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Importance of Thiol-Functionalized Molecules for the Structure and Properties of Compression-Molded Glassy Wheat Gluten Bioplastics

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ABSTRACT: High-temperature compression molding of wheat gluten at low water levels yields a rigid plastic-like material. We performed a systematic study to determine the effect of additives with multiple thiol (SH) groups on gluten network formation during processing and investigate the impact of the resulting gluten network on the mechanical properties of the glassy end product. To this end, a fraction of the hydroxyl groups of different polyols was converted into SH functionalities by esterifying with 3-mercaptopropionic acid (MPA). The monofunctional additive MPA was evaluated as well. During low-temperature mixing SH-containing additives decreased the gluten molecular weight, whereas protein cross-linking occurred during high-temperature compression molding. The extent of both processes depended on the molecular architecture of the additives and their concentration. After molding, the material strength and failure strain increased without affecting the modulus, provided the additive concentration was low. The strength decreased again at too high concentrations for polyols with low SH functionalization. Attributing these effects solely to the interplay of plasticization and the SH-facilitated introduction of cross-links is inadequate, since an improvement in both strength and failure strain was also observed in the presence of high levels of MPA. It is hypothesized that, regardless of the molecular structure of the additive, the presence of SH-containing groups induces conformational changes which contribute to the mechanical properties of glassy gluten materials.

KEYWORDS: *wheat gluten, rigid materials, mechanical properties, thiol-containing additives, protein reactions*

INTRODUCTION

Wheat gluten is important for the characteristics of several food products. It also has potential as a renewable resource for biodegradable materials. In general, production of gluten plastics consists of breaking up the intermolecular bonds followed by rearrangement of the polymer chain conformation and, finally, formation of new intermolecular bonds that stabilize the three-dimensional network.[1] In research, gluten-based materials are often produced by solution casting. However, 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 to be preferred from an environmental point of view.

High-temperature compression molding of gluten at low moisture or plasticizer content produces stiff but brittle material.[2] To reduce the brittleness, plasticizers are frequently added prior to molding.[3] They increase the fracture strain of gluten-based materials at the expense of both modulus and strength.[4] The materials tend to be rubbery rather than glassy at room temperature typically when plasticizer levels in excess of 15% are used.[1] With molding temperatures exceeding 100 °C, the strength and Young's modulus increase for gluten rubbers while their elongation at break decreases.[5,6] The fact that material properties improve with increasing molding temperature has been attributed to increased cross-linking densities.[6] Reducing agents such as cysteine have also been applied for increasing the strength and elongation at break of glycerol-plasticized wheat gluten.[7]

However, the mechanical properties of rigid gluten-based materials—the case of interest of the present paper—are quite different from those of rubbery materials. Altering primary bonds (e.g., disulfide bonds) in glassy gluten materials by introducing cross-links does not heavily affect their modulus. However, the strength and failure strain of such materials increase with increasing molding temperature, which corresponds to an increase in the degree of cross-linking.[8] According to Meijer and Govaert[9] in their review of the mechanical performance of polymer systems, the modulus of nonoriented polymers in the glassy state is determined by the polymer's weak bonds (hydrogen bonds and van der Waals bonds) and free volume kinetics (involving aspects of thermal history and aging), while the network's strong bonds (ionic bonds and covalent bonds such as peptide and disulfide bonds) may bring toughness by delocalizing local strains.

The type of protein cross-links in rigid gluten materials depends on the molding conditions and the initial gluten powder moisture content. Disulfide bonds are the predominant gluten cross-links, but at molding temperatures exceeding 130

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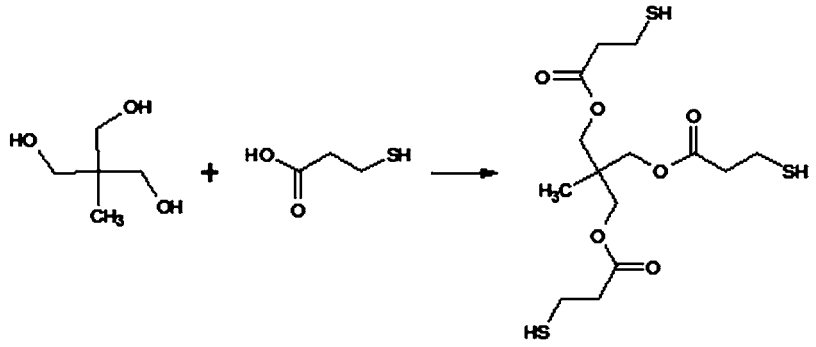

Figure 1. Thiol (SH) functionalization of tris(hydroxymethyl)ethane by esterification of its hydroxyl groups with the carboxyl group of 3-mercaptopropionic acid (MPA).

°C, the dehydroalanine-derived cross-link lanthionine is also formed.[10] Formation of dehydroalanine-derived cross-links can be stimulated with alkali, but apparently the ratio of disulfide to nondisulfide cross-links does not affect the mechanical properties of rigid gluten materials.[11] To improve the strength and strain at failure of rigid, glassy gluten-based materials, Woerdeman et al.[2] incorporated a star-branched molecule with three terminal thiol (SH) groups into the protein structure. The lower water absorption of the resultant material suggests that this additive increases the cross-link density. Dicharry et al.[12] improved the mechanical properties of gluten Bioplastic with poly(vinyl alcohol) (PVA) containing low levels of SH groups. Water absorption of gluten Bioplastic with the additive was higher than that of a control sample without additive, suggesting a lower degree of gluten cross-linking in the presence of the additive.[12] The authors suggested that the improvements in mechanical properties with these two SH-containing additives result from different mechanisms.[12] Whereas the improved mechanical properties with the star-branched additive in Woerdeman et al. could be related to the increased degree of cross-linking with that additive,[2] the improved mechanical properties with PVA containing low levels of SH groups seems to be unrelated to the degree of cross-linking but possibly rather to a complex morphology.[12]

