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Importance of Crosslinking and Disulfide Bridge Reduction for the Mechanical Properties of Rigid Wheat Gluten Bioplastics Compression Molded with Thiol and/or Disulfide Functionalized Additives

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ABSTRACT: Thiol (SH) containing additives improve the mechanical properties of rigid, glassy gluten materials. However, the underlying molecular mechanism is still unclear. In particular, the importance of the preceding gluten-additive mixing conditions remains to be investigated. Here, different additives containing either only SH, only disulfide or both SH and disulfide functionalities were synthesized and their impact on the gluten network using different mixing conditions prior to subsequent molding were assessed. All SH containing additives decreased the gluten molecular weight (MW) during mixing to a degree depending on the conditions. Additives with only disulfide functionality did not significantly affect protein size during mixing irrespective of the conditions used. Only when mixing induced sufficient MW reduction did the strength and failure strain of rigid gluten materials increase. This shows that factors other than the degree of cross-linking affect the strength of rigid gluten materials. These results support our hypothesis that altered molecular conformations and improved molecular entanglements contribute to material strength. The extent to which such conformational changes occur may depend on the additive and the way of mixing. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41160.

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INTRODUCTION

Ecological concerns over petroleum-based products have stimulated research on biodegradable materials derived from biopolymers. Wheat gluten is an interesting raw material for bioplastics because of its annually renewable, abundant availability as coproduct of the starch industry, its low cost, biodegradability, and ability to crosslink upon heating.[1] It consists of single-chained gliadins and polymeric glutenins, which are, respectively, soluble and insoluble in alcoholic media. Glutenins consist of subunits connected by disulfide bonds, which have gliadin-like solubilities.[2] In research, both wet and dry processes are used to produce gluten-based materials.[3,4] Fast techniques that require little if any solvent such as thermomolding or, more specifically, high temperature injection or compression molding are industrially more relevant and preferred from an environmental point of view.[5,6]

Depending on the level of plasticizer, high temperature compression molding yields rubbery or glassy materials.[5,7] Rubbery gluten materials are flexible and ductile.[7] Glassy gluten materials—the case of interest in the present article—are stiff and brittle.[5,8] Both strength and failure strain of rigid glassy materials increase with molding temperature, which corresponds to increased degrees of crosslinking. The introduction of crosslinks does not heavily affect the modulus of glassy gluten materials.[5] These observations align very well with what is observed for synthetic polymers. The modulus of nonoriented polymers in the glassy state is determined by the polymer's weak bonds (e.g., hydrogen bonds) and free volume kinetics (involving aspects of thermal history and ageing), while the toughness is governed by the network's strong bonds (e.g., covalent bonds such as peptide and disulfide bonds) and their ability to delocalize local strains.[9]

Different types of gluten crosslinks are present in rigid, glassy gluten materials, but disulfide bonds are predominant.[10,11] To improve the strength and failure strain of rigid gluten materials, molecules with multiple terminal thiol (SH) groups can be

incorporated into the protein structure.[8,12] These additives can act as reducing agents during blending with gluten and as cross-linking agents during molding.[8] The improved mechanical properties obtained when using polythiol additive were initially related to an increased degree of crosslinking,[8] but experiments with the monothiol 3-mercaptopropionic acid (MPA) indicated a more complex relationship between gluten network characteristics and material properties in the presence of SH containing additives.[13] Although a decrease in crosslinking degree with increasing MPA concentration was observed, the strength of the rigid gluten materials did not depend on the MPA concentration. This led to the hypothesis that, besides cross-linking, also molecular conformations and improved molecular entanglements may contribute to the material strength.[13] Similar improvements in mechanical properties were obtained at low concentrations of either MPA (106 μmol SH/g protein) or molecules with multiple SH groups at the same concentration [SH-functionalized polyglycerol (PG) and tris(hydroxymethyl)ethane (THME)]. Since only minimal protein extractability was obtained after molding gluten containing low concentrations of additives at 150°C, the degree of crosslinking could not be assessed.[13]

At high concentrations of SH-functionalized PG, plasticization or the presence of distinct phases seems more important for the mechanical properties than the contribution of entanglements or altered conformations.[13] Interestingly, high levels of free SH groups were still present after molding at 150°C with different additive concentrations (106, 265, and 530 μmol SH/g protein).[13] Since from a theoretical point of view, all those SH groups can be oxidized to intermolecular disulfide bonds, this clearly demonstrates the potential for additional crosslinking. It should also be pointed out that, in literature, all additives were mixed with gluten in a solvent [i.e., 70% ethanol in Jansens et al.,[13] 0.05*M* acetic acid in Woerdeman et al.[8] and Dicharry et al.[12]] and that the effect of the additives may (in part) be related to their solubility in the solvent.

To efficiently improve the performance of gluten materials, it is important to understand the impact that additives have on the gluten network and, as a result, on the mechanical properties of the end product. Therefore, in this research, we molded gluten in the presence of a low concentration (106 μmol sulfur/g protein) of several SH containing additives with different chemical structure at a molding temperature (130°C), which allows evaluating the crosslinking degree based on protein extractability measurements. Since our previous research showed that high levels of free SH groups are still present after molding samples with SH containing additives,[13] we here investigated the effect of additives containing either only disulfide functionality or both SH and disulfide functionalities and compared their effect with that of the additive with similar chemical structure but only SH functionality. In this research, (hyper)branched additives were selected since the functional groups are in principle readily available for reaction. To evaluate the impact of the mixing step, additives were mixed with gluten in different ways. First, gluten was mixed in two different ethanol concentrations (70 and 95%). Whereas gliadin and glutenin subunits are soluble in 70% ethanol (glutenin is not), gluten proteins as such are not soluble in 95% ethanol. However, gluten was also mixed with additives in an extruder without adding solvent. The mechanical properties of the molded materials were determined with a 3-point bending test. The gluten network was then analyzed by determining both the protein extractability in sodium dodecyl sulfate (SDS) containing medium and the levels of free SH groups. Finally, the obtained network characteristics were related with the mechanical properties.

