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Mechanisms of Heat-Mediated Aggregation of Wheat Gluten Protein upon Pasta Processing

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ABSTRACT: During pasta processing, structural changes of protein occur, due to changes in water content, mechanical energy input, and high temperature treatments. The present paper investigates the impact of successive and intense thermal treatments (high temperature drying, cooking, and overcooking) on aggregation of gluten protein in pasta. Protein aggregation was evaluated by the measurement of sensitivity of disulfide bonds toward reduction with dithioerythritol (DTE), at different reactions times. In addition to the loss in protein extractability in sodium dodecyl sulfate buffer, heat treatments induced a drastic change in disulfide bonds sensitivity toward DTE reduction and in size-exclusion high-performance liquid chromatography profiles of fully reduced protein. The protein solubility loss was assumed to derive from the increasing connectivity of protein upon heat treatments. The increasing degree of protein upon aggregation would be due to the formation of additional interchain disulfide bonds.

KEYWORDS: Pasta processing, protein aggregation, heat treatment, drying, cooking, disulfide bonds

INTRODUCTION

Pasta is a traditional and highly popular cereal-based food. Pasta is obtained after kneading durum wheat semolina with water, extruding, and drying. The quality of cooked pasta is attributed to its specific structure, obtained after successive structural changes of the two main wheat components, starch, and protein, due to changes in water content, mechanical energy input, and high temperature treatments. It is well admitted that the molecular structure of pasta is built during the extrusion stage, through the formation of a protein network around the starch granules. The protein network has to be reticulated (during the drying stage when conducted under high temperatures) to be able to restrict starch swelling and gelatinization during the final cooking stage and thus to contribute to end product qualities.[1−3]

The gluten protein content and characteristics of wheat semolina are known to be critical for end properties of pasta.[3−8] Different methods based on the measurement of the protein solubility have been used to characterize changes in protein aggregation levels during pasta processing.[9−17]

In freshly extruded pasta dough, protein is aggregated to form a continuous viscoelastic network.[12,13,18,19] The protein network is transient as it mainly arises from entanglement of the glutenin subunits, as well as from dynamic thiol-disulfide exchange reactions. Extrusion leads to a slight loss of protein solubility.[12,13,20]

During pasta drying, heat treatments affect both biochemical characteristics and rheological properties of products.[9−11,14,15,21,22] High temperature (HT) conditions generate large changes in structure and, more particularly, lead to protein network insolubilization. HT drying causes wheat protein disulfide cross-linking, leading to large protein aggregates. The behavior of gluten protein to relatively high temperatures has been extensively studied by a number of workers.[15,23−27] It is established that when glutenin is heated above 55 °C or gliadins above 70 °C, disulfide/sulphydryl (SH) exchange reactions occur. At very high temperature conditions (up to 90 °C), gliadin links to glutenin through a sulfhydryl-disulfide exchange mechanism.[14] The ability of the wheat protein to reticulate and form large and insoluble protein aggregates during high temperature drying is the main reason of improving cooked pasta quality.[28] Pasta dried at high temperature is superior (less sticky and firmer) to pasta dried at low temperatures, because protein aggregation limits the water absorption of starch during gelatinization.

Pasta cooking in boiling water induces two structural changes: an increase in protein network reticulation extent and gelatinization and swelling of the starch granules.[29] The protein network, which has been largely aggregated during high temperature drying, reduces starch granule swelling, which will make the pasta firmer and not sticky. The additional aggregation of the protein network due to cooking (at 100 °C) has been characterized by a decrease in protein solubility in dilute acetic acid[30] or in sodium dodecyl sulfate (SDS).[10,22]

The objective of the present study is to investigate the impact of processing on aggregation of gluten protein in pasta and, more particularly, to evaluate the impact of successive thermal treatments (i.e., cooking after high temperature drying) and strong thermal treatments (i.e., overcooking) on pasta structure. Determination of the sensitivity of disulfide bonds toward reduction with dithioerythritol (DTE) (using various reactions times in 6 mM DTE) was considered because thiol-reducing agents are very efficient in promoting protein extractability from heat-treated gluten.[26] Measurements are based on the follow-up of changes in protein solubility in SDS buffer, after progressive reduction of disulfide bonds by DTE.

MATERIALS AND METHODS

Raw Materials. Durum wheat semolina of industrial quality (Panzani, Marseille, France) was used to prepare pasta. The water content of semolina

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(14.1 g water/100 g semolina) was determined by an oven drying method at 105 °C for 24 h according to AACC Method 44-15.02. The total nitrogen content (TN) was determined by the Kjeldahl method, and the crude protein content (13.3 g protein/100 g dry matter) was calculated according to TN × 5.7, based on the AFNOR method (NFV 03-050).

Pasta Processing. Spaghetti was processed with a batch pilot-scale pasta extruder (Sercom, Montpellier, France). Pasta making was conducted using 800 g of semolina, which was first mixed with tap water inside the horizontal mixing tank of the equipment (to obtain a water content of 47 g/100 g db) for 5 min at 120 rpm and then for 15 min at 60 rpm. The product was then extruded at 30 rpm and 40 °C to obtain fresh spaghetti (1.55 mm diameter). The fresh spaghetti was dried in a pilot-scale drier (Afrem, Lyon, France), to reach 12% water content. The drying was conducted at very high temperature (90 °C) according to the following drying program: starting conditions = 35 °C and 88% relative humidity (RH); first period, 60 min to reach 90 °C and 86% RH; second period, 120 min to reach 90 °C and 77% RH; and third period, 40 min to reach 35 °C and 70% RH (the end conditions).