In spite of the improvements obtained by adding SH-functionalized molecules, current gluten-based materials are still outperformed by their synthetic polymer counterparts.[1] As reported by Woerdeman et al.[2] and Dicharry et al.,[12] additives containing multiple SH groups hold promise for toughening rigid gluten Bioplastic. However, it is still unclear how these additives impact the mechanical properties. As stated above, it has been suggested that the improvement in strength is due to an increased degree of cross-linking, but this does not appear valid for all SH-containing additives.[2,12] Furthermore, it should be stressed that Woerdeman et al.[2] reported on the effect of only one branched additive and that Dicharry et al.[12] investigated PVA with four different MWs and low levels of SH groups. Comparison of the work of Woerdeman et al.[2] and Dicharry et al.[12] suggests that the actual mechanism also depends on the molecular architecture of the additive. To efficiently affect the performance of gluten materials, it is important to understand the molecular level changes induced by the use of such additives and establish the impact of these changes on the mechanical properties of the end product. Therefore, the aim of this research was to investigate the effect of the chemical structure and functionality of SH-containing additives on gluten cross-linking during mixing and compression molding and establish the impact of additive-induced gluten network alterations on the mechanical properties of rigid gluten materials. Hereto, a systematic study was set up which included construction of additives with different molecular architecture and SH functionality. This was done by (partly) esterifying branched additives with multiple hydroxyl groups with MPA. As such, we synthesized additives with either low or high levels of SH groups. To further elucidate the importance of SH functionalization, the effects of additives with SH functionalization and their unfunctionalized counterparts were compared. To determine the importance of the cross-linking potential of molecules containing multiple SH groups, the effect of such molecules was compared with that of the monothiol MPA. The impact of the additives on the gluten network was analyzed by determining both the protein extractability in sodium dodecyl sulfate-containing medium and the levels of free SH groups. The obtained network characteristics were then confronted with the mechanical properties and the water absorption of the Bioplastic.

EXPERIMENTAL SECTION

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.[13] Protein content (N × 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).[14] Hyperbranched polyglycerol (PG) 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.[15]

All other chemicals, solvents, and reagents were from Sigma-Aldrich (Steinheim, Germany) unless specified otherwise and at least of analytical grade.

Synthesis of SH-Functionalized Molecules. Tris(hydroxymethyl)ethane (THME) and PG with average MWs of about 700, 2000, and 5000 further referred to as PG700, PG2000, and PG5000, respectively, were SH functionalized by esterifying their hydroxyl groups with 3-mercaptopropionic acid (MPA) (Figure 1). SH-functionalized THME and PG are further referred to as fTHME and fPG, respectively. THME (10.00 g, 83.2 mmol) was mixed with MPA (22.5 mL, 258.2 mmol), toluene (40.0 mL), and catalytic amounts of *p*-toluenesulfonic acid (1.43 g, 8.3 mmol). Azeotropic distillation was then performed for 24 h under argon atmosphere with removal of water with a Dean−Stark apparatus. Toluene was then removed by evaporation, and the reaction mixture was dissolved in 150 mL of diethyl ether. It was then washed 10 times with deionized water using a separation funnel. The organic layer was dried over magnesium sulfate, and the solvent was removed by evaporation.

Samples with a different degree of esterification (DE), i.e., the percentage of the initial hydroxyl groups on the unfunctionalized

molecules that is esterified with MPA, were produced with PG. To synthesize samples with low DE, PG (10.0 g) was mixed with MPA (10.0 mL, 114.8 mmol), toluene (20.0 mL), and *p*-toluenesulfonic acid (0.35 g, 2.03 mmol). The reaction mixture was heated and refluxed under argon atmosphere for 60 min. After reflux, toluene was evaporated and 100 mL of diethyl ether was added. The mixture was stirred for 48 h, and during this time the diethyl ether was replaced at least 4 times. Finally, the diethyl ether phase was decanted, and the remaining viscous oil was dried in a vacuum oven for at least 16 h at 40 °C.

Samples with high DE were synthesized by mixing PG (10.0 g) with MPA (25.0 mL, 286.9 mmol), toluene (30.0 mL), and *p*-toluenesulfonic acid (0.35 g, 2.03 mmol). For PG2000 and PG5000, the reaction mixtures were refluxed under argon atmosphere for 72 h. After reflux, toluene was evaporated and 100 mL of cold methanol was added. The mixture was stirred for 48 h, and during this time the cold methanol was replaced at least 4 times. Finally, the methanol phase was decanted and the remaining viscous oil was dried in a vacuum oven. To obtain a high DE with PG700, the reaction mixture was distilled azeotropically under argon atmosphere for 19 h. fPG was then recovered with the procedure used for the fPG with low DE. The resultant fPG with low and high DE are further referred to as fPG700L (low DE), fPG700H (high DE), fPG2000L, fPG2000H, fPG5000L, and fPG5000H.

Characterization of SH-Functionalized Additives. Additives were characterized by nuclear magnetic resonance (NMR) and mass spectrometry (MS). ^{1}H NMR spectra (300 MHz) were recorded at room temperature on a Bruker Avance 300 (Bruker, Billerica, MA) in CD_3OD for unfunctionalized molecules and fPG with a low DE, and in $CDCl_3$ for fTHME and fPG with a high DE. Tetramethylsilane 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. Gluten was mixed with different SH-containing additives, including pure MPA, in 70% aqueous ethanol (10% w/v). The same level of SH groups was added for each additive (Table 1). Three concentrations (106, 265, and 530 μmol SH/g protein) were tested. The lowest concentration corresponds to about 2/3, the middle to about 5/3, and the highest to about 10/3 of the cysteine residues present in gluten. After mixing overnight at room temperature, ethanol was evaporated and the remaining mixture was freeze dried. Samples were then ground to pass a 250 μm sieve, and their moisture content was adjusted to 8% by storing them over saturated potassium carbonate solution. As a control, gluten was mixed with 70% ethanol without any additive.