EXPERIMENTAL

Materials

Wheat gluten with a protein content of 77.8% (dry basis) and a moisture content of 5.6% was obtained from Tereos Syral (Aalst, Belgium). The moisture content was determined according to the AACC Approved Method 44-19.[14] Protein content ($N \times 5.7$) was determined using an adaptation of the AOAC Official Method to an automated Dumas protein analysis system (EAS Variomax N/CN Elt, Gouda, The Netherlands).[15] Hyperbranched PG with an average molecular weight (MW) of about 2000 (PG2000) was obtained from the Institute of Organic Chemistry of the Johannes Gutenberg University Mainz (Mainz, Germany). Its synthesis involves a ring-opening multibranching polymerization of glycidol with 1,1,1 tris(hydroxymethyl)propane as initiator.[16] Hyperbranched poly(ester amide) hybrane with an average MW around 1500 (H1500) was obtained from DSM research (Geleen, The Netherlands). Its synthesis involves in a first step the reaction of diisopropanolamine with cyclohexane dicarboxylic anhydride. The hyperbranched structure is then formed in a second step.[17] All other chemicals, solvents and reagents were from Sigma-Aldrich (Steinheim, Germany) unless specified otherwise and were at least of analytical grade.

Synthesis of SH Functionalized Molecules

THME, PG2000 and H1500 were SH functionalized by esterifying (part of) their hydroxyl groups with the carboxyl group of MPA. The SH functionalization of THME and PG was performed as in Jansens et al.[13] with minor modifications. Briefly, THME (10.00 g, 83.2 mmol) was mixed with MPA (22.5 mL, 258.2 mmol), toluene (40.0 mL), and *p*-toluenesulfonic acid (1.43 g, 8.3 mmol). The reaction mixture was then subjected to azeotropic distillation for 24 h under argon atmosphere. Toluene was removed by evaporation and the obtained SH functionalized THME (fTHME) was dissolved in 150 mL diethyl ether and extensively washed with deionized water. PG2000 (10.0 g) was mixed with MPA (12.5 mL, 143 mmol), toluene (25 mL), and *p*-toluenesulfonic acid (0.35 g, 2.03 mmol). The reaction mixture was refluxed under argon atmosphere for 60 min, followed by evaporation of toluene and washing of SH functionalized PG2000 (fPG2000) with diethyl ether. H1500 (50.00 g) was mixed with MPA (17.0 g, 160.5 mmol), toluene (600 mL) and catalytic amounts of *p*-toluene sulfonic acid (0.60 g, 3.4 mmol). Azeotropic distillation was then performed for 24 h under argon atmosphere. After 24 h, toluene was evaporated and the reaction mixture was dissolved in about 50 mL methanol. This solution was added drop wise to 5 l water under continuous stirring, resulting in a white precipitate. The latter was collected by filtration, redissolved in methanol and the precipitation was

