

Biochemical Analysis and Rheological Properties of Gluten Modified by Transglutaminase

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ABSTRACT

Cereal Chem. 77(2):121–127

A transglutaminase from *Streptovorticillium* sp. was used to create new covalent intermolecular cross-links between proteins in gluten. This modification induced drastic changes in its physicochemical properties as well as in its rheological behavior. To understand these changes, we characterized the gluten extractability in acetic acid and identified the proteins of supernatant and pellet by immunoblotting using antibodies specific for each prolamin class. The proportion of soluble proteins decreased drastically after transglutaminase treatment due to the formation of large insoluble polymers as shown by SDS-PAGE. Among the constitutive proteins of gluten, the high molecular weight glutenin subunits were the most affected in the

transglutaminase reaction. The rheological behavior of gluten after 18 hr of incubation with transglutaminase was studied in shear by dynamic measurements over 10^{-3} – 10^1 Hz frequency range and by creep and recovery tests. The behavior of treated glutes remained that of a transient network, but the viscoelastic response was shifted toward shorter times and the steady-state viscosity was greatly increased. The enzymatic treatment caused a considerable reinforcement of the network. The modified glutes were also less sensitive to thermal processing than unmodified glutes, as shown by a lower amplitude of variation of storage modulus G' with temperature after enzymatic treatment.

Dough properties are related to the viscoelastic behavior of gluten. Gluten proteins contain gliadins, which are monomeric, and glutenin subunits, which are linked together through disulfide bridges to form high molecular weight polymers. Association by hydrophobic and hydrogen bonds forms the gluten network, where the viscoelastic properties are related to the type and size of glutenin polymers (Popineau et al 1994)

Oxidizing and reducing agents affect the mechanical properties of dough or gluten very strongly by modifying the redox status of SH and SS groups of prolamins. It is likely that most of these effects are due to changes induced in the polymerization status of the glutenin subunits. However, the mechanism and patterns of such changes remain unknown and, indeed, much about the effects of redox agents on dough or gluten viscoelasticity is still unclear and remains a matter for investigation. This is true for potassium bromate and ascorbic acid, which have been used for decades in baking as dough improvers. Their action has been the subject of many studies. Dong and Hosney (1995) showed that potassium bromate slightly increases the storage modulus G' of dough. Similar positive effects on G' of dough and on the loss modulus G'' were observed with ascorbic acid (Berland and Launay 1995), but the ratio $\tan \delta = G''/G'$ did not vary. These improvers are believed to oxidize SH groups to SS bonds, thus strengthening the gluten network, but the precise processes are still obscure. Transglutaminase, an enzyme able to cross-link proteins, could also improve dough elasticity (Losche 1995) and crumb strength (Gerrard et al 1998). This enzyme catalyzes protein cross-linking through the formation of inter- or intramolecular $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptidic bonds. Intermolecular $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptidic bonds cause the polymerization of proteins. In the case of gluten, this enzyme induced the formation of high molecular weight polymers (Larré et al 1998) despite the low lysine content in gluten proteins.

The formation of such polymers as the result of transglutaminase action could modify the rheological properties of the gluten network. The objectives of this study were 1) to analyze the composition of polymers formed by transglutaminase using SDS-PAGE and immunoblotting, and 2) to investigate the rheological behavior of the enzymatically modified glutes. The viscoelastic

properties of these systems in shear under small strain values were characterized by combining dynamic measurements with creep and recovery tests. The effect of temperature was also considered.

MATERIALS AND METHODS

Materials

Analyses were performed on three near-isogenic lines (NIL 2+12, NIL 5+10, NIL double-null) of the wheat cultivar Sicco. Samples were provided by P. I. Payne. High molecular weight glutenin subunit (HMW-GS) compositions were 1/7+9/2+12, 1/7+9/5+10, and null/7+9/null, respectively (Payne et al 1987). Gluten was extracted according to Cornec et al (1994). Unless otherwise specified, the results reported concern the NIL 2+12 Sicco line.

Transglutaminase (EC 2.3.2.13) derived from *Streptovorticillium* sp. No. 8112 was kindly provided by Ajinomoto Co., Inc., Japan. Transglutaminase activity was determined by the hydroxamate procedure of Folk and Cole (1966).

Transglutaminase Reaction

Freeze-dried gluten (300 mg) was mixed with transglutaminase solution of various activities in 0.1M tris-HCl buffer, pH 7.5, at 20°C, and the reaction mixture was incubated for 18 hr at 40°C. Preliminary measurements showed that in all conditions tested, the enzymatic reaction was finished within this period. For the control experiments, the enzyme solution was replaced by the same volume of buffer. The exact volume needed to rehydrate the gluten was determined beforehand as described by Cornec et al (1994). Various enzyme concentrations were used in this study: 0.003, 0.006, 0.03, and 0.3 enzyme activity U/mg of dry gluten. Reaction products were then freeze-dried or immediately used for the rheological characterization.

Selective Gluten Solubilization

Freeze-dried native and treated glutes were dispersed for 18 hr in 0.1M acetic acid at a concentration of 1 mg/mL. The suspensions were centrifuged at $16,000 \times g$ for 20 min. The supernatants and pellets were recovered and freeze-dried. Protein content was determined by Kjeldahl procedure.