Table 1. Degree of Esterification (DE) of the SH-Functionalized Additives Calculated from Their Proton Nuclear Magnetic Resonance Spectra and Concentration of the Different Additives Expressed as Mass Percentage of Gluten Dry Matter for 106, 265, and 530 μmol SH/g Protein[a]

		concentration of additive (%)		
sample[a]	DE (%)	106 μmol SH/g protein	265 μmol SH/g protein	530 μmol SH/g protein
MPA		0.9	2.1	4.2
fTHME	98	1.1	2.6	5.1
fPG700L	16	4.4	10.2	18.5
fPG700H	70	1.6	3.8	7.4
fPG2000L	14	5.0	11.6	20.8
fPG2000H	76	1.6	4.0	7.7
fPG5000L	17	4.0	9.4	17.3
fPG5000H	70	1.6	3.8	7.4

[a]3-Mercaptopropionic acid: MPA. SH-functionalized tris(hydroxymethyl)ethane: fTHME. SH-functionalized polyglycerol: fPG. fPG*xxx*L and fPG*xxx*H codes are such that *xxx* refers to the average molecular weight of the unfunctionalized PG prior to SH functionalization, and the letters L and H, respectively, refer to samples with a low and high DE.

High-Temperature Compression Molding. Gluten with and without additives was 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 150 °C for 5 min. Before removing the samples, the mold was allowed to cool to 30−35 °C (about 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 5 samples were tested in a three-point bending test according to ASTM D790-03. Samples were tested with an Instron Universal instrument model 4467 equipped with 1kN 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 0.17 cm). Modulus, strength, and failure strain were determined from the acquired stress−strain curves. The remaining fragments were used to determine the water absorption. 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 Molecular Weight Distribution. The level of protein extractable with SDS-containing medium (SDSEP) was determined as in Jansens et al.[10] Samples containing 1.0 mg of protein were extracted with 1.0 mL of 0.05 M sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS (Acros Organics, Geel, Belgium). All extractions were performed in triplicate. To evaluate SDSEP contents under reducing conditions (SDSEPred), samples prepared as above were extracted under nitrogen atmosphere with the same buffer also containing 1.0% (w/v) dithiothreitol (Acros Organics) and 2.0 M urea. After centrifugation (10 min, 10 000 *g*) and filtration over polyethersulfone (0.45 μm, Millex-HP, Millipore, Carrigtwohill, Ireland), extracted proteins were separated with SE-HPLC as described by Lagrain et al.[16] using a LC-2010HT system (Shimadzu, Kyoto, Japan) with automatic injection. 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.[17] 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 M urea and 1.0% (w/v) DTT.

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 of sample buffer [0.05 M sodium phosphate buffer (pH 6.5) containing 2.0% (w/v) SDS, 3.0 M urea, and 1.0 mM tetrasodium ethylenediamine tetraacetate]. Then, 100 μL of DTNB reagent [0.1% (w/v) in sample buffer] was added, and 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 to concentrations of free SH using a calibration curve with reduced glutathione.[17] Controls containing either no DTNB or no sample were used to correct for background extinction of DTNB and sample.

Water Absorption. In order to indirectly evaluate the cross-linking density, water absorption was determined on compression-molded samples which were typically 30 mm long, 11 mm wide, and 1.7 mm thick. Each sample was weighed, and its dry mass m_1 was calculated. The sample was then submerged in deionized water (containing 20 mg/L sodium azide to avoid microbial growth) at 20 °C. At specified time intervals, samples were withdrawn and droplets were removed from the surface with paper tissue. Samples were then immediately weighed (m_2) and submerged again. Water absorption was monitored for 72 h. Samples were dried for 24 h at 130 °C and their dry mass determined (m_3).

Water absorption due to submersion was expressed as the increase in sample mass divided by its dry weight after submersion for 72 h and multiplied by 100. Thus

$$\text{water absorption} = \frac{(m_2 - m_3)}{m_3} \times 100$$

Weight loss after submersion was expressed as the difference between the dry weight before submersion and that after submersion for 72 h divided by the initial dry weight and multiplied by 100. Thus

$$\text{weight loss} = \frac{(m_1 - m_3)}{m_1} \times 100$$

This value represents all material that may have been detached from the gluten platelets during the experiment. In addition, it is of note that water absorption represents an approximate value since it neglects potentially detached material. Water absorption and weight loss were determined in duplicate.

Glass Transition Temperature. Differential scanning calorimetry (DSC) measurements were performed in a Q2000 DSC instrument (TA Instruments, New Castle, DE) using hermetically closed aluminum pans. An empty pan was used as reference, and the system was calibrated with indium. Samples were first cooled from 20 to −75 °C at 10 °C/min and kept at −75 °C for 10 min. Next, samples were heated to 110 °C at 10 °C/min, kept at 110 °C for 10 min, and cooled again to −75 °C at the same rate. After equilibration at −75 °C, samples were scanned a second time. The glass transition temperature (T_g) was determined for the two heating runs using the enthalpy method.[18] This method involves integration of the DSC heat capacity signal to yield a function which follows the temperature-dependent sample enthalpy. The T_g is read at the intersection of two extrapolated second-order polynomials describing the stable glassy and liquid enthalpies, respectively, below and above the glass transition region. In contrast to the first heating runs, the second heating runs did not display endothermic aging effects.[18] Therefore, T_g values based on the second heating runs were reported.

Statistical Analysis. Statistical analyses were conducted with the Statistical Analysis System software 9.2 (SAS Institute, Cary, NC). Significant differences ($P < 0.05$) for several variables were determined by the ANOVA procedure, and mean values were compared with the Tukey test ($P < 0.05$).

