Protein Insolubilization and Thiol Oxidation in Sulfite-Treated Wheat Gluten Films during Aging at Various Temperatures and Relative Humidities

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Gluten films were prepared by casting an acidic and ethanolic solution of gluten previously treated with sodium sulfite. The effects of sulfitolysis on proteins were investigated by SE-HPLC and thiol/disulfide content measurements. During sulfitolysis, insoluble glutenin macropolymer was converted into its constitutive subunits. About 10% of gluten disulfide bonds were cleaved, of which three-fourths originated from interchain disulfide bonds. Oxidation of thiol groups released during sulfitolysis was followed for various temperatures (T) and relative humidities. Oxidation was shown to be a second-order rate process occurring below the glass transition temperature (T_g) and related to $T - T_g$. Thiol oxidation ensured the formation of interchain bonds between specific classes of gluten proteins according to an ordered process. Intrachain bonds were also formed and through thiol/disulfide-exchange reactions were finally converted to interchain bonds. Thus, fully oxidized gluten films had more insoluble glutenin macropolymers than native gluten.

Keywords: *Wheat gluten film aging; sulfite; thiol oxidation; protein size distribution; glass transition*

INTRODUCTION

Among renewable agricultural resources, plant proteins are commercially available at low cost and have film-forming capabilities. Advantages, types, formation, and properties of agricultural packaging materials based on proteins have been recently reviewed by Cuq et al. (1998). The mechanical and barrier properties of protein-based films are dependent mainly on the hydrophilic/hydrophobic balance of proteins and on the structure of the macromolecular three-dimensional network. Among forces which stabilize the structure of films, disulfide bonds can play a key role (Okamoto, 1978). They are highly relevant in determining the properties of wheat gluten proteins (Shewry and Tatham, 1997).

Wheat gluten proteins include two contrasted fractions, namely gliadin and glutenin. Gliadin provides the viscous component of gluten, while glutenin contributes to the elastic properties (Orth and Bushuk, 1972; Huebner and Wall, 1976). Glutenin, described as a macropolymer (glutenin macropolymer, GMP), consists of subunits joined by interchain disulfide bonds. GMP exhibits a wide range of molecular size from 150 000 to more than 5 000 000. The differences in mixing properties and baking qualities of wheat varieties have been related to the quantity and size distribution of GMP (Gupta et al., 1993). Cornec et al. (1994), studying the rheological properties of gluten subfractions, observed an increase of G' modulus with protein molecular size. Heat treatment of gluten subfractions improved their elastic behavior by increasing their average molecular weight (Tsiami et al., 1997). As reviewed by Weegels and Hamer (1998), upon heating, disulfide/thiol exchange and thiol oxidation took place, which led to an increase of the covalent coupling by disulfide bonds of gluten proteins. Covalent coupling of proteins stabilized the network structure rendering films more resistant to strain and more elastic (Okamoto, 1978). Protein disulfide bonding could also decrease the solubility of gluten films in various solvents. For instance, the water solubility of glutenin is lower than that of gliadin, because of its larger molecular size. Reduction of disulfide bonds increased glutenin solubility in water/ethanol mixtures (Wieser et al., 1989).

Several studies attempted to improve the mechanical properties of protein films by using chemical cross-linkers or by applying thermal treatments (Marquié et al., 1995; Ali et al., 1997; Galietta et al., 1998). Nevertheless, straightforward coupling of gluten proteins may also be obtained through disulfide/thiol-interchange reactions mediated by chemicals or alkaline treatment. Gennadios et al. (1993) showed that addition of sodium sulfite in gluten film-forming solution strengthened gluten films. According to Gennadios et al. (1993), the thiol groups liberated during sulfitolysis would have been converted back into disulfide during film drying resulting in the reinforcement of the gluten network structure. Sulfite ions displace the less nucleophilic sulfur from the disulfide bond of cystine peptides (RSSR′) according to the following equation:

$$RSSR' + SO_3^{2-} \leftrightarrow RSSO_3^- + R'S^- \qquad (1)$$

Disulfide cleavage and formation depend on the pH of the film-forming solution. According to Cecil and McPhee (1955), during sulfitolysis, SO_3^{2-} is the active species whereas HSO_3^- does not react ($SO_3^{2-} + H^+ \leftrightarrow HSO_3^-$, $pK_a = 6.8$). Reversibility of sulfitolysis and thiol oxidation into disulfide also depends on pH because of the

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acid–base equilibrium of thiol group ($RS^- + H^+ \leftrightarrow RSH$, $pK_a \gg 8.5$).

Okamoto (1978) noticed a remarkable increase of strength for gluten and keratin films prepared from basic (pH > 10) film-forming solutions. At high pH disulfide bonds are cleaved and the released thiol groups would become available for formation of new disulfide linkages. Nevertheless, Herald et al. (1995) noted that films made from an acidic dispersion of gluten showed higher tensile strength than those from an alkaline dispersion. The authors postulated that at alkaline pH, breakdown of disulfide weakened the gluten network allowing the gluten film to stretch further. Thus, oxidation of the released thiol groups into interchain disulfide bonds during film drying would not always occur, and conditions ensuring it remain to be studied.

