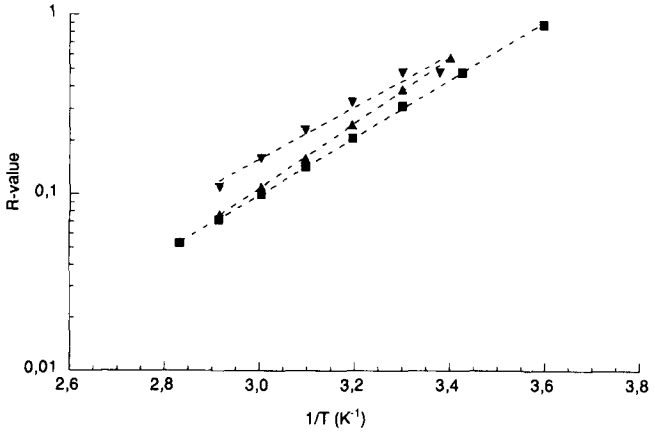

Fig. 4. Arrhenius plot of the R-value versus the inverse of the absolute temperature (T). Squares, 4MT-labelled non-prolamin protein (NPP) depleted gluten; reversed triangles, 4MT-labelled fraction of low molecular weight glutenin subunits; triangles, 4MT-labelled fraction of high molecular weight glutenin subunits.

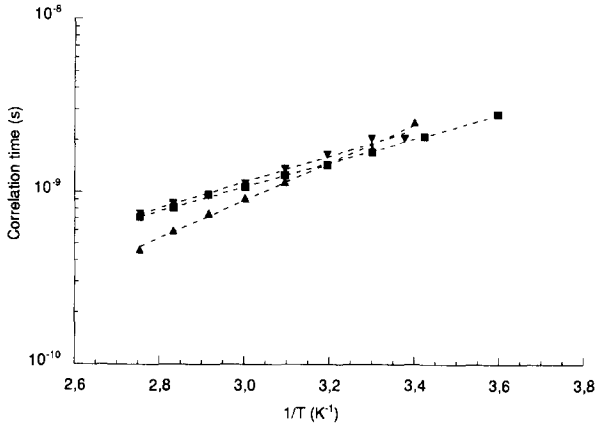

Fig. 5. Arrhenius plot of the rotational correlation time (τ_c) versus the inverse of the absolute temperature (T). Symbols as in Fig. 4.

[20]. These results also indicate that the network structure is at least partly responsible for the reduced movement of TEMPO in the aqueous phase of gluten compared to a water solution.

4. Discussion

We observed no transition in the segmental flexibility of hydrated gluten proteins in the temperature range explored. This is opposite to the viscoelastic behaviour of gluten, where the height of the rubbery plateau ($G_N°$) has been reported to decrease upon heating from 10 to 50°C (10^3 to 10^2 N/m^2), then to increase strongly reaching 10^4 N/m^2 at 80°C [21]. The size of glutenin polymers increase strongly for samples having undergone a heating above about 50°C [21], showing a reorganisation of intermolecular covalent bonds between the glutenin subunits and possibly the gliadins [22]. This has been explained by the sulfhydryl–disulfide interchange between wheat proteins, reported around 60°C, and not by an increase in the total number of disulfide bonds [23]. When a sulfhydryl blocking agent is added to gluten, the height of $G_N°$ is reported to scarcely vary with temperature, slightly increasing [21]. The authors therefore explained the heat related variations of $G_N°$ to two events: from 10 to 50°C the disruption of low energy inter-molecular bonds is observed; above this temperature, the sulfhydryl–disulfide interchange imposes an increase in $G_N°$, due to the larger size of the glutenin polymers. On the other hand, the retardation time (τ_0) associated to the loss compliance peak of the mechanical spectra, which is very close to the onset of the rubbery plateau [24], followed an Arrhenius law, with an activation energy of 90 kJ/mol in gluten [21]. These results indicate that the retardation time is independent of the glutenin size distribution, and could be related to the displacement of a theoretical unit polymer length, corresponding to the average peptide length between two covalent bonds [24]. The unit polymer length is not modified as the total number of disulfide bonds is constant in gluten upon heating [23]. In this case, the activation energy of 90 kJ/mol would represent the contribution of low energy interactions (hydrogen bonds, hydrophobic interactions and entanglement points) between two covalent bonds, whilst the E_a determined by ESR spectroscopy (13.5 kJ/mol) is related to low energy bonds in the immediate surrounding of

the spin-label, which corresponds to about 1/6th of the unit polymer bond.

The transfer of the nitroxides from the slow to the fast moving population and the increase in the fexibility of the mobile labels we observed when gluten is heated or exposed to urea indicates that the effect of hydrogen bonds is dominant as compared to hydrophobic interactions in maintaining the prolamin conformations, at least in the α-helix rich environment of the cysteine residues in their native and functional state. The hydrogen bonds can concern the peptide backbone or the numerous glutamine side chains of the storage proteins [25].

The similarity in the slope of the R-value in Arrhenius plot and of the E_a determined for all the samples indicates that a similar increase in segmental flexibility takes place in a gliadin rich and in a glutenin rich system. Ablett et al. [8] also observed this in NMR experiments. The dynamics of segmental motion in heating therefore seem related to intrinsic properties of prolamin rather than to the organisation of gluten network. In solution, the heating of gliadin and glutenin has been reported to decrease their α-helix content, which corresponds to the spin-label environment [26,27]. The similarity of behaviour to heat of the gliadin possessing cysteine residues and of the LMW subunits, which represent a large majority of glutenin, can be related to their analogies in structure [28].

The slightly higher value of E_a determined in the fraction of HMW – as compared to the LMW – glutenin subunits shows that their structure is more sensitive to temperature. This can explain their higher reactivity to disulfide interchange in heating [23]. An interesting point is the incomplete reversibility of the heat denaturation of the purified LMW and HMW fractions, whilst the NPP-depleted gluten presents a good reversibility, in terms of molecular flexibility. Since the purification steps alter the structure of the proteins and may modify the organisation of the intramolecular covalent links, no firm conclusions can be made from this observation. It has been found by IR spectroscopy that the reduction and alkylation has little effect on the secondary structure of γ-gliadin in solution [26], or on the conformation of A-gliadin reflected by intrinsic viscosity [29]. Fluorescence studies showed that the reduction/alkylation and the denaturation by 8 M urea of γ-gliadin transferred their tryptophane residues to a more polar environment [30]. The denaturation was reversible upon dialysis, suggesting that the protein goes back to its native structure. Because of the resemblance in primary structure and conformation between γ-gliadins and glutenin subunits, especially of LMW, the conformation of the polypeptides in the purified glutenin fractions may be quite close to the native state. We found that upon hydration the purified LMW and HMW glutenin fractions presented a dough-type texture, confirming size exclusion chromatography results that showed they were crosslinked. The main difference between native glutenin and purified subunits would be the organisation of the cross-linking. The difference in heat-setting between gluten and purified fractions could then reflect the importance of the history of gluten to its properties. It suggests that functional glutenins are organised polymers, and not randomly cross-linked polypeptides, and although the heat creates a reticulation between glutenin polymers, the initial organisation remains, so upon cooling the low energy interactions re- form. The gliadins probably play a part in maintaining the organisation of gluten in heat treatments by maintaining an extended conformation of the subunits. On the contrary, the purified glutenin subunits that are polymerised in an unorganised way do not recover their low energy interactions immediately upon cooling.

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