RESULTS AND DISCUSSION

A. Synthesis and Characterization of SH-Functionalized Additives. The success of SH functionalized (Figure 1) was evaluated 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 with 88. fTHME was characterized by ^{1}H NMR (in $CDCl_3$) with the following proton peak assignments: δ (ppm) ≈ 1.0 (CH_3), 1.7 (SH), 2.6−2.8 (CH_2CH_2SH), 3.4 (CH_2OH), and 3.9−4.1 (CH_2OCO). Integration of the areas of the signals at 1.0 and 3.9−4.1 ppm allowed concluding that 98% of the hydroxyl groups had been esterified. This indicates that for most THME molecules all 3 hydroxyl groups carried ester groups (Figure 1). fPG was also characterized by ^{1}H NMR ($CDCl_3$ for samples with a high DE), and the following proton peaks were assigned: δ (ppm) ≈ 0.8 [CH_3 of tris(hydroxymethyl)propane, the core of PG], 1.4 [CH_2 of tris(hydroxymethyl)propane], 1.7 (SH), 2.6−2.8 (CH_2CH_2SH), 3.2−4.5 (CH_2 of PG and CH not attached to OCO), 5−5.2 (OH and CH of PG attached to OCO). Integration of the areas of the signals at 2.6−2.8 and 3.0−4.5 ppm allowed calculating the DE values listed in Table 1. The average levels of hydroxyl groups for PG700, -2000, and -5000 are, respectively, 11, 29, and 70. The amount of SH groups on a single fPG molecule thus depends on both MW and DE.

B. Impact of SH-Functionalized Additives on Molecular Properties of Gluten. *Protein Extractability.* Gluten was mixed in 70% ethanol with additives. The SDSEP contents of gluten before and after mixing (references 1 and 2) were 88.3% and 75.0%, respectively. The loss of extractability indicates that mixing (without compression molding) increased cross-linking between gluten proteins. The SE-HPLC profiles of the reference samples revealed that the decrease in SDSEP content as a result of mixing was in essence a decrease in glutenin extractability (Figure 2). Figure 3 shows the SDSEP content of

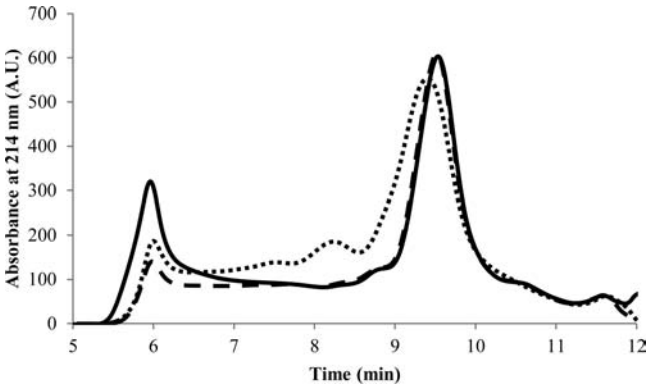

Figure 2. Size-exclusion HPLC chromatograms of the SDS extracts of gluten (reference 1) (—), gluten mixed without additives (reference 2) (- - - -), and gluten mixed with 106 μmol MPA/g protein (···). Two peaks can be distinguished in the chromatogram of gluten. Peak around 6 min corresponds to extractable glutenins, while extractable gliadins elute around 9.5 min au, arbitrary units.

gluten mixed with three concentrations of each SH-containing additive. Mixing gluten with MPA resulted in a higher SDSEP content than that of the reference. This increase together with the observed decreased protein MW in SE-HPLC (Figure 2) indicates that MPA served as a reducing agent. MPA released glutenin subunits which eluted in the same region as gliadins (Figure 2). The maximal extractability was already attained at the lowest concentration of MPA (106 μmol SH/g protein). However, further MW reduction of the SDS extractable glutenins into their monomeric subunits was observed with increasing MPA concentrations. The SDSEP content of gluten mixed with fTHME was also higher than that of gluten mixed without additives. This demonstrated that these SH-functionalized additives also act as reducing agents. However, the maximal SDSEP content was only reached when a higher concentration (265 μmol SH/g protein) of fTHME was added. On one hand, this may be linked to the low solubility of this molecule in 70% ethanol which could hinder its reactivity. On the other hand, it may have to do with the higher number of SH groups on a single molecule than in the case of MPA and, thus, the lower number of molecules with SH groups at a certain additive concentration. The effect of fPG depends on both its MW and its DE. At low DE, the SDSEP content of gluten mixed with fPG700L and fPG2000L was already maximal at the lowest concentration, while a higher concentration of fPG5000L (265 μmol SH/g protein) was necessary to obtain a SDSEP content near 100%. As evidenced by the SE-HPLC profiles, the glutenins extractable in SDS-containing medium were reduced with increasing concentration and this for all samples of low DE. In general, additives with a high DE were less effective as reducing agents during mixing,

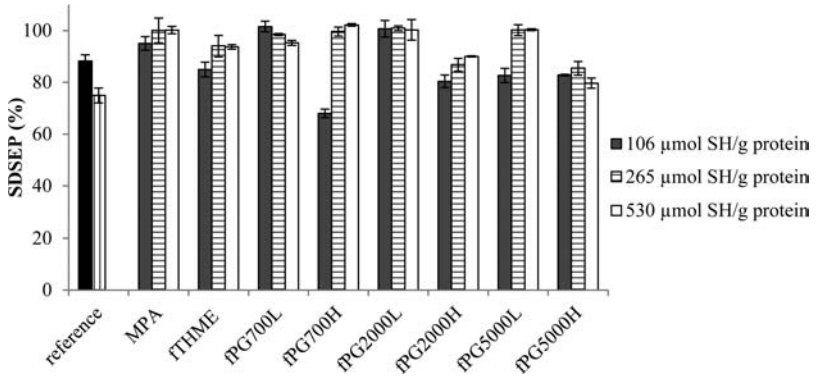

Figure 3. Levels of extractable protein with SDS-containing medium (SDSEP), expressed as percentages of the protein extractability of unmolded gluten in SDS-containing medium under reducing conditions for reference samples (black bar before mixing (reference 1) and white bar after mixing (reference 2)) and gluten mixed with different additives at 3 different concentrations.