We initially investigated the effect of sulfitolysis of gluten from a biochemical point of view. Release of thiol groups and modification of the size distribution of proteins were assayed. Then, subsequent formation of disulfide bonds upon drying and during film aging was studied. Aging was studied under various temperatures and relative humidities.

MATERIALS AND METHODS

Wheat Gluten. Vital gluten was prepared from cultivar Soissons by the Institut Technique des Céréales et des Fourrages (Boigneville, France). Protein content (72.98 ± 0.5%, db) (N × 5.7) was determined in triplicate by the Dumas method (NA 2000, Fisons Instruments, France). Moisture content (4.11 ± 0.5%, db) was determined in triplicate by weight loss after 2 h of drying at 130 °C.

Gluten Film Preparation. The film-forming solution was adapted from Gontard et al. (1992) except it was supplemented with sodium sulfite. The gluten (15 g) was placed in a hermetic box with 45 mL of a sulfite solution (0.66 $g{\cdot}L^{-1}$). The mixture of the components was ensured by briefly shaking the box. After 2 h resting at room temperature, 32 mL of ethanol (95%), 3 g of glycerol (99.5%), and 1.8 mL of acetic acid (100%) were successively added under magnetic stirring. The solution was adjusted to 100 mL with distilled water and heated to 60 °C under magnetic stirring. The film-forming solution was spread out over a level Plexiglas plate using a thin-layer chromatography spreader (Braive 4000, Liege, Belgium) regulated to 0.8 mm. The films were dried for 10 h in a ventilated oven at 25 °C and then stored as described below.

Gluten Film Storage. The gluten films were cut in 6 pieces of 8 cm × 1.5 cm and stored over saturated salt solutions and silica gel at 25 and 50 °C, for duration ranging from 24 to 656 h. Saturated salts solutions included NaCl and $MgCl_2$. Before biochemical analyses, films were conditioned for 20 h in a room thermostated at 20 °C and 60% relative humidity (RH).

Moisture and Protein Content of Gluten Films. Triplicate specimens of gluten film were rapidly weighted and stored for 1 week in an airtight and ventilated desiccator at 40 °C over P_2O_5. Average moisture was calculated from weight loss of the 3 specimens. Moisture contents were determined as 1.14% (±0.17), 4.86% (±0.38), and 14.78% (±0.14) for gluten films respectively stored for 24 h at 25 °C under silica gel and saturated solutions of $MgCl_2$ and NaCl. For gluten films equilibrated for 20 h at 20 °C and 60% RH, moisture was 11.35% (±0.98). Protein content was calculated assuming that gluten films contained glycerol (3 g/14.40 g of dry gluten) but no remaining acetic acid or ethanol.

Thiol Content. Solution A included propan-2-ol, Tris/HCl buffer (250 mM, pH 8.5), and 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) solution (4 $g{\cdot}L^{-1}$, in ethanol) (1/1/0.2, V/V/V). Solution B had the same composition, except pure ethanol replaced the DNTB solution. The solutions were saturated with helium and stored under argon. Four weighted gluten samples (10, 20, 30, and 40 mg) were suspended in 1.6 mL of solution A. A control series was prepared using solution B. After 10 min of vortexing, tubes were centrifuged for 5 min (at 15000*g*) and absorbance of supernatants was read at 412 nm. The concentration of the released thionitrobenzoate ion (TNB^{2-}) was calculated using a molar absorption coefficient of 13600 $M^{-1}{\cdot}cm^{-1}$. The absorbance calculated per gram of sample was corrected from that of the control series. Results were expressed in μmol of thiol/g of proteins. A coefficient of variation of 2% was calculated for gluten film from triplicate experiments. For a freshly made gluten film equilibrated for 20 h at 20 °C and 60% RH, an average thiol content of 8.90 ± 0.178 $\mu mol{\cdot}g^{-1}$ was measured, while it was 0.94 ± 0.01 $\mu mol{\cdot}g^{-1}$ for the vital gluten.

