Controlled Reduction Study of Modifications Induced by Gradual Heating in Gluten Proteins

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The stepwise reduction with dithiothreitol (DTT) of proteins in native and heat-treated gluten (1 h, 45−110 °C) was studied by SDS−PAGE. In native gluten and after heating at 45 °C, up to 0.02 mM DTT led to no apparent change. Subunits from glutenins and high-molecular weight (HMW) albumins were released with 0.4−10 mM DTT, indicating the presence of disulfide bonds with different susceptibilities to reduction. Monomeric proteins were more resistant to reduction; low-molecular weight (LMW) albumins/globulins were reduced at 2 mM DTT and α- and γ-type gliadins at 4 mM DTT. At 65 °C, only the HMW albumins were affected; they lost water solubility and amylase activity and were released at higher DTT concentrations. When heated above 90 °C, all the proteins, except the ω-gliadins, formed disulfide-bonded aggregates. The supposed D glutenin subunits were released at 0.4 mM DTT, the HMW glutenin subunits at 2 mM DTT, and the other proteins at 4 mM DTT. Gluten proteins therefore appeared to be involved differently in heat-induced aggregation.

Keywords: *Gluten; heat treatment; disulfide-dependent aggregation; controlled disulfide reduction*

INTRODUCTION

Sulfhydryl (SH)/disulfide (S−S) groups play important roles in the structure and reactivity of gluten and thus in the technological properties of wheat (Pomeranz, 1988; Schofield, 1994). The presence, number, and sites of SH and S−S groups as well as the surface properties and noncovalent interactions of gluten proteins are crucial.

A detailed knowledge of the location of inter- and intramolecular S−S bonds of gluten proteins is still being developed. In fact, the state of aggregation of glutenin, the polymeric component of gluten, has been shown to depend on multiple intermolecular S−S bonds linking the low-molecular weight (LMW) and high-molecular weight (HMW) subunits to each other and to the γ-type gliadins (Kohler et al., 1993). Furthermore, the location of cysteines in the N- and C-terminal domains of HMW glutenin subunits has a profound effect on glutenin polymerization behavior (Shewry et al., 1992). In α-type gliadin polypeptides [which according to Shewry et al. (1986) embrace α- and β-gliadins), only intramolecular disulfides are present. Their location is homologous to the location of intramolecular S−S bonds in γ-type gliadins and LMW glutenin subunits (Kohler et al., 1993). ω-Gliadins lack cysteine (Booth and Ewart, 1969).

Some flour albumins and globulins, residual in hand-washed gluten, have been identified as a LMW cysteine-rich fraction with a high affinity for flour lipids; this is called the S protein fraction (Zawistowska et al., 1986). HMW albumins occurring in gluten were found to correspond to β-amylase (Curioni et al., 1994).

Studies involving controlled reduction of dough by DTT have led to the formulation of a block polymer model of polymeric glutenin; also demonstrated was the presence of rheologically effective disulfide bonds (Gao et al., 1992). An extra strong and a weaker wheat flour were shown to have different resistance to reduction by DTT (Kim and Bushuk, 1995). These studies elucidate the relationship existing between glutenin molecular structure and functionality.

The S−S bonding structure of gluten is not static. Technologically important reduction and oxidation reactions involving these groups occur in flour, and especially in dough, during mixing and baking (Pomeranz, 1988). Exogenous redox reagents, such as ascorbic acid and potassium bromate, which for many years have been used in baking as flour improvers, are thought to act on the S−S structure of glutenin (Schofield and Chen, 1995). Also, the involvement of redox active components which occur endogenously in flour is important as such components modify the functional properties of gluten proteins, affecting product quality. Recently, considerable interest has been expressed in the involvement of glutathione, which is able to cleave protein intermolecular S−S bonds, specifically those in glutenin, in the redox reactions occurring in gluten (Schofield and Chen, 1995). Moreover, wheat flour has an active redox transfer system, the NADP/thioredoxin system, which acts on the intra- and intermolecular S−S bonds in proteins (Kobrehel et al., 1992).

Physical processes affect the breakdown and reassembly of S−S structure. During mixing, glutenin is partly depolymerized, while it repolymerizes during resting (Weegels et al., 1993). Drastic changes in the S−S structure of gluten are induced by heat treatment. Schofield and co-workers (1983) report that the conformational changes caused by heat treatment rearrange the S−S bonds also in the monomeric proteins of gluten, especially gliadins. Weegels and co-workers (1994a,b) observed S−S binding and irreversible conformational changes upon heating, at 80 °C, gluten containing more than 20% moisture. Jeanjean and co-workers (1980) suggest that the formation of S−S bonds during heating is the most probable mechanism for the changes in gluten heated at 100 °C. However, not all the salt-soluble proteins and gliadins participate in the formation of the insoluble protein network. Our previous work has shown that the reactions involving the S−S/

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SH system are preceded by changes in protein conformation, reflected in surface hydrophobicity (Cerletti et al., 1994).

The use of gluten in any food product involves at least a heating step, during which the gluten proteins are set. Heating causes the setting of the loaf structure during baking and the generation of the textural properties of pasta (Schofield et al., 1983). In the preparation of commercial gluten, heating during the drying step can lead to the loss of gluten functionality (Booth et al., 1980; Schofield et al., 1983; Wadhawan and Bushuk, 1989; Weegels et al., 1994a).

