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Changes in wheat kernel proteins induced by microwave treatment

Carmela Lamacchia *, Loretta Landriscina, Paola D'Agnello

Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università degli Studi di Foggia, Via Napoli, 25, 71122 Foggia, Italy

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ABSTRACT

Wheat kernels were subjected to microwave treatment, and the proteins were characterized by size exclusion high-performance liquid chromatography (SE-HPLC) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Using this process, the proteins polymerize, forming intermolecular bonds among the same classes of proteins. Furthermore, the polymerization occurs only through disulphide bonds. Although SDS–PAGE did not show any differences for either the number or intensity of protein bands between flour samples before and after microwave treatment, gliadins from treated flours showed significantly reduced cross-reactivity with the R5 antibody. Moreover, the gluten became soluble in an aqueous saline solution, and it was not possible to isolate it using the Glutomatic apparatus. However, the treated flour, in the presence of water, was able to form dough and leaven and produce bread.

1. Introduction

Gluten is one of the earliest protein fractions described by chemists. It is defined as the "cohesive, visco-elastic proteinaceous material" that remains when wheat dough is washed to remove starch granules and water-soluble constituents (Shewry, 2009). Gluten contains hundreds of proteins, which are present either as monomers or as oligomers and polymers linked by inter-chain disulphide bonds (Shewry & Halford, 2002) and characterized by high contents of glutamine and proline and low contents of charged amino acids.

Traditionally, gluten proteins have been grouped according to their solubility in alcohol-water solutions, such as 60% ethanol, as the soluble gliadins and the insoluble glutenins (Osborne, 1924). The interchain disulphide bonds largely determine these properties, with the glutenin consisting of disulphide-stabilized polymers. Reduction of these inter-chain bonds allows the separation of the glutenin subunits into low molecular weight (LMW) and high molecular weight (HMW) groups. By contrast, the alcohol-soluble gliadin fraction consists mainly of monomeric proteins, which either lack cysteine (ω-gliadins) or contain only intra-chain disulphide bonds (α-type and γ-type gliadins). Gluten proteins are susceptible to heat treatment, and their behaviour under relatively high temperatures has been primarily evaluated in model systems. When glutenin is heated above 55 °C or gliadins are heated above 70 °C, disulphide/sulphydryl (SH) exchange reactions occur (Schofield, Bottomley, Timms, & Booth, 1983). Lavelli, Guerrieri, and Cerletti (1996) showed that 65 °C particularly influenced the S–S structure of HMW albumins and possibly their linkage to glutenin oligomers. Furthermore, LMW albumins and gliadins are affected at higher temperatures. In addition, Singh and MacRitchie (2004) found that glutenins polymerize below 100 °C but that the polymerization of gliadins occurs only at higher temperatures. The molecular size of the glutenin aggregates increases, decreasing their extractability (Lamacchia et al., 2007; Schofield et al., 1983; Weegels, Verhoek, de Groot, & Hamer, 1994a). At 100 °C, gliadins undergo similar changes. The extractability of gliadins from bread, using 70% ethanol, is much lower than that of gliadins from flour, and α- and γ-gliadins are more affected than are ω-gliadins (Wieser, 1998). The effects have been ascribed to sulphydryl (SH)-disulphide interchange reactions induced by heat, which affect all gluten proteins except the cysteine-free ω-gliadins (Schofield et al., 1983). Morel, Redl, and Guilbert (2002) suggested that, at temperatures below 60 °C, no changes in free sulphydryl groups occur. Heating to at least 90 °C leads to disulphide bond linked aggregates and conformational changes, primarily affecting gliadins and low-molecular-weight albumins and globulins (Guerrieri, Alberti, Lavelli, & Cerletti, 1996). Although, Kokini, Cocero, Madeka, and de Graaf (1994) proposed that cross-links among gliadin molecules are formed above 70 °C in the absence of glutenins, others have hypothesized that gliadins cross-link only with glutenins (Redl, Morel, Bonicel, Vergnes, & Guilbert, 1999; Singh & MacRitchie, 2004) and that the incorporation of gliadin monomers in the glutenin network leads to a three-dimensional structure (Morel et al., 2002). In a study performed on pasta, Lamacchia et al. (2007) showed that gluten proteins undergo changes induced by drying cycles, which

* Corresponding author.
E-mail address: carmela.lamacchia@unifg.it (C. Lamacchia).