Nature of the Gluten Film Reducing Power. DNTB reacts with thiol groups or sulfite ions generating TNB^{2-} according to a 1/1 stoichiometry. Sulfitolysis of protein disulfide bonds also generates *S*-sulfocysteine residues, which do not react with DNTB. To identify the nature of the reducing groups (remaining sulfite or thiol), gluten film samples were treated to remove any sulfite present. The procedure allowed the elimination of more than 99.5% of sulfite ions as verified with 45 μL of a sodium sulfite solution at 5 mM. Besides, the reducing power of a cysteine solution (45 μL at 5 mM) remained unchanged all through the procedure. Samples of freshly made gluten film were weighed (10–40 mg) and vortexed for 10 min in an open test tube with 1 mL of sulfuric acid (0.2 N). Ethanol (0.2 mL) was added and tubes were incubated for 1 h at 60 °C. The acidic (pH < 1) medium converts sulfite into sulfurous acid ($SO_3^{2-} + H^+ \leftrightarrow HSO_3^-$, $pK_a = 6.8$; $H^+ + HSO_3^- \leftrightarrow H_2SO_3$, $pK_a = 1.81$). Sulfur dioxide (SO_2) in equilibrium with sulfurous acid (H_2SO_3) is then driven out from the solution during incubation. Then, DNTB (0.1 mL, 4 $mg{\cdot}mL^{-1}$ in ethanol) was added and the mixture was buffered to pH 8.1 with 0.8 mL of Tris/HCl (1 M, pH 8.8). The absorbance of the released TNB^{2-} was read as above. For gluten films, similar levels of TNB^{2-} were determined using this procedure or the standard procedure described above. All the reducing power of gluten films can therefore be attributed to thiol groups.

Thiol Plus Disulfide Content. Thiol plus disulfide content of gluten was measured after reduction of disulfide bonds with dithioerythritol according to Morel and Bonicel (1996). Triplicate measurements gave an average content of 162.8 ± 4.5 $\mu mol{\cdot}g^{-1}$ of thiol equivalent for the vital gluten. Once corrected for its thiol content, the disulfide content of gluten was calculated as 81.18 ± 0.51 $\mu mol{\cdot}g^{-1}$.

SE-HPLC Samples of Film-Forming Solution, Gluten, and Gluten Films. Samples of film-forming solution (80 μL) were taken at variable time intervals and added to 1 mL of H_2SO_4 (0.2 N) to stop sulfitolysis. Thiol groups were alkylated with iodoacetamide (IAM) in the presence of DNTB, to oxidize the remaining sulfite ions. Then, 800 μL of Tris/HCl (1 M, pH 8.5), 200 μL of DNTB (4 $g{\cdot}L^{-1}$ in ethanol), and 1 mL of IAM (10 mM) were added. The mixture was incubated for 1 h at ambient temperature before being adjusted to 20 mL with sodium phosphate buffer (pH 6.8, 0.1 M) containing 1% sodium dodecyl sulfate (SDS) (extracting buffer). Gluten (10 mg) and gluten film (20 mg) samples were directly suspended in 20 mL of extracting buffer containing 3 mM IAM. Samples, prepared in duplicate, were shaken for 80 min at 60 °C to extract the SDS-soluble proteins, which were recovered by centrifugation (30 min at 65316*g*). The residue was suspended in 5 mL of extracting buffer, sonicated for 180 s using a tip sonicator (Vibracel 72434, Bioblock Scientific, Illkirch, France) to solubilize SDS-insoluble proteins (Singh et al., 1990), and centrifuged as above. Aliquots (20 μL) of SDS-soluble and SDS-insoluble extracts were kept at −18 °C before analysis.

Size-Exclusion HPLC. Size-exclusion HPLC analyses were carried out using a Waters LC Module-1-Plus system according to Redl et al. (1999). The chromatographic profiles were integrated using the Millenium software (Waters, Saint-Quentin-les-Yvelines, France). Once corrected for their different solid-to-solvent ratio during extraction, areas of soluble and insoluble proteins (in arbitrary units) were added and the sum was assumed to correspond to the total protein content

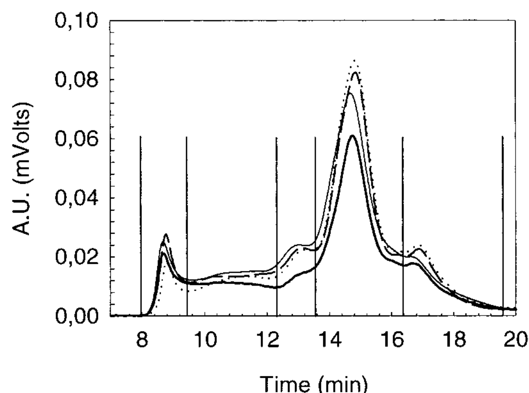

Figure 1. SE-HPLC profiles of SDS-soluble proteins from gluten, gluten film, and samples of film-forming solution: (—) gluten film; (thick —) native gluten; (- - -) and (···) samples of film-forming solution respectively taken 5 min after combining gluten and sulfite solution and just before spreading; (F1) polymeric proteins ranging from M_r 680 000 to 7 000 000; (F2) polymeric proteins ranging from M_r 145 000 to 680 000; (F3) oligomeric and monomeric proteins ranging from M_r 78 000 to 145 000; (F4) monomeric proteins ranging from M_r 17 000 to 78 000; (F5) monomeric proteins of M_r < 17 000.