SDS-PAGE and Immunoblotting

The supernatants and pellets were subjected to SDS-PAGE under reducing conditions. Supernatants were analyzed on vertical 12% polyacrylamide gel and the pellets on 10% polyacrylamide gel according to Laemmli (1970). Immunoblotting was performed after SDS-PAGE on both fractions (supernatants and pellets). We

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used polyclonal and monoclonal antipeptide antibodies directed against the constitutive gluten proteins (Denery-Papini et al 1994, 1995, 1996). The anti-NT2 ω antiserum was obtained by immunization with a 12-amino acid peptide (Table I) corresponding to the N-terminal sequence of ω 5-type gliadin (Kasarda et al 1983).

Rheological Measurements

The viscoelasticity of the reaction mixtures and the control was studied in shear with a Carrimed SL 100 rheometer in the con-

plate geometry (4° cone angle, 2 cm diameter) (Cornec et al 1994). After 18 hr of incubation at 40°C, the samples were placed into the measuring device of the rheometer and covered with silicon oil to avoid evaporation.

For the first step, the frequency dependence of the storage (G') and loss (G'') moduli (mechanical spectrum) was recorded at 20°C over the frequency range 0.001–10 Hz; the strain amplitude was kept close to 3% to be in the linear region (Lefebvre et al 1994).

Next, the sample was submitted at 20°C to a retardation test (creep and creep recovery): constant stress σ_0 was applied for 10 hr and then removed. The strain γ was recorded against time during the 10-hr creep period and for 30 hr after stress removal (recovery). For σ_0 , the value of the stress that gave a strain amplitude of 3% at 0.001 Hz was chosen. This kept the maximum strain value at the end of the creep period to reasonable values.

To study the effect of temperature, native and treated glutes were loaded onto the rheometer at 20°C, heated to 70°C, held at 70°C for 30 min, and cooled back to 20°C. The heating and cooling steps were conducted at 1°C in 3 min. During this temperature cycle, G' and G'' were monitored at 1 Hz under a strain amplitude of 3%.

TABLE I
Reactivity of Antipeptide Antisera

Domain	Peptide	Antibodies	Specific Detection ^a
N-terminal	NIQVDPSGY	Anti-NT1- γ	γ -Gliadins
C-terminal	GIDAGIGGQ	Anti-CT2- γ	γ and ω -Gliadins
N-terminal	SRLSPRGKELGC	Anti-NT2- ω	ω 5-type Gliadins
N-terminal	SHIPGLERPSGC	Anti-NT-LMG (MAb 6x1)	LMW-GS
Rep	GYYPSTPQQPGC	Anti-R2-HMG	HMW-GS

^a GS = glutenin subunits.

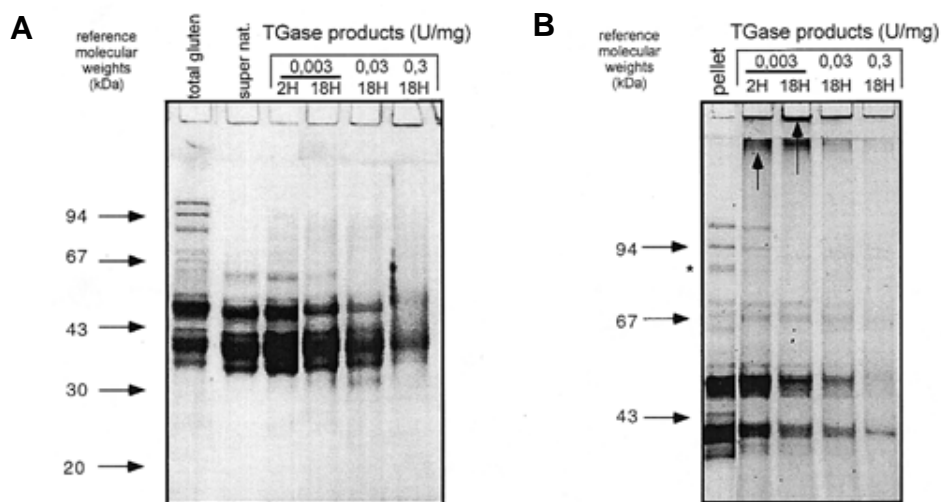


Fig. 1. SDS-PAGE in reducing conditions for 12% (A) and 10% (B) polyacrylamide of acetic soluble and insoluble fractions of native gluten from Sicco NIL 2+12 or glutes treated with transglutaminase (TGase) enzyme as indicated.