The protein network developed during heating at high temperatures is insoluble even in strong dissociating media; after reduction, solubility is recovered, but the information on the S−S bond arrangement is lost. In the present work, a tentative investigation on the heat-formed protein network was made by studying the S−S bond arrangement in heat-treated and native gluten, through a stepwise reduction as done for native glutenin by Gao et al. (1992) and Kim and Bushuk (1995). A SDS-containing medium was adopted to allow the dispersion of the sample and to improve the S−S bond accessibility to the reducing agent. The behavior of native gluten was taken as the reference. At baking, protein−protein interactions involve all gluten proteins; therefore, we considered gluten in our studies, not just the isolated proteins.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT) and iodoacetamide (IAM) were from Fluka. Zulkowsky starch was from Sigma. Coomassie Brillant Blue R-250 was from Bio-Rad. Ethanol and *n*-propanol were Baker Analyzed reagents.

Isolation and Heat Treatment of Gluten. Soft wheat flour of the Italian cultivar Mec (*Triticum aestivum*) was supplied by the Istituto Nazionale della Nutrizione, Rome. Gluten was prepared by the AACC method of hand washing number 38-10 using a 30 min resting time.

Small samples of gluten (10 g, 60% moisture) were wrapped in aluminum foil, folded, and sealed to prevent water loss during heating. The glutens were heated for 1 h in an oven at 45, 65, 90, or 110 °C. After this, they were frozen in liquid nitrogen, freeze-dried, ground, sieved (60 mesh), and stored at 4 °C under vacuum. Unheated gluten was also considered.

Preparation of Protein Fractions. Water- and salt-soluble proteins were extracted from 10 mg of gluten with 1 mL of 0.05 M NaCl, at 25 °C for 30 min, and in the same conditions including 10 or 20 mM DTT in the solvent. Ethanol-soluble proteins were extracted from 5 mg of gluten with 2 mL of 70% ethanol, at 25 °C for 1 h, dried under vacuum in a Savant Speed-Vac SC-100 apparatus, and redissolved in 1 mL of 50% *n*-propanol. This procedure allowed the elimination of ethanol which in this experiment disturbed SDS−PAGE analysis. Isolated water/salt- and alcohol-soluble fractions were reduced by adding DTT to a final concentration of 20 mM (corresponding to 10 mmol of DTT per milligram of protein). HMW and LMW glutenin subunits were isolated from 5 mg of gluten in 50% propanol containing 65 mM DTT (corresponding to 10 mmol of DTT per milligram of protein) and derivatized with 135 mM 4-vinylpyridine, according to the two-step procedure of Singh et al. (1991). The following modification was adopted to achieve a better separation on SDS−PAGE; after derivatization with 4-vinylpyridine, the protein solution (0.2 mL) was precipitated by adding cold acetone (0.8 mL) and centrifuged (4000*g*). The supernatant was discarded, and proteins were redissolved in 50% propanol.

Reductive Titration of Gluten. Heat-treated glutens (10 mg) were suspended in 1 mL of 50 mM Tris/HCl and 2% SDS at pH 7.5 containing 0−40 mM DTT (0−5 mmol of DTT per milligram of protein). The extraction was carried out at 25 °C for 15 min, or 4 h when indicated, with gentle stirring. The slurry was then clarified by centrifugation (4000*g*). Part of the supernatant (0.45 mL) was alkylated by adding 25 mL of 200 mM IAM at 25 °C for 40 min in the dark as described by Crestfield et al. (1963).

Protein Determination. Total proteins of the gluten sample were determined in a Carlo Erba NA 1500 automatic nitrogen analyzer, with an atropine standard; the conversion factor was 5.7 (Tkachuk, 1969). Insoluble proteins were measured in the extraction residues, washed with water, of samples heated at various temperatures. The difference in the proteins (total insoluble) gave the amount of solubilized proteins.

Electrophoresis. SDS−PAGE was carried out according to Laemmli (1970) on 12% polyacrylamide gels. Protein markers were the Sigma standard mixture of proteins (myosin, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme).

Proteins were stained with Coomassie Brilliant Blue R-250. Scans of SDS−PAGE gels were carried out by a Panasonic videocamera interfaced with a personal computer; image analysis was done with Cream software (Kem-en-Tec, Copenhagen, Denmark). The areas in the reduced and unreduced SDS extract tracings were normalized by assuming that the LMW albumins/globulins remained quantitatively unmodified by reduction. The areas of peaks are expressed as the percent of total area of the reduced extract tracing; when peaks overlapped, the quantitation was only indicative. HMW glutenin subunits and HMW albumins + D glutenin subunits were evident in the reduced extract. Gliadins were determined in the unreduced extract. The amount of LMW glutenin subunits was calculated as the difference between the area in the reduced extract, corresponding to gliadins + LMW glutenin subunits, and the area of gliadins. For the sake of brevity, the figures of electrophoretic separations omit samples with identical patterns.

Determination of Amylase Activity. Amylase activity was assayed in the NaCl extracts, using Zulkowsky starch as substrate (Bernfeld, 1955).

RESULTS

Solubility of Proteins. The protein content of gluten was 80 ± 1.0 %. Proteins extracted from native gluten by 50 mM Tris/HCl and 2% SDS at pH 7.5 were 90% of the total. The yield did not change greatly in gluten treated up to 65 °C, whereas it significantly decreased after treatment at higher temperatures (inset of Figure 1). As shown in Figure 1, extraction from gluten treated at the highest temperature, 110 °C, depended on the amount of DTT added and on the extraction time. When the reducing agent was 0.8 mM, the proteins extracted in 15 min were 30% of the total while 89% of proteins were solubilized in a 4 h extraction. With DTT concentrations of up to 0.4 mM, or above 4 mM, the extraction was not time-dependent. In all cases, longer extractions, up to 16 h, did not modify the amounts extracted.