of the sample. Chromatographic profiles of SDS-soluble proteins were typically divided into five fractions expressed in percent (%) of total proteins (Dachkevitch and Autran, 1989). Relative standard deviations for total protein area or for fraction percent were below 2%, so that reliable measurements could be obtained from duplicate samples. Standards comprising thyroglobulin (669 000), bovine serum albumin (66 000), chymotrypsin A (25 700), and cytochrome *c* (11 700) (from Sigma) were run to assist in determining the molecular weight range of gluten proteins. From the earliest to the latest eluted fraction, fractions F1 and F2 included GMP with molecular weight (M_r) ranging from 145 000 up to 7 000 000 (the manufacturer claimed exclusion M_r), fractions F3 and F4 corresponded mainly to gliadins, while fraction F5 likely corresponded to remaining wheat flour-soluble proteins (Singh et al., 1990). Calibration of the column allowed us to estimate the median M_r's of the distribution range of fractions F2, 267 200; F3, 98 035; F4, 33 750; and F5, 8 325. For fraction F1, which was partly eluted at the void volume of the column (7 000 000 according to the manufacturer) we have taken an arbitrary value of 2 500 000 for median M_r. These M_r values were used to estimate the fraction contents in terms of moles instead of percent of total proteins.

RESULTS AND DISCUSSION

Effect of Sulfitolysis on Wheat Gluten Protein. SE-HPLC analyses of samples, taken all through the preparation of the film-forming solution, were carried out to follow the effect of sulfitolysis on gluten proteins. Chromatographic profiles of SDS-soluble proteins extracted from gluten (thick line), from two film-forming solutions (dotted lines), and from a freshly made gluten film (thin line) are displayed in Figure 1. Sulfitolysis reaction appeared to be very efficient and rapid since a large increase in the area of SDS-soluble proteins took place within 5 min. The SDS-insoluble GMP dropped from about 25.41% for gluten to 1.39% for the film-forming solution. The change coincided with a marked increase in fractions F3 and F4, whose molecular weights (M_r) ranged from 145 000 to 17 000. Sulfitolysis promoted extensive depolymerization of SDS-insoluble GMP, leading to the release of protein monomers such as low molecular weight ($M_r \cong 45\,000$) and high molecular weight ($M_r \cong 90\,000$) glutenin subunits.

No further change in SE-HPLC profiles was observed for samples taken from the film-forming solution within the following 2 h (result not shown), indicating that a steady-state balance between sulfite ions and protein disulfide was quickly reached. The gluten film-forming solution had a pH of 7.70. Since the dissociation constant of HSO_3^- is $10^{-6.8}$, sulfite was mostly in the form of SO_3^{2-} while thiols groups were almost nonionized ($RS^- + H^+ \leftrightarrow RSH$, p$K_a \approx 8.5$). As a consequence, it was expected that the forward reaction was highly supported and that sulfitolysis proceeded readily. Addition of acetic acid, which lowered the pH of film-forming solution to 4, and of glycerol and ethanol had no effect on the SE-HPLC profile (not shown). Heating of the film-forming solution just before spreading shifted the reaction forward since the content of monomers increased slightly at the expense of SDS-soluble GMP (F1 and F2) (Figure 1). At the end of the preparation of film-forming solution, SDS-soluble GMP still accounted for 19% of total proteins indicating that sulfitolysis did not result in the breakdown of all interchain bonds.

Drying of film-forming solution resulted in a slight decrease of F4 at the benefit of soluble GMP. Content of insoluble GMP remained almost unchanged at about 2.25% of total proteins. These results indicated that disulfide bond formation was prevented during film drying. Freshly made gluten films were able to reduce Ellman's reagent, and the reducing power was attributed exclusively to the presence of thiol groups (see Materials and Methods). Bisulfite (HSO_3^-), which is in equilibrium with sulfite at pH 4 ($SO_3^{2-} + H^+ \leftrightarrow HSO_3^-$, p$K_a = 6.8$), loses some SO_2 and is gradually oxidized to sulfate, upon exposure to air. Sulfinic acid, in equilibrium with bisulfite ion ($HSO_3^- + H^+ \leftrightarrow H_2SO_3$, p$K_a$ = 1.81), is unstable and decomposes into SO_2 and H_2O. These reactions would occur during the drying of the film-forming solution, leading to the disappearance of all sulfite species through oxidation or decomposition reactions.

For freshly made gluten films, a thiol content of 8.90 ± 0.178 μmol·g^{-1} was measured. According to Stricks and Kolthoff (1951), the equilibrium constant for eq 1 in a cystine system is 8.9×10^{-2} at pH 7.75. Taking this equilibrium constant into account we have calculated that 9.25 μmol·g^{-1} (i.e. 11.4%) of the gluten disulfide bonds should react with sulfite ions. During film drying, thiol groups were protected from oxidation, because they were nonionized ($RS^- + H^+ \leftrightarrow RSH$, p$K_a$ ≈ 8.5).

