Crosslinks in wheat gluten films with hexagonal close-packed protein structures

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Abstract

Wheat gluten/glycerol (WGG) films were extruded with aqueous ammonia/salicylic acid or urea to investigate the reactions contributing to their hexagonal close-packed protein structures and material properties. The addition of aqueous ammonia and salicylic acid increased the pH, which, in turn, increased the level of intermolecular disulfide and lanthionine cross-links in the WGG films. Increased protein cross-linking reactions resulted in higher material strength and tensile modulus. These cross-linking reactions and the resulting material properties were similar for WGG films with 7.5 and 10% aqueous ammonia. Added urea into WGG film partially degraded into cyanate and ammonium. Cyanate subsequently reacted with lysine and cysteine to ε-carbamyllysine and S-carbamylcysteine, respectively. Even though these reactions resulted in a more alkaline reaction environment, hereby favoring disulfide bond formation and decreasing protein extractability, they also prevented the involvement of cysteine and lysine in protein cross-linking. The alkylation of these reactive amino acids, together with the plasticizing effect of urea, led to lower material strength and modulus with increasing levels of urea.

Keywords:

wheat gluten, hexagonal close-packed structure, biofilms, urea, homocitrulline, S-carbamylcysteine

Abbreviations:

WGG	wheat gluten/glycerol
SE-HPLC	size-exclusion high-performance liquid chromatography
SDS	sodium dodecyl sulfate
HPAEC-IPAD	high-performance anion-exchange chromatography with integrated pulsed amperometric detection

1. Introduction

The use of plant proteins for renewable bio-based plastics is intensively researched. Gluten, the storage proteins of common wheat, is an attractive co-product of the starch industry. It is environment-friendly, relatively inexpensive and has been explored for various bio-based materials such as injection-molded nanocomposites, packaging films, house insulation foams and protein-hemp composites (Micard et al., 2000; Blomfeldt et al., 2010; Wretfors et al., 2010; Cho et al., 2011). Wheat gluten consists of monomeric and polymeric protein fractions, named gliadin and glutenin, respectively. During thermal processing, gluten forms large networks, of which the structure is partially determined by various cross-links (Rombouts et al., 2012). In native gluten, the most important amino acid involved in covalent bonding is cysteine. It forms cystine, and thus disulfide bonds in and between glutenin subunits, and in gliadins (Shewry and Tatham, 1997). Heat induces additional disulfide bonds in and between gliadins and glutenins by sulfhydryl oxidation and sulfhydryl-disulfide interchange reactions **(Figure 1)** (Schofield et al., 1983; Singh and MacRitchie, 2004; Johansson et al., 2013). Thermal processing of gluten under alkaline conditions induces β-elimination of cystine, leading to uncommon amino acids such as dehydroalanine, lanthionine (when dehydroalanine reacts with cysteine) and lysinoalanine (when dehydroalanine reacts with lysine) **(Figure 2)** (Lagrain et al., 2010a; Rombouts et al., 2010). Molecular changes due to these heat-induced reactions have been noted both in plasticized wheat gluten films (Kayserilioglu et al., 2001) and in glassy gluten bioplastic (Jansens et al., 2011). The formation of isopeptide bonds such as ε-(γ-glutamyl) lysine and ε-(β-aspartyl) lysine occurs during heating of gluten at low moisture contents (Feeney and Whitaker, 1984; Rombouts et al., 2011b), which is the case during the production of wheat gluten films (Olabarrieta et al., 2006).

The present paper focuses on wheat gluten/glycerol (WGG) films, which can be extruded in the presence of alkali (sodium hydroxide or aqueous ammonia) and salicylic acid, or urea.