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were consistent with the results found in the model system. Furthermore, the albumins and globulins were completely denatured at 60 °C and incorporated into high-molecular-weight polymers, whereas, because of the conformational structure and low thiol availability, gliadins were only partially denatured at 90 °C and aggregated into polymers of higher molecular weight. In a recent study, Lamacchia, Baiano, Lamparelli, La Notte, and Di Luccia (2010) reported that high temperature applied to durum wheat kernels induced significant changes in proteins but these were different from those reported in a gluten model system and pasta. In particular, proteins were denatured and polymerized, and the albumins and globulins tended to coagulate and interact with gliadins instead of HMW glutenins to form protein aggregates of intermediate molecular weight, revealed as a new intermediate protein peak. The explanation for this phenomenon was that, in the kernel, gluten is not formed and gluten proteins are deposited in different protein bodies (Rubin, Levanony, & Galili, 1992). Tosi, Gritsch, He, and Shewry (2011) confirmed that, in wheat kernels, gluten proteins may form protein bodies by two mechanisms, either transport via the Golgi and Golgi-derived vesicles into the vacuole or by direct accumulation within the lumen of the ER. Additionally, the segregation of protein types into specific populations of protein bodies can occur within the same cell. Differences in the proportions of different types of gluten proteins in protein bodies within the same cell was also demonstrated by immunofluorescence, using double antibody labelling. Therefore, cells of the central starchy endosperm clearly contained protein bodies, which contained HMW subunits of glutenin but little or no gliadin, and this segregation may be maintained, even after protein body fusion and matrix formation.

Apart from fundamental interests, the effects of heating gluten proteins are relevant to practical processes, such as the drying of rain-damaged wheat, drying of gluten from starch/gluten manufacturing plants, and in relationship to effects on gluten proteins during baking and extrusion.

Because Lamacchia et al. (2010) showed, for the first time, that the application of high temperature to wheat grains generates protein polymerization different from that reported in a gluten model system or in bread or dried pasta, the effects of heating gluten proteins in wheat grain have potential for producing modified gluten with unique properties.

In the present study, we examined the behaviour of gluten proteins in wheat kernels subjected to high temperature for a short time, using a high percentage of seed moisture and microwave energy (Italian Patented Method N. 0001414717, 2015; Patent Cooperation Treaty n. PCT/IB2013/000797, 2013). The polymerization of the different protein classes was ascertained, as were the changes in the glutenin and gliadin fractions separated by SDS–PAGE. In addition, the effects of changes in the protein fractions induced by the microwave treatment on both the binding ability of the R5 antibody and the bread-making properties were also evaluated.

2. Materials and methods

2.1. Raw materials and microwave treatment

The wheat kernels (Blasco, Adamello, Ofanto, and Simeto varieties and mixtures of soft and durum wheat Canadian grains) used in this study were supplied by CRA (Foggia, Italy) and by the Casillo group S.p.a (Corato, Italy), respectively. Treated wheat flour (TWF) was obtained by milling the microwave-treated caryopses (Italian Patented Method N. 0001414717, 2015; Patent Cooperation Treaty n. PCT/IB2013/000797, 2013) that were previously harvested and threshed. In particular, 100 g of cleaned wheat grains were dampened for at least two hours, until reaching 18–20% humidity, and then measured with a halogen thermal balance (Mettler Toledo, HB43-S, Swiss). These were then drained and subjected to 1000 watt power for 2 min in a microwave apparatus (Delonghi, Italy) to reach a temperature of approximately 110–120 °C and then measured with a thermal camera (FLUKE i 20 model, Italy).

These parameters were set on the basis of the work of Lamacchia et al. (2010), who induced polymer protein changes but eliminated the negative effects due to the burning of the caryopses. After microwave treatment, the wheat kernels were cooled and dried at room temperature (24 °C) for 12–24 h and then ground, using a roller mill. The particle size of the TWF used was in the range of 100–200 μm. Experiments on TWF were performed with the flour obtained from wheat kernels before the microwave treatment as the control wheat flour (CWF). For each grain sample, (Adamello, Blasco, Ofanto, Simeto, mixture of Canadian durum wheat grains and mixtures of Canadian soft wheat grains), three independent experiments were performed.

2.2. Proteins extraction for SE-HPLC

Proteins from the CWF and TWF samples were extracted, using the method of Gupta, Khan, and MacRitchie (1993). Soluble proteins from 10 mg of samples were extracted with 1 ml of 0.5% SDS–phosphate buffer (pH 6.9). The suspension was shaken for 30 min and the solubilized protein (“soluble” or “extractable” protein) was recovered by centrifugation for 10 min.

The resulting residues were extracted with 1 ml of 0.5% SDS-phosphate buffer (pH 6.9) by sonication for 15 s (Microson Ultrasonic cell distributor), ensuring that the samples were completely dispersed within the first 5 s, and then heated to 35 °C for 30 min. The supernatants after centrifugation (10 min at 17,000*g*) were named “unextractable” proteins or “insoluble” proteins.

Total proteins (10 mg) were extracted in 1 ml of the same buffer, vortexed, and sonicated for 30 s, and the supernatants (“total” protein) were recovered for SE-HPLC analysis.

All extracts were filtered through a 0.45-μm PVDF filter prior to injection on the column.