From native gluten to freshly made gluten film, the thiol content increased by about 8 μmol·g^{-1}. According to eq 1 this means that sulfitolysis ensured the breakdown of 8 μmol·g^{-1} of disulfide bonds. Compared with the native gluten, freshly made gluten film displayed more soluble proteins (F3 + F4 + F5), which in terms of moles accounted for 5.83 μmol·g^{-1}. These proteins are subunits released from GMP. Since GMP is assumed to be a linear branched polymer, each subunit being linked to the following one by a single interchain disulfide bond, the release of one subunit should be accompanied by the cleavage of one interchain disulfide bond (Graveland et al., 1985; Ewart, 1968, 1979). SDS-soluble GMP (F1 + F2) increased by 0.16 μmol·g^{-1} from gluten to gluten film. Because of the huge size of SDS-insoluble GMP (Wrigley, 1996) the release of soluble counterparts could require the breakdown of several interchain disulfide bonds. Despite this uncertainty, we could estimate that the changes observed from gluten to gluten film implied the breakdown of at least 6

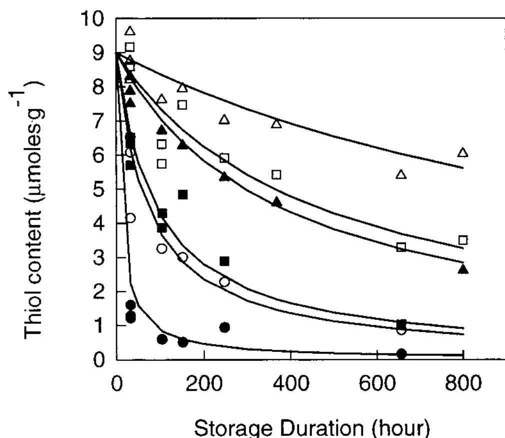

Figure 2. Changes in thiol content of gluten films during storage at various temperatures and relative humidities (RH). Storage conditions: (filled symbols) temperature 50 °C; (open symbols) temperature 25 °C; (○) RH 75.3; (□) RH 33.0; (△) RH 5. Thiol content is expressed in μmol·g^{-1} of proteins. Solid lines represent results fitted according to eq 3 using k values given in Table 1 and an initial thiol content of 9 μmol·g^{-1}.

μmol·g^{-1} (5.83 + 0.16 μmol·g^{1}) of interchain disulfide bonds. A maximum of 2 μmol·g^{-1} of intrachain bonds would also have been cleaved. On the basis of the quantitative distribution of the different gluten protein types, Grosch and Wieser (1999) calculated that approximately 10% of gluten disulfide bonds are involved in interchain bonds. In that respect, interchain disulfide bonds appeared to be much more sensitive to sulfitolysis than the intrachain bonds as already shown for several soluble proteins (Cecil and Wake, 1962).

Thiol Oxidation during Storage. Thiol content of the gluten films was measured at various intervals after storage at different relative RHs and temperatures (Figure 2). Storage conditions largely affected the rate of thiol oxidation. Temperature increased the oxidation rate at all RH levels. Thiol oxidation rate remained slow when gluten films were stored in relatively dry conditions (RH = 0−5%). Combination of high RH (75%) and high temperature (50 °C) led to the most drastic effect with thiol groups almost disappearing after aging of only 130 h.

The rate of thiol oxidation was assumed to be a second-order rate process, depending upon two thiol groups (SH) according to the following equation:

$$-\mathrm{d(SH)}/\mathrm{d}t = k(\mathrm{SH})^2 \quad (2)$$

We could also imagine that S-sulfonate groups and thiol groups reacted together according to eq 1. Nevertheless, in this case, eq 2 is still valid since thiol and S-sulfonate groups are balanced. Data were fitted linearly according to:

$$1/[\mathrm{thiol}] = k \times t + 1/[\mathrm{thiol}_0] \quad (3)$$

where t is the aging duration in hours, [thiol] is the thiol content per g of proteins, k is the thiol oxidation second-order rate constant (in h^{-1}), and [thiol$_0$] is the thiol content (per g of proteins) at the beginning of the aging period. For gluten film stored at 25 °C, at RH = 0−5%, the rate of thiol oxidation was slow and allowed a good estimation of the slope intercept with the y-axis (1/[thiol$_0$]). A value of 9 μmol·g^{-1} of protein was calculated, close to that measured on freshly made gluten films (8.90 ± 0.18 μmol·g^{-1}). All the linear regressions were

Table 1. Second-Order Rate Constant (k) for Thiol Oxidation in Gluten Films Stored at Various Conditions

storage conditions					
salt	RH[c]	T (°C)	k[a]	R^2	film moisture[b]
silica gel	0−5.0	25	83	0.75	1.14
silica gel	0−5.0	50	302	0.95	nd
$MgCl_2$	33.0	25	245	0.89	4.86
$MgCl_2$	30.5	50	1240	0.97	nd
NaCl	75.3	25	1572	0.98	14.78
NaCl	74.4	50	10545	0.83	nd

[a] Experimental data fitted according to eq 3 with [thiol$_0$] = 9 μmol·g^{-1} of protein; k is given in h^{-1}. [b] Film moisture content (%, db) according to storage conditions. [c] Relative humidity (RH) (%) of the saturated salt solutions according to Rockland (1960) and Greenspan (1977) and silica gel according to manufacturer specifications.