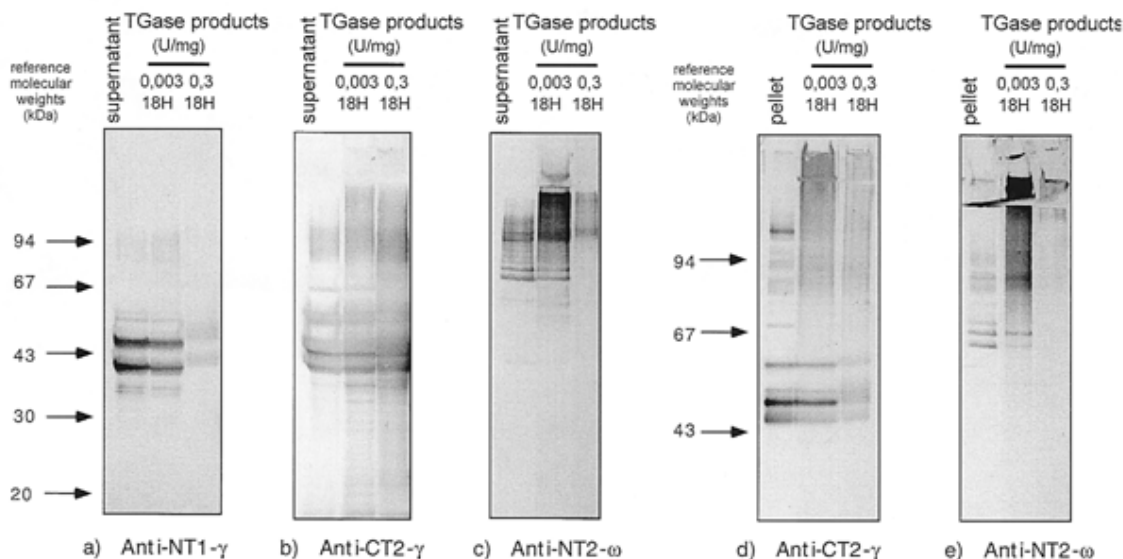


Fig. 2. Immunoblotting analysis of acetic soluble (a, b, c) and insoluble (d, e) fractions of Sicco NIL 2+12 native glutes or gluten treated with transglutaminase (TGase) enzyme as indicated, with three antipeptide antisera against γ and ω gliadins.

RESULTS

SDS PAGE and Immunoblotting Analyses

The transglutaminase reaction on gluten induced the formation of polymers insoluble in acetic acid (Larré et al 1998). Various ratios of enzyme and substrate were chosen to modify gluten in order to obtain different levels of modification at the end of the reaction. Preliminary SDS-PAGE analysis of the total reaction products obtained at various enzyme concentrations and reaction times (2 and 18 hr) clearly showed that polymerization occurred, and that the quantity of polymerized products was related to the quantity of enzyme used and the reaction time (results not shown). Besides the formation of isopeptidic bonds, transglutaminase can also catalyze the incorporation of primary amines or deamidation. In this study, the amine incorporation can be neglected because we can assume that no reactive amino groups are present in gluten except those of the proteins, meaning that the only bonds formed will be between gluten proteins. The question of deamidation as secondary reaction of transglutaminase can be raised because of the high glutamine content of gluten. The pH level used for the enzymatic reaction favors the reactivity of the ϵ amino groups of lysine and not that of water molecules, making this secondary reaction almost improbable (Larré et al 1993). Moreover, this polymerization is accompanied by a decrease of gluten solubility. Such a large increase of the insoluble fraction after enzymatic reaction was indeed a proof that deamidation, if present, had no significant effect on the reaction product. The changes of gluten behavior were dominated by cross-linking.

To have a better resolution of the different types of proteins, we chose to separate the reaction products into two fractions according to their solubility in acetic acid (Larré et al 1998). Electrophoresis results for both fractions, soluble and insoluble, are presented in Fig. 1A and B.

SDS-PAGE patterns obtained for native gluten showed that gliadins and low molecular weight glutenin subunits (LMW-GS) were mainly found in the soluble fraction (lane 2, Fig. 1A) with molecular weights in the range of 30,000–60,000. But few LMW-GS still remained in the pattern of the insoluble fraction (lane 1, Fig. 1B). HMW-GS were only present in the insoluble fraction. The enzyme reaction led to products that are less soluble in acetic acid. The patterns relative to the soluble fractions obtained with different enzyme concentrations showed a progressive decrease in

the intensity of the bands corresponding to native gliadins and LMW-GS when the enzyme to substrate (E/S) ratio increased. At the same time the analysis of the insoluble fraction patterns (Fig. 1B) showed that the intensity of the bands corresponding to HMW-GS decreased and, simultaneously, new bands corresponding to higher molecular weight molecules appeared, some of them unable to penetrate the stacking gel. When reaction times were extended to 18 hr for enzyme concentrations of 0.03 and 0.3 U/mL (Fig. 1B), the intensity of the bands corresponding to native gluten decreased and almost disappeared, but no additional bands were observed. This was related to the formation of polymers of very high molecular weight that became totally insoluble in SDS.

Immunoblotting analysis with antibodies directed against the different proteins followed electrophoresis (Fig. 2). Using antibodies able to recognize γ and ω -gliadins, we were able to detect in the soluble fraction, after the enzymatic reaction, bands corresponding to native proteins and also bands corresponding to proteins of higher molecular weight. For γ -gliadins, antibodies directed against the N-terminal peptide did not recognize any molecules other than the native residual proteins after the enzymatic reaction, while the antibodies directed against the C-terminal peptide detected bands of higher molecular weight. This selective recognition can be related to the accessibility of the epitopes to the antibodies. As both sequences contain one glutamine and no lysine, this loss of recognition by anti-NT1 γ antibodies could imply that the N-terminal region of γ -gliadins is more implicated than the C-terminal in cross-linking, and that the epitope was modified by the formation of a bond between Gln 3 and a lysyl residue. This would indicate that the N-terminal region of γ -gliadins is very accessible to the enzyme and that Gln 3 is very reactive. Some gliadins were also revealed in the insoluble fraction with molecular weights $>70,000$ Da.

After the enzymatic reaction, MAb 6x1 directed against LMW-GS still detected these proteins in the insoluble fraction at their original molecular weights and also recognized polymers of higher molecular weights (Fig. 3). The anti-R2 HMG antibodies detected very few of the HMW-GS at their original molecular weight after the enzymatic reaction, whereas they reacted strongly with bands located at very high molecular weights, even in the stacking gel.