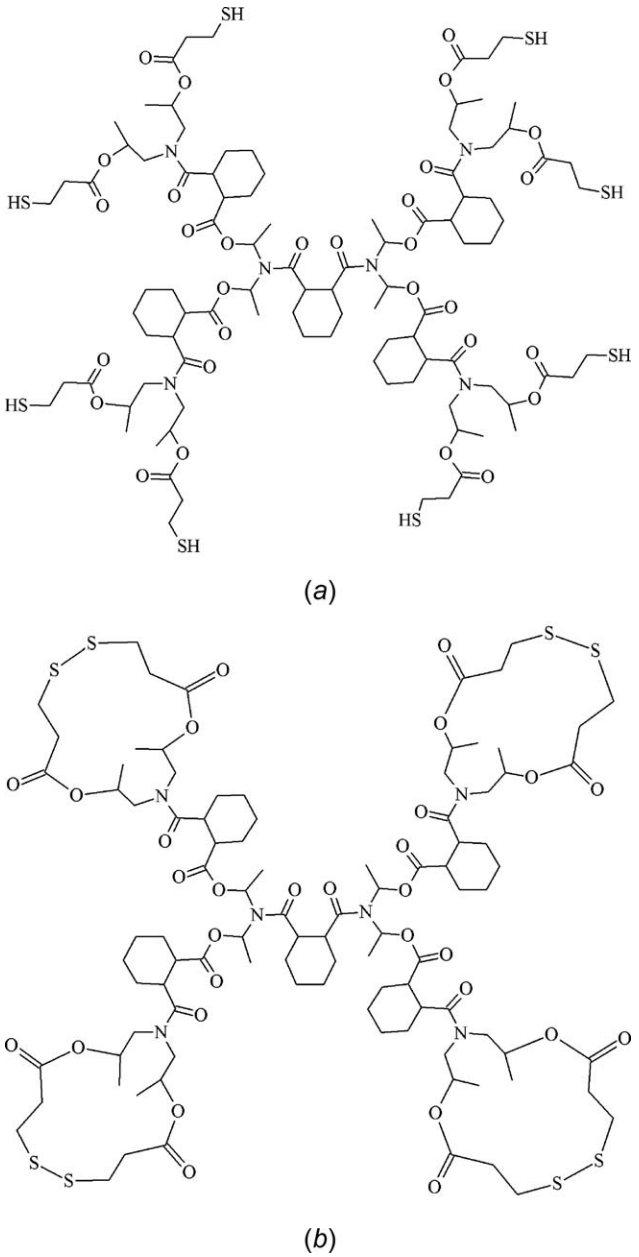

Figure 1. A possible chemical structure of SH functionalized hybrane H1500 (fH1500; A) and of oxidized fH1500 with a high degree of oxidation (oxfH1500H; B). Since the oxidized fH1500 remained soluble, most disulfide bonds were presumably intramolecular.

repeated two times. Finally, the white precipitate SH functionalized H1500 (fH1500) [Figure 1(a)] was dried overnight in a vacuum oven at 40°C.

Oxidation of SH Functionalized Additives

To prepare soluble fH1500 with an intended degree of oxidation of about 100% (oxfH1500H) [Figure 1(b)], fH1500 (0.50 g) was dissolved in dichloromethane (10 mL) and the solution was slowly added drop wise (10 min) under intense stirring to dichloromethane (400 mL) containing N-bromosuccinimide (0.20 g, 1.12 mmol). Soluble fH1500 with an intended degree of oxidation of about 50% (oxfH1500L) was prepared by dissolving fH1500 (1.5 g) in dichloromethane (10 mL). The solution was slowly added drop wise (10 min) under intense stirring to dichloromethane (800 mL) containing N-bromosuccinimide (0.24 g, 1.34 mmol). After 60 min, part of the dichloromethane was evaporated and the remaining solution (about 100 mL) was washed two times with deionized water using a separation funnel. The resulting white product was dried overnight in a vacuum oven at 40°C. The solubility of the oxidized additives indicates that the disulfide bonds formed by oxidation of free SH groups are mainly intramolecular.

Characterization of Functionalized Additives

Additives were characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS). ^{1}H and ^{13}C NMR spectra (300 MHz) were recorded at room temperature on a Bruker Avance 300 (Bruker, Billerica, MA) in $CDCl_3$ for fTHME and fPG and in DMSO for fH1500 and oxfH1500. Tetramethyl silane was used as chemical shift reference. Mass spectra were recorded with an electrospray ionization mass spectrometer (HP 5989A, Agilent Technologies, Santa Clara, CA).

Modification of Gluten Proteins with Functionalized Additives

Gluten was mixed with different SH and disulfide containing additives both in 70% ethanol and in 95% ethanol (10% w/v). The same level of sulfur atoms (106 μmol/g protein, in the form of SH or disulfide groups) was added for each additive (Table I). This concentration corresponds to about 2/3 of the cysteine residues present in gluten. After mixing overnight, ethanol was evaporated and the remaining mixture was freeze dried. The samples were then ground and sieved (250 μm). Their moisture content was adjusted to 7% by adding appropriate amounts of crushed ice, which itself was prepared by sprinkling water in a mortar with liquid nitrogen and grinding the resulting mixture to a fine ice powder with a pestle. The gluten sample was then added and mixed with the ice using the pestle. Finally, to homogenize it, the sample was shaken overnight with

Table I. The DE of the Functionalized Additives Calculated from Their Proton or Carbon NMR Spectra, the Number of Sulfur Atoms for Each Additive and the Concentration of the Different Additives Used, Expressed as Mass Percentage of Gluten Dry Matter

Sample[a]	DE (%)	Sulfur (atoms/mol)	Concentration of additive (%)
MPA	-	1	0.9
DTT	-	2	0.6
fTHME	100	3	1.1
fPG2000	37	11	2.4
fH1500	100	8	2.2
oxfH1500L	100	8	2.2
oxfH1500H	100	8	2.2

[a] MPA: 3-Mercaptopropionic acid; DTT: dithiothreitol; fTHME: SH-functionalized tris(hydroxymethyl)ethane; fPG: SH functionalized polyglycerol; fH1500: SH-functionalized hybrane; oxfH1500L: oxidized fH1500 with low degree of oxidation; oxfH1500H: oxidized fH1500 with high degree of oxidation.

a head-over-head shaker. As a reference, gluten was also mixed in both solvents without any additive.

Gluten was also mixed with the same additives using a corotating twin-screw extruder with a recirculation channel that allows controlling the mixing time (DSM Xplore, Geleen, The Netherlands). Hereto, it was first manually blended with 40% water and the additive. The mixture was then extruded for 5 min at 30°C and 100 rpm under nitrogen atmosphere. As a reference, gluten was extruded with 40% water and no additives. After extrusion, the samples were freeze dried, ground, sieved (250 μm), and the moisture content was adjusted to 7%.

High-Temperature Compression Molding

Control gluten and the same with additives were compression molded in a preheated mold between two Teflon sheets with a Pinette Press Zenith 2 (Pinette Emidecau Industries, Chalon sur Saône, France) at 5 bar. Samples were molded at 130°C for 5 min. Before removing the samples, the mold was allowed to cool to 35–30°C over 30–40 min.