The purpose of such films is to inhibit migration of moisture, gases and lipids, to carry food ingredients, and to improve the mechanical integrity or handling characteristics of food. They can be used as recyclable wraps, pouches, bags, casings, and sachets (Lagrain et al., 2010b). During extrusion processes with additives, gluten proteins build complex polymers with hierarchically ordered close-packed structures, which were either tetragonal or hexagonal (Kuktaite et al., 2011). The additive type impacts both the structural and the functional material properties (Ullsten et al., 2009; Kuktaite et al., 2011). In WGG films produced with sodium hydroxide and salicylic acid, tetragonally packed protein structures are formed (Kuktaite et al., 2011). When aqueous ammonia/salicylic acid or urea are used as additives, hexagonal close-packed structures are formed (Kuktaite et al., 2011; Kuktaite et al., 2012). The presence of ammonia/salicylic acid and urea both result in lower extractability, suggesting an increased protein aggregation (Gällstedt et al., 2011; Türe et al., 2011). Interestingly, the material properties of both types of gluten films strongly differ (Türe et al., 2011). The addition of urea yields softer, weaker and more extensible films than that of aqueous ammonia/salicylic acid-based. Also, the extractability loss is greater for the WGG films with aqueous ammonia and salicylic acid than for those with urea, despite the higher processing temperature of the latter. The molecular origin of this difference remains to be investigated.

Given the great potential of wheat gluten for producing biobased films, knowledge of the reactions during production of such films, which may very well be related to material properties, is necessary. The present study investigates reactions in WGG films with an hexagonal close-packed protein structure produced using aqueous ammonia/salicylic acid or urea. In addition, the impact of these reactions on protein extractability and structural properties of wheat gluten are discussed.

2. Materials and methods

2.1. Materials

Commercial wheat gluten was supplied by Lantmännen Reppe (Lidköping, Sweden). It contained 77.7% protein (N x 5.7) according to the NMKL Kjeldahl method (no. 6) (NMKL, 1976). Glycerol (≥99.5 wt.%) was provided by Tefac (Karlshamn, Sweden). Aqueous ammonia (33%, w/v), salicylic acid (99% wt.%) and urea (≥99.5 wt.%) were purchased from Merck (Darmstadt, Germany). Lanthionine, lysinoalanine, ε–carbamyllysine and S-carbamylcysteine standards were from TCI Europe (Zwijndrecht, Belgium), Bachem (Weil am Rhein, Germany), Chemos (Regenstauf, Germany) and ABIChem (Munich, Germany), respectively. All other chemicals, solvents and reagents were at least of analytical grade and purchased from Sigma–Aldrich (Steinheim, Germany) or VWR International (Leuven, Belgium).

2.2. Sample preparation

The starting material for all WGG films was a mixture of wheat gluten and glycerol (weight ratio 70/30). Two types of WGG films were extruded as described in Kuktaite *et al.* (2011) and Türe et al. (2011). WGG films with two concentrations of aqueous ammonia (7.5 and 10.0 wt %) and 1.5 wt % salicylic acid were extruded at 120 °C. WGG films with three concentrations of urea (10, 15, 20 wt %) were extruded at 130°C. Extruded films were cooled to room temperature and stored at -20 °C until further analysis.

The sample names were abbreviated as WGG followed by the amount of aqueous ammonia (A) or urea (U) added. For instance, WGG-7.5A refers to a sample produced from 91.0 wt % wheat gluten/glycerol (weight ratio 70/30), 7.5 wt % aqueous ammonia and 1.5 wt % salicylic acid. WGG-10U refers to a sample produced from 90.0 wt % wheat gluten/glycerol (weight ratio 70/30) and 10 wt % urea. The control samples for the films with aqueous

ammonia/salicylic acid and urea are named WGG-A-control and WGG-U-control, respectively.