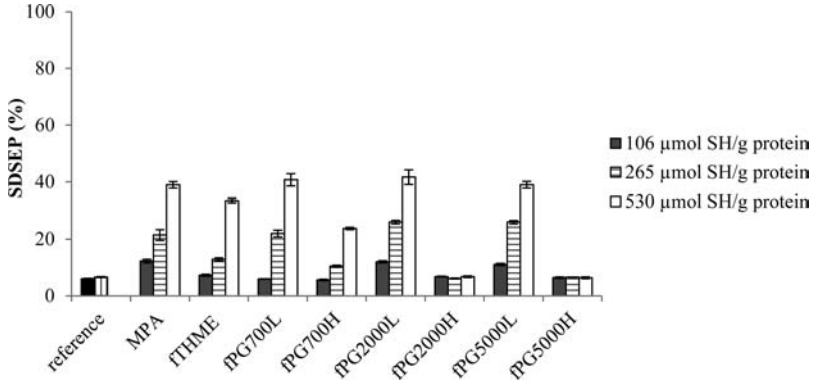

Figure 4. SDSEP contents, expressed as percentages of the protein extractability of unmolded gluten in SDS-containing medium under reducing conditions, for reference samples and gluten with different additives at 3 different concentrations compression molded at 150 °C for 5 min.

since even with increasing concentrations they did not yield an SDSEP content of 100%. These additives are likely less effective because they have a high number of SH groups on a single molecule and, hence, as noted above are present in lower numbers. Indeed, a high DE at a certain MW means a lower number of molecules containing SH groups, because, for comparison reasons, the total number of SH groups per mass unit of gluten proteins was kept constant for all additives at the three concentrations chosen, i.e., 106, 265, and 530 μmol SH/g protein. Likewise, a higher MW at a certain DE corresponded to a lower number of additive molecules with SH groups at any of these concentrations. The number of molecules with SH groups was thus the highest for PG with a low MW and low DE (fPG700L). Furthermore, with increasing DE, the fPG becomes more apolar and, thus, less soluble in the solvent. It can be concluded that both smaller molecules and a higher amount of SH-containing molecules result in more intermolecular disulfide bond reduction during mixing of gluten proteins. The importance of the SH groups for the reducing effect of fPG was further demonstrated by mixing gluten with different amounts of unfunctionalized PG in 70% ethanol. The SDSEP contents of these samples were similar to that of reference sample 2 (data not shown).

Compression molding decreased the SDSEP content (Figure 4), indicating that gluten proteins cross-link during this unit operation. After molding at 150 °C for 5 min, a minimum extractability of 6−8% was obtained for the reference samples.[10] Cross-linking of gluten proteins also took place in the presence of additives. The SDSEP content of compression-molded samples increased with the concentrations of additives except for fPG2000H and fPG5000H. For the latter, the SDSEP content remained low irrespective of the added concentration. This could be explained by their limited reducing effect during mixing (Figure 3).

The SDSEPred content of the reference samples was about 78%, indicating that not only disulfide bonds but also nonreducible cross-links were formed.[10] The SDSEPred content was not affected by adding any of the applied concentrations of fPG2000H and fPG5000H. The SDSEPred content at the lowest concentration of MPA, fPG700L, fPG2000L, and fPG5000L was similar to that of the reference samples and increased with increasing additive concentrations. The latter indicates that the contribution of nondisulfide bonds in gluten cross-linking diminishes with increasing concentration of these additives.

Free SH Content. The free SH content of the used gluten powder was 2.5 μmol/g protein. Mixing in 70% ethanol did not significantly affect it. Molding increased the free SH content of the reference samples (Figure 5). This can be explained by β-elimination reactions.[10]

The level of free SH after mixing of gluten with additives was lower than that added (106 μmol/g protein), suggesting some oxidation of free SH (Figure 5). Mixing without additives did not decrease the free SH content, possibly because it is not very probable that free SH groups encounter each other. The level of free SH after mixing gluten with additives was additive dependent. For fPG2000H and fPG5000H, the detected free SH content was much lower than with the other additives. It is

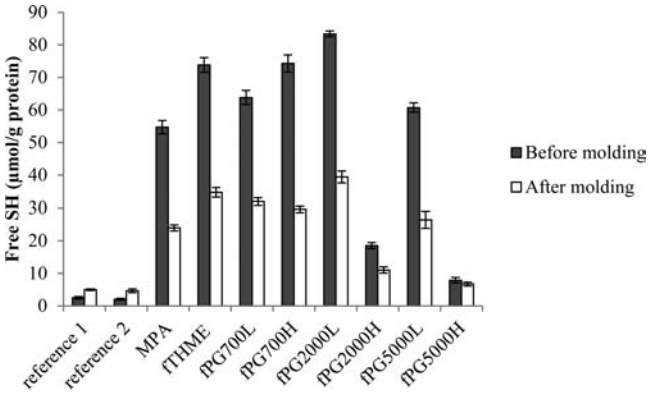

Figure 5. Free SH content of gluten mixed without and with different additives at the lowest concentration (106 µmol SH/g protein) before and after molding.

possible that more oxidation occurred for samples with a high DE. However, a more straightforward explanation may well be that less free SH are detected at higher DE due to steric hindrance and/or a poor solubility of fPG2000H and fPG5000H in the sample buffer.