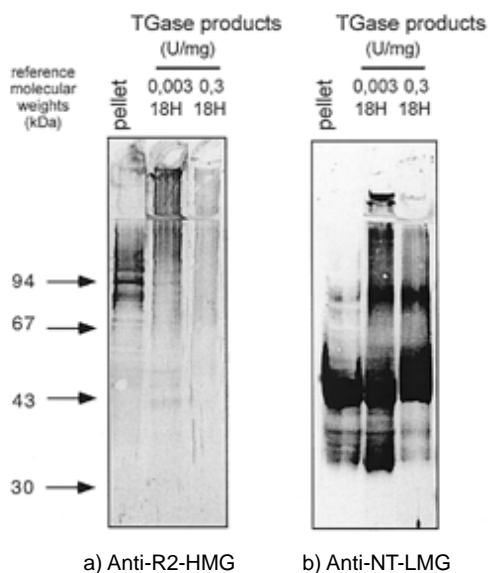


Fig. 3. Immunoblotting analysis of acetic insoluble fractions of Sicco NIL 2+12 native gluten or Sicco NIL 2+12 glutens treated with transglutaminase (TGase) enzyme as indicated, with two antipeptide antisera against high (a) and low (b) molecular weight glutenin subunits.

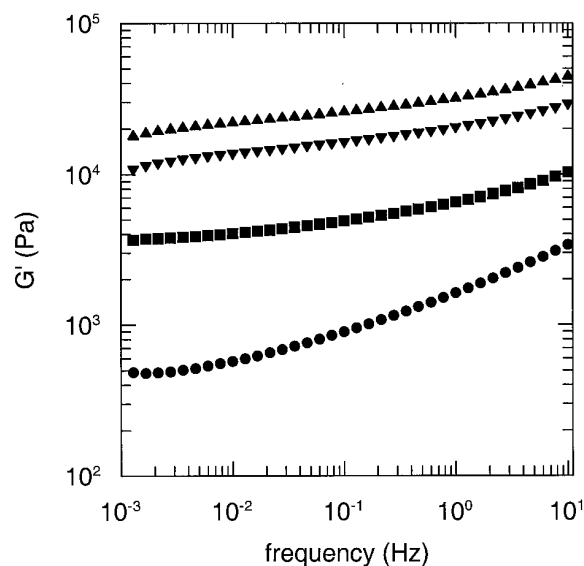


Fig. 4. Mechanical spectra (storage moduli G' only) of Sicco NIL 2+12 native gluten and gluten treated at various enzyme and substrate ratios (expressed in units of transglutaminase/mg of gluten). Strain amplitude 0.03; temperature 20°C. ▲ = 0.3 U/mg; ● = without enzyme; ▼ = 0.006 U/mg; ■ = 0.003 U/mg.

Viscoelastic Behavior at 0.001–10 Hz

The mechanical spectra obtained for native and three enzymatically treated glutes are presented in Fig. 4. After treatment, G' increased all over the experimental window and appears to be less frequency-dependent. The tangent of the loss angle $\tan \delta$ is plotted against frequency in Fig. 5. This figure shows that the values of $\tan \delta$ obtained for the treated glutes are lower than those obtained for native gluten and that the minimum in $\tan \delta$ was shifted toward higher frequencies.

As we explained previously (Cornec et al 1994), the mechanical spectra of glutes lend themselves more conveniently to a quantitative analysis when plotted as the variations with frequency (f) of the storage compliance $J' = G'/(G'^2 + G''^2)$ and the loss compliance $J'' = G''/(G'^2 + G''^2)$. A loss peak (maximum in J'') is framed in the high frequency region of the experimental window, but it is more or less visible because it is strongly overlapped in the low frequency region by slower retardation processes. In the high frequency region of the spectrum, J' and J'' curves can be fitted by Cole-Cole functions (Cornec et al 1994):

$$J'(f) - J_g = \left(J_N^0 - J_g \right) \frac{\left[\left(f_0/f \right)^n + \cos(\pi n/2) \right]}{\left[\left(f_0/f \right)^n + 2 \cos(\pi n/2) + \left(f/f_0 \right)^n \right]} \quad (1)$$

$$J''(f) = \left(J_N^0 - J_g \right) \frac{\sin(\pi n/2)}{\left[\left(f_0/f \right)^n + 2 \cos(\pi n/2) + \left(f/f_0 \right)^n \right]} \quad (2)$$

where: J_N^0 is the compliance associated with the viscoelastic plateau, the high frequency limit of which is marked by the loss peak; f_0 is the frequency of the maximum in J'' (central frequency of the retardation process considered); n is the frequency spread parameter that measures the broadness of the retardation times distribution corresponding to the loss peak; and J_g is the glassy compliance that can be neglected as compared to J' and J_N^0 values. The modulus corresponding to the viscoelastic plateau is $G_N^0 = 1/J_N^0$.

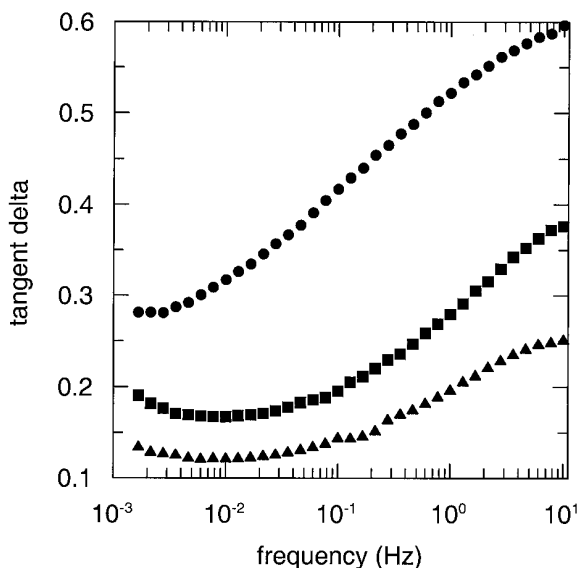


Fig. 5. Variation with frequency of the tangent of the loss angle for Sicco NIL 2+12 native gluten and gluten treated at various enzyme and substrate ratios (expressed in units of transglutaminase/mg of gluten). Strain amplitude 0.03; temperature 20°C. \blacktriangle = 0.3 U/mg; \bullet = without enzyme; \blacksquare = 0.003 U/mg.