2.3. Size-exclusion high-performance liquid chromatography

Size-exclusion high-performance liquid chromatography (SE-HPLC) was performed as described by Lagrain *et al*. (2005), using an LC-2010 system (Shimadzu, Kyoto, Japan) with automated injection. To evaluate extractability in sodium dodecyl sulfate (SDS) containing media, freeze dried samples [1.0 mg protein/ml] were extracted (60 min, 20 °C) with 50 mM sodium phosphate buffer (pH 6.8) containing 2.0 % (w/v) SDS. To evaluate the extractability under reducing conditions, extraction was carried out under N_2 atmosphere in the same SDS containing buffer, but now also containing 2.0 M urea and 1.0 % dithiothreitol. All analyses were performed in duplicate. After centrifugation (10 min, 11,000 g) and filtration (Millex-HP, 0.45 µm, polyethersulfone; Millipore, Carrigtwohill, Ireland), supernatants were loaded (60 µl) on a Biosep-SEC-S4000 column with separation range from 15 to 500 kDa (Phenomenex, Torrance, CA). The elution solvent was acetonitrile/water (1:1, v/v) containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1.0 ml/min and the column temperature 30 °C. Protein elution was monitored at 214 nm. Extractability in SDS containing buffer (under non-reducing and reducing conditions) was calculated from the corresponding peak area, and expressed as a percentage of total extractability, *i.e.* of the unheated sample under reducing conditions.

2.4. Amino acid analysis and determination of protein cross-links

Amino acids and the amino acid cross-links lysinoalanine and lanthionine in the WGG films were liberated by acid hydrolysis and separated by high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD) as described by Rombouts *et al.* (2009). Freeze dried samples (5.0 mg protein) were heated (24h, 110 °C) in 1.0 ml 6.0 M HCl containing 0.1% phenol and 3.0 mM norleucine (as internal standard).

Reaction mixtures were subsequently diluted (200-fold) in deionised water and filtered (Millex-GP, 0.22 µm, polyethersulfone; Millipore). Amino acids (injection volume 25 µl, flow rate 0.25 ml/min, 30 °C) were separated on an AminoPac PA10 column (250 x 2 mm; Dionex Benelux, Amsterdam, The Netherlands) using a Dionex BioLC system (Dionex, Sunnyvale, CA) equipped with Dionex Chromeleon Version 6.70 software. Gradient conditions and detection waveform were as previously described (Rombouts et al., 2009). Amino acid levels were expressed on dry matter protein (µmol/g protein) based on the relative peak areas of standard solutions. The amino acid cysteine was not determined, only its oxidized counterpart cystine was determined.

2.5. Dehydroalanine determination

Samples (100 mg) were heated in sealed reaction tubes (12 ml) in 0.5 ml 1.5 N HCl at 110 °C for 120 min to liberate dehydroalanine as pyruvic acid, which was quantified colorimetrically (Rombouts et al., 2011a).

2.6. Statistical analyses

Amino acid levels and protein extractabilities were analyzed by one-way analysis of variance using Statistical Analysis System software 8.1 (SAS Institute, Cary, NC), with the mean values compared using the Tukey test ($P<0.05$).

3. Results and discussion

3.1. Reactions during the production of WGG films with additives

Amino acids levels were determined in hydrolyzed wheat gluten (control), WGG films extruded at 120 °C (with aqueous ammonia and salicylic acid), and WGG films extruded at 130 °C (with urea) (**Table 1**).

The amino acid levels of WGG films were not significantly different ($P<0.05$) from those of wheat gluten. In addition, neither lanthionine nor lysinoalanine was detected in these samples. The lack of lanthionine and lysinoalanine formation in the WGG films without additives is in agreement with the outcome of kinetic studies of β-elimination reactions and lanthionine formation (Lagrain et al., 2010a). The pH of these samples (6.0 prior to processing) was too low and the residence time of the samples in the extruder (about 1 min) was too short for substantial β-elimination to occur.