Oxidized and Reduced Polypeptide Identification. The effect of reduction on the individual protein types was shown by extracting the protein fractions of gluten in different solvents, with or without DTT, as indicated in Scheme 1, and examining the component polypeptides by SDS−PAGE (Figures 2 and 3). The results are commented below.

NaCl Extract. This displayed, almost exclusively, LMW albumins/globulins (apparent M_r values of 14 400 and 13 000). When reduced with 20 mM DTT, these proteins, particularly the M_r = 13 000 components, displayed a slower mobility. An HMW albumin with a M_r of 59 000 and a minor one with a M_r of 63 000 became evident after reduction (Figure 2A); they probably correspond to β-amylase (Gupta et al., 1991).

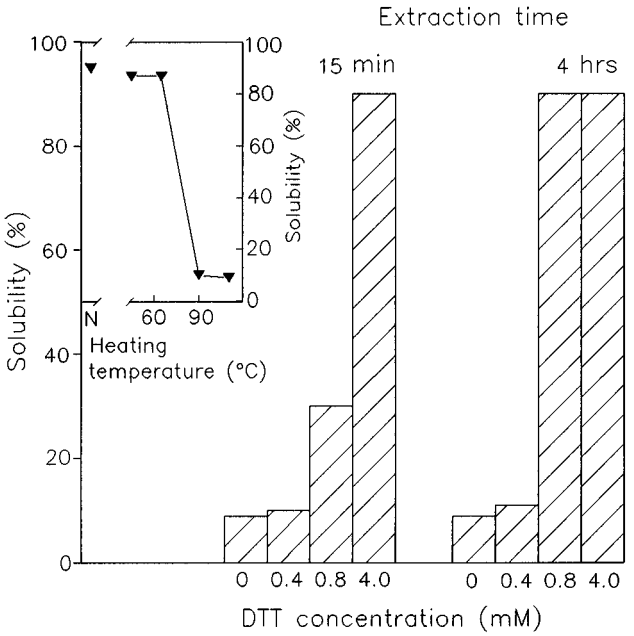


Figure 1. Effect of reduction and extraction time on protein solubility of gluten heated at 110 °C. The extracting buffer was 50 mM Tris/HCl and 2% SDS at pH 7.5. The concentration of DTT indicated on the abscissa is the amount added in the extracting SDS buffer. The extraction time was 15 min or 4 h. (Inset) Protein extraction from native gluten (N) and glutens heated at various temperatures by the SDS buffer.

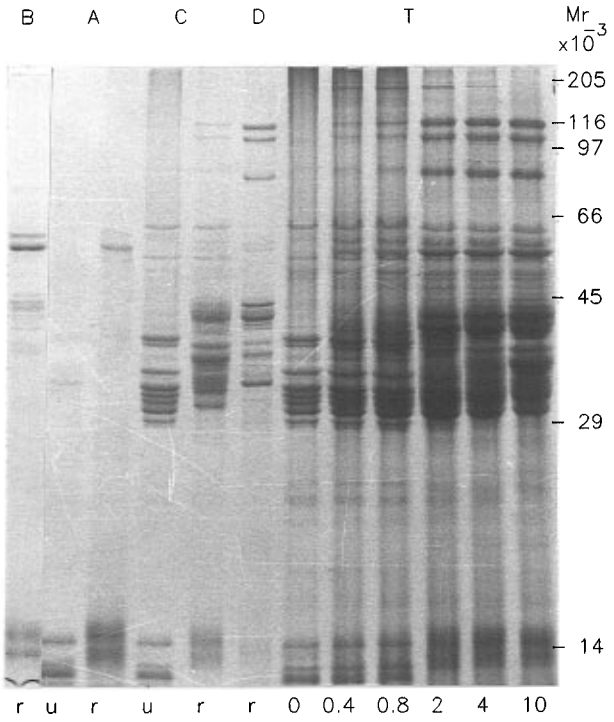


Figure 2. Effect of DTT on the extraction and the SDS−PAGE separation of native gluten proteins. Extractant A = 0.05 M NaCl, B = 0.05 M NaCl + 10 mM DTT, C = 70% ethanol, and D = 50% propanol + 65 mM DTT. Extracts A and C were analyzed prior to and after reduction with 20 mM DTT; u and r = unreduced and reduced fractions, respectively. T is extraction of native gluten with 2% SDS buffer containing increasing concentrations of DTT. The DTT concentration (millimolar) is indicated below each lane. The positions and M_r values of standard proteins are given.