After molding, the free SH content of all gluten samples processed with additives, except for gluten processed with fPG5000H, was drastically lower than after mixing. This could be attributed to oxidation of free SH groups, resulting in disulfide bonds.

C. Impact of SH-Functionalized Additives on Material Properties of Compression-Molded Gluten. *Water Absorption.* During submersion, gluten plates take up most of the water they absorb during the first 24 h. After 48−72 h, a constant water absorption was attained for most samples (results not shown). Table 2 summarizes water absorption after

Table 2. Water Absorption of Gluten Compression Molded with Different Concentrations of Additives

	water absorption[a] (%)		
sample	106 µmol SH/g protein	265 µmol SH/g protein	530 µmol SH/g protein
MPA	91.7 (0.3) abc	92.7 (0.1) abc	94.9 (1.3) ad
fTHME	90.3 (2.4) bce	88.5 (0.2) bef	86.1 (0.5) f
fPG700L	98.2 (0.8) d	109.1 (0.3) g	115.6 (1.6) h
fPG700H	87.2 (0.2) ef	87.2 (1.1) ef	84.9 (0.1) f
fPG2000L	109.6 (1.9) g	129.2 (0.7) i	116.8 (1.5) hj
fPG2000H	91.7 (2.2) abc	88.4 (0.9) bef	86.3 (1.1) f
fPG5000L	106.2 (1.8) g	120.3 (0.6) j	125.6 (1.4) i
fPG5000H	90.5 (2.6) bce	90.8 (0.9) bce	86.3 (1.3) f

[a]Range of duplicate measurements is given in brackets. Values with the same letter are not significantly different ($P < 0.05$). Sample codes as in Table 1.

72 h of submersion for the different samples. The equilibrium water absorption for some polymeric matrices (including rigid gluten Bioplastic) provides a measure for the degree of cross-linking: the lower the water absorption, the higher the degree of cross-linking.[8,19,20]

However, when additives are used, they influence not only the degree of cross-linking but also the weight loss during submersion in water. In addition, the presence of additives can also impact water absorption to a degree depending on their concentration and affinity for water. Furthermore, there seems to be no relationship between water absorption and the MPA concentration (Table 2), whereas the SDSEP content data indicated a decreased degree of cross-linking with increasing MPA concentrations. This shows that water absorption is not suitable for comparing the degree of cross-linking of rigid gluten materials in the presence of different additives.

However, water absorption and weight loss still do provide information on the behavior of the material when exposed to water. Even at the highest additive concentration, none of the samples disintegrated during submersion. Significant differences in water absorption were observed depending on the additives and their concentration (Table 2). For some samples, water absorption was maximal after 24−48 h and decreased with longer submersion time (data not shown). This was observed for gluten molded with the highest concentrations of fPG700L and fPG2000L. The decrease in water absorption was likely caused by loss of fPG to the water phase during submersion. Indeed, the weight loss of gluten materials with fPG with a low DE increased with fPG concentration (Table 3). This indicates

Table 3. Weight Loss of Gluten Compression Molded with Different Concentrations of Additives

	weight loss[a] (%)		
sample	106 µmol SH/g protein	265 µmol SH/g protein	530 µmol SH/g protein
MPA	2.8 (0.2) abc	3.1 (0.1) abd	3.8 (0.0) de
fTHME	2.5 (0.0) bcf	2.6 (0.0) abcf	2.9 (0.0) abc
fPG700L	5.3 (0.0) g	9.0 (0.0) h	16.8 (0.2) i
fPG700H	3.2 (0.2) abd	3.3 (0.0) ad	3.8 (0.1) de
fPG2000L	4.7 (−) gj	9.3 (0.0) h	20.2 (−) k
fPG2000H	2.8 (−) abd	2.6 (0.0) abc	2.8 (0.0) abc
fPG5000L	4.1 (0.1) ej	7.3 (0.1) l	14.5 (0.1) m
fPG5000H	1.6 (0.9) n	2.7 (0.6) abc	2.3 (0.0) cfn

[a]Range of duplicate measurements is given in brackets. Values with the same letter are not significantly different ($P < 0.05$). Sample codes as in Table 1.

that a large proportion of these additives was not covalently attached to the gluten and displayed more affinity for water than for gluten. Weight loss for functionalized additives was lower than that for the corresponding unfunctionalized additives which were added in the same amount (data not shown). Since the functionalized additives are more apolar than the unfunctionalized ones, they show less affinity for water. In addition, functionalized additives can be covalently attached to gluten.

Flexural Properties. Figure 6 shows the mechanical properties of the compression-molded gluten samples. The mechanical properties of the reference samples were not significantly different, demonstrating that the mixing procedure did not affect the mechanical properties. At the lowest concentration, MPA, fTHME, and the other additives had little if any impact on the flexural modulus (Figure 6a). Furthermore, the modulus was not affected by the MPA and fTHME concentration, even though the SDSEP contents pointed to differences in cross-linking degrees (Figure 4). This is in line with results which showed that the modulus of rigid gluten materials without additives does not strongly depend on covalent cross-linking.[8] For fPG with a low DE, the modulus decreased with increasing additive concentration. A decrease in modulus was also observed with increasing concentrations of unfunctionalized PG700 (Table 4). To check whether the