In the WGG films produced with aqueous ammonia and salicylic acid, the lysine level (121 μmol/g protein) prior to extrusion was not significantly different ($P<0.05$) from that after extrusion, but the cystine level decreased from 85 to 75 and 73 μmol/g protein for WGG-7.5A and WGG-10A, respectively (**Table 1**). The cystine loss was the result of the formation of dehydroalanine-derived cross-links (**Figure 2**). Dehydroalanine itself, a reactive intermediate of these reactions, was not found in any of the samples. However, about 14.4 μmol lanthionine/g protein was detected in the WGG films produced with 7.5 or 10.0% aqueous ammonia and 1.5% salicylic acid (**Table 1**). The addition of aqueous ammonia and salicylic acid (which evidently because of not being in excess was totally converted into ammonium salicylate) increased the pH from 6.0 to 10.2 (±0.1) and created more favorable conditions for β-elimination reactions and subsequent lanthionine formation. The addition of ammonia and the resulting pH increase are most essential for lanthionine formation to occur, as no lanthionine was detected in the WGG-A-control. In contrast, no lysine was lost and no

179 lysinoalanine was found in any of the samples (**Table 1**), probably because formation of
180 lysinoalanine requires an even higher pH than that of lanthionine (Rombouts et al., 2010). For
181 the WGG films produced using aqueous ammonia and salicylic acid, β-elimination of cystine
182 and subsequent formation of lanthionine (**Figure 2**) explain the observed cystine loss as well
183 as the lanthionine formation .
184 In the WGG films produced using urea, lysine levels decreased to about 40 μmol/g protein
185 during extrusion, irrespective of the urea concentration. Cystine levels decreased more than in
186 WGG films produced with aqueous ammonia. Also, higher urea concentrations resulted in
187 higher cystine losses. No lysinoalanine, but about 4.5 μmol/g protein lanthionine was found in
188 WGG films produced with urea, irrespective of urea concentration. These results indicate that
189 β-elimination and lanthionine formation occur during the production of WGG films with urea,
190 but to a lesser extent than in WGG films with aqueous ammonia. For the WGG films
191 produced with urea, cystine losses during extrusion were greater than expected based on the
192 formation of lanthionine. Furthermore, lysine levels decreased drastically during processing,
193 while no lysinoalanine was detected. Hence, other reactions must have occurred in these
194 films, which involved cystine and lysine. Sweetsur and Muir (1981) indicated that upon
195 heating, urea is decomposed into cyanate, which can then react with lysine in proteins to
196 produce homocitrulline.
197 It has indeed been reported that lysine and cysteine react readily with cyanate to ε-
198 carbamyllysine (homocitrulline) and S-carbamylcysteine, respectively (Stark et al., 1960;
199 Stark, 1965; Kraus et al., 1994) (**Figure 3**). For instance, when wheat gluten is suspended in
200 buffer (pH 8.0, 25 °C) containing 0.5 M cyanate, approximately 95% of the lysine residues
201 react with cyanate within 3 h (Batey, 1983). Isocyanic acid, the reactive form of cyanate, is
202 spontaneously formed in aqueous urea solutions (Dirnhuber and Schütz, 1948; Kraus et al.,
203 1994). To verify the potential occurrence of these reactions in WGG films produced using

urea we tried to detect ε-carbamyllysine. The WGG films with urea indeed contained 58 ± 1 μmol ε-carbamyllysine/g protein, irrespective of the urea concentration confirming the intermediate presence of cyanate (**Table 1**). Cyanate reacts even more rapidly with cysteine than with lysine (Stark et al., 1960), but the reaction product, S-carbamylcysteine, degrades during acid hydrolysis (Stark et al., 1960). Hence, it was not detected in any of the samples. However, additional cysteine residues are released during β-elimination of cystine. So, it is reasonable to assume that S-carbamylcysteine is also formed during production of WGG films with urea, especially since substantial cystine losses were noted. S-carbamylcysteine formation and β-elimination reactions have a positive impact on each other. The reactions with cyanate increase the pH (**Figure 3**) (Stark et al., 1960) and thus create more favorable conditions for disulfide cross-linking and β-elimination reactions. The latter release additional cysteine residues, which can be consumed in S-carbamylcysteine formation and so on. This explains why lanthionine and more disulfide cross-links were found in the WGG films with urea than in the corresponding control sample. However, the reactions with cyanate consume cysteine, a precursor for lanthionine formation. So, the impact of urea addition on lanthionine formation is mainly positive (it increases the pH), but also a bit negative (it consumes a cross-link precursor). In contrast, the impact of aqueous ammonia and salicylic acid addition on lanthionine formation is only positive. This explains why less lanthionine is formed in the WGG films with urea than in those with aqueous ammonia.