A convenient method to determine J_N^0 , f_0 , and n (the classical one indeed) consists of plotting J'' against J' . The retardation process shows as an arc of circle passing through the origin, the equation of which is obtained by eliminating f/f_0 between Equations 1 and 2:

$$J'' = \left(\frac{J_N^0}{2 \tan(\pi n/2)} \right) \left[\left[1 + 4 \frac{J'}{J_N^0} \left(1 - \frac{J'}{J_N^0} \right) + \tan^2(\pi n/2) \right]^{\frac{1}{2}} - 1 \right] \quad (3)$$

The fit of Equation 3 to the results gives the Cole-Cole parameters J_N^0 and n , and a simple geometrical construction yields f_0 (Tschoegl 1989). Figure 6 shows the application of this classical representation to some of our results. Table II collects the values of the Cole-Cole parameters obtained for the different enzyme concentrations with the NIL 2+12 line. The Cole-Cole parameters relative to native glutes and glutes treated at 0.03 U/mg for three near-isogenic lines are given in Table III.

The action of the enzyme on NIL 2+12 gluten increases the height of the viscoelastic plateau G_N^0 by a factor of ≈ 30 and results in a tremendous shift of the upper frequency limit of this plateau. The value of f_0 for enzymatic treatment at 0.3 U/mg was 1,000 \times higher than that of the control. The exponent n (the values of which are necessarily between 0 and 1) does not vary much. However, it is somewhat lower after enzyme reaction, indicating that the distribution of retardation times becomes slightly broader. As far as G_N^0 is concerned, the effect of the enzyme seems to be completed for ≈ 0.03 U/mg. The moderate decrease observed at the highest concentration used is probably not significant. On the contrary, f_0 does not show such a plateau or maximum, but increases steadily with enzyme concentration.

The native gluten of NIL 2+12 can be considered weak; the value of its viscoelastic plateau modulus $G_N^0 = 359$ N/m². The effect of transglutaminase treatment at 0.03 U/mg of gluten was also characterized on NIL 5+10 and NIL double-null, which can be considered as stronger and slightly weaker than NIL 2+12, respectively. In both cases, enzymatic cross-linking modifies in the same way as the Cole-Cole parameters (Table III). Moreover, the enzymatic treatment raises them to almost the same level of viscoelasticity as it does for NIL 2+12. The G_N^0 values for the three lines are very high, such values are higher than values obtained for strong gluten. However, similar values have been obtained on gluten mainly consisting of large glutenin polymers (Cornec et al 1994). This confirmed that in all cases, the enzymatic treatment induced very large polymers and left only a few small-sized proteins free in the network.

Steady-State Compliance and Viscosity

The low frequency slope of the dissipation peak analyzed above is embedded in slower retardation mechanisms (Figs. 4 and 5). They extend visibly below the lower limit of the frequency window and their study, therefore, would require the analysis of the step response to transient experiments such as creep and recovery tests. Figure 7 shows the creep and recovery curves of native glutes and glutes treated at 0.3 U/mg. The fact that a large part of the steady-state compliance is not recovered at the end of the recovery period, although the compliance has reached a plateau value or nearly so, indicates without ambiguity that gluten keeps its liquid character after the enzymatic cross-linking reaction. The response also spans a shorter time range after this reaction. The detailed study of gluten long-term viscoelastic response was not within the scope of this work.

We limited ourselves to the creep and recovery curves of native and gluten treated at 0.3 U/mg for the values of the steady-state compliance (J^0) and the steady-state viscosity (η), which characterize the overall elastic and viscous contributions, respectively, to the deformation (Table IV).

The values of the rheological quantities determined from the creep and recovery curves agree fairly well, and this could be taken as an indication that the results were obtained in a linear regime and steady-state conditions, or at least close to them. J_e^0 should satisfy the condition $J_e^0 \geq J_N^0$. In fact, one expects J_e^0 to be somewhat higher than J_N^0 because of the slower retardation modes mentioned earlier. Tables II and IV show that this is indeed the case for the native sample. In cross-linked gluten, the value of J_e^0 is slightly lower than J_N^0 . This anomaly could result from errors in the rheological measurements or in the determination of J_N^0 and J_e^0 because of the difficulty in reaching the steady-state in the retardation experiments. In addition, a nonreversible hardening of gluten is induced by flow during creep (J. Lefebvre, *personal communication*). This phenomenon results in a lower apparent J_e^0 value. Both explanations imply that the difference between J_e^0 and J_N^0 is greatly reduced after enzymatic reaction. Relatively, the slower retardation processes make a much smaller contribution to the total strain after cross-linking.