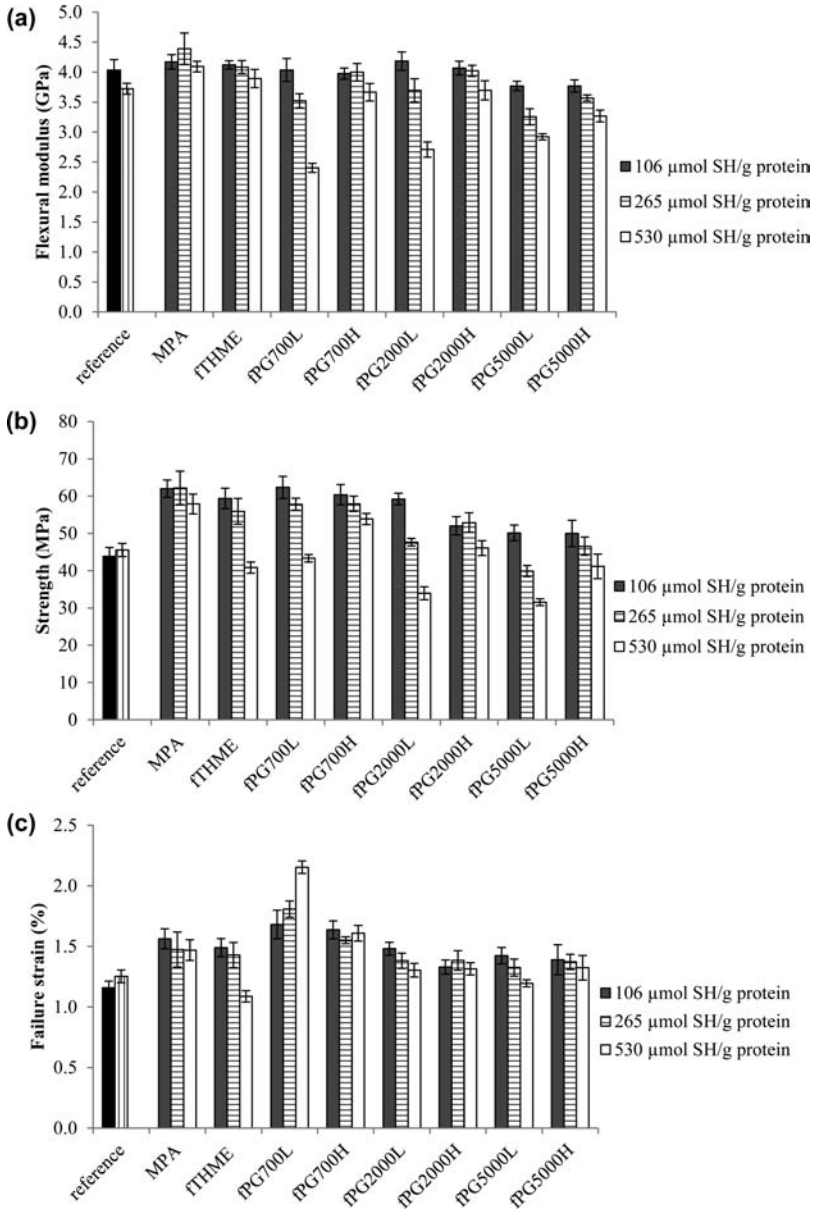

Figure 6. Mechanical properties for reference samples and gluten with additives at 3 different concentrations compression molded at 150 °C for 5 min: (A) flexural modulus, (B) strength, and (C) failure strain.

Table 4. Mechanical Properties of the Reference Samples and Samples with Unfunctionalized Polyglycerol (PG) Compression Molded at 150 °C for 5 min

	amount of additive (%)	flexural modulus[a] (GPa)	strength[a] (MPa)	failure strain[a] (%)
reference 1		4.0 (0.2) a	43.8 (2.4) a	1.2 (0.1) a
reference 2		3.7 (0.2) ab	45.5 (1.7) a	1.3 (0.1) af
PG700	1.3	4.1 (0.1) a	55.9 (2.3) b	1.5 (0.0) b
PG700	8.3	3.7 (0.1) b	62.7 (2.7) c	2.0 (0.1) c
PG700	15.5	2.3 (0.1) c	51.5 (1.3) d	3.2 (0.2) d
PG700	18.5	1.7 (0.1) d	34.9 (0.9) e	4.8 (0.1) e
PG2000	20.8	1.4 (0.2) e	19.7 (0.3) f	1.9 (0.1) c
PG5000	17.3	1.6 (0.0) de	17.0 (0.8) f	1.4 (0.1) bf

[a]Standard deviation of 5-fold measurements is given in brackets. Values with the same letter are not significantly different ($P < 0.05$). Sample codes as in Table 1.

decrease in modulus with increasing concentration of PG and fPG with low DE could be due to plasticization, the T_g of gluten in the Bioplastic samples was determined (data not shown). The T_g of gluten decreased with increasing concentration of PG700 and fPG700L, demonstrating that these additives indeed act as plasticizer, which can explain the decreased modulus. However, the T_g of gluten was not lower in the presence of high concentrations of PG2000, PG5000, fPG2000, and PG5000. Since the T_g of these additives is much lower than that of gluten at the applied moisture content, the lack of decrease in gluten T_g indicates that these additives have little or no plasticizing effect and that gluten is very poorly miscible with these additives. It follows that these additives likely form a separate phase in the gluten matrix. The modulus of such system depends on the volume fractions and moduli of the respective phases. PG and fPG are viscous materials with a modulus much lower than that of pure rigid gluten. Their combination in a blend is thus expected to yield materials with

a lower overall modulus compared to pure gluten, irrespective of whether the moduli of the phases are coupled in series, parallel, or a hybrid way.