In conclusion, conditions during the extrusion of WGG films with aqueous ammonia and salicylic acid induced cleavage of disulfide bonds by β-elimination, followed by lanthionine formation. Conditions during the extrusion of WGG films with urea induced the formation of ε-carbamyllysine, S-carbamylcysteine and lanthionine. Highest lanthionine levels were detected in the WGG films with aqueous ammonia and salicylic acid

229 **3.2. Protein extractability loss during production of WGG films with additives**

230 To determine the potential impact of lanthionine cross-links on the protein network of WGG
231 films, protein extractabilities in SDS containing medium were determined under conditions
232 that reduced the disulfide bonds (**Figure 4, bars with pattern fill**). For all WGG films
233 (extruded), protein extractability under reducing conditions was not significantly different
234 ($P<0.05$) from that of wheat gluten. However, in the case of the WGG films produced with
235 aqueous ammonia and salicylic acid, the SE-HPLC chromatograms of the WGG films
236 extracted with SDS containing media under reducing conditions (**Figure 5**) showed an
237 increased amount of high molecular weight compounds as compared to wheat gluten. This
238 indicates formation of larger molecules by non-disulfide cross-links, which were nevertheless
239 still extractable under reducing conditions. These compounds represent oligomers (di/-
240 trimers) of glutenin subunits and/or gliadins, linked by intermolecular non-reducible cross-
241 links.

242 In contrast, protein extractability under non-reducing conditions (**Figure 4, bars with solid**
243 **fill**) decreased during WGG film production for all samples, and thus even for the control
244 samples. That reduction of disulfide bonds restored the protein extractability completely,
245 indicates the importance of disulfide cross-links for the protein networks. WGG-A-control
246 and WGG-U-control had remaining protein extractabilities of 72.2% and 26.0%, respectively.
247 Protein extractabilities of WGG films with aqueous ammonia and salicylic acid were about
248 6%, while those of the films with urea were 8%. Thus, a higher extrusion temperature (130 °C
249 for WGG-U-control versus 120 °C for WGG-A-control) resulted in a greater extractability
250 loss. Also, the reduction of disulfide bonds led to a greater extractability increase for WGG
251 films with additives, than for the corresponding control WGG films. In other words, the
252 addition of ammonia/salicylic acid or urea increased disulfide cross-linking. Sulfhydryl-
253 disulfide interchange reactions are favored by alkaline pH, with the pK_a of cysteine being

about 8.5 (Lagrain et al., 2010b). A pH shift upon addition of aqueous ammonia and salicylic acid from 6.0 to 10.2 (±0.1), explains why more disulfide cross-linking occurs in the WGG films with aqueous ammonia and salicylic acid. The addition of urea did not directly affect the pH, but it initiated reactions with cyanate which increased the pH.

In conclusion, the addition of aqueous ammonia and salicylic acid led to non-reducible lanthionine cross-links in WGG films, but the most important cross-links are the reducible disulfide cross-links. The addition of urea increased the formation of disulfide bonds, and also some non-reducible lanthionine cross-links, although to a lower degree then in aqueous ammonia and salicylic acid WGG films.