Pasta Cooking and Overcooking. Pasta cooking and overcooking were conducted in boiling water according to the French norm NF ISO 7303. A 100 g sample of pasta was cooked in standard bottled water (French brand: Evian) water containing 0.7% (w/v) sodium chloride with a water:solid ratio of 20. The optimum cooking time for dried pasta (9 min) was determined according to the AACC method (66-50), defined as when the white core of the pasta disappeared after it was squeezed between two glass plates. Pasta cooking was conducted for 10 min, that is, the optimum cooking time + 1 min, as classically done for sensorial analysis. Pasta overcooking was conducted for 18 min (optimum cooking time × 2). At the cooking (or overcooking) time, pasta was drained and immediately cooled with 200 mL of Evian water at 20 °C to stop thermal affects. Pasta samples were then collected, freeze-dried, ground using a ball-mil (Dangoumau, Prolabo, France), and sieved to pass through a 250 μm mesh screen. Dried powders were stored at ambient temperature in small hermetic pans until characterization.

Thiol and Disulfide Contents. The aggregation level between protein was estimated by the values of disulfide SS contents, which were calculated by the difference between the measured contents of accessible thiol (SH_{free}) and the measured contents of total thiol (SH_{eq}). Contents in accessible thiol (SH_{free}), total thiol equivalent (SH_{eq}), and disulfide groups (SS) were assayed as described by Morel et al.[26,31,32] For the determination of accessible thiol (SH_{free}), 60 mg of ground samples was shaken under argon (in hermetic small pans) for 15 min with 1.3 mL of a thiol reagent solution (50% propan-2-ol, 80 mM Tris−HCl buffer, pH 8.5, and 0.4 mg/mL 5,5′-dithiobis-2-nitrobenzoic acid). After centrifugation (5 min, 20800*g*), absorbency by the nitro-thiobenzoate anion (NTB^-) was measured at 412 nm (ε = 13 600 M^{-1} cm^{-1}). For measurement of the total thiol equivalent groups (SH_{eq}) (i.e., thiol content measured after total reduction of SS bonds), 30 mg of sample was exhaustively reduced with dithioerythritol (40 mM DTE in 80 mM Tris/HCl, pH 8.5, 0.3 mL) for 2 h at 60 °C, before adding 1.6 mL of glacial (−18 °C) acetic acid (100 mM) in acetone, to precipitate and stop the reaction. Samples were centrifugated at 959*g* for 4 min at 4 °C. The pellet was suspended in 160 μL of acetic acid (100 mM), before being precipitated again with 1.6 mL of acetone acid (see above), and centrifugated. This washing was performed twice. The pellet was finally suspended in 1.3 mL of thiol reagent solution and treated as previously described for (SH) determination. The disulfide groups (SS) content was calculated from SH_{free} and SH_{eq} determinations (SH_{eq} = 2 SS + SH_{free}). Measurements were carried out in triplicate. Results are expressed in μmol per g of protein.

Stepwise Reduction of Disulfide Bonds. Stepwise reduction with DTE was performed according to modified method of Morel and Bonicel[33] and Morel et al.[26] Typically, a series of six samples (30 mg) were incubated for 0, 4, 8, 12, 16, or 20 min with 300 μL of 4-morpholineethanesulfonic acid (80 mM MES/NaOH buffer at pH 6.5, containing 20 mM DTE). Reduction was stopped by adding 1.6 mL of glacial acetone acid, and excess DTE was removed by two washing steps as described above for the total thiol equivalent groups determination. The thiol content of the washed pellet was determined as above with 1.3 mL of thiol reagent solution. Alternatively, the protein was extracted from the washed pellet and analyzed by size-exclusion high-performance liquid chromatography (SE-HPLC) (see below). In any case, DTE consumption was limited to 10%, so that we may assume that disulfide bond (SS) reduction proceeded according to a pseudofirst-order kinetic.[26]

$$\frac{d(SS)}{dt} = -k[DTE_0][SS] \quad (1)$$

where k is the rate constant, DTE_0 is the initial concentration in DTE, and SS is the sample disulfide content. Accordingly, data are plotted as a function of time-per-DTE concentration (in min M).

Protein Extraction Procedure. The protein was extracted in triplicate from semolina and pasta and from DTE-reduced samples according to a modified method of Morel et al.[31] For semolina and pasta samples, the extraction of SDS-soluble protein (fraction F_s) was conducted for 80 min at 60 °C with 160 mg of sample in 20 mL of a sodium phosphate buffer containing 1% SDS. For the washed pellets, 1.6 mL of buffer was used. After centrifugation, the pellets were suspended at 60 °C for 60 min with 5 mL (1 mL for DTE reduced samples) of the SDS-phosphate buffer, containing 20 mM dithioerythritol (DTE), and sonicated for 5 min (Vibra Cell sonificator, 20 kHz). The combination of sonication and DTE broke down disulfide bonds and brought the insoluble protein fraction into solution (fraction F_i). Both fractions were analyzed by SE-HPLC. [34] The SE-HPLC apparatus was a Waters model (LC Module1 plus) controlled by Millenium software (Waters). A TSK G4000-SWXL (TosoHaas) size exclusion analytical column (7.5 mm × 300 mm) was used with a TSK G3000-SWXL (TosoHaas) guard column (7.5 mm × 75 mm). The columns were eluted at ambient temperature with 0.1 M sodium phosphate buffer (pH 6.9) containing 0.1% SDS. The flow rate was 0.7 mL/min, and protein was recorded at 214 nm. Apparent molecular weights were estimated by calibrating the column with protein standards.

Determination of total amounts of soluble protein was conducted in triplicate. The chromatograms of SDS-soluble protein (F_s) and DTE-soluble protein (F_i) (expressed in V s) are summed once corrected for their different solid to solvent ratios, to evaluate the amount of total extractable protein expressed in g soluble protein/100 g protein, according to Morel and Bar-L'Helgouac'h.[35]

Table 1. Impact of Pasta Processing on SDS-Soluble Protein Content[a]

	SDS-soluble protein content (g/100 g dry basis)
semolina	77.8 (1.0) c
extruded pasta	75.5 (1.4) c
dried pasta	22.6 (1.8) b
cooked pasta	18.2 (2.0) ab
overcooked pasta	14.5 (1.9) a

[a] Values in parentheses are standard deviations calculated from triplicate. Means of triplicates with the same letter within a column are not significantly different ($P > 0.05$).