Indeed, reduced and unreduced NaCl extracts had amylase activity (7.8 u/mL). The quantitative relations derived from the densitometric profiles of SDS−PAGE separations agree with the data of Gupta et al. (1991),

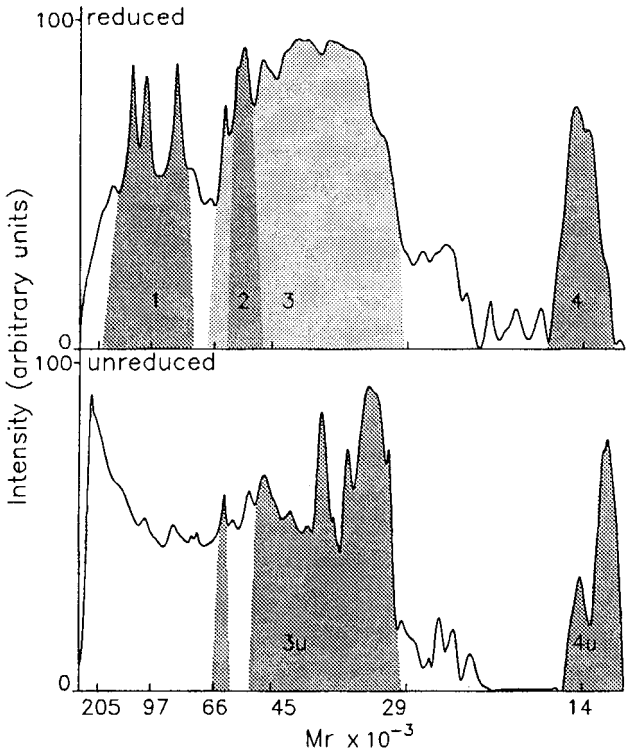
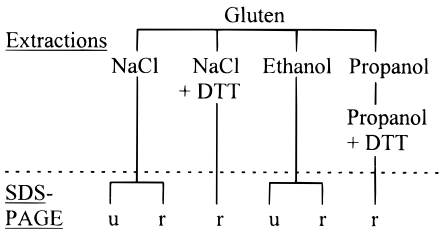


Figure 3. Densitometric scans of SDS−PAGE separation of reduced (20 mM DTT) and unreduced 2% SDS buffer extracts from native gluten. Protein percentages were calculated as described in the text. The peaks considered are shaded. Reduced extract: 1 = HMW glutenin subunits (12%), 2 = HMW albumins + D glutenin subunits (6%), 3 = gliadins (40%) + LMW glutenin subunits (29%), and 4 = LMW albumins/globulins (13%). Unreduced extract: 3u = gliadins and 4u = LMW albumins/globulins. Values on the abscissa are the M_r values of standard proteins.

Scheme 1. Gluten Fractionation[a]



[a] u and r are unreduced and reduced samples, respectively.

who reported that HMW albumins represent about 10% of the polymeric proteins of flour (Figure 3).

NaCl ± DTT Extracts. The solubilization of the components with M_r values of 59 000 and 63 000 was increased in the presence of 10 mM DTT (Figure 2B). Amylase activity also increased to 45 u/mL. In the presence of 20 mM DTT, the extract was the same.

Ethanol Extract. This mostly contained α- and γ-type gliadins (M_r values from 40 000 to 30 000), ω-gliadins (M_r values 65 000 and 55 000), and LMW albumins/globulins (Figure 2C). The α- and γ-type gliadins, but not the ω-ones, and the LMW albumins/globulins were slowed by 20 mM DTT; this behavior indicates that intramolecular disulfides were reduced and the molecules were more expanded. The bands were less sharp, probably because of the high DTT concentration in the sample, as occurs with an excess of salt (Hames, 1990), and/or because of superimposition of bands. Some polymers were diffused in the upper lane in the unreduced extract; in the presence of DTT, they disappeared

and weak HMW and LMW glutenin subunits became visible.

Extract with Propanol ± DTT, following Propanol Extraction. HMW (M_r values of more than 66 000) and LMW (M_r values from 45 000 to 34 000) glutenin monomers were clearly separated (Figure 2D). A component with a M_r of 61 000, which may correspond to the D glutenin subunits identified by Masci et al. (1993), and the M_r 59 000 HMW albumin also appeared.

SDS Buffer Extract. This contained all the protein monomers mentioned in the other unreduced extracts and increased polymers at the left end of the densitometric profile (Figure 3). This indicates the disaggregating power of SDS; i.e., the presence of aggregation depends on noncovalent forces. When the extract was reduced with 20 mM DTT, the polymers disappeared, releasing glutenin subunits and the HMW albumins with M_r values of 59 000 and 63 000. The mentioned mobility changes of LMW albumins/globulins and α- and γ-type gliadins were also evident. The separation and quantities of the various components can be readily seen in the densitometric tracings (Figure 3). The overlap of the gliadins and LMW glutenin subunits is evident in the reduced extract.

Redox Titration. The susceptibility of disulfide bonds to reduction was studied by extracting native gluten and glutens heated at various temperatures (45−110 °C) with the SDS buffer containing increasing amounts of DTT. Part of the reduced samples was alkylated with IAM before electrophoretic analysis so as to avoid sulfhydryl/disulfide exchanges or oxidation during the analysis; however, alkylation had no effect on the pattern of separation.

Native Gluten and Gluten Treated at 45 °C. The behavior of samples unheated or heated at 45 °C did not differ. A DTT concentration of 0.02 mM did not modify the electrophoretic pattern (Figure 2T, lane 0).

A DTT concentration of 0.4 mM released the HMW albumin with a M_r of 63 000 and, in part, the one with a M_r of 59 000, together with weak HMW and LMW glutenin subunits, and one component with a M_r of 205 000. The unfocalized trace along the upper electrophoresis lane is probably due to the gradual breakdown of the block polymer structure of glutenin-producing monomers and oligomers (Figure 2T).

LMW albumins/globulins displayed the slower mobility of their reduced forms around 2 mM DTT; in α- and γ-type gliadins, this happened with 4 mM DTT. This locates the stability regions for the intramolecular disulfides. Diffuse background color at 2 mM DTT in the α- and γ-type gliadins region and the less sharp bands can be interpreted as incomplete reduction (Figure 2T).