Effect of Temperature on Viscoelasticity of Gluten

We have not attempted a detailed analysis of the effect of cross-linking on the temperature-dependence of gluten viscoelasticity, but just compared the response of native and treated NIL 2+12 gluten to temperature cycles monitored at a fixed frequency (1 Hz). The changes in G' observed through the temperature cycles $20^\circ\text{C} \rightarrow 70^\circ\text{C} \rightarrow 20^\circ\text{C}$ for native and treated (0.3 U/mg) NIL 2+12 gluten are illustrated in Fig. 8. In the native sample, G' first decreases as temperature increases and then reaches a well-marked minimum at $\approx 50^\circ\text{C}$. At $>50^\circ\text{C}$, G' increases steeply. Up to 50°C , the decrease in G' is essentially reversible and can be attributed to the weakening of the hydrogen bonds that contribute to the cohesion of the gluten network (Lefebvre et al 1994). Above 50°C , the effect of heating becomes predominantly irreversible as chemical changes affect the SH groups and SS bonds, especially those of the HMW-GS (Schofield et al 1983, Lefebvre et al 1994, Hargreaves et al 1995). This irreversibility shows in the difference between heating and cooling of the G' vs. temperature curve (Fig. 8). During cooling from 70 to 20°C , G' values increase monotonously (due to the reformation of rheologically efficient, low-energy interactions in the network) and are considerably higher than those displayed during heating, which is probably a consequence of network reorganization triggered by the SH/SS exchange reactions. In treated gluten, the effect of temperature is greatly reduced, although it remains qualitatively similar: the amplitude of variations of G' with temperature and the difference between the heating and cooling parts of the cycle are both much smaller than for native gluten.

DISCUSSION

We have demonstrated that the addition of covalent bonds by transglutaminase treatment modifies the gluten network properties. Enzymatic treatment of glutes, presenting either high or low viscoelastic properties, gives rise to modified glutes with very similar high viscoelasticity (Tables II and III). In all cases, this improvement of the viscoelastic properties was accompanied by the formation of polymers. Characterizing polymers by SDS-PAGE in reducing conditions showed that all types of constitutive proteins of Sicco NIL 2+12 gluten were able to form polymers through isopeptidic bonds catalyzed by transglutaminase. However, immunoblotting assays show that even in the strongest reaction conditions (high E/S ratio or 18 hr of reaction time), some of the α - and β -gliadins and LMW-GS were not engaged in an isopeptidic bond. Less participation of LMW-GS as compared with HMW-GS was also pointed out. The examination of glutamine and lysine contents of both types of glutenin subunits revealed that the glutamine content of both types of glutenins is very high and comparable, but HMW-GS contain 6–8 mol of lysine/mol of protein (Thompson et al 1985, Anderson et al 1989) but only 1 mol of lysine/mol of protein for LMW-GS (Pitts et al 1988,

Colot et al 1989). This very low lysine content of LMW-GS could explain their lesser participation in the formation of intermolecular isopeptidic bonds.

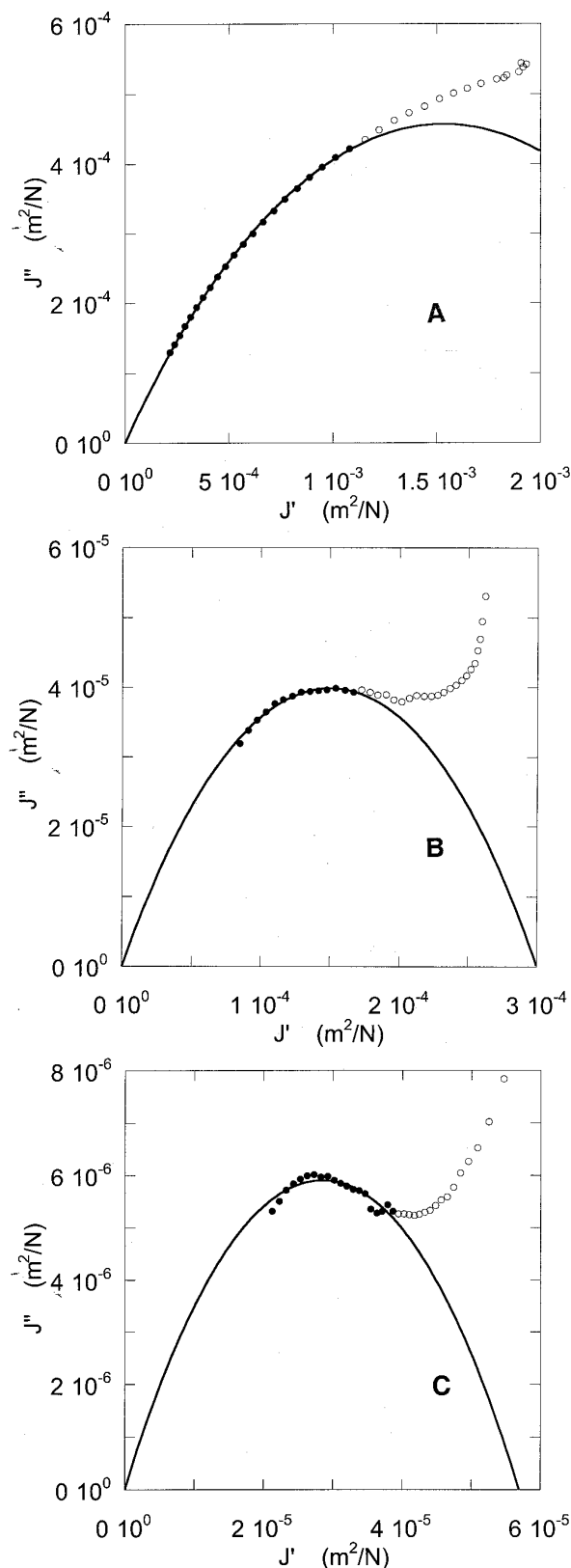


Fig. 6. Mechanical spectra plotted in the complex plane $J' - J''$ for Sicco NIL 2+12 native gluten and gluten treated at various enzyme and substrate ratios (expressed in units of transglutaminase/mg of gluten). **A**, native gluten; **B**, 0.003 U/mg; **C**, 0.03 U/mg. Solid lines show arc of circle obtained by fitting Equation 3 to results in the high frequency region. ● = Experimental points used for the fit.