2.3. SE-HPLC analysis

SE-HPLC was performed, using an LC Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) and Phenomenex Biosep SEC S-4000 column (300 × 7.8 mm, Phenomenex, Torrence, CA, USA).

Each sample (20 μl) was injected onto the column, and the eluted proteins were monitored at 214 nm. Three replicates of each sample were used to investigate the protein composition. The mobile phase was 50% acetonitrile containing 0.05% trifluoroacetic acid with a flow rate of 0.7 ml/min. The SE-HPLC column was calibrated, using protein standards with a range of molecular weights (kDa) as follows: ribonuclease A (13.7), chymotrypsinogen (25.0), ovalbumin (43.0), bovine serum albumin (67.0), aldolase (158), catalase (232), ferritin (440) and thyroglobulin (669).

The percentage of unextractable polymeric protein (UPP) was calculated as described by Gupta et al. (1993). Briefly, the percentage of total UPP was calculated as [peak LPP (Large Polymeric Proteins) + SPP (Small Polymeric Proteins) area (unextractable)/peak LPP + SPP area (total)] × 100. Peak LPP + SPP area (total) refers to the total of peak LPP + SPP (extractable) and peak LPP + SPP (unextractable) (Kuktaite, Larsson, & Johansson, 2003).

2.4. Determination of SH and S–S groups

The protein disulphide and sulphydryl contents in the CWF and TWF samples were estimated by colorimetric determination of the

free SH groups, using the solid phase assay with $NTSB^{2-}$ according to the method of Chan and Wassermann (1993).

2.5. SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) analyses

Total proteins from the samples (1 g) were extracted using 10 ml of an extraction buffer containing 0.0625 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol (v/v), and 5% dithiothreitol (DTT) (w/v). CWF and TWF samples were incubated in the extraction buffer for 2 h and were then centrifuged at 9500 rpm for 15 min at 10 °C. The supernatants containing proteins were carefully removed and stored at −20 °C until used. Gliadins and glutenins from flours (1 g) were extracted, using the Osborne sequential extraction method (Osborne, 1924).

To separate the extracted proteins, SDS–PAGE was performed on a 12.5% gel under reducing and non-reducing conditions, using a vertical electrophoresis system, Hoefer SE 600 (GE Healthcare, Milan, Italy). SDS–PAGE analysis was performed at 25 mA for 3 h at room temperature. The gels were stained with 0.25% w/v Coomassie Brilliant Blue (CBB) overnight.

Gel images were taken, using a Chemi-Doc imager (Chemidoc MP, Bio-Rad Laboratories).

Quantification of the bands was carried out, using image analysis software (Phoretix Total Lab, Phoretix International).

2.6. Gluten antigenicity assessment

An R5-sandwich ELISA analysis (Valdés, Garcìa, Llorente, & Méndez, 2003) of the CWF and TWF samples was performed by the Istituto di Ricerche Agrindustria (Modena, Italy). Samples were treated in accordance with the Ridascreen_Gliadin ELISA kit (R-Biopharm AG Darmstadt, Germany) which is a sandwich enzyme immunoassay for the quantitative analysis of contaminations by prolamins from wheat (gliadin), rye (secalin), and barley (hordein) in raw products, such as flours (buckwheat, rice, corn, oats, teff) and spices, as well as in processed food and heat-processed wheat- and barley-based products. This kit is able to identify gliadins, hordeins and secalins with assay sensitivities of 0.78, 0.39 and 0.39 ng/ml, respectively. The assay's detection limit was 1.5 ng gliadins/ml (1.56 ppm gliadins, 3.2 ppm gluten). For gliadin extraction, a 25 mg sample was dispersed in 2.5 ml of Cocktail Solution (Art. No. R7006, official R5-Mendez method), provided by the Ridascreen Gliadin Kit, for 40 min at 50 °C and then extracted with 80% ethanol, mixed for 1 h at room temperature and centrifuged at 3000*g* for 10 min at room temperature. The supernatant was diluted with sample diluent and 100 µl of diluted samples or standards were added to each separate duplicate well pre-coated with gliadin antibody. After 30 min of incubation at room temperature, the liquid was poured out and the plate was tapped upside down against absorbent paper. Then the plate was washed three times with washing buffer (R-Biopharm AG Darmstadt, Germany) to remove unbound antigen. Thereafter, 100 µl of diluted horse radish peroxidase (HRP) conjugated detecting antibody (R-Biopharm AG Darmstadt, Germany) were added to each well, which were incubated at room temperature for 30 min followed by three times washing. Then the wells were filled with 50 µl of substrate and 50 µl of chromogen (R-Biopharm AG Darmstadt, Germany), and incubated for 30 min at room temperature in the dark to develop the colour. The reaction was stopped by adding 100 µl of stop solution (R-Biopharm AG Darmstadt, Germany), to each well and absorbance was measured with a microplate reader (SEAC, Sirio model, Calenzano, Florence, Italy). The antigenicity was calculated from the standard curve, by using the Ridasoftwin software (R-Biopharm AG Darmstadt, Germany), as equivalent mg of gluten/kg of sample. The cocktail extraction procedure for heat-processed food samples has the advantage that aggregated α- and γ- fractions are solubilized and extracted and can still react specifically with R5 antibody.