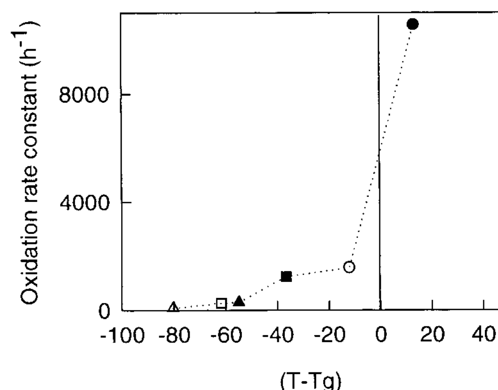

Figure 3. Oxidation rate (k) of thiol groups of gluten films as a function of the difference between the storage temperature (T) and the glass transition temperature (T_g) calculated for each storage condition. Storage conditions: (filled symbols) temperature 50 °C; (open symbols) temperature 25 °C; gluten film moisture contents (○) 14.78%, (□) 4.86%, (△) 1.14%. T_g was estimated for each storage condition, according to the data reported by Gontard and Ring (1996); i.e. T_g is equal to 384 K for a dry gluten film plasticized with glycerol (20 g·g^{-1} gluten) and T_g decreases by 5 °C when the gluten film water mass fraction increases by 1%.

forced into the same intercept (1/[9 μmol·g^{-1}]) (Table 1). The oxidation rates (k) ranged from 133 to 17850 h^{-1} according to gluten film storage conditions and were especially high for films stored at high temperature and high moisture content.

To establish the effect of gluten plasticization on oxidation rate, we plotted k values as a function of the difference between storage temperature (T) and the glass transition temperature of gluten (T_g) (Figure 3). As amorphous polymers move from the glassy to the rubbery state, the viscosity falls dramatically from 10^{12} to 10^3 Pa·s at the glass transition temperature (T_g) (Levine and Slade, 1988; Slade and Levine, 1993). Since viscosity is related to molecular mobility, the glass transition is characterized by an increase in rotational mobility (Rozen and Hemminga, 1990; Rozen et al., 1991). Also, the reduced viscosity allows for reactant translational mobility and for faster diffusion reactions (Franks, 1985; Le Meste and Duckworth, 1988).

T_g values of the gluten films studied here could be estimated from the data published by Gontard and Ring (1996). They determined, using DSC and DMTA, that gluten film plasticized with glycerol (20 g/g gluten, as our study) had a glass transition temperature of 111 °C. They have established that T_g dropped by 5 °C as the moisture of gluten film increased by 1%. T_g values were estimated from these data for each storage condi-

tion. It can be seen in Figure 3 that the oxidation rate began to increase at −40 °C below T_g and then rose notably above T_g. This indicates that thiol oxidation occurs even though gluten films are stored within their glassy state and that only short range mobility is allowed for thiol groups. Above T_g, segmental movements of protein chains increase abruptly and the probability of molecular collisions may rise leading to a sudden increase of the thiol group oxidation rate. Gontard et al. (1996) have showed that the oxygen permeability of gluten films increases exponentially as gluten proteins undergo the glass to rubbery transition. Thus, the abrupt increase of thiol oxidation rate above T_g might follow the increase in oxygen concentration in the vicinity of proteins, whereas the increase in segmental mobility would be less essential.

Change in Gluten Protein Molecular Size Distribution. Formation of interchain disulfide bonds during gluten film aging was estimated by following changes in the molecular weight distribution of the SDS-soluble proteins. Evolutions of GMP and F4 fractions as a function of storage duration for each storage condition are presented in Figure 4. As a general trend, solubility of gluten protein in SDS decreased with storage time, F4 and GMP fractions being mainly affected while fractions F3 and F5 remained almost unchanged (not shown). Drops of SDS-soluble GMP (fractions F1 + F2) and of protein monomers (F4) were especially noticeable for gluten films stored at $T - T_g$ values above −40 °C (i.e. high humidity irrespective of temperature, combination of medium humidity and high temperature). Rate constants of F4 and GMP decays could not be determined since it was not possible to describe the time decay of any fractions of SDS-soluble proteins by simple rate laws, indicating that complex interactions between proteins from different fractions were involved.

Decrease in protein solubility led to a gradual increase in the content of SDS-insoluble GMP (Figure 5). For gluten films stored at $T - T_g$ below −40 °C, the drop of fraction F4 was accompanied by an increase in Fi and to a lesser extent in F1, while areas of intermediate fractions F2 and F3 remained unchanged (Figure 5a). This suggested that proteins from fraction F4 did not interact. Otherwise a gradual shift of the SE-HPLC profiles at the benefit of all the nearby larger fractions (F3, F2, and F1) would have been observed. In addition, it is interesting to note that F4 protein monomers reacted specifically with polymers from fractions Fi and F1, and not at all with polymers from F2. Oxidation of gluten thiol groups does not seem to proceed at random and could not be controlled merely by the mobility of chemical groups.