Mechanical Property Determination

Compression molded specimens were stored for 48 h at 50% RH and 20°C prior to testing. For each molding condition, at least five samples were tested in a three-point bending test according to ASTM D790-03. The samples were tested with an Instron Universal instrument model 4467 equipped with 1 kN load cell (Instron, High Wycombe, United Kingdom) and a crosshead speed of 1.0 mm/min. The specimen support length was at least 16 times the thickness of the plates (about 1.7 mm). Modulus, strength, and failure strain were determined from the acquired stress-strain curves. The modulus was determined as the slope of the stress-strain curve at 0.2% strain. The strength and failure strain were respectively the highest stress and strain before the sample failed. Prior to all other analyses, the molded samples were ground to pass a 250 μm sieve.

Determination of Protein Extractability in SDS Containing Medium and MW Distribution

The level of proteins extractable with SDS containing medium (SDSEP) was determined as in Jansens et al.[11] Samples containing 1.0 mg protein were extracted with 1.0 mL 0.05 mol/L sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS (Acros Organics, Geel, Belgium). All extractions were performed in triplicate. After centrifugation (10 min, 10,000 × *g*) and filtration over polyethersulfone (0.45 μm, Millex-HP, Millipore, Carrigtwohill, Ireland), extracted proteins were separated with size-exclusion high-performance liquid chromatography (SE-HPLC) using a LC-2010HT system (Shimadzu, Kyoto, Japan) with automated injection. The extracts (60 μL) were loaded on a BioSep SEC-S4000 column (300 × 7.8 mm, Phenomenex, Torrance, CA) and eluted with acetonitrile/water (1 : 1, v/v) containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1.0 mL/min at a temperature of 30°C.[18] Eluted protein was detected at 214 nm.

The SDSEP content was calculated from the peak areas and expressed as percentage of the peak area of unmolded gluten extracted with the SDS containing medium in the presence of 2.0 mol/L urea and 1.0% (w/v) dithiothreitol (DTT, Acros Organics).

Free SH Determination

Free SH groups were determined colorimetrically after reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Samples (0.8–1.3 mg protein) were shaken for 60 min in 1.0 mL sample buffer [0.05 mol/L sodium phosphate buffer (pH 6.5) containing 2.0% (w/v) SDS, 3.0 mol/L urea and 1.0 mmol/L tetrasodium ethylenediamine tetraacetate]. Then, 100 μL DTNB reagent [0.1% (w/v) in sample buffer] was added and the samples were shaken for 10 min. After filtration over a polyethersulfone membrane (0.45 μm, Millex-HP, Millipore), the extinction at 412 nm was read exactly 45 min after adding DTNB reagent. Extinction values were converted into concentrations of free SH using a calibration curve with reduced glutathione.[18] Controls containing either no DTNB or no sample were used to correct for background extinctions.

Statistical Analysis

Statistical analyses were conducted with the Statistical Analysis System software 9.3 (SAS Institute, Cary, NC). Significant differences ($P < 0.05$) for several variables were determined by the ANOVA procedure.

RESULTS AND DISCUSSION

Synthesis and Characterization of SH Functionalized Additives

Additives containing hydroxyl groups were SH functionalized by esterification with MPA. Successful SH functionalization of the different molecules was confirmed by comparing the MS spectra of SH functionalized and unfunctionalized additives (data not shown). For each hydroxyl group esterified with MPA, the MW of the additive increases by 88. The ^{1}H NMR spectra of fTHME and fPG2000 discussed elsewhere allow calculating the degree of esterification (DE).[13] The DE is the percentage of the initial hydroxyl groups in the unfunctionalized molecule which is esterified with MPA. All three hydroxyl groups of fTHME were esterified, and for fPG2000 a DE of 37% was obtained. Since unfunctionalized PG2000 contains on average 29 hydroxyl groups, fPG2000 contains on average 11 SH groups. The ^{1}H NMR spectrum of fH1500 showed several overlapping multiplets. This prevented an accurate calculation of the DE. Therefore, the DE was approximated with ^{13}C NMR based on the difference in chemical shift of methyl groups of fH1500 [Figure 1(a)] in proximity to an hydroxyl group and those close to an ester group. This showed esterification of all eight hydroxyl groups. Two oxidized soluble fH1500 (oxfH1500) samples were prepared from fH1500. Their degrees of oxidation, defined as the percentage of the initial amount of free SH groups oxidized, was calculated based on the difference in chemical shift of methylene groups next to an SH group and those next to a disulfide bond and were 48% (oxfH1500L) and 100% [oxfH1500H, Figure 1(b)].

Flexural Properties of Compression Molded Gluten with Additives

Gluten mixed with and without additives was compression molded at 130°C to form rigid materials with mechanical properties such as listed in Tables II, III and IV. The properties for

Table II. Flexural Modulus (GPa) of Gluten Molded After Either Solvent Mixing (in 70% Ethanol or 95% Ethanol) or Extrusion Mixing with or without Additives[a]

	70% Ethanol	95% Ethanol	Extrusion
Reference	3.5 (0.2) a,b,c	3.3 (0.2) b,c,e	4.1 (0.1) f
MPA	3.8 (0.2) d	3.4 (0.1) a,b,c,e	3.9 (0.1) d,f
DTT	3.6 (0.2) a,b,d	3.6 (0.1) a,b,c,d	3.5 (0.1) a,b,c,d
fTHME	3.7 (0.1) a,d	3.4 (0.2) a,b,c,f	4.2 (0.2) f
fPG2000	3.7 (0.1) a,d	3.1 (0.1) c,e	3.4 (0.1) a,b,c,e
fH1500	3.7 (0.2) a,d	3.6 (0.1) a,b,d	3.6 (0.3) a,b,c,d
oxfH1500L	3.8 (0.1) d	3.4 (0.2) a,b,ce	3.4 (0.1) a,b,c
oxfH1500H	3.5 (0.1) a,b,c	3.5 (0.2) a,b,c	3.3 (0.1) c,e