The release of the M_r = 59 000 HMW albumin increased gradually with DTT concentration up to 2 mM.

The increase in glutenin monomers with DTT concentrations between 0.4 and 2 mM and the slow disappearance of background color indicate that intermolecular disulfides in glutenins are sensitive to DTT in this concentration range. However, these disulfides vanished completely only with 10 mM DTT. Also, the band with a M_r of 205 000 disappeared, thereby confirming its nature as a disulfide-sustained oligomer (Figure 2T). The electrophoretic pattern did not change upon increasing DTT concentration up to 40 mM.

Gluten Treated at 65 °C. The HMW albumins were extracted by the SDS buffer with or without added DTT.

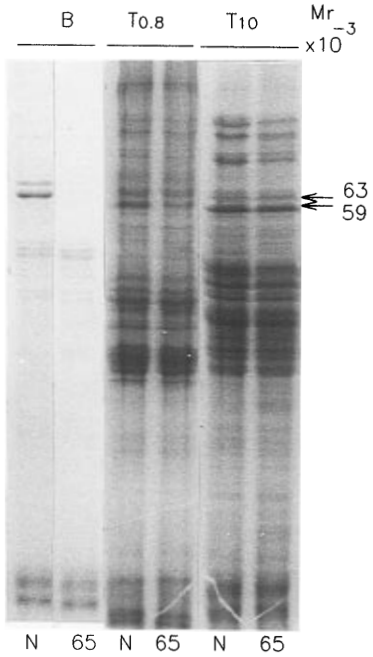


Figure 4. Effect of heat treatment at 65 °C on gluten proteins. SDS−PAGE analysis was done on native gluten (N) and gluten treated at 65 °C (65) extracted in various buffers: B = 0.05 M NaCl + 10 mM DTT and $T_{0.8}$ and T_{10} = buffer containing 2% SDS and 0.8 or 10 mM DTT, respectively. The apparent M_r values of HMW albumins are given.

However, for release in the monomeric form, higher levels of DTT were required than for native gluten (parts $T_{0.8}$ and T_{10} of Figure 4). The NaCl extractant did not solubilize these albumins, not even in the presence of 10 mM DTT (Figure 4B); furthermore, there was no amylase activity. Heating at 65 °C also deactivated the amylase solubilized in NaCl from native gluten. The other components behaved as in native gluten, except that bands with M_r values above 55 000 were less clearly defined when DTT was below 0.8 mM.

Glutens Treated at 90 and at 110 °C. These samples had similar behavior and differed considerably from those treated at lower temperatures. The most drastically heated sample is considered here. All the polypeptides, except ω-gliadins, formed insoluble aggregates. The aggregation was disulfide-based since it was resolved by reduction but not by SDS. Components with a M_r of 61 000, probably D glutenin subunits, were the first to be released, with 0.4 mM DTT (Figure 5). Prior to heating, the HMW albumins which have been shown to be quantitatively more abundant than the D glutenin subunits (Gupta et al., 1991; Masci et al., 1993) probably partly mask the D glutenin subunits on SDS−PAGE of SDS extracts. After heating at high temperatures, the D glutenin subunits became visible, probably since HMW albumins require higher DTT concentrations for their release.

SDS−PAGE analysis was performed on 15 min and 4 h extracts so as to account for the time-dependent extraction yield with DTT concentrations between 0.4 and 4 mM (Figure 1). In 15 min extractions with 0.8 mM DTT, HMW glutenins were weakly apparent; they became clearly perceptible with 2 mM DTT. At this DTT concentration in the regions of LMW glutenins, α- and γ-type gliadins, and LMW albumins/globulins, only faint shadows were apparent. The background increased when DTT was 4 mM; also, α- and γ-type gliadins/LMW glutenin subunits and LMW albumins/

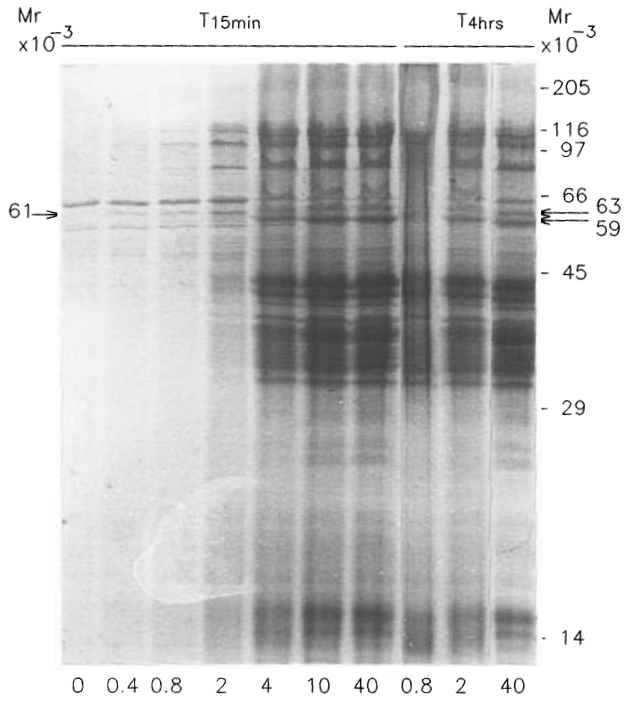


Figure 5. SDS−PAGE analysis of proteins extracted from gluten treated at 110 °C with 2% SDS buffer containing DTT, for 15 min (T_{15min}) or 4 h (T_{4hrs}). The DTT concentration (millimolar) is indicated below each lane. The patterns of T_{4hrs} extracted with ≤0.4 mM DTT were identical to corresponding extracts of T_{15min}. T_{4hrs} extracted with 4−10 mM DTT had the same patterns as with 2 mM DTT. The M_r values and positions of standard proteins are reported. The arrows indicate the supposed D glutenin subunits (M_r = 61 000) and the HMW albumins (M_r = 59 000 and 63 000). Differences between the M_r = 61 000 component and the M_r = 59 000 and 63 000 components can be appreciated on comparing patterns of T_{15min} with 2 and 4 mM DTT.

globulins were strongly present, and the M_r 59 000 HMW albumin became quite distinct (part T_{15min} of Figure 5).