2.7. Gluten index and baking process

The gluten index was determined according to the ICC procedure 137/1. Bread was baked as "Francesini" or "Ciabatta" from the CWF and TWF samples in a bakery (LA.PA s.r.l., Crema, Italy). Dough was prepared by mixing all ingredients in a dough mixer as follows: 1.5 kg of flour, 5% sourdough, 57% water and 1.8% salt. The doughs were leavened for approximately 20 min (at 28/30 °C and 70% humidity) and then shaped into either "Francesini" or "Ciabatta" and leavened again for 40 min. The leavened bread was placed on Teflon perforated trays and baked in an oven at 220 °C for 20 min.

2.8. Statistical analysis

Data are reported as the means of three replicates. Mean differences were compared by unpaired Student's *t*-test. The statistical packaged SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) was used. A p-value <0.05 was considered significant.

3. Results and discussion

3.1. Effect of microwave treatment on flour glutenin polymers

The proportion of large glutenin polymers is an important determinant of end-use quality because it relates directly to gluten and dough elasticity (strength) (Gupta, Masci, Lafiandra, Bariana, & MacRitchie, 1996). Size-exclusion (SE)-HPLC was used to determine changes in the glutenin polymer size distribution after the microwave treatment described in Section 2.1.

The method uses sonication in 2% (v/v) SDS solution to render all glutenin polymers soluble and within the size fractionation range for SE-HPLC. During the extraction, some cleavage of the polymers occurs and the size distribution observed is therefore not identical to that of the native polymers. However, the procedure is highly reproducible, and good correlations between the polymer size distribution and functional properties are observed (Gupta et al., 1993).

A typical size-exclusion profile (Fig. 1) shows some peaks corresponding to the gluten protein fractions (called LPP, SPP and LMP in Fig. 1) and additional peaks that correspond to other components (SMP in Fig. 1). Analysis of these fractions by SDS–PAGE showed that LPPs (large polymeric proteins) consist mainly of high-molecular-mass polymers enriched in HMW subunits of glutenin and that SPPs (small polymeric proteins) are mainly low-molecular-mass glutenin polymers. LMPs (large monomeric proteins) consist mainly of ω-, α-, β- and γ-type gliadins (Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'h, 2000), and SMPs (small monomeric proteins) are mainly non-gluten proteins and low-molecular-mass components. The sums of LPP + SPP + LMP + SMP and LPP + SPP + LMP provide an estimate of the total proteins and total gluten proteins, respectively. The SE-HPLC charts for the samples before (CWF) and after microwave treatment (TWF) are shown in Fig. 1. Table 1 shows the change in the amount of total protein, which was measured as the chromatogram area and the two area ratios %LPP/%SPP and %LMP/%LPP for the two different treatments. The proportion of large polymers LPP may correlate with dough strength but the ratios of %LPP/%SPP and %LMP/%LPP usually show stronger correlations (Millar, 2003).

There are no clear differences in the profiles (Fig. 1) of the two samples (CWF *vs* TWF), whereas significant changes are detected

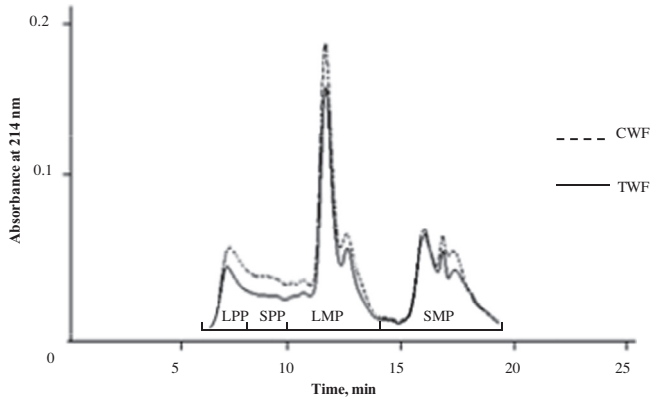

Fig. 1. SE-HPLC elution profile of total proteins ("extractable" and "unextractable") of CWF (control wheat flour) and TWF (treated wheat flour) of the Blasco variety, representative of the other wheat varieties and wheat grain mixtures, before and after the microwave treatment described in this study. LPP: large polymeric proteins; SPP: small polymeric proteins; LMP: large monomeric proteins; SMP: small monomeric proteins.