Additive codes as in Table I.
[a] Standard deviation of 5-fold mechanical property determinations on single samples is given in brackets. Values with the same letter are not significantly different ($P < 0.05$).

molded gluten mixed without additives in either 70% or 95% ethanol were not significantly different. The strength of molded extruded gluten without additives was slightly higher than that of molded gluten mixed without additives in these solvents.

The additives had little if any effect on the modulus, irrespective of the mixing system (Table II). This is in line with earlier findings that the modulus in the presence of such SH containing additives was only affected at higher mass concentrations.[13] The effect of the additives on both strength and failure strain depended on the mixing system. After mixing in 70% ethanol, all nonoxidized SH containing additives improved the strength to a similar extent (Table III). The strength was also higher when a partly oxidized additive (oxfH1500L) was used, while the fully oxidized additive oxfH1500H did not yield mechanical properties which exceeded those of the reference samples. All nonoxidized SH containing additives improved the strength when mixed with gluten in 95% ethanol (Table III). However, the improvement in strength with MPA, DTT, and fTHME when 95% ethanol was used as mixing solvent was higher than that with fPG2000 and fH1500, although the differences were not always significant. Oxidized additives had no significant effect on the strength when mixed with gluten in 95% ethanol. When the samples were extruded, an improved strength was observed for all samples with nonoxidized additives, whereas the strength of samples with oxidized additives was not significantly different from that of the reference sample.

Since the stress-strain relation of all samples is linear and since the moduli are hardly affected by the presence of SH containing additives, higher strength values also imply higher failure strains and higher toughness for the materials (Table IV).

Chemical Changes of the Gluten Network when Mixing and Compression Molding of Gluten with Additives

Protein Extractability After Mixing. The SDSEP content of gluten mixed without additives (reference) in 70% ethanol was lower than that of untreated gluten (Table V), indicating that during mixing gluten crosslinking took place. The SE-HPLC profiles of these extracts revealed this decreased SDSEP content due to mixing in 70% ethanol was in essence a decreased glutenin extractability (data not shown). For gluten mixed without additives in 95% ethanol, no significant change in SDSEP content compared with untreated gluten was observed.

Mixing gluten with MPA increased the SDSEP content (Table V). At the same time, it decreased the SDSEP average MW in

Table III. Flexural Strength (MPa) of Gluten Molded After Either Solvent Mixing (in 70% Ethanol or 95% Ethanol) or Extrusion Mixing with or without Additives[a]

	70% Ethanol	95% Ethanol	Extrusion
Reference	31.2 (1.9) a	31.2 (2.8) a	37.0 (3.4) a,f,i,j
MPA	50.7 (1.2) b,c,d,e	47.5 (1.3) b,d,e	48.9 (3.6) b,d,e
DTT	54.1 (3.3) c,d	46.5 (2.4) b,d,e,g	56.9 (2.9) c
Fthme	52.1 (5.5) b,c,d	45.1 (3.6) b,e,g,h	47.3 (2.2) b,d,e
fPG2000	48.5 (3.2) b,d,e	38.9 (2.8) f,h,i,j	52.5 (2.3) b,c,d
fH1500	50.4 (4.2) b,c,d,e	39.8 (2.3) g,h,i,j	52.3 (3.7) b,c,d
oxfH1500L	51.7 (3.8) b,c,d	37.9 (2.2) a,f,h,i,j	43.8 (3.3) e,g,h,j
oxfH1500H	32.3 (3.0) a,f	33.0 (1.8) a,f,i	36.7 (2.9) a,f,i,j

Additive codes as in Table I.
[a] Standard deviation of 5-fold mechanical property determinations on single samples is given in brackets. Values with the same letter are not significantly different ($P < 0.05$).

Table IV. Failure Strain (%) of Gluten Molded After Either Solvent Mixing (in 70% Ethanol or 95% Ethanol) or Extrusion Mixing with or without Additives[a]

	70% ethanol	95% ethanol	Extrusion
Reference	0.9 (0.0) a,b	1.0 (0.1) abi	0.8 (0.1) a
MPA	1.3 (0.1) c,d,e,f	1.5 (0.1) f,g,h	1.2 (0.1) c,d,e,j
DTT	1.5 (0.1) f,g,h	1.3 (0.1) d,f	1.7 (0.1) g
fTHME	1.4 (0.2) d,f,h	1.4 (0.2) d,f	1.1 (0.0) b,c,i,j
fPG2000	1.4 (0.1) d,f	1.3 (0.1) d,f	1.6 (0.1) g,h
fH1500	1.4 (0.1) d,f	1.1 (0.1) c,e,i,j	1.5 (0.2) f,g,h
oxfH1500L	1.3 (0.1) d,e,f	1.1 (0.1) c,e,i,j	1.3 (0.1) c,d,e
oxfH1500H	0.9 (0.1) a,b,i	1.0 (0.1) a,b,i,j	1.1 (0.1) c,e,i,j

Additive codes as in Table I.
[a] Standard deviation of 5-fold mechanical property determinations on single samples is given in brackets. Values with the same letter are not significantly different ($P < 0.05$).