With extractions lasting 4 h, the nearly complete solubilization of α- and γ-type gliadins, and LMW albumins/globulins together with glutenin subunits occurred even with 0.8 mM DTT, whereas the M_r 59 000 and 63 000 HMW albumins required 2 mM DTT; the components were the same as those in 15 min extractions with high DTT concentrations (part T_{4hrs} of Figure 5).

With at least 4 mM DTT, all disulfides affecting solubility were rapidly removed (part T_{15min} of Figure 5); the smearing of bands, even with 40 mM DTT, indicates, however, that the proteins were not restored to their native state.

DISCUSSION

Disulfide bonds in gluten proteins vary widely in their susceptibility to reduction by DTT. Breakage can be limited by the accessibility which depends on the three-dimensional conformation. Buried S−S bonds are reduced more slowly than those that are accessible, due to greater free energies of the transition states for their reduction (Creighton et al., 1995). S−S bonds in proteins are generally stabilizing and nonreactive; however, there are exceptions. The S−S bond in the active site of disulfide isomerase is very reactive due to a strained conformation of the oxidized form of the protein which is less stable than the reduced form (Martin et al., 1993). The reactivity of S−S bonds is also a reflection of their local environment, i.e., charge distribution and hydrophobicity (Halberlein et al., 1994; Martin et al., 1993).

In the present work, the susceptibility to reduction of S−S bonds of gluten proteins was studied in a SDS medium so as to overcome the low solubility of these proteins. An effect of SDS on protein conformation is not to be excluded. However, it was demonstrated that SDS has no effect on the secondary structure of gliadin; the secondary structure of glutenin was determined only in the presence of SDS, and it was not possible to establish whether it corresponded to the actual structure of glutenin (Weegels et al., 1994b).

In native gliadins, the reduction of S−S bonds, which are intramolecular, occurred at high DTT concentrations and caused changes in the SDS−PAGE mobility of the proteins. The faster mobility of the unreduced forms indicates a more compact structure compared to the reduced form. The disulfides in these proteins connect distant residues of the polypeptide chain (Kohler et al., 1993) and probably play a role in the folded conformation. This suggests that the folded conformation of gliadins influences their S−S bond reactivity. LMW albumins/globulins, which are S-rich proteins (Gautier et al., 1994), displayed similar behavior.

The gradual liberation in native glutenin of the monomeric subunits occurred over a wide range of DTT concentrations. Some of the S−S bonds were very labile, while others required high DTT concentrations. This reduction pattern fits the block polymer model of glutenin worked out on flour pastes by Gao et al. (1992). These authors identified, among the products of partial reduction, HMW glutenin dimers which were especially resistant to reduction. Also, Werner et al. (1992) identified and characterized HMW dimers which were liberated in the partial reduction of isolated glutenin. The component with a M_r of about 205 000 shown in the present study, disappearing at high DTT concentrations, might correspond to the above HMW dimers. More detailed structural information is necessary to explain the different reactivity of S−S bonds of glutenin.

The observed release of the HMW albumins occurring in parallel with the reduction of glutenin oligomers fits the data obtained by size exclusion chromatography by Gupta et al. (1991), who found that in native flour of the Chinese Spring variety HMW albumins with an apparent M_r from 60 000 to 65 000 may exist both free and bound together or to glutenin molecules through disulfide bridges. Similar results were obtained by Curioni et al. (1994) after fractionation of gluten from durum wheat on controlled pore glass beads.

At 65 °C, the amylase activity disappeared and, in parallel, HMW albumins lost their water solubility. This thermal lability corresponds to that described for the β-amylase of flour (Kruger and Reed, 1988).

All gluten proteins, except ω-gliadins which lack cysteine, formed insoluble S−S-bonded aggregates on heating at 110 °C. Polypeptides were released from the heat-formed aggregates at different DTT concentrations. In our opinion, this depends on the different cross-linking levels resulting from the number of cysteines in the polypeptides and on the different accessibilities or reactivities of S−S bonds due to aggregation of the unfolded proteins. The component with a M_r of 61 000, which was released at low DTT concentrations, may be identified as a D glutenin subunit described by Masci et al. (1993). This subunit possesses only one cysteine;

therefore, it cannot form additional disulfides on heating. This may explain its easy splitting. The time-dependent solubilization of heated proteins with 0.8 mM DTT most likely involves a reshuffle of the S−S/SH groups, also related to changes in the polypeptide arrangement, gradually making all insolubilizing S−S bonds available to reduction. Lower DTT concentrations had no effect, and at high DTT concentrations, the reduction was obviously more rapid. The redox potential of all heat-induced disulfides corresponds therefore to a DTT concentration of 0.8 mM DTT. The easier liberation of HMW glutenin subunits in 15 min extractions compared to that of gliadins and albumins/globulins may indicate that in monomeric proteins heat induces a higher level of cross-linking or conformational changes than in HMW glutenin subunits, which are protected by their network structure. The similar behavior of LMW glutenin subunits and α- and γ-type gliadins may depend on the homologous distribution of intramolecular S−S bonds in the two protein groups (Koler et al., 1993).