for either the total peak area or the two area ratios (Table 1). The two SE-HPLC charts indicate that the microwave treatment applied to mature wheat grains may not allow intermolecular bonding between proteins of different classes, leading to the formation of larger polymers and increasing the LPP peak. Protein profiles instead suggest the formation of intramolecular bonds into a single class of proteins that could be explained by the segregation of wheat proteins into different protein bodies (Lamacchia et al., 2010), which accumulate simultaneously and independently in wheat starchy endosperm cells, and by the qualitative and quantitative protein gradients in wheat endosperm. In fact, sub-aleurone cells are enriched in LMW-GS and gliadins, whereas HMW-glutenins are only present in significant amounts in the inner part of the starchy endosperm (Tosi et al., 2011). However, the SE-HPLC chart of proteins treated by the microwave process (TWF) does not show the aggregation of SMPs and their interaction with gliadins as seen by Lamacchia et al. (2010). This may be because the temperature applied in this study was significantly lower (110–120 °C) than that used in the previous study (Lamacchia et al., 2010) in which the seeds were arranged on a steel bearing placed into direct contact with a wood-fire for 120 s until the kernel browned.

The decrease of total peak area indicates that microwave treatment reaches high temperatures and denatures protein fractions, making them unextractable, or allows the polymerization of proteins in larger polymers that become unextractable (Lagrain, Brijs, Veraverbeke, & Delcour, 2005). The ratios of %LPP/%SPP and %LMP/%LPP, beside the correlation with dough strength, allow also, for a better understanding of which fraction of gluten protein was more susceptible to the microwave treatment. The significant decreases in the %LPP/%SPP value and the increase in the %LMP/%LPP, respectively, indicate that, among the different classes of proteins, the large polymeric proteins, LPPs, were more denatured or polymerized. This is consistent with the observation that glutenins (LPP and SPP) are susceptible to an increase in temperature and that glutenin denaturation is complete at 60 °C, whereas gliadins (LMP), because of their conformational structure, are more stable to the increase in temperature, and denaturation begins at 60 °C and increases significantly at 90 °C (Lamacchia et al., 2007; Schofield et al., 1983; Singh & MacRitchie, 2004).

The proteins with the highest molecular weight have the highest correlation with strong dough properties (MacRitchie, 2014). A certain amount of these polymers remains unextractable in various extracting systems (acetic acid solution or sodium dodecyl sulphate phosphate buffer). The %UPP (percentage unextractable polymeric protein in total polymeric protein) is often used as a measurement of the amount and size distribution of the polymeric protein (Gupta et al., 1993). A high %UPP value is related to a greater proportion of glutenin that is insoluble in sodium dodecyl sulphate without sonication and is likely associated with the highest molecular weight (Singh & MacRitchie, 2004). Therefore, wheats with a greater percentage of UPP, are expected to have a greater dough resistance (elasticity) and a longer mixing requirement than have those with a greater proportion of extractable polymeric proteins (Gupta et al., 1993). Because the amount of UPP is influenced by different quaternary structures, which result from polymers involving disulphide bridges and strong cross-linking bonds through dityrosine formation (Tilley et al., 2001) and aggregates involving hydrogen bonding (Aussenac, Carceller, & Kleiber, 2001), both intra- (formed by gliadins and glutenins) and inter- (formed by glutenins) chain disulphide bonds, as well as non-covalent bonds, are predicted to be important for the formation of the gluten polymer complex. Table 2 shows the proportion of UPP and total cysteine and S–S bonds of the grains before and after microwave treatment. The percentage of total UPP in the treated sample decreases significantly, consistent with the SE-HPLC chart and with the two area ratio values, but a concomitant increase in the S–S bonds is also detected. These results suggest that this microwave treatment induces modification in the SH and disulphide bonds, moving polymeric proteins, and in particular, the HMW (LPP) subunits towards larger insoluble protein aggregates, which become unextractable, even after sonication. This is consistent with the conclusion reached by Singh and MacRitchie (2004). In addition, Table 2 shows an increase in the total cysteine, suggesting that microwave treatment induces a major exposure of the cysteine residues due to the dissociation and unfolding of proteins. In fact, the modification of proteins by heat usually results in changes in the secondary and tertiary structure of the protein molecules that become denatured. The process of denaturation involves the dissociation and unfolding of proteins

Table 1
Values of the total peak areas for the investigated CWF (control wheat flour) and TWF (treated wheat flour) samples computed from SE-HPLC profiles of "total" proteins ("extractable and "unextractable"). LPP/SPP% ratio and LMP/LPP% ratio: differences between CWF and TWF. The data are reported as the mean values ± standard deviation ($n = 3$ independent experiments for each grain sample used; Blasco, Ofanto, Adamello, Simeto, mixtures of Canadian durum wheat grains and mixtures of Canadian soft wheat grains). Different letters indicate significant differences at $P < 0.01$ (capital letters) and $P < 0.05$ (small letters).