SE-HPLC (data not shown). This indicated that MPA acts as a reducing agent.[13] Its SH group reacts with disulfide crosslinks of gluten aggregates, resulting in the release of gluten fragments, which are extractable with SDS containing medium. In the presence of MPA, the SDSEP content was maximal after mixing in the solvents. Similar results were obtained after mixing gluten with DTT. The SDSEP content readings obtained for the materials produced with all other additives was below the maximum value after mixing in at least one of the solvents (Table V). For fTHME, a maximum SDSEP content was noted when gluten was mixed in 95% ethanol, but not when mixed in 70% ethanol. Nevertheless, the SDSEP content after mixing gluten in 70% ethanol with fTHME was higher than that of gluten mixed without additives in the same solvent indicated that fTHME also acted as a reducing agent in this solvent. The SDSEP content after mixing gluten with fPG2000 was clearly higher than that of the reference after mixing in 70% ethanol, while no significant difference between the reference sample and the sample containing fPG2000 was observed after mixing in 95% ethanol.

The SDSEP data clearly demonstrate that the effects of additives on gluten crosslinking strongly depend on the mixing solvent. It is likely that the solubility of additives and proteins affect their reactivity. Whereas gliadins and glutenin subunits are soluble in 70% ethanol, glutenin is not. Furthermore, both gliadins and glutenins are insoluble in 95% ethanol. The additives react with glutenins and thus even with proteins that are not soluble in the mixing solvent. Whether these additives also react with gliadins evidently cannot be observed with the applied technique.

fH1500 had a reducing effect during mixing with gluten in either one of the ethanol concentrations as evidenced by the higher SDSEP contents (Table V). A reducing effect was also observed for the partly oxidized additive oxfH1500L, while no significant reduction was observed with the fully oxidized additive oxfH1500H in the solvents, in line with it not having free SH groups and thus no role as reducing agent.

The SDSEP content after extrusion of gluten without additives was lower than that of untreated gluten (Table V), indicating that gluten cross-linking had occurred. In the case of extrusion in the presence of MPA, DTT, fTHME, or fPG2000, a maximal extractability was obtained. Interestingly, a maximal extractability was not reached with fPG2000 after mixing in the solvents. This shows that the additive is more reactive during extrusion than during solvent-based mixing. Possibly, the intermolecular disulfide bonds of gluten are more accessible for the additive during extrusion as a result of the shear forces, which tend to align the proteins. The reducing effect of

Table V. Levels (%) of Protein Extractable with SDS Containing Medium (SDSEP) of Gluten After Either Solvent Mixing (in 70% Ethanol or 95% Ethanol) or Extrusion Mixing with or without Additives [Untreated Gluten 86.1% (1.9) def][a]

	70% Ethanol	95% Ethanol	Extrusion
Reference	75.2 (0.6) a	82.4 (0.5) e,f,g	77.2 (1.1) a,h
MPA	98.5 (1.3) b	99.3 (0.5) b	99.3 (1.5) b
DTT	99.9 (1.3) b	100.9 (1.2) b	101.6 (1.6) b
fTHME	88.8 (1.0) c,d	100.1 (1.2) b	101.2 (2.6) b
fPG2000	92.7 (1.5) c	85.2 (1.1) d,e,f	100.5 (1.8) b
fH1500	85.3 (1.6) d,e,f	89.1 (1.3) c,d	91.9 (0.3) c
oxfH1500L	82.7 (0.2) e,f,g	89.3 (1.2) c,d	83.6 (1.1) e,f
oxfH1500H	77.9 (0.8) a,g,h	86.8 (2.6) d,e	81.7 (2.9) f,g,h

Additive codes as in Table I.
[a] Standard deviation of 3-fold protein extractability determinations is given in brackets. Values with the same letter are not significantly different ($P < 0.05$).

Table VI. Free Sulfhydryl (SH) Content (μmol/g Protein) of Gluten After Either Solvent Mixing (in 70% Ethanol or 95% Ethanol) or Extrusion Mixing with or without Additives and After Molding [Untreated Gluten 2.4 μmol/g Protein (0.6) ab][a]

	70% Ethanol		95% Ethanol		Extrusion	
	Mixing	Molding	Mixing	Molding	Mixing	Molding
Reference	2.2 (0.4) a,b	2.8 (0.1) a,b	2.3 (0.1) a,b	2.1 (0.1) a,b	3.5 (0.3) a,b	0.8 (0.3) a
MPA	96.8 (1.5) c	72.1 (1.3) f,g	77.1 (1.5) e	39.9 (0.2) p	41.0 (2.3) p	27.1 (0.6) s
DTT	101.9 (1.3) d	79.1 (1.9) e	85.2 (0.9) o	70.5 (0.7) f,g	73.5 (2.0) f	46.3 (0.8) t
fTHME	78.7 (0.4) e	65.8 (1.3) j	69.2 (2.0) j,g	54.9 (0.3) q	60.3 (0.2) r	34.7 (1.1) h
fPG2000	72.6 (1.8) f,g	50.2 (1.9) k	39.1 (0.9) p	20.2 (0.5) i,l	59.3 (0.7) r	32.1 (0.8) h
fH1500	35.2 (0.8) h	23.3 (0.4) l	34.3 (1.3) h	20.9 (0.9) i,l	21.0 (0.1) i,l	10.9 (0.2) m,n
oxfH1500L	19.4 (1.9) i	11.1 (0.4) m,n	19.7 (0.2) i,l	9.5 (0.2) n	14.0 (0.6) m,n	5.3 (0.2) b
oxfH1500H	1.6 (0.0) a	2.0 (0.1) a,b	1.5 (0.1) a	1.7 (0.1) a	2.2 (0.3) a,b	1.5 (0.2) a

Additive codes as in Table I.
[a] Standard deviation of 3-fold free SH determinations is given in brackets. Values with the same letter are not significantly different ($P < 0.05$).

fH1500 was less pronounced than that of the other additives and decreased with increasing degree of oxidation.