RESULTS

Protein Solubility. To evaluate the extend of protein aggregation in pasta during processing, we first evaluated the SDS-

Table 2. Impact of Pasta Processing on Contents in Accessible Thiol (SH_{free}), Total Thiol Equivalent (SH_{eq}), and Disulfide Groups (SS)[a]

	accessible thiol [SH_{free}] (μmol/g)	total thiol equivalent [SH_{eq}] (μmol/g)	disulfide groups [SS] (μmol/g)
semolina	6.05 (0.22) c	145.4 (4.5) a	69.7 (4.7)
extruded pasta	5.97 (0.12) c	161.9 (2.9) bc	78.5 (4.2)
dried pasta	2.63 (0.06) b	163.1 (0.7) c	80.2 (2.3)
cooked pasta	2.43 (0.21) b	160.9 (1.1) bc	79.2 (7.6)
overcooked pasta	1.83 (0.15) a	154.9 (5.1) ab	76.5 (8.7)

[a] Values in parentheses are standard deviations calculated from triplicate. Means of triplicates with the same letter within a column are not significantly different ($P > 0.05$).

soluble protein content of extruded, dried, cooked, and overcooked pasta. Values were compared to those obtained for the initial durum wheat semolina (Table 1). As expected, protein from durum wheat semolina is largely SDS-soluble (77.8%). Similarly high values of protein solubility (79−93%) for durum wheat semolina are available in the literature.[10,13,22] During the pasta processing, protein insolubilization mainly occurs during stages of heat treatments. When compared to protein in semolina, pasta extrusion, which is conducted at low temperature (35−40 °C) does not significantly ($P < 0.05$) affect protein solubility. The wheat protein remains mostly soluble (75.5%) in freshly extruded pasta. The application of ultra high temperature (90 °C) during the drying leads to a sharply significant ($P < 0.05$) drop in protein solubility. Only 22.6% remains soluble protein in dried pasta. The subsequent heat treatments during cooking (10 min at 100 °C) or overcooking (18 min at 100 °C) only induce slight but not significant ($P < 0.05$) subsequent decreases in protein solubility. The soluble protein content is still 18.2% in cooked and 14.5% in overcooked pasta. The main loss of protein solubility thus occurs at first heat treatment (UHT drying), and only slight decreases are observed during the subsequent heat treatments (cooking and overcooking). Similar effects of heat treatments during pasta processing on gluten protein solubility have already been published. A high residual content in soluble protein has been found in pasta submitted to moderate heat treatments, for instance, pasta dried at low (55 °C) or medium (70 °C) temperature, with, respectively, 70 and 64% SDS-soluble protein.[22] On the other hand, low contents in SDS-soluble protein have been found in pasta submitted to intense heat treatments, for instance, after drying at 90 °C (21−28%) or after cooking in boiling water (14−17%).[10,22]

The present results demonstrate that the content of SDS-soluble protein does not allow the discrimination of pasta that has been submitted to successive or intense heat treatments. Only slight not significant decreases in SDS-soluble protein exist between dried and cooked pasta or between cooked and overcooked pasta (Table 1). A simple measurement of the contents of SDS-soluble protein is not sufficient to give an accurate description of the molecular changes generated by successive or intense heat treatments. The protein network, which is formed during extrusion, is mostly insolubilized during the first heat treatment. The additional molecular aggregation of the gluten protein upon the successive heat treatments conducts to strengthen the protein network density through the formation of additional intermolecular covalent cross-link but with only a slight decrease of the protein SDS-soluble content.

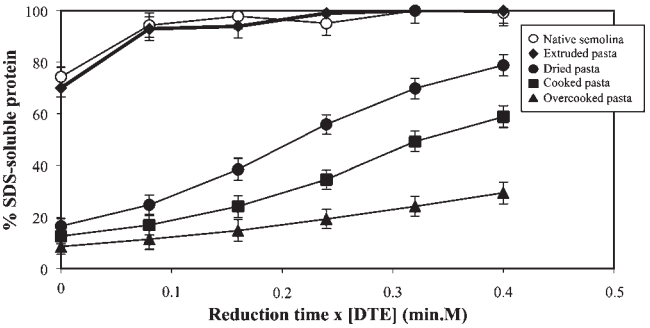

Figure 1. Time course of SDS-soluble protein content for native semolina and extruded, dried, cooked, or overcooked pasta during stepwise reduction of disulfide bonds.

Contents in Thiol and Disulfide Groups. A description of aggregation mechanisms of wheat gluten protein during pasta processing was considered by evaluating the contents in accessible thiol (SH_{free}), total equivalent thiol (SH_{eq}), and disulfide bonds (SS) in semolina and pasta products (Table 2). In the native semolina, values of accessible thiol (SH_{free}), thiol equivalent (SH_{eq}), and disulfide (SS) contents are considered as slightly low, because of difficulties in the extraction procedure. The initial unfolded state of native protein reduces the sensibility of the intrachain SS bonds that are poorly accessible during thiol measurement, because the experimental protocol does not use any denaturing agent. After extrusion, the thiol equivalent content (SH_{eq}) for extruded pasta (162 μmol/g) is higher because of the possible folding effects of mechanical stress.

Pasta processing first induces a gradual decrease in accessible thiol (SH_{free}) content, from 6.05 μmol/g protein in semolina to 1.83 μmol/g protein in overcooked pasta. No significant difference in disulfide (SS) content is considered between the semolina, extruded pasta, and dried pasta, due to the lack of accuracy of SS dosage. The total content of SS bonds remained almost constant during pasta processing, until the cooking stage. The disulfide content (SS) in dried pasta (80.2 μmol/g protein) has been considered as the real disulfide content in pasta products.

On the other hand, we observe a slight not significant decrease on disulfide content (SS) after overcooking (76.5 μmol/g protein). The slight decrease in SS groups is not balanced by an increase in SH_{free} content (Table 2). Results should indicate a slight irreversible loss in thiol equivalent groups, during severe heat treatment that could be associated with the possible formation of intermolecular C−S bonds with a cystine residue. A similar finding was reported by Anderson and Ng,[36] who noticed a net decrease in SH_{eq} in high temperature-extruded flour products. Morel et al.[26] also observed a loss of thiol equivalent groups in gluten after long mixing time at 40−80 °C. These C−S bonds could be responsible for the presence of nonreductible polymers. When compared to the total content in SDS bonds, we could hypothesize that the content of intermolecular C−S bonds is about 1.2% in cooked pasta and 4.6% in overcooked pasta.