3.3. The relationship between protein cross-linking and material properties

The addition of either 7.5 or 10.0% aqueous ammonia resulted neither in different protein extractabilities, nor in varied levels of amino acids or cross-links in the gluten films. This is in agreement with the outcome of the morphological characterization of the WGG films produced with aqueous ammonia and salicylic acid. Similar SAXS profiles and characteristic dimensions between the hexagonal close-packed scattering objects were obtained for WGG-7.5A and WGG-10A (70 Å and 71.4 Å, respectively), indicating that both films had a similar supramolecular organization (Kuktaite et al., 2011; Kuktaite et al., 2012). In addition, tensile properties such as Young's modulus, maximum stress and strain at maximum stress were not different for both films (Gällstedt et al., 2011; Türe et al., 2011). The concentration of urea had no impact on protein extractability nor on levels of lysine, lanthionine or ε-carbamyllysine. However, increasing urea concentrations resulted in decreased cystine levels, suggesting increased S-carbamylcysteine formation. With increasing urea concentration, a decrease of stiffness and strength, and an increase of extensibility were noted (Türe et al.,

279 2011). The distances between the hexagonal close-packed scattering objects increased with
280 increasing urea concentration from 68.2 Å for WGG-10U to 74.8 Å for WGG-20U (Kuktaite
281 et al., 2012). The plasticizing action of urea, but also the level of S-carbamylcysteine may
282 have resulted in deteriorated mechanical properties. To the best of our knowledge, the
283 formation of ε-carbamyllysine and S-carbamylcysteine during the extrusion of gluten-based
284 biomaterials has not yet been reported.
285
286 The reactions occurring during processing of WGG films with aqueous ammonia/salicylic
287 acid or urea clearly impact their molecular and mesoscale protein structures. Controlling these
288 reactions may very well lead to enhanced material properties.
289
290

4. Conclusions

Results in this paper relate the reactions that occur during processing of WGG films to their material properties, and the impact of additives hereupon. From the above, it is clear that various chemical reactions occur in the WGG films with aqueous ammonia/salicylic acid or urea having hexagonal close-packed structures. Overall, these reactions increase intermolecular bonding in the protein network in the WGG films. In both types of WGG films, disulfide cross-links are the most important cross-links, but the non-reducible cross-link lanthionine was also formed. Both types of additives led to more alkaline conditions, either directly (in the case of aqueous ammonia/salicylic acid) or due to reactions initiated by the additive (in the case of urea). However, in the case of WGG films produced with urea, S-carbamylcysteine formation consumed cysteine, which is required for lanthionine formation. Hence, β-elimination followed by lanthionine formation only substantially contributed to the protein network in WGG films produced with aqueous ammonia and salicylic acid. This, together with the fact that urea acts as a plasticizer, may well explain why WGG films with urea are softer and weaker but more extensible than those produced with aqueous ammonia and salicylic acid even though the latter were produced at a lower processing temperature (Türe et al., 2011).

The understanding of the molecular origin of differences between WGG films with different additives can help industry to better tailor the production of WGG films for specific applications.

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Tables

Table 1. Level of cross-linked amino acids in µmol/g protein. Standard deviations are given between brackets. Means in the same column with the same letter are not significantly different (*P*<0.05).

	Cystine	Lysine	Lanthionine	ε-Carbamyllysine
Wheat gluten	85 (7) ^{A}	121 (13) ^{A}	0 (-) ^{C}	0 (-) ^{B}
WGG-A-control	87 (6) ^{A}	126 (1) ^{A}	0 (-) ^{C}	0 (-) ^{B}
WGG-7.5A	75 (6) ^{AB}	121 (1) ^{A}	15 (0) ^{A}	0 (-) ^{B}
WGG-10A	73 (5) ^{AB}	119 (3) ^{A}	14 (0) ^{A}	0 (-) ^{B}
WGG-U-control	85 (4) ^{A}	113 (2) ^{A}	0 (-) ^{C}	0 (-) ^{B}
WGG-10U	69 (2) ^{BC}	40 (3) ^{B}	5 (0) ^{B}	57 (0) ^{A}
WGG-15U	55 (1) ^{CD}	43 (1) ^{B}	4 (1) ^{B}	59 (2) ^{A}
WGG-20U	46 (2) ^{D}	36 (1) ^{B}	5 (1) ^{B}	57 (0) ^{A}

418 **Figure captions**

419 **Figure 1**. Reactions leading to the formation of disulfide bonds and occurring in WGG films
420 produced using either aqueous ammonia/salicylic acid or urea.