Samples	Total peak areas (mm^2)	LPP/SPP (%)	LMP/LPP (%)
CWF	9349 A	29.1 A	1.55 A
TWF	7112 B	22.3 B	2.36 B

A and B = $P < 0.001$.

Table 2
Percentages of total UPPs (unextractable polymeric proteins), SH-free (total free cysteines), S–S (disulphide bond) and total cysteine concentration. Differences between the CWF and TWF samples. Data are reported as the mean values ± standard deviation ($n = 3$ independent experiments for each grain sample used as follows: Blasco, Ofanto, Adamello, Simeto, mixtures of Canadian durum wheat grains and mixtures of Canadian soft wheat grains). Different letters indicate significant differences at $P < 0.01$ (capital letters) and $P < 0.05$ (small letters).

Samples	UPP (%)	S–S concentration (μmol/g of protein)	SH-free concentration (μmol/g of protein)	Total cysteine concentration (μmol/g of protein)
CWF	37.3 A	51.4 A	71.7 A	175 A
TWF	28.4 B	78.1 B	66.3 B	223 B

A and B = $P < 0.001$.

and is often accompanied by the formation of disulphide linkages and the exposure of hydrophobic amino acids on the surface. The degree of heat treatment results in differences in the extent of the denaturation and functionality of the proteins. The more the hydrophobic amino groups are exposed, the more insoluble the proteins become.

3.2. Effect of microwave treatment on flour protein fractions

To better understand this phenomenon, total proteins (Fig. 2A) and the glutenin and gliadin fractions (Fig. 2B) of CWF and TWF were separated by SDS–PAGE under reducing and non-reducing conditions. Analysis of these fractions by SDS–PAGE (Fig. 2) showed that the microwave treatment did not cause either a decrease in the number of bands or a decrease in the intensity

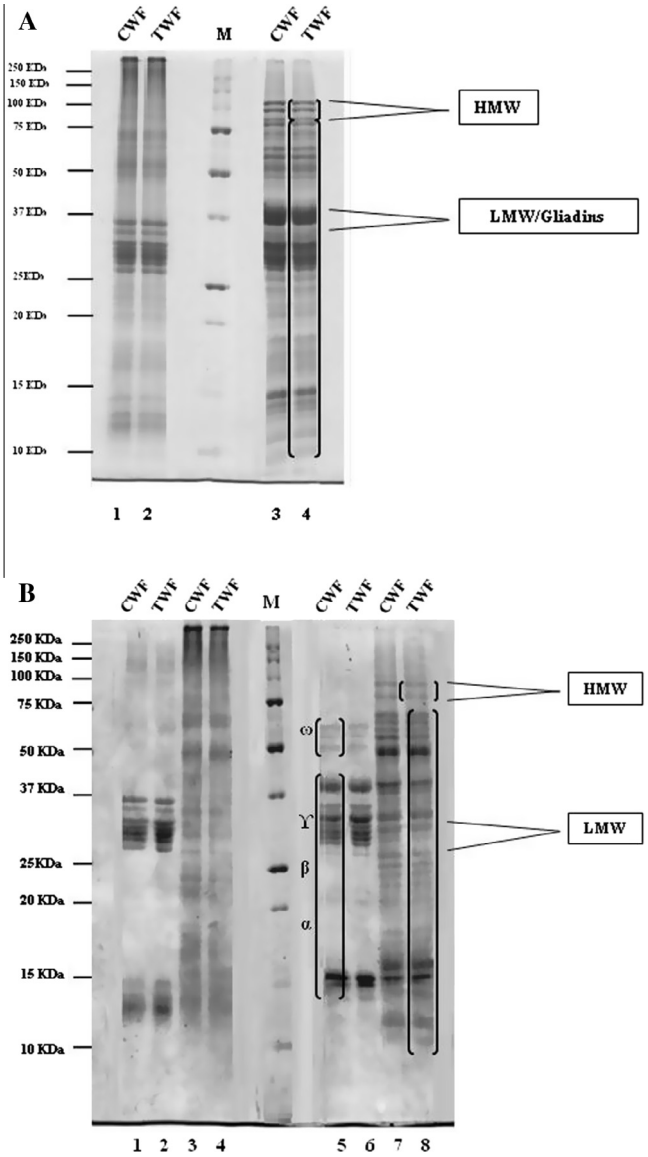

Fig. 2. SDS–PAGE separation of CWF (control wheat flour) and TWF (treated wheat flour) of the Blasco variety, representative of the other wheat varieties and wheat grains mixtures, before and after microwave treatment described in this study. (A) Total protein under non-reducing (1–2) and reducing (3–4) conditions. M: marker. (B) Protein fractions under non-reducing (1–4) and reducing (5–8) conditions. Lane 1: CWF gliadins; lane 2: TWF gliadins; lane 3: CWF glutenins; lane 4: TWF glutenins; lane 5: CWF gliadins; lane 6: TWF gliadins; lane 7: CWF glutenins; lane 8: TWF glutenins M: marker.