The rheological behavior of enzymatically treated gluten is qualitatively quite similar to that of native gluten but was shifted to much larger values of storage G' and loss moduli G'' . The transglutaminase reaction increased the viscoelastic plateau already present in the gluten. The enzyme effect lead to the formation of isopeptidic bonds between the constitutive proteins and, therefore, between polymers or polymers and gliadin. The size of the large glutenin polymers is increased. For NIL 2+12, values calculated for the viscoelastic plateau modulus G_N^0 increased with enzyme concentration to 15,600 N/m². The maximum obtained for G_N^0 could be accounted for in three ways: 1) all available lysine present in the gluten has reacted and the remaining monomeric proteins, as well as the polymerized protein, are no longer able to participate in the reaction; 2) when a certain number of covalent bonds is formed in the network, the enzyme is no longer able to diffuse and find any more substrate in its vicinity; 3) there is a limit to the number of covalent bonds added in the gluten, past which no further effect on viscoelasticity could be seen.

Comparing the viscoelastic characteristics of three NIL lines with different glutenin subunits after enzymatic treatment showed that the values measured for G_N^0 are quite similar. This indicates the existence of a threshold number of connections in the network.

TABLE II
Viscoelastic Characteristics for Sicco NIL 2+12 Glutens^a

Enzyme Concentration ^b (U/mg of gluten)	J_N^0 (m ² /N)	G_N^0 (N/m ²)	f_0 (Hz)	n
0*	0.00280	359	0.019	0.380
0.003	30×10^{-5}	3,330	0.73	0.330
0.006	8.7×10^{-5}	11,530	0.26	0.280
0.03**	6.5×10^{-5}	15,600	1.9	0.266
0.3	10.1×10^{-5}	9,910	22	0.306

^a Obtained by fitting the Cole and Cole model to the high frequency region of the experimental $J'(f)$ and $J''(f)$ functions. J_N^0 = compliance associated with the viscoelastic plateau; f_0 = frequency of the maximum in J'' (central frequency of the retardation process considered); n = frequency spread parameter that measures the broadness of the retardation times distribution corresponding to the loss peak; G_N^0 = modulus corresponding to the viscoelastic plateau.

^b * = mean of three replicates; ** = mean of two replicates.

TABLE III
Viscoelastic Characteristics of Three Glutens Obtained from Sicco Near-Isogenic Lines^a

Enzyme Concentration (U/mg)	Line	J_N^0 (m ² /N)	G_N^0 (N/m ²)	f_0 (Hz)	n
0	NIL 2+12	2.8×10^{-4}	359	0.019	0.380
	NIL 5+10	8.6×10^{-4}	1,170	0.12	0.320
	NIL 00	3.6×10^{-3}	278	$<10^{-3}$	0.447
0.03	NIL 2+12	6.5×10^{-5}	15,600	1.9	0.266
	NIL 5+10	7.2×10^{-5}	13,950	1.7	0.235
	NIL 00	7.7×10^{-5}	12,900	0.60	0.267

^a Obtained by fitting the Cole and Cole model to the high frequency region of the experimental $J'(f)$ and $J''(f)$ functions. J_N^0 = compliance associated with the viscoelastic plateau; f_0 = frequency of the maximum in J'' (central frequency of the retardation process considered); n = frequency spread parameter that measures the broadness of the retardation times distribution corresponding to the loss peak; G_N^0 = modulus corresponding to the viscoelastic plateau. 00 = double-null.

TABLE IV
Steady-State Compliance (J_e^0) and Steady-State Viscosity (η) of Treated and Untreated Gluten

Enzyme Concentration (U/mg)	From Creep Curve		From Recovery Curve	
	J_e^0 (m ² /N)	η (Pa·sec)	J_e^0 (m ² /N)	η (Pa·sec)
0	9.88×10^{-3}	1.97×10^6	11.1×10^{-3}	2.11×10^6
0.3	8.56×10^{-5}	490×10^6	8.00×10^{-5}	460×10^6

The trend for f_0 to shift toward higher frequencies when the enzyme concentration was increased can be compared with results obtained by Cornec et al (1994) on gluten fractions varying in their content in the largest polymers. They observed a similar displacement of f_0 as this content increased, suggesting that the shift we observed could be related to the formation of larger size polymers.