In general, the strength was higher in the presence of the lowest concentration of additives than without additives (about 44 MPa) (Figure 6b), which could be the result of an increased degree of cross-linking. Whether the degree of cross-linking was indeed increased with these additives could not be concluded with our other tests since the SDSEP content (Figure 4) was low at this concentration for all additives and the swelling behavior did not provide a reliable measure for the cross-linking degree. The highest strength (about 60 MPa) was observed when molding in the presence of MPA, fTHME, fPG700L, fPG700H, and fPG2000L. In the presence of PG2000H and fPG5000, a strength of about 51 MPa was obtained. For fTHME and fPG, a decrease in strength with increasing concentration was observed. This decrease was stronger for the fPG with low DE than for the fPG with high DE and was in line with the higher SDSEP for these materials. However, besides a lower degree of cross-linking also an increased degree of plasticization may contribute to the reduced strength of samples containing fPG700. The idea that this may well be the case is inspired by the effects that were observed when unfunctionalized PG700 was added: with increasing PG700 concentration the strength first increased to 62 MPa at 8.3% additive and decreased with further increasing concentrations (Table 4). Possibly, a low level of added PG700 can enhance gluten cross-linking during compression molding by moderate plasticization, i.e., by increasing the mobility of the protein chains. An increased degree of cross-linking with increasing plasticizer (water) content from 5.6% to 18.0% during compression molding was previously reported.[10] Similarly, for extruded gluten rubbers, Türe et al.[21] reported an increased degree of cross-linking in the presence of urea. It was not possible to obtain an indication for the degree of cross-linking here, since a minimal SDSEP content was obtained after molding for all samples containing unfunctionalized PG. At higher concentrations, plasticization may well overtake the side effect it can have on gluten cross-linking. This observation not only implies that plasticization may lead to lower strength values but also that the positive effect of adding fPG700 is not necessarily only due to the effect of SH groups of fPG700 on the cross-linking. Adding a low amount of fPG700 may also contribute to the gluten cross-linking via moderate plasticization. The decrease in strength with increasing concentration of fPG2000 and fPG5000 with low DE is likely related to the presence of a distinct phase in the sample.

Finally, it is argued that besides cross-linking (potentially enhanced by moderate plasticization), also altered molecular conformations and improved molecular entanglements may contribute to the strength. This hypothesis follows from the experiments in which MPA was added. The strength did not depend on the concentration of MPA, although the SDSEP content after molding increased with increasing MPA concentration. This suggests that the loss of strength to be expected from a lower degree of cross-linking is compensated by another factor. It is hypothesized that SH/disulfide interchange reactions between gluten and SH-containing additives facilitate conformational changes which result in a rearrangement of the weak bonds between protein chains and/or in an increased entanglement degree and that this improves the strength. The extent to which such conformational changes occur could depends on the additive and its concentration. Note that the reducing effect of additives on the gluten network during mixing indeed depended on the additive and its concentration (Figure 3). At the lowest MPA concentration, the SDSEP content was already maximal after mixing, indicating a high number of SH/disulfide interchange reactions. For some other additives higher concentrations were required to obtain the maximal SDSEP content. As previously mentioned, at higher concentration of additives, plasticization or demixing can also play a role.

The failure strain of gluten compression molded with the lowest additive concentrations was higher than for the reference samples (Figure 6c). For gluten molded with fPG700L, the failure strain increased with increasing concentrations. It also increased with increasing concentration of PG700 (up to 4.8%) at the same mass concentration as 530 μmol SH/g protein fPG700L (see Tables 1 and 4). At this concentration, the sample with fPG700L had a failure strain of 2.2%, which was lower than that of its unfunctionalized counterpart. However, it had a higher modulus and strength. The failure strain of samples containing PG2000 and PG5000 at the high mass concentration was, respectively, 1.9% and 1.4%. For gluten molded with MPA, fPG2000, and fPG5000 no effect of concentration on failure strain was observed. The failure strain of gluten molded with fTHME decreased with increasing concentration of the additive, which corresponds to the decrease in strength with increasing concentration, while the modulus remained unaffected.

The improvements in mechanical properties obtained for the lowest concentration of SH-containing additives were similar to those obtained by Woerdeman et al.[2] and Dicharry et al.[12] at comparable additive levels. At this concentration, an improved strength and failure strain was obtained for all SH-containing additives without the penalty of a decreased modulus. However, a striking result is that a similar improvement can be obtained using a simple monothiol instead of the more expensive additives with multiple SH groups tested in the present research and that of Woerdeman et al.[2] and Dicharry et al.[12] While additives with multiple SH groups may act as cross-linker, this does not seem to be the most important factor determining the mechanical properties of rigid gluten-based materials. The presence of SH functionality which can undergo SH/disulfide interchange seems to suffice. Such interchange reactions can affect both cross-linking and the gluten conformation.

Overall, the effect of SH-containing additives on the gluten network depended on the molecular architecture of the additive and its concentration. In general, small additives and additives with low amounts of SH groups on a single molecule had the most pronounced reducing effect during solvent mixing. The degree of cross-linking after molding decreased with increasing reducing effect of additives during mixing. Our results show that the beneficial effect of SH-containing additives can not only be ascribed to protein cross-linking. It is hypothesized that SH/disulfide interchange reactions between gluten and SH-containing additives not only affect protein cross-linking but also induce conformational changes, which rearrange the weak bonds between protein chains, and/or formation of entanglements and that this affects the mechanical properties. Depending on the additive and its concentration, plasticization or the presence of distinct phases can also contribute to the mechanical properties. In order to toughen rigid, glassy gluten materials small monothiols are more relevant from an economic viewpoint than branched polythiols.

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ABBREVIATIONS USED

ACN, acetonitrile; DE, degree of esterification; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); fTHME, functionalized tris(hydroxymethyl)ethane; fPG, functionalized polyglycerol; MS, mass spectrometry; MPA, 3-mercaptopropionic acid; MW, molecular weight; NMR, nuclear magnetic resonance; PG, polyglycerol; SDS, sodium dodecyl sulfate; SDSEP, extractable protein with SDS-containing medium; SDSEPred, SDSEP under reducing conditions; SE-HPLC, size-exclusion high-performance liquid chromatography; SH, thiol; THME, tris(hydroxymethyl)ethane; PG, unfunctionalized polyglycerol; T_g, glass transition temperature

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