Protein Solubility upon Stepwise Disulfide Bond Reduction. Native semolina and the different pasta products were treated with stepwise reduction for different times (from 4 to 20 min) with 20 mM DTE at pH 6.5. Changes in % SDS-soluble protein after different times of DTE reduction for semolina and pasta are presented in Figure 1.

The application of successive heat treatments induces a gradual decrease in the content of SDS-soluble protein. For

native semolina and extruded pasta, we observe a rapid increase in SDS-soluble protein content, toward reduction with DTE. About 100% of the protein are soluble in SDS after only 0.2 min M reduction in DTE. Heat treatments during pasta drying—overcooking denatures the protein to an extent that DTE reduction no longer solubilizes them in SDS buffer. After 0.4 min M stepwise reduction in DTE, the content of SDS-soluble protein is 78.8% for the dried pasta, 58.9% for the cooked pasta, and only 29.3% for the overcooked pasta. Successive and severe heat treatments during pasta processing significantly modify the solubility behavior of protein.

Sensitivity of Disulfide Groups to Reduction. The sensitivity of disulfide bonds in semolina and in pasta products toward DTE was investigated during stepwise reduction. Figure 2 shows the reduction profiles, with the changes in % reduced disulfide bonds (as compared to the initial values of 80.2 μmol/g protein), as a function of time-per-DTE concentration.

For semolina and pasta products, we observe an exponential sensitivity of disulfide bonds toward reduction with DTE. Although we only determined five experimental points for each stepwise reduction, we tried to fit experimental data according to a biexponential model (eq 2).

$$SS_{reduced} = a_1[1 - \exp(-k_1 t)] + a_2[1 - \exp(-k_2 t)] \quad (2)$$

where $SS_{reduced}$ is the % reduced disulfide content upon stepwise reduction; t is the reduction time-per-DTE concentration (min M); and a_1, a_2, k_1, and k_2 are the model parameters. The model parameters (Table 3) were calculated to fit experimental data from a nonlinear optimization procedure (Gauss−Newton procedure) using the software Excel 2004 (Microsoft). The proposed equation fitted well with experimental data for pasta products (R^2 from 0.990 to 0.998 and mean absolute deviation between experimental and calculated values ranging from 0.28 to 1.20%).

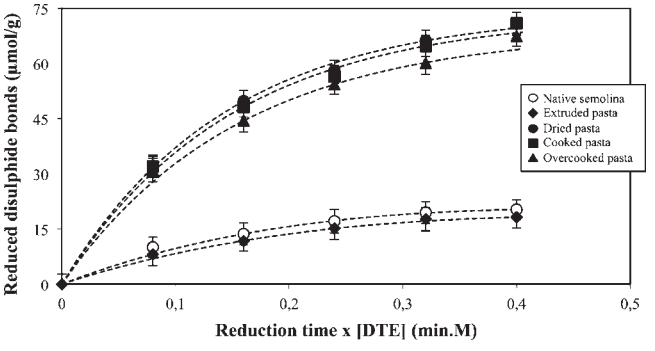

Figure 2. Time course of disulfide bonds reduction for native semolina and extruded, dried, cooked, or overcooked pasta during stepwise reduction of disulfide bonds. Percent reduced disulfide bonds are calculated as compared to the initial values of 80.2 μmol/g protein. The dotted line represents the first order kinetic model (eq 1).

The exponential rise below 0.25 min M would correspond to the reduction of the more sensitive bonds. On the other hand, the apparent steady increase above 0.25 min M would mainly be due to the reduction of the less sensitive bonds. Similar exponential sensitivity of disulfide bonds toward reduction with DTE has been reported by Morel et al.[31] in native and heat-treated wheat gluten products. The exponential rise has been explained by the reduction of the more sensitive SS bonds. Morel et al.[26] indicated that disulfide bond sensitivity toward chemical reduction depends on their accessibility. In the absence of a chemical denaturing agent (urea or SDS), intrachain disulfide bonds from wheat protein have been shown to be protected from chemical reduction and are thus less sensitive to DTE. Intrachain bonds are likely to be buried into the core of the native molecule. In contrast, the interchain disulfide bonds are more sensitive to DTE. An increase in the amount of disulfide bonds sensitive to DTE would therefore indicate the formation of new interchain disulfide bonds and/or the intrachain disulfide bonds exposure following the alteration in protein conformation. Morel et al.[26] stated that an increase in disulfide susceptibility to reduction can be related to the mechanism, leading to protein solubility loss. The two rate constants of the biexponential model (eq 1) can be considered to describe the reaction rates for SS bonds reduction (Table 3). The high values (2.75−6.91 min^{-1} M^{-1}) of the k_1 rate constant are associated with rapid reduction of interchain SS bonds. Low values (5×10^{-5} to 1.12×10^{-2} min^{-1} M^{-1}) of the k_2 rate constant are associated to slow reduction of intrachain SS bonds.