Protein Extractability After Compression Molding. The SDSEP content decreased after compression molding (Table VII), indicating that gluten proteins cross-link. A similar SDSEP content was obtained after molding of gluten mixed without additives in the ethanol solutions (reference samples). The decrease in SDSEP content during molding depended on both the additive and the mixing solvent. Molding of gluten mixed with MPA and DTT in 95% ethanol yielded a lower SDSEP content than molding of gluten mixed without additives in this solvent. For MPA this was not the case when mixed with gluten in 70% ethanol. The SDSEP content after molding in the presence of fTHME, fPG2000, fH1500, and the partly oxidized additive oxfH1500L was lower than that of the reference samples and lower than that with DTT. This indicates a higher degree of crosslinking for these samples. Overall, samples containing additives with multiple SH groups have a lower SDSEP content after molding than gluten with MPA. These additives potentially act as cross-link agents connecting two or even more protein chains.[8] However, the lower SDSEP content when using additives with multiple SH groups does not necessarily mean that these additives act as crosslinkers. Indeed, low concentrations of disulfide reducing agents can facilitate gluten crosslinking as demonstrated by Lagrain et al.,[19] while a high concentration of SH groups can restrict crosslinking during molding.[13] Furthermore, we can reasonably assume that not all SH groups in additives containing multiple such groups (e.g., fPG2000 with on average 11 SH groups) are reactive during mixing and molding. Indeed, one can expect that, due to the steric hindrance exerted by protein chains when linked to the additive, some of the SH groups of additives with multiple such groups will become unavailable for reaction. Thus, even though the same absolute quantity of SH groups were added for all additives, the concentration of reactive SH groups is likely lower for additives containing multiple SH groups. It is possible that the lower concentrations of reactive SH groups facilitate gluten crosslinking, whereas higher such concentrations may partially restrict crosslinking, which is probably the case for MPA.

The SDSEP content after molding of the sample with fully oxidized fH1500 (oxfH1500H) was higher than that of all other samples mixed in the same solvent (Table VII). This demonstrates that the additive interferes with gluten cross-linking. It has little effect during mixing but results in a lower degree of crosslinking after heat treatment. Similar observations were made by Lagrain et al.[19] when heat-treating gluten in the presence of an oxidizing agent. They postulated that oxidants hinder gluten crosslinking due to a decreased level of reactive free SH groups. Use of the fully oxidized additive oxfH1500H (an additive with only disulfide groups) did not change the total level of free SH groups, but clearly decreased the ratio of SH to disulfide groups (gluten + additive). In this way, the effect of the additive resembles the action of an oxidant because the likelihood that a free SH group reacts with a gluten disulfide bond is lower when the additive is present.

The SDSEP content after molding of extruded gluten was lower than that of molded gluten mixed in ethanol solutions indicating a more pronounced cross-linking during molding after extrusion. This could be related to a change in the protein conformation, which can be expected under conditions of high shear[20] and which may well expose previously buried reactive groups. With the exception of the sample containing fully oxidized oxfH1500H, all samples extruded with additives and molded had either a similar or a lower SDSEP content than the reference sample. The SDSEP content after molding gluten with the different nonoxidized additives was lower when extrusion mixing was used rather than mixing in the alcohol containing media, indicating an enhanced crosslinking during molding in the former case. Possibly, this is related to an enhanced availability of reactive groups after extrusion.

Free SH Content After Mixing and Compression Molding. The free SH content of the used gluten powder was 2.4 μmol/g protein. None of the mixing procedures significantly affected it when no additives were used. The level of free SH after mixing gluten with additives in 70 or 95% ethanol was generally lower than that added (106 μmol/g protein for non-oxidized additives), suggesting partial oxidation of free SH (Table VI). For

Table VII. Levels (%) of Protein Extractable with SDS Containing Medium (SDSEP) of Gluten Molded After Either Solvent Mixing (in 70% Ethanol or 95% Ethanol) or Extrusion Mixing with or without Additives [Molded Untreated Gluten 28.6% (0.9) k][a]

	70% Ethanol	95% Ethanol	Extrusion
Reference	33.5 (0.6) a,b	28.1 (1.5) k	12.7 (0.7) e,i
MPA	33.6 (0.8) a	21.5 (0.6) f,l	9.2 (0.2) h,n
DTT	22.7 (0.1) c,d	24.0 (0.8) c	7.8 (0.8) n
fTHME	13.9 (0.4) e,f	19.2 (0.7) m	11.1 (0.8) h,i
fPG2000	15.4 (0.4) f,g	11.9 (0.6) e,i	8.4 (0.9) n
fH1500	11.0 (0.4) h,i	16.4 (0.4) g	4.8 (0.3) o
oxfH1500L	12.2 (0.5) e,i	20.0 (0.1) l,m	11.6 (0.4) e,i
oxfH1500H	38.4 (0.4) j	31.4 (0.8) b	20.2 (1.5) l,m