of the bands. These results, confirmed by quantification of band intensities obtained via image analysis., suggest that TWF proteins are not denatured, do not interact with other compounds in wheat endosperm and that the decrease of the total peak area observed by SE-HPLC analysis is primarily due to protein polymerization that occurs only through disulphide bonds. These last results are consistent with the work of Weegels et al. (1994a) and Weegels, de Groot, Verhoek, and Hamer (1994b) but are in contrast to the work of Guerrieri and Cerletti (1996). Guerrieri et al. (1996) and Lamacchia et al. (2010) suggest that, at temperatures higher than 100 °C, changes other than disulphide bridging are involved. This may be because heat-induced changes in the properties of gluten proteins may be a function, not only of the temperature reached, but also of both the heating time applied and the sample moisture content (Singh & MacRitchie, 2004; Weegels et al., 1994a,b).

Because an increase in the number of cysteine residues was observed, we attempted to determine whether this microwave treatment induced unfolding of the proteins with exposure of the amino acids to increase gluten antigenicity; so the amount of the potential celiac-toxic most repetitive pentapeptide epitope, gluta mine–glutamine–proline–phenylalanine–proline (QQPFP), in gluten proteins was determined. CWF and TWF gliadins were tested for their ability to bind a specific monoclonal antibody, R5, using the high sensitivity of the R5-ELISA (Valdés et al., 2003), which is used to monitor residual gluten levels as low as 3.2 ppm in all commercial gluten-free foods.

Interestingly, the R5-ELISA indicated a significant reduction (~99%) in the levels of detectable gluten protein, showing that with this microwave treatment, gliadin significantly decreases its cross-reactivity with the R5 monoclonal antibody. In particular, the amount of gluten detected decreased from 10–12% (CWF) to 60 ppm ± 3 (TWF) in durum wheat grains and to 40 ppm ± 3 (TWF) in soft wheat grains. The R5-ELISA, used in this work, includes an extraction procedure allowing aggregates of gliadins generated by the heat-process to be solubilised and it is well known that heat treatment leaves QQPFP epitopes unchanged (Valdés et al., 2003). The R5 antibody also recognizes the epitopes-LQPFP, QLPYP and PQPFP in the sequence of the recently described potent inducer of gut-derived human T-cell lines in coeliac patients, the A gliadin 33-mer peptide of residues 57–89 (LQ LQPFPQPQLPYPQPQLPYPQPQL–PYPQPQPFP) (Arentz-Hansen et al., 2000). The reduced gluten antigenicity observed with the R5-Elisa is in accordance with preliminary results showing that flours treated by this patented method did not induce production of the inflammatory cytokine, interferon gamma, when tested, after digestion and deamidation, on gut-derived human T-cell lines of celiac patients highly reactive to 33-mer, omega and gamma peptides (Lamacchia, Di Luccia, & Gianfrani, 2015). To the best of our knowledge, only Kwak et al. (2012) have tried to reduce the antigenicity of gliadins in medium wheat dough by using microwave energy at 1000 watts for 1, 5 and 10 min. The results showed that the microwave treatment did not reduce the antigenicity of wheat gliadins, which was measured as the ability of anti-gliadin IgG to bind gliadins, and that the antigenicity increased as the time of microwave exposure and the degree of protein denaturation increased. It can be argued that the denaturation of wheat proteins and the increase of antigenicity during microwave treatment occur after the gluten is already formed. For wheat proteins subjected to microwave energy while present in the endosperm, denaturation of the different protein classes occurs minimally and may be caused by their deposition in different protein bodies in their native form, allowing for chemical changes otherwise impossible in the already formed gluten. This dramatically reduces the antigenicity of gluten.

Fig. 3. Bread-making properties of TWF (treated wheat flour) samples. (A) Dough before leavening. (B) Dough after leavening. (C) Bread baked as "Ciabatta". (D) Bread baked as "Francesino". Dough and breads in the picture were obtained from a mixture of Canadian soft wheats treated by the microwave process described in this study and are representative of the other treated grain samples.