For the native semolina and the extruded pasta, curves show only reduction of high sensitive SS bonds, with, respectively, 17.9 and 15.8% of the total SS bonds. The reduction of the less sensitive bonds above 0.25 min M is almost negligible. Protein aggregation is based on low contents of interchain SS bonds in semolina (about 18%) and in extruded pasta (about 16%). The most part (about 82−84%) of the disulfide bonds could be considered as intrachain SS bonds that are less sensitive to DTE reduction. Similarly low values of intrachain disulfide bonds content in wheat protein (20−23%) have been found in native gluten by Morel et al.[26]

The application of heat treatment during pasta processing induces an increase in the content of SS bonds that are sensitive to stepwise DTE reduction. For dried pasta, more than 60% of the total SS bonds are sensitive to reduction during the exponential rise (below 0.25 min M). Above 0.25 min M, we only observe a gradual reduction of the less sensitive SS bonds (i.e., 70.8% SS bonds are reduced at 0.4 min M DTE). Most of the SS bonds in heat-treated pasta thus behave as interchain SS bonds, as they are accessible and sensitive to DTE reduction. Heat

Table 3. Model Parameters of the Biexponential Model (eq 2) Used To Describe the Time Course of Relative Disulfide Bonds Reduction (as Compared to the Initial Values of 80.2 μmol/g Protein) for Native Semolina and Pasta Samples[a]

	a_1 (% reduced SS)	k_1 (min^{-1} M^{-1})	a_2 (% reduced SS)	k_2 (min^{-1} M^{-1})	R^2	average absolute deviation
semolina	50.2	3.17	−69.7	0.0113	0.990	0.37
extruded pasta	51.0	2.75	−69.5	0.0112	0.995	0.28
dried pasta	74.3	6.91	−68.9	0.00002	0.998	0.65
cooked pasta	73.6	6.66	−68.9	0.00009	0.996	1.09
overcooked pasta	69.1	6.43	−73.0	0.00005	0.995	1.19

[a] Where a_1, a_2, k_1, and k_2 are the model parameters defined in eq 2. The absolute deviation is the difference between experimental and calculated values.

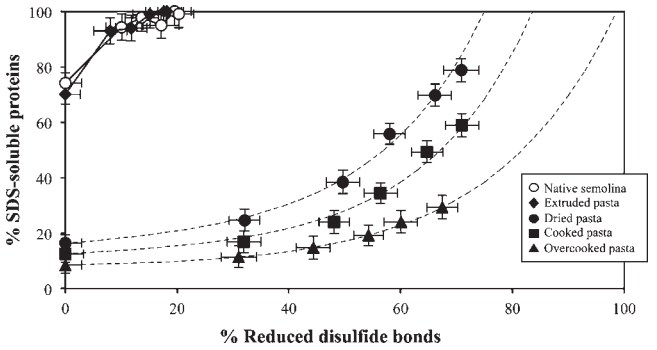

Figure 3. Impact in protein extractability (% SDS soluble protein) of reduced disulfide bonds (as compared to the initial values of 80.2 μmol/g protein) during stepwise reduction DTE, from native semolina and different pasta samples: extruded, dried, cooked, and overcooked pasta. The dotted line represents the biexponential model (eq 2).

treatment could thus favor −SS− exchanges reactions that transform most part of the intrachain SS bonds (in extruded pasta) to interchain SS bonds (in dried pasta).

The application of successive heat treatments does not significantly change the reduction behavior, when compared to dried pasta. We only observe that the application of successive heat treatments after the drying induces a slight decrease in the content SS bond sensitive to DTE. For cooked pasta, 60.7% of total SS bonds are reduced after the exponential rise (below 0.25 min M), and 71.0% of total SS bonds are reduced after the steady increase (at 0.4 min M). For overcooked pasta, 56.7% of total SS bonds are reduced after the exponential rise (below 0.25 min M), and 67.5% of total SS bonds are reduced after the steady increase (at 0.4 min M). Similar decreases in content of SS bond sensitive to DTE were found by Morel et al.[26] for wheat gluten heat treated up to 60 °C. As already supposed, these results should indicate a slight irreversible loss in SS bonds after severe heat treatments, which could be associated with the possible formation of intermolecular C−S bonds with cystine residue.

Protein Solubility versus Disulfide Bond Reduction. Pasta processing induces significant changes in protein solubility (Figure 1) and in the sensitivity of SS bonds to DTE reduction (Figure 2). Relationships between these two parameters are presented in Figure 3. Figure 3 shows that reduction of disulfide bonds increased protein solubility in SDS-phosphate buffer. For native semolina and extruded pasta, almost total protein was brought into solution after the reduction of less than 10−15% of the disulfide bonds. These disulfide bonds belong to the 18−20% of interchain SS bonds, which maintain the polymeric black bone of glutenin. It was shown that this type of bonds is readily accessible to reduction.[33] Further reduction of disulfide bonds did not lead to any change in protein solubility. These bonds correspond to the intrachain SS bonds, which were reduced at a very slow rate by DTE (Table 3). As expected, intrachain SS bonds play no role on protein solubility in SDS buffer.

In contrast, an increase in solubility was slower for heat-treated pasta samples, especially for the successive heating conditions. For instance, for an overcooked pasta sample, reduction of more than 30% of the total disulfide bonds does not lead to very significant increase in protein extractability. Whereas successive heat treatments increase the sensitivity of disulfide bonds to DTE reduction, less and less protein was brought into solution when the bonds were broken. In pasta products after heat treatments, the release of SDS-soluble protein contents with increased