Previous work showed that the modifications in the aggregation state induced on heating prolonged to 18 h at 110 °C are not resolved by reduction. This indicates that groups other than S−S/SH are affected (Guerrieri et al., 1996).

Schofield et al. (1983) found that heat-induced disulfide/sulfhydryl exchange reactions occur in glutenin above 55 °C and in gliadins above 75 °C. The present study indicates that heating at 65 °C particularly affects the S−S bonding structure of HMW albumins and probably their linkage to glutenin oligomers. LMW albumins/globulins were affected at higher temperatures, as were gliadins. The loss, at 65 °C, of water solubility of the HMW albumins, a significant proportion of polymeric proteins (Gupta et al., 1991), indicates changed polarity and may account for the different interactions with water-soluble components of flour. Similarly, the changes in the aggregation state of LMW albumins/globulins, which include lipid binding proteins (Gautier et al., 1994), probably lead to different interaction with flour lipids. These results show that water-soluble proteins are also modified together with gliadins and glutenins at the temperatures which affect the functional (Booth et al., 1980; Hay and Every, 1990), rheological (Dreese et al., 1988), and surface behavior (Eliasson and Silverio, 1993) of gluten. This parallel suggests that these proteins also may play a role in the above properties.

It is apparent that as S−S formers gluten proteins behave differently upon heating, becoming "reactive" at different temperatures and showing a different ability to form cross-links. Control of the time and temperature conditions in the process and of the moisture level may affect the formation of the protein network and the interactions between flour components and, thereby, the rheological properties. This opens up the possibility of developing products with novel characteristics.

LITERATURE CITED

American Association of Cereal Chemists. Method 38-10. In *Approved methods of the AACC*, 8th ed.; American Association of Cereal Chemists: St. Paul, MN, 1983.

Bernfeld, P. Amylases, α and β. In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Academic Press: New York, 1955; Vol. 1, pp 149−150.

Booth, M. R.; Ewart, J. A. D. Studies on four components of wheat gliadins. *Biochim. Biophys. Acta* **1969**, *181*, 226−233.

Booth, M. R.; Bottomley, R. C.; Ellis, J. R. S.; Malloch, G.; Schofield, J. D.; Timms, M. F. The effect of heat on gluten-physico-chemical properties and baking quality. *Ann. Technol. Agric.* **1980**, *29*, 399−408.

Cerletti, P.; Guerrieri, N.; Lavelli, V.; Alberti, E. Heat modification of gluten proteins. In *Proc. International Symposium on Wheat Kernel Proteins−molecular and functional aspects*; Università degli Studi della Tuscia−Consiglio Nazionale delle Ricerche: St. Martino al Cimino (VT), 1994; Vol. I, pp 69−74.

Creighton, T. E.; Zapun, A.; Darby, N. J. Mechanisms and catalysts of disulphide bond formation in proteins. *Tibtech*. **1995**, *13*, 18−23.

Crestfield, A. M.; Moore, S.; Stein, W. H. The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. *J. Biol. Chem.* **1963**, *238*, 622−627.

Curioni, A.; Furegon, L.; Peruffo, A. D. B.; Pogna, N. E.; Zamorani, A. A 60 kDa protein is a component of glutenin aggregates. In *Proceedings of the International Symposium on Wheat Kernel Proteins−molecular and functional aspects*; Università degli Studi della Tuscia−Consiglio Nazionale delle Ricerche: St. Martino al Cimino (VT), 1994; Vol. I, pp 345−348.

Dreese, P. C.; Faubion, J. M.; Hoseney, R. C. Dynamic rheological properties of flour, gluten, and gluten-starch doughs. I. Temperature-dependent changes during heating. *Cereal Chem.* **1988**, *65*, 348−353.

Eliasson, A. C.; Silverio, J. Interfacial behaviour of gluten proteins after heat treatment. In *Gluten Proteins*; Association of Cereal Research, Eds.; Association of Cereal Research Publishers: Detmold, 1993; pp 11−20.

Gao, L.; Ng, P. K. W.; Bushuk, W. Structure of glutenin based on farinograph and electrophoretic results. *Cereal Chem.* **1992**, 69, 452−455.

Gautier, M. F.; Lullien-Pellerin, V.; Dieryck, W.; De Lamotte, F.; Ihorai, T.; Pahin, V.; Alary, R.; Marion, D.; Joudrier, P. Molecular studies of cysteine-rich proteins from wheat. In *Proceedings of the International Symposium on Wheat Kernel Proteins−molecular and functional aspects*. Università degli Studi della Tuscia−Consiglio Nazionale delle Ricerche: St. Martino al Cimino (VT) 1994; Vol. I, pp 89−94.

Guerrieri, N.; Alberti, E.; Lavelli, V.; Cerletti, P. Use of spectroscopic and fluorescence techniques to assess heat induced molecular modifications of gluten. *Cereal Chem.* **1996**, *73*, 368−374.

Gupta, R. B.; Shepherd, K. W.; MacRitchie, F. Genetic control and biochemical properties of some high molecular weight albumins in bread wheat. *J. Cereal Sci.* **1991**, *13*, 221−235.