Additive codes as in Table I.
[a] Standard deviation of 3-fold protein extractability determinations is given in brackets. Values with the same letter are not significantly different ($P < 0.05$).

samples containing fH1500, the detected free SH content was much lower than that of samples containing other nonoxidized additives. A plausible explanation is that less free SH are detected due to steric hindrance and/or poor solubility of fH1500 in the sample buffer. Generally, the free SH content was lower when gluten was mixed with additives by extrusion than when ethanol solutions were used, indicating more oxidation during extrusion. Evidently, the free SH content after mixing was lower for the oxidized fH1500 additives, irrespective of the mixing procedure.

Molding had little if any effect on the free SH content of gluten mixed without additives in either of the solvents or via extrusion (Table VI). The free SH content of all gluten samples molded with SH containing additives was drastically lower than after mixing. The decrease can be attributed to the oxidation of free SH groups, resulting in disulfide bonds. In general, the free SH content was lower when gluten was mixed with additives by extrusion than when solvent mixing was used. The free SH content was already lower after extrusion of gluten with additives than when solvents were used and remained lower after the molding step. The lower free SH content indicates that more disulfide bonds are present in molded samples when gluten was extruded with additives. This observation agrees with the protein extractability results (Table VII), where a lower SDSEP content after molding was observed when gluten was mixed with additives via extrusion.

Relationship Between Gluten Network Properties and Mechanical Properties of Gluten with Additives

In previous work, an increase in strength with increasing degree of crosslinking for gluten molded in the absence of additives was observed.[5] Here, the strength also seems to be related to the degree of crosslinking for samples mixed without additives (Table VII). However, whereas after mixing in 70% ethanol all nonoxidized SH containing additives improved the strength to a similar extent (Table III), their degree of crosslinking differed. The degree of crosslinking in the presence of MPA and to a lesser extent that with DTT was lower than that of the other samples with nonoxidized additives obtained when molding samples mixed in 70% ethanol. This demonstrates that the degree of crosslinking is not the only factor that determines the strength of rigid, glassy gluten materials. Furthermore, whereas a partly oxidized additive (oxfH1500L) improved the strength, use of the fully oxidized additive oxfH1500H resulted in mechanical properties similar to those of the reference, even though this additive affected the cross-linking (Table VII). It appears that the presence of SH groups in the additives is important if they are to improve the mechanical properties. The SDSEP data of the samples mixed in 70% ethanol (Table V) showed that all additives with SH groups had a reducing effect during mixing, which was not the case for the fully oxidized additive (oxfH1500H). Hence, this reducing effect during mixing seems crucial for obtaining materials with improved mechanical properties.

The improvement in strength with MPA, DTT, and fTHME when 95% ethanol was used as mixing solvent was higher than that with fPG2000 and fH1500, although the differences were not always significant. Interestingly, the degree of crosslinking of molded gluten containing fPG2000 or fH1500 was higher than that of gluten mixed with MPA, DTT, and fTHME. This further supports the view that the degree of crosslinking is not the only factor affecting the strength of rigid gluten materials. The smaller increase in strength as a result of use of fPG2000 or fH1500 than as a result of the use of MPA, DTT or fTHME appears related to the more limited reduction during mixing of gluten with fPG2000 and fH1500 in ethanolic media (Table V). It seems that reduction of gluten during mixing is more important for obtaining high strength of rigid gluten materials than impacting the crosslinking occurring during molding.

When the samples were mixed by extrusion, an improved strength was observed for all samples with nonoxidized additives (Table III). As indicated by the SDSEP content after extrusion (Table V), all these additives had a reducing effect during extrusion. This effect was less pronounced for fH1500 than for the other samples, but this apparently had no effect on the strength of the molded sample. This suggests that a certain reducing effect is important, but it is not necessary to obtain a SDSEP content of 100%. With increasing degree of oxidation of fH1500, the reducing effect during extrusion decreased and this was accompanied with a decrease in strength.

Overall, small commercially available SHs without cross-linking potential and polythiols with crosslink potential improved the strength of rigid, glassy gluten materials, which appeared related to the importance of the reducing effect during mixing. This supports our view that besides crosslinking during molding, also altered molecular conformations before and during molding contribute to the strength.[13] SH containing additives can undergo SH/disulfide interchange reactions with gluten which results in conformational changes. It is hypothesized that the additive-induced conformational changes result in a gluten network with rearranged weak bonds between protein chains and/or with increased entanglement and that this improves the strength. The extent to which such conformational changes occur may depend on the additive and the mixing step. Note that the reducing effect of additives on the gluten network during mixing indeed depended on the additive and the mixing step (Table V).

CONCLUSION

Flexural strength and failure strain of rigid gluten materials can be improved with SH containing additives, whereas additives with only disulfide functionality have no effect on these mechanical properties. Both additives with SH and disulfide functionality affect the cross-linking during processing. However, our results demonstrate that the degree of crosslinking is not the only factor that determines the strength of rigid, glassy gluten materials. The effect of the additives depends on the system used to mix the additives with gluten. Mechanical properties were improved when additives acted as a reducing agent during mixing. The importance of the reducing effect during mixing supports our view that besides crosslinking, also altered molecular conformations and improved molecular entanglements contribute to the strength.

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