421 **Figure 2**. Reactions involving dehydroalanine-derived cross-linking and occurring in WGG
422 films produced using either aqueous ammonia/salicylic acid or urea. Cystine reacts to the
423 intermediate dehydroalanine (β-elimination of cysteine), which subsequently reacts with
424 cysteine or lysine to the end products lanthionine or lysionalanine, respectively.

425 **Figure 3**. Reactions involving cyanate and occurring in WGG films produced using urea.
426 Cyanate is spontaneously formed in aqueous urea. Cyanate reacts with lysine or cysteine to ε-
427 carbamyllysine or S-carbamylcysteine, respectively.

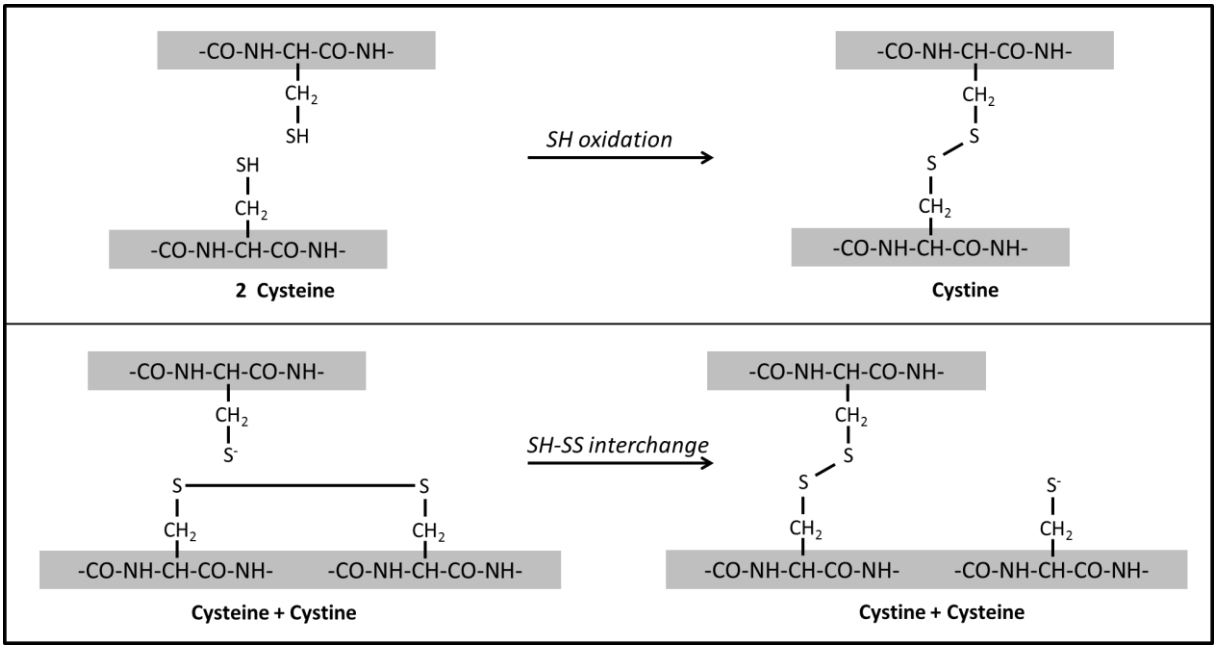
428 **Figure 4**. Protein extractability of WGG films in SDS containing media under non-reducing
429 (solid fill) and reducing (pattern fill) conditions, expressed as a % of the protein in wheat
430 gluten extractable under reducing conditions.

431 **Figure 5**. SE-HPLC chromatograms of WGG films extracted with SDS containing media
432 under reducing conditions.