Haberlein, I. Structure requirements for disulfide bridge sulfitolysis of oxidised *Escherichia coli* thioredoxin studied by fluorescence spectroscopy. *Eur. J. Biochem.* **1994**, *223*, 473−479.

Hames, B. D. One dimensional polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins. A Practical Approach*; Hames, B. D., Rickwood, D., Eds.; Oxford University Press: Oxford, U.K., 1990; Chapter 1.

Hay, R. L.; Every, D. A simple glutenin turbidity test for the determination of heat damage in gluten. *J. Sci. Food Agric.* **1990**, *53*, 261−270.

Jeanjean, M. F.; Damidaux, R.; Feillet, P. Effect of heat treatment on protein solubility and viscoelastic properties of wheat gluten. *Cereal Chem.* **1980**, *57*, 325−331.

Kim, H. R.; Bushuk, W. Changes in some physicochemical properties of flour proteins due to partial reduction with dithiothreitol. *Cereal Chem.* **1995**, *72*, 450−456.

Kobrehel, K.; Wong, J. H.; Balogh, A.; Kiss, F.; Yee, B. C.; Buchanan, B. B. Specific reduction of wheat storage proteins by thioredoxin h. *Plant Physiol.* **1992**, *99*, 919−924.

Kohler, P.; Belitz, H. D.; Wieser, H. Disulphide bonds in wheat gluten: further cystine peptides from high molecular weight and low molecular weight subunits of glutenin and from γ-gliadins. *Z. Lebensm.-Unters.-Forsch.* **1993**, *196*, 239−247.

Kruger, J. E.; Reed, G. Carbohydrate-degrading enzymes. In *Wheat: Chemistry and Technology*; Pomeranz, Y., Ed.;

American Association of Cereal Chemists: St. Paul, MN, 1988; Vol. 2, pp 442−453.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Martin, J. L.; Bardwell, J. C. A.; Kuriyan, J. Crystal structure of the DsbA protein required for disulphide bond formation in vivo. *Nature* **1993**, *365*, 464−468.

Masci, S.; Lafiandra, D.; Porceddu, E.; Lew, E. J. L.; Tao, H. P.; Kasarda, D. D. D-Gutenin subunits: N-terminal sequence and evidence for the presence of cysteine. *Cereal Chem*. **1993**, *70*, 581−585.

Pomeranz, Y. Composition and functionality of wheat flour components. In *Wheat: Chemistry and Technology*; Pomeranz, Y., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1988; Vol. 2.

Schofield, J. D. Wheat proteins: structure and functionality in milling and breadmaking. In *Wheat−Production, Properties and Quality;* Bushuk, W., Rasper, V. F., Eds.; Blackie Academic and Professional: Glasgow, U.K., 1994; Chapter 7.

Schofield, J. D.; Chen, X. Analysis of free reduced and free oxidised glutathione in wheat flour. *J. Cereal Sci*. **1995**, *21*, 127−136.

Schofield, J. D.; Bottomley, R. C.; Timms, M. F.; Booth, M. R. The effect of heat on wheat gluten and the involvement of sulphydryl-disulphide interchange reactions. *J. Cereal Sci*. **1983**, *1*, 241−253.

Shewry, P. R.; Tatham, A. S.; Forde, J.; Kreis, M.; Miflin, B. J. The classification and nomenclature of wheat gluten proteins: a reassessment. *J. Cereal Sci*. **1986**, *4*, 97−106.

Shewry, P. R.; Halford, N. G.; Tatham, A. S. High molecular weight subunits of wheat glutenin. *J. Cereal Sci*. **1992**, *15*, 105−120.

Singh, N. K.; Shephered, K. W.; Cornish, G. B. Rapid communication. A simplified SDS-PAGE procedure for separating LMW subunits of glutenins. *J. Cereal Sci*. **1991**, *14*, 203−208.

Tkachuk, R. Nitrogen to protein conversion factors for cereal and oilseed meals. *Cereal Chem*. **1969**, *46*, 419−423.

Wadhanvan, C. K.; Bushuk, W. Studies on vitality of commercial wheat gluten. I. Physical, chemical and technological characteristics. *Cereal Chem*. **1989**, *66*, 456−461.

Weegels, P. L.; Hamer, R. J.; Schofield, J. D. Depolymerization and polymerization of individual glutenin subunits in situ in dough. Implications for the structure of gluten. In *Gluten Proteins*; Association of Cereal Research, Eds.; Association of Cereal Research Publishers: Detmold, 1993; pp 57−66.

Weegels, P. L.; Verhoek, J. A.; De Groot, A. M. G.; Hamer, R. J. Effects on gluten of heating at different moisture contents. I. Changes in functional properties. *J. Cereal Sci*. **1994a**, *19*, 31−38.

Weegels, P. L.; De Groot, A. M. G.; Verhoek, J. A.; Hamer, R. J. Effects on gluten of heating at different moisture contents. II. Changes in physico-chemical properties and secondary structure. *J. Cereal Sci*. **1994b**, *19*, 39−47.

Werner, W. E.; Adalsteins, A. E.; Kasarda, D. D. Composition of high-molecular-weight glutenin subunit dimers formed by partial reduction of residue glutenin. *Cereal Chem*. **1992**, *69*, 535−541.

Zawistowska, U.; Bietz, J. A.; Bushuk, W. Characterization of low-molecular-weight protein with high affinity for flour lipid from two wheat classes. *Cereal Chem*. **1986**, *63*, 414−419.

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