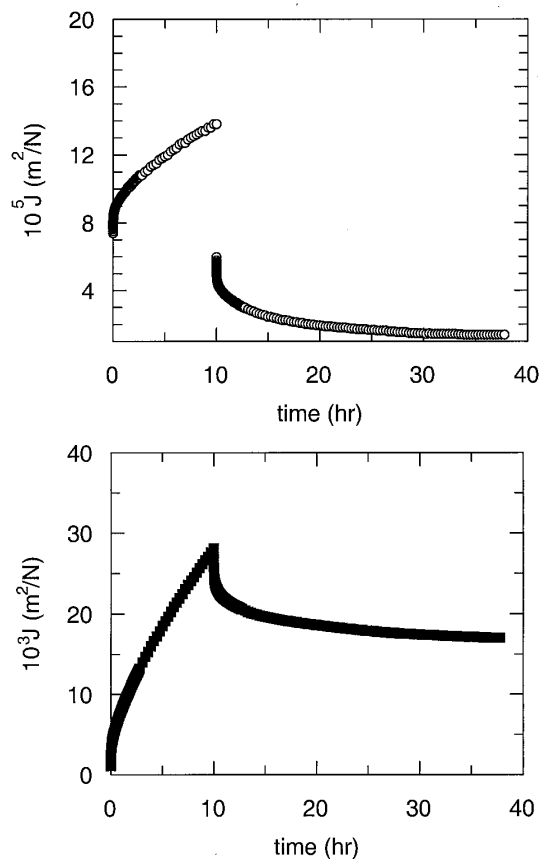


Fig. 7. Creep and recovery curves obtained for Sicco NIL 2+12 native gluten and gluten treated at various enzyme and substrate ratios (expressed in units of transglutaminase/mg of gluten). Applied stress 15 Pa for native gluten, 500 Pa for enzymatically treated gluten; ■ = without enzyme; ○ = 0.3 U/mg.

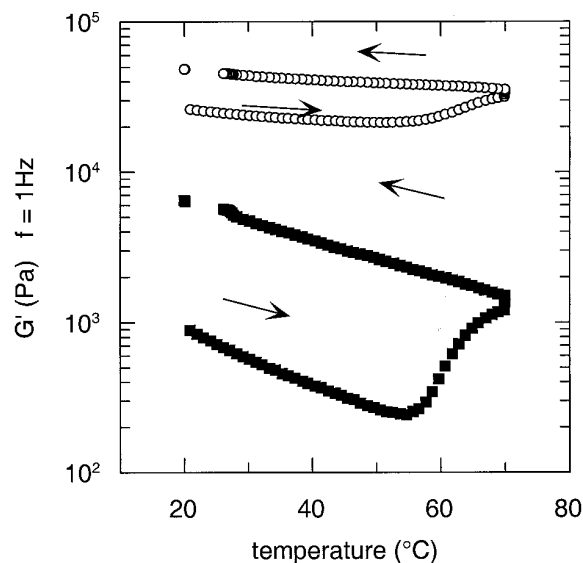


Fig. 8. Storage moduli G' of Sicco NIL 2+12 native gluten and gluten treated at various enzyme and substrate ratios (expressed in units of transglutaminase/mg of gluten) as a function of temperature during heating and cooling cycle; ■ = without enzyme; ○ = 0.3 U/mg.

We can assume that the increase in the height of the viscoelastic plateau observed after enzyme reaction reflects an increase in the connectivity of the gluten network from the formation of covalent bonds. However, despite the creation of these supplementary covalent intermolecular bonds within the initial network, enzymatically treated gluten keeps its viscous character, as shown by the creep and recovery experiments, which means that gluten remains a transient network system. Therefore, the covalent intermolecular bonds formed by the enzyme reaction are probably not directly responsible for the increase in cross-linking density of the network, but rather involve structural modifications within the building blocks, which result in an increase of the number or the strength of nonpermanent cross-links between them. The increase of G_N^0 and the decrease of J_N^0 , as well as the increase of the steady-state viscosity after enzymatic treatment, resulted from the apparition of larger size polymers, partly at the expense of monomeric proteins, as shown by immunochemical results.

Glutenin subunits are associated through SS bonds to form large polymers that constitute basic elements of the network. The reversible decrease in G_N^0 observed as temperature increases up to 50°C indicates that the contribution of hydrogen bonds to connectivity is probably predominant (Lefebvre et al 1994). At >50°C, the irreversible effects of temperature on gluten viscoelasticity, which are inhibited when an SH-blocking agent is added, seem to involve the SH/SS interchange reactions (Schofield et al 1983, Lefebvre et al 1994). The formation of isopeptidic bonds visibly stabilizes the network against temperature effects. Part of this stabilizing action is probably indirect and could be a consequence of the stiffening of the structure (Hargreaves et al 1995) that would hamper the SH/SS exchange reactions, which decreases the irreversible changes induced by heating at >50°C.

CONCLUSIONS

Protein cross-linking by transglutaminase was efficient in hydrated gluten despite its low lysine content, relatively low water content, and very high viscosity of the medium. All the constitutive gluten proteins were substrates, but the HMW-GS presented the highest reactivity. The level of polymerization was dependent on the quantity of enzyme and the reaction time. The formation of new covalent bonds in the gluten network deeply affected its rheological behavior. Addition of transglutaminase made it possible to transform a very weak gluten into a very strong one. The increase of the viscoelastic plateau values and the shift of f_0 toward higher frequencies were related to an increase in the connectivity of the gluten network. Nevertheless, our data showed that the gluten network keeps its liquid character. Heat treatment affected native and treated gluteins in a similar way, but the transglutaminase cross-linked gluten appeared to be less sensitive. The superimposition of covalent bonds to the initial gluten network stabilized it against effects of temperature.

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[Received May 24, 1999. Accepted August 25, 1999.]

NOTE FROM THE PUBLISHER: The article by C. Larré, S. Denery-Papini, Y. Popineau, G. Deshayes, C. Desserme, and J. Lefebvre that appeared in the January-February issue (77:32-38) "Biochemical Analysis and Rheological Properties of Gluten Modified by Transglutaminase" contains errors and omissions in text. The corrected article is reprinted here in its entirety.