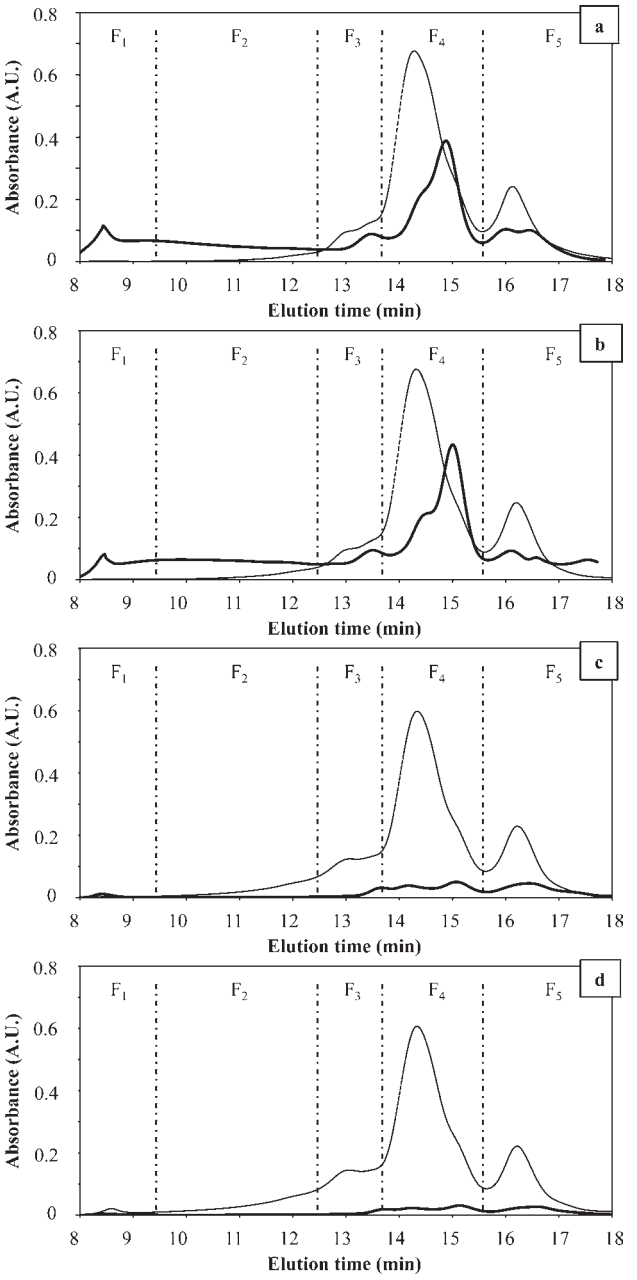

Figure 4. Size exclusion distribution profiles of SDS-soluble gluten protein from native samples (solid line) and fully reduced samples (dotted line) in semolina (a), extruded pasta (b), dried pasta (c), and cooked pasta (d). Fractions F_1−F_5, respectively, included glutenin polymers above 680000 (F_1) and ranging from 95000 to 680000 (F_2), gliadin monomers ranging from 55000 to 95000 (F_3) and from 20000 to 55000 (F_4), and salinosoluble protein from 6000 to 20000 (F_5).

disulfide bonds reduction was described using a mathematical biexponential model (Figure 3). The proposed equation fit well with experimental data for pasta products (with R^2 ranging from 0.791 to 0.891 and average absolute deviation ranging from 0.22 to 1.94%).

In heat-treated pasta, it clearly appears that the selected conditions for the stepwise reduction in DTE at 0.4 min M are not sufficient to reduce enough interchain SS bonds and solubilize all of the protein in DTE. In dried pasta, only 70.8% of the SS bonds are reduced at 0.4 min M DTE and only give 78.8%

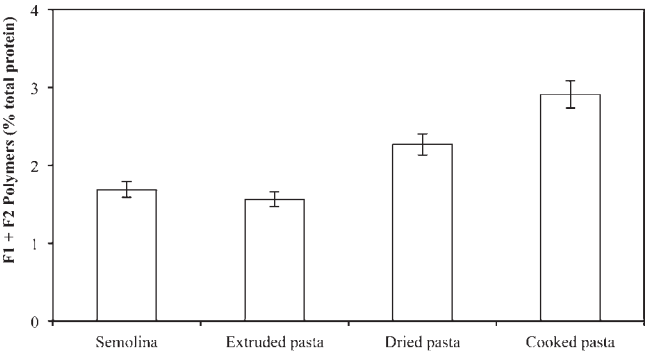

Figure 5. Change in protein fractions $F_1 + F_2$ (in % of total protein) after exhaustive reduction in DTE from native semolina and different pasta samples.

content of SDS-soluble protein. The successive heat treatments strengthen this effect. For instance, in overcooked pasta, 67.5% of the SS bonds are reduced at 0.4 min M DTE and only give 29.3% content of SDS-soluble protein.

Extrapolation of the calculated exponential curves was considered to estimate a "theoretical" content in SDS bonds that has to be reduced to obtain 100% soluble protein (Figure 3). The theoretical content in SDS bonds is calculated at 75% for dried pasta, 84% for cooked pasta, and 98% for overcooked pasta. The difference between the theoretical value and the maximum content of reduced SS bond after the stepwise reduction (4% for dried pasta, 13% for cooked pasta, and 31% for overcooked pasta) can estimate the residual content of interchain SS bonds, not yet reduced after 0.4 min M DTE reduction. Successive and severe heat treatments thus generate a high content of interchain SS bonds, by exchange reactions from the initial intrachain SS bonds, which greatly contribute to protein aggregation and insolubility.

Protein Extractability after Total or Stepwise Disulfide Bond Reduction. Typical SE-HPLC profiles from native semolina and pasta samples, before (plain line) and after (dotted line) total DTE reduction, are shown in Figure 4. The SE-HPLC profiles for samples before total reduction confirm the significant decrease in protein overall solubility after the drying stage. In the native semolina SE-HPLC profile, fractions F_1 and F_2 consist of glutenin polymer and fractions F_3−F_5 consist mainly of gliadin monomers of decreasing size (ω, γ, β, and α) and of some remaining soluble protein.[35]

Total DTE reduction ensures that all of the disulfide SS bonds are reduced in the samples, irrespective of their accessibility. Total reduction affects both the interchain and the intrachain SS bonds. For all samples, total reduction of disulfide bonds leads to total protein extractability in SDS buffer, as attested from the constancy of total SE-HPLC areas, from native semolina to harshly heat-treated pasta. Total reduction of disulfide bonds alters the size distribution of protein, which eluted in three peaks. The first one, which coincided with fraction F_3 of native semolina, would consist of high molecular weight glutenin subunits (HMW-GS) and ω-gliadin (M_r = 55000−95000). The following fractions would consist of reduced gliadin and low molecular weight glutenin subunits (LMW-GS). As compared with original SE-HPLC profile (for native semolina), the exhaustive DTE reduction induces a shift toward shorter retention times for fraction F_4. This suggested that gliadin monomers unfolded, as a consequence of intrachain disulfide bond reduction.