When gluten films were stored in conditions favoring thiol oxidation (i.e. at $T - T_g > -40$ °C), SDS-insoluble polymers (Fi) accumulated at the expense of SDS-soluble proteins from F4, F2, and F1 (Figure 5b). Drops of F2 and F1 were monotonic until Fi reached 30% of total proteins and then stopped, although 10% of F2 was still remaining. It is noteworthy that the stop coincided with the almost total disappearance of F1. The insolubilization of GMP from F2 would necessarily involve GMP from F1. These polymers might differ one from the other by more than their molecular weight and would have distinct subunit compositions. After Fi reached 30% of total proteins, F4 dropped suddenly at its benefit, F3 and F2 areas remaining unchanged. As

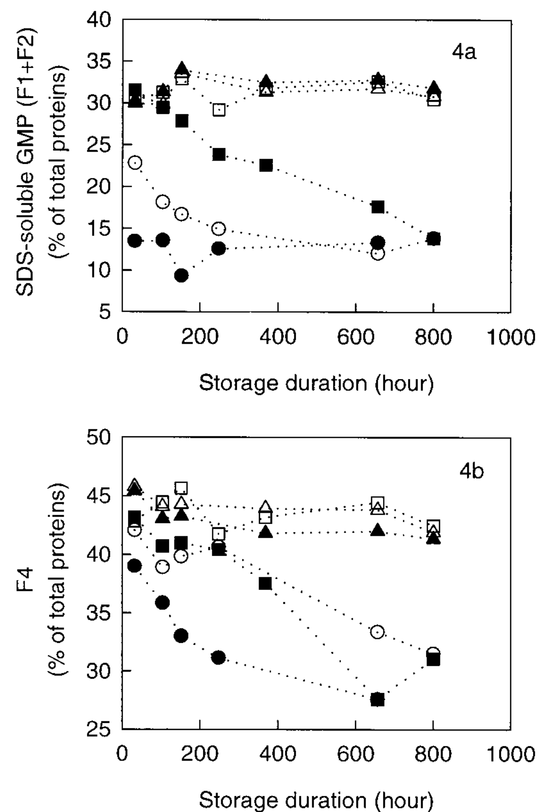

Figure 4. (a,b) Changes in SDS-soluble GMP and in monomeric proteins (F4) during the storage of gluten films. Storage conditions: (filled symbols) temperature 50 °C; (open symbols) temperature 25 °C; gluten film moisture contents (○) 14.78%, (□) 4.86%, (△) 1.14%.

has been already discussed for films stored at $T - T_g <$ −40 °C, proteins from F4 seemed to react specifically with insoluble polymers (Fi).

Changes in SDS-soluble proteins from fractions F4, F2, and F1 were plotted as a function of thiol group content (Figure 6). Data were grouped according to whether the $T - T_g$ value during storage was above (filled symbols) or below (open symbols) −40 °C. The alteration of the molecular weight distribution of proteins was consistent with thiol oxidation, and irrespective of storage conditions, for each protein fraction, data overlapped to give a unique curve. This suggests that a single sequence of events accounts for protein insolubilization through the formation of interchain disulfide bonds. Transition temperature and thus protein chain mobility would not have an influence on the sequence of events.

Three steps have been distinguished. At first, the only notable event was the monotonic decay of F4 coinciding with thiol oxidation from 9−3.5 μmol·g^{-1}. From the decreasing slope we calculated that the insolubilization of 1 mol of protein from F4 was coupled to the oxidation of 3.73 mol of thiol groups, i.e. to the formation of 3.73 mol of disulfide bonds. Since the formation of a single interchain disulfide bond between a monomer from F4 and a GMP from Fi should be enough to ensure F4 monomer insolubilization, it was likely that intrachain bonds were also formed. In a second phase and as thiol content dropped below 3.5 to 2 μmol·g^{-1}, an abrupt decrease of fractions F2 and F1 was observed. Hence, insolubilization of 20% of total proteins was achieved while less than 2% (1.50 μmol·g^{-1}) of the gluten disulfide bonds (81.18 μmol·g^{-1}) were oxidized. In terms of moles,