433

434

435 **Figure 1**



436

437

438

Figure 2

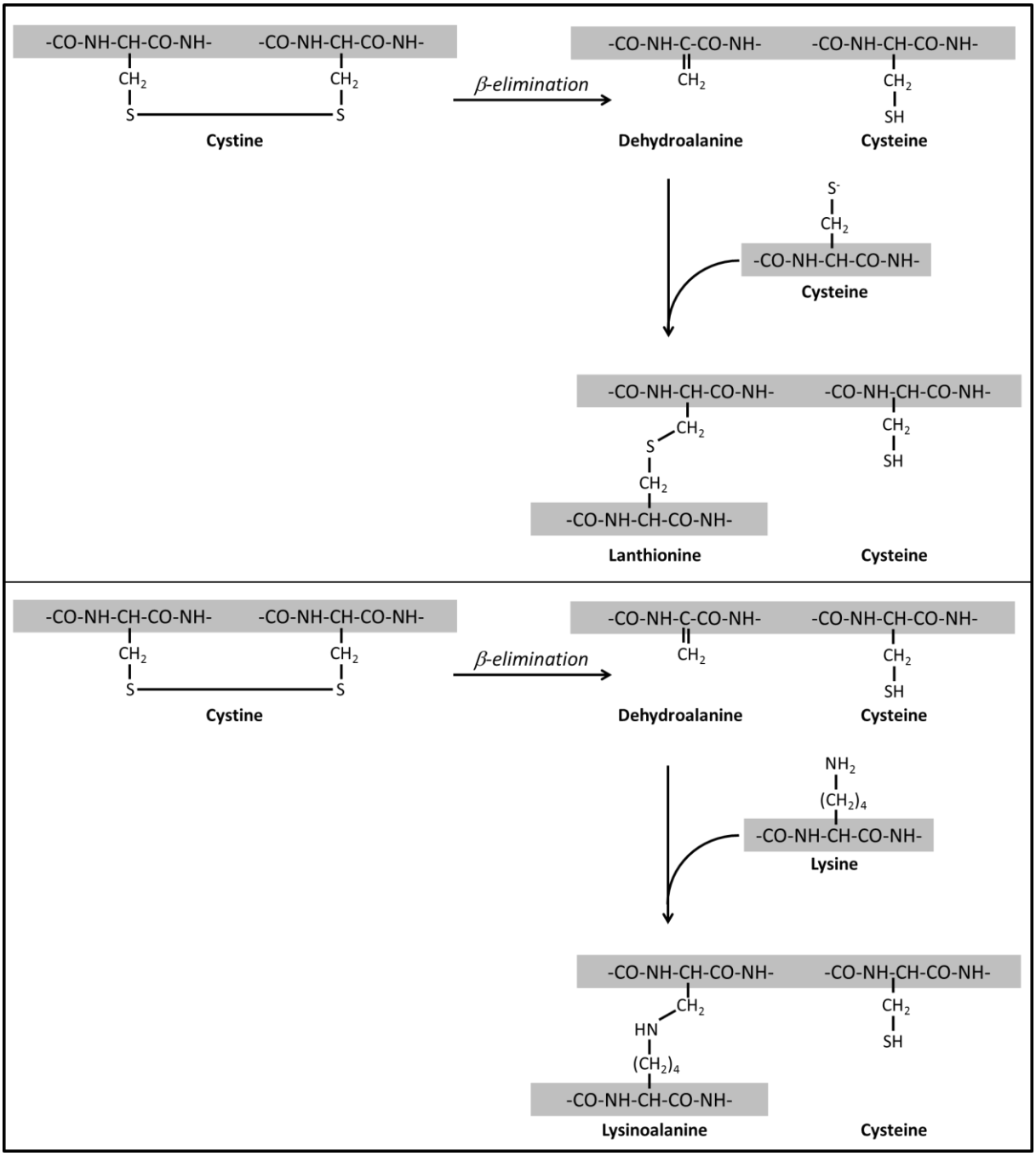