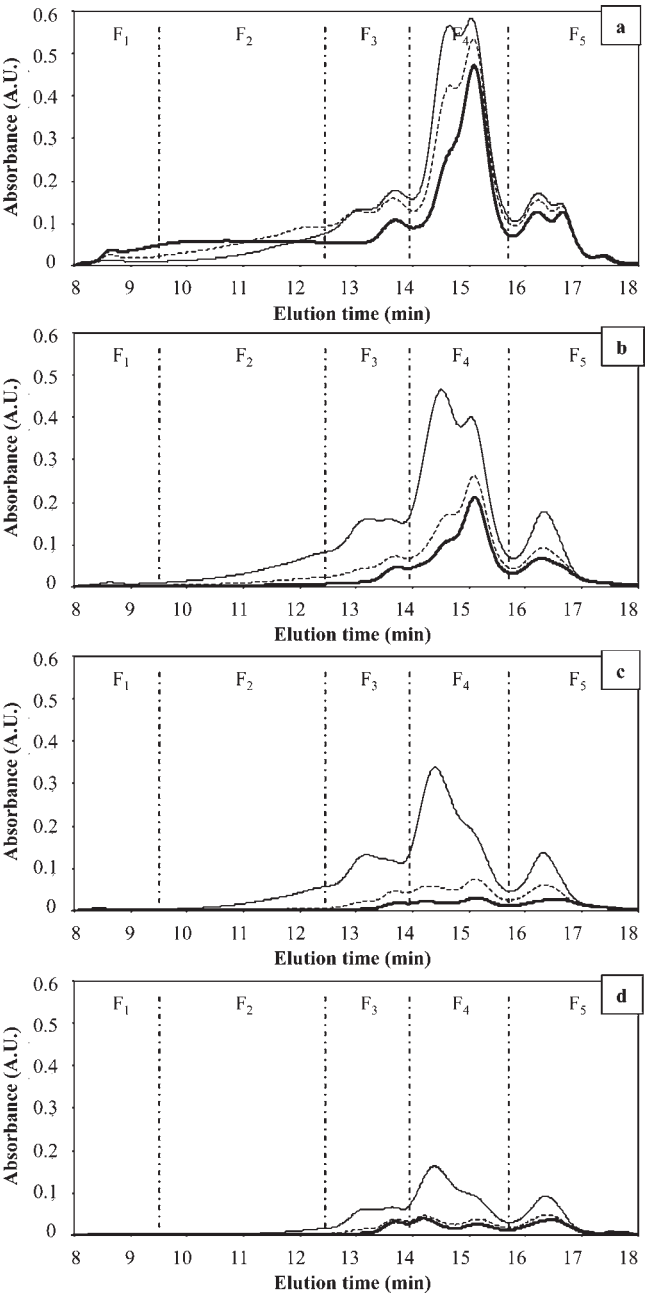

Figure 6. Size exclusion distribution profiles of SDS-soluble gluten protein from native semolina (a), dried pasta (b), cooked pasta (c), and overcooked pasta (d) before (solid line) and after stepwise DTE reduction (with 20 mM DTE at pH 6.5) for 0.08 (dotted line) or 0.4 min M (dashed line). Fractions F_1−F_5, respectively, included glutenin polymers above 680000 (F_1) and ranging from 95000 to 680000 (F_2), gliadin monomers ranging from 55000 to 95000 (F_3) and from 20000 to 55000 (F_4), and salinosoluble protein from 6000 to 20000 (F_5).

Slight differences in SE-HPLC profiles exist for pasta that has been submitted to heat treatment (Figure 4). SE-HPLC chromatograms revealed that even after total disulfide bond reduction, protein polymers (eluted within fractions F_1 and F_2) are still present in heat-treated samples, in contrast with native semolina. Accumulation of nonreducible polymers, estimated from the sum areas of fractions F_1 and F_2 (high molecular weight fractions with $M_r \gg 100000$, at retention times below 12.5 min) is shown in

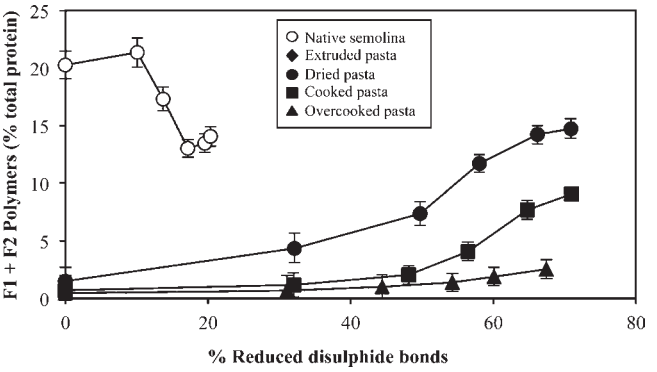

Figure 7. Change in protein fractions $F_1 + F_2$ upon disulfide bond reduction in DTE from native semolina and different pasta samples: extruded, dried, cooked, or overcooked pasta.

Figure 5. A slight increase in area of fractions F_1 and F_2 is observed after the successive heat treatments (2.3% in dried pasta and 2.9% in cooked pasta, as compared to 1.6% in native semolina and extruded pasta). Heat treatment of pasta generates a portion of the polymer that resists exhaustive DTE reduction. Apart from the formation of new interchains disulfide bonds, heat treatments would induce the formation of intermolecular isopeptide bonds responsible for the accumulation of nonreducible polymers.[26]

Figure 6 shows the typical changes in SE-HPLC profiles upon stepwise reduction in DTE, from native semolina and pasta samples. For native semolina upon stepwise reduction (for 0, 4, or 20 min), the release of LMW-GS was evidenced as the growing F_4 shoulder, while HMW-GS eluted within F_3. Also, a short transient increase in fractions F_1 and F_2 was registered at the beginning. Initially, reduction brought SDS-insoluble glutenin polymers into solution as large polymers, which were in turn reduced into smaller ones. In contrast with native semolina, protein from the heat-treated pasta samples (dried, cooked, or overcooked pasta) was mostly brought into solution as monomers, whereas changes within fractions F_1 and F_2 remained small.