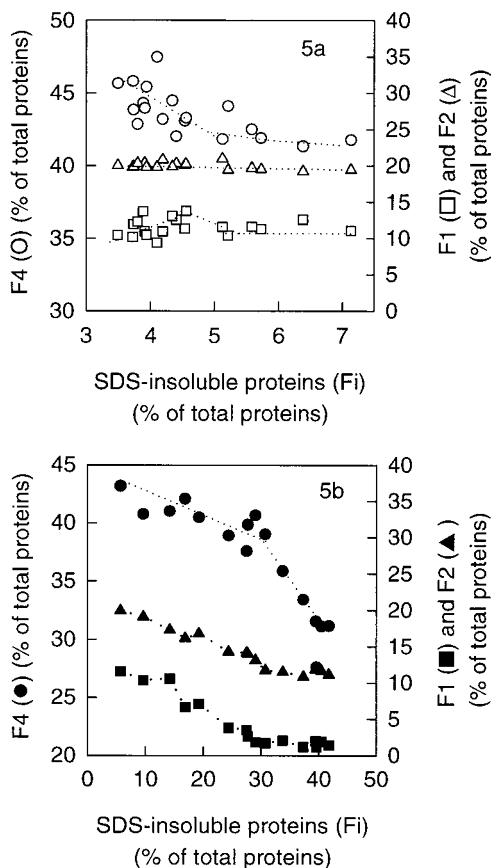

Figure 5. (a,b) Balance between SDS-soluble proteins from fractions F4, F2, and F1 and SDS-insoluble GMP in gluten films respectively stored below (a) or above (b) a $T - T_g$ value equal to −40 °C. Storage conditions: (open symbols) $T - T_g < -40$ °C; (filled symbols) $T - T_g > -40$ °C; SDS-soluble proteins of F4 (○), F1 (□), and F2 (△). T_g was estimated as in Figure 3.

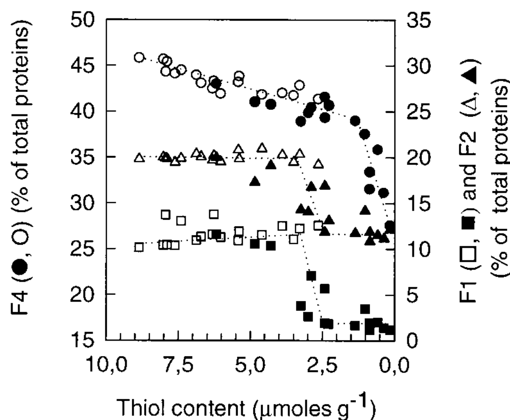

Figure 6. Relationship between SDS-soluble proteins from fractions F4, F2, and F1 and the thiol group content of gluten films stored at various temperatures and relative humidities. Storage conditions: (open symbols) $T - T_g < -40$ °C; (filled symbols) $T - T_g > -40$ °C. T_g was estimated as in Figure 3.

the drop of SDS-soluble GMP accounted for less than 0.32 μmol·g^{-1}. As previously calculated for F4, the balance between protein insolubilization and disulfide formation was far from the expected ratio of 1. During the last phase, contribution of fraction F4 to Fi rose suddenly. In terms of moles, F4 decreased by about 3.5 μmol·g^{-1} while only 1.5 μmol·g^{-1} of thiol groups were oxidized. This ratio strongly suggested that protein insolubilization was involving thiol/disulfide-interchange reactions which would convert intrachain bonds into interchain bonds. When oxidation reached completion, a network structure different from gluten was obtained. Gluten films comprised more insoluble proteins (up to 41.8% instead of 25.3% for native gluten) and less monomeric proteins (27.6% of F4 instead of 38.5%) and soluble GPM (1.21% of F1 and 10.8% of F2 instead of respectively 5.5% and 12.5% for gluten). Compared to native gluten, the fully oxidized gluten film had about 2 μmol·g^{-1} fewer SDS-soluble proteins. It was previously suggested that sulfitolysis led to the cleavage of 8 μmol·g^{-1} of disulfide bonds, among which 2 μmol·g^{-1} were intrachain. Intrachain bonding was shown to occur during the oxidation process. Nevertheless, these type of bonds would not be stable and would finally be converted into interchain bonds, thus resulting in the insolubilization of 2 μmol·g^{-1} of soluble proteins more for the fully oxidized gluten film than for native gluten.

CONCLUSION

At the level used in our study sulfite anions contributed mainly to the breakdown of interchain bonds of GMP. During drying of the film-forming solution, the acidic pH prevented oxidation of sulhydryl and *S*-sulfonate groups released during sulfitolysis. Nevertheless, thiol oxidation occurred even in storage conditions for which the mobility of chemical groups was highly restricted. We have shown that thiol oxidation, although not allowing the rebuilding of the initial structure of gluten, was not a random process. In particular, disulfide bond formation took place between quite specific protein fractions that remained the same, whereas gluten film moved from the glassy to the rubbery state. As gluten films were stored mainly in conditions which hindered the translational mobility of proteins (i.e. within glassy state), it is likely that these specific protein interactions were set up during the drying of the film-forming solution and were initially stabilized by weak bonds. Those interactions would bring thiol and *S*-sulfonate groups of proteins closer, so that in the presence of some oxidizing agent, disulfide covalent coupling can occur. Thus, the oxygen permeability would be the parameter that governs thiol oxidation of gluten films, whereas the molecular mobility and in particular segmental or translational mobility of protein chains would not be requisite. Further research is needed to study the oxidation effects on the mechanical properties of gluten films. If the mechanical properties were changed during storage, more attention would have to be paid on the history of gluten film before performing mechanical testing.

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