3.3. Effect of microwave treatment on gluten index (GI) and bread-making properties of wheat flours

The gluten index (GI) is a measure of the gluten characteristics, which indicates whether the gluten is weak, normal or strong. Gluten is separated from wheat flour using the Glutomatic equipment, taking advantage of the insolubility of gluten proteins in aqueous saline solutions (Osborne, 1924). Next, gluten is centrifuged to force the wet gluten through a specially constructed sieve under standardized conditions. The total weight of gluten is defined as the gluten quantity. The percentage of wet gluten remaining on the sieve after centrifugation is defined as the gluten index. If the gluten is very weak, all of the gluten may pass through the sieve, and the gluten index is 0. When nothing passes through the sieve, the index is 100. CWF and TWF samples were analysed for GI and results showed that the isolation of gluten from the TWF samples was not possible. Consequently, it was also not possible to calculate a GI to determine its strength. These results suggest that microwave treatment induces significant modification of gluten proteins such that they become soluble in aqueous solution, although these modifications are not observed in the SDS–PAGE analysis (Fig. 2A and B).

The main reasons for the water-insoluble characteristics of gluten are the hydrophobic nature of its proteins, consisting of a large percentage of uncharged amino acid residues (glutamines Gln; asparagines Asn) and the presence of hydrogen bonds. By transforming the amides of Gln and Asn into carboxyl groups through deamidation, the solubility of proteins in wheat gluten is transformed with effects such as changing of the charge density, increasing the electrostatic repulsion and breaking the hydrogen bonds (Liao et al., 2010; Riha, Izzo, Zhang, & Ho, 1996). In our case, the solubility of gluten proteins cannot be due to the deamidation of amino acids because no difference between the CWF and TWF proteins bands was detected during the SDS–PAGE separation (Fig 2A and B). However, high temperature for short time, generated by the microwave treatment applied to the caryopses, may break the hydrogen bonds between protein glutamine residues (Shewry, Popineau, Lafiandra, & Belton, 2001) when proteins are in their native form in protein bodies, allowing conformational and/or structural changes (Liao et al., 2010), as evidenced by the increase in the total cysteine levels (Table 1). This would explain the significant decrease in gliadin cross-reactivity towards the R5 monoclonal antibody because it recognizes the repetitive pentapeptide QQPFPepitope.

Because the GI analysis did not allow for gluten separation for the TWF samples and because gluten is the proteinaceous mass that confers cohesive and visco-elastic properties to dough, allowing the production of bread, baked goods and pasta, bakery tests were performed to understand the degree of gluten protein modification.

Breads were baked in a bakery (LA.PA s.r.l, Crema, Italy), and although deep modification of the gluten properties was detected using the Glutomatic analysis, the TWF samples absorbed 10% more water than do the CWF samples when forming a dough (Fig. 3A), which preserves most of the plastic characteristics of the gluten, allowing the leavening and production of bread (Fig. 3B–D). TWF breads showed all of the characteristics of the control bread (CWF); however, a decrease in the loaf volume was detected and expected because of a decrease of dough elasticity.

The elasticity of dough is due to HMW glutenin subunits and, in particular, to the regions associated with its β-turn structure that is rich in glutamines and proline (Shewry et al., 2001). Numerous studies suggest that hydrogen bonds between glutamines of glutenin subunits and polymers may also contribute to elasticity (Shewry et al., 2001), as shown in a new model, which is now termed the "loop and train" model (Belton, 1999). The decrease in the elasticity of the TWF samples is consistent with the observation that breaking the hydrogen bonds between gluten proteins and conformational changes induced by microwave treatment in the kernel allow polymers in the flour to be hydrated but not to form a high amount of loop to train structures that are important for determining dough elasticity. Furthermore, breaking the hydrogen bonds between protein glutamines in wheat kernels induced by microwave treatment may be consistent with the increase in the

percentage of water adsorbed by the flour during mixing. In fact, if more glutamines are available to form hydrogen bonds with water for the plasticization of dough, a higher percentage of water must be added to the flour.

4. Conclusions

The microwave treatment used in this study and applied in hydrated wheat kernels reaching a high temperature for a short time induced significant changes in gluten proteins. Among these changes, the most important are the reduced antigenicity (by 99% in comparison with the control samples) and the solubility of gluten in saline aqueous solution. These changes suggest that the microwave treatment applied to wheat kernels may allow the breaking of hydrogen bonds between glutamine residues when proteins are in their native form in protein bodies, thus favouring protein conformational and/or structural changes. The present study, for the first time, shows the mechanism by which a chemical-physical treatment abolishes the antigenic capacity of gluten such that it is unrecognizable by the antibody used worldwide to determine the amount of gluten in flour for celiac patients and also shows how this modification allows flour to be processed to make bread. As proposed by the Codex Alimentarius Committee, the flour described in this study should be classified as a very low gluten content (21–100 ppm of gluten) for use in the production of food with superior sensory properties for the diet of patients with gluten-correlated disorders (intolerant and sensitive to the gluten) or for those genetically predisposed to this pathology. However, it is subject of further investigation to understand both the molecular changes occurring and how these changes affect digestibility, availability and characterisation of potential antigenic fragments *post*-digestion. Further studies should also focus on the reactivity of the products in gluten-sensitive patients by more complex immunological and clinical trials.

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