Figure 7 shows that for native semolina, the reduction of 5−10% of the total disulfide bonds results in an apparent slight transient increase in F_1. In the case of the heat-treated pasta, the release of polymers was more and more delayed as heat treatment severity increased. Also, less and less polymer seemed to be released upon reduction.

DISCUSSION

Heat treatment is clearly the driving unit operation of change in protein structure upon pasta processing. The present work attempted to clarify the involvement of successive and intense heat treatments on protein solubility characteristics. It appears that one of the first noticeable events upon heat treatment during pasta processing is the drop of protein solubility in SDS. Concurrently, the disulfide bond susceptibility to DTE reduction increases, respectively. A SDS solubility drop decrease indicates a growing connectivity between polypeptide chains and also development of insoluble polymer structures. We showed that the amount but also the structure of insoluble protein changed upon heat treatments during pasta processing. Indeed, for heat-treated pasta and in contrast with native semolina, limited reduction of disulfide bonds did not allow the extraction of large polymers.

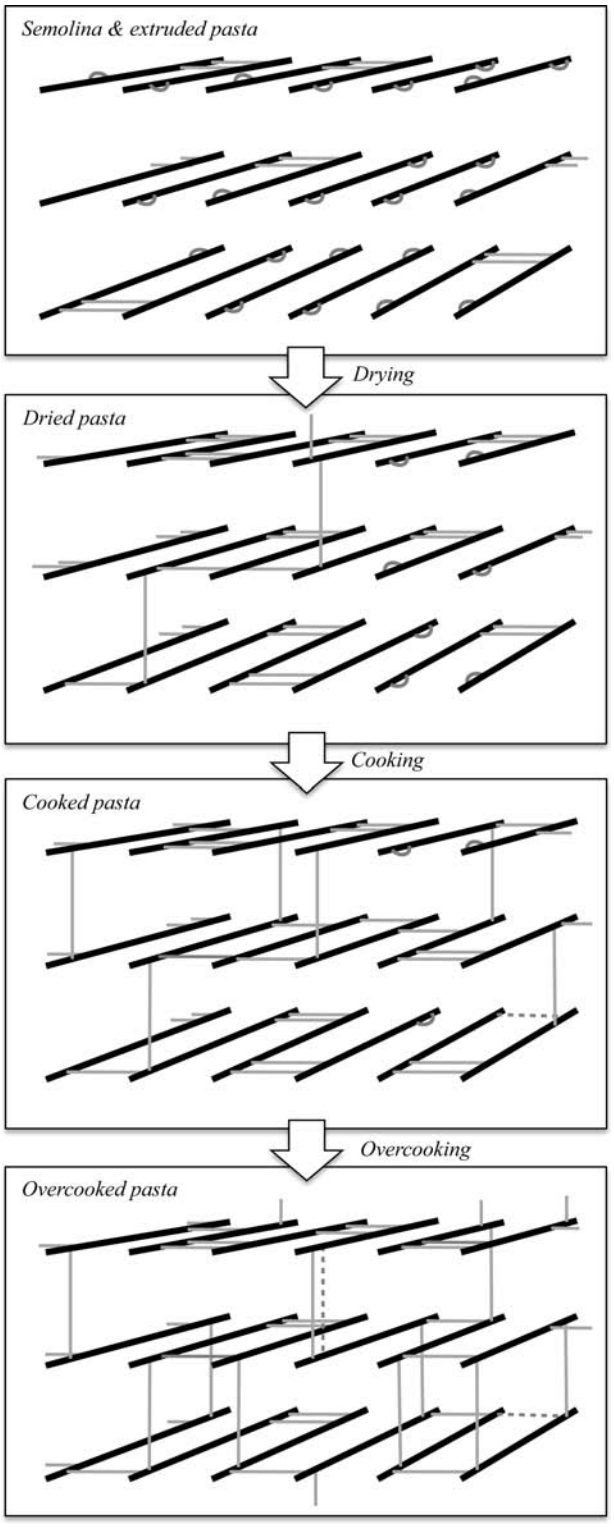

Figure 8. Schematic representation of possible molecular changes in gluten protein aggregation during pasta processing (where the black line represents the protein chain, the gray curved line represents the intrachain disulfide bonds, the gray line represents the interchain disulfide bonds, and the gray dashed line represents the CS bonds).

Even if the glutenin polymer structure is still under discussion, we consider one of the most favored models that is a linear backbone of glutenin subunits end-to-tail disulfide bonded.[37] For

linear polymers, effective reduction in size can be expected after only a few scissions along the chain. Conversely, if polymer chains are connected into a three-dimensional network structure, random scissions along chains will be rather ineffective at the beginning (Figure 8). The presented results indicated that heat treatments during pasta processing induced the buildup of a three-dimensional connected structure. Hydrophobic, disulfide, or isopeptide bonds could also be involved in the growing connectivity of protein. Indeed, a delayed response upon disulfide reduction may be expected from a three-dimensional network stabilized by any one of those types of bonds. Nevertheless, the isopeptide bond role would be negligible; otherwise, reduction of total disulfide bonds would not have brought total protein into solution. Gluten protein comprises cysteine residues supposed to be involved in either intra- or interchain disulfide bonds, without free sulfhydryl groups available.[38,39] Thus, additional interchain disulfide bonds would be formed at the expense of intrachain bonds via interchange reactions. Conversion of intrachain disulfide bonds into interchain ones might be significant. Free sulfhydryl groups are obligatory intermediates of disulfide interchanges, but Morel et al.[26] suggested that free thiol content might not be the driving variable of disulfide interchanges.

Heat treatments induced a drastic change in thiol and disulfide contents, in disulfide bonds sensitivity toward DTE reduction, and SE-HPLC profiles of fully reduced protein. From the comparative analysis of the rate of changes, protein solubility loss was assumed to derive from the increasing connectivity of gluten protein upon pasta processing. The increasing degree of gluten protein polymerization would predominantly be due to the formation of additional intermolecular disulfide bonds and/or to increasing hydrophobic interactions between gluten protein. The formation of nonreducible polymers cross-linked by isopeptide bonds would play a minor role in the strengthening of the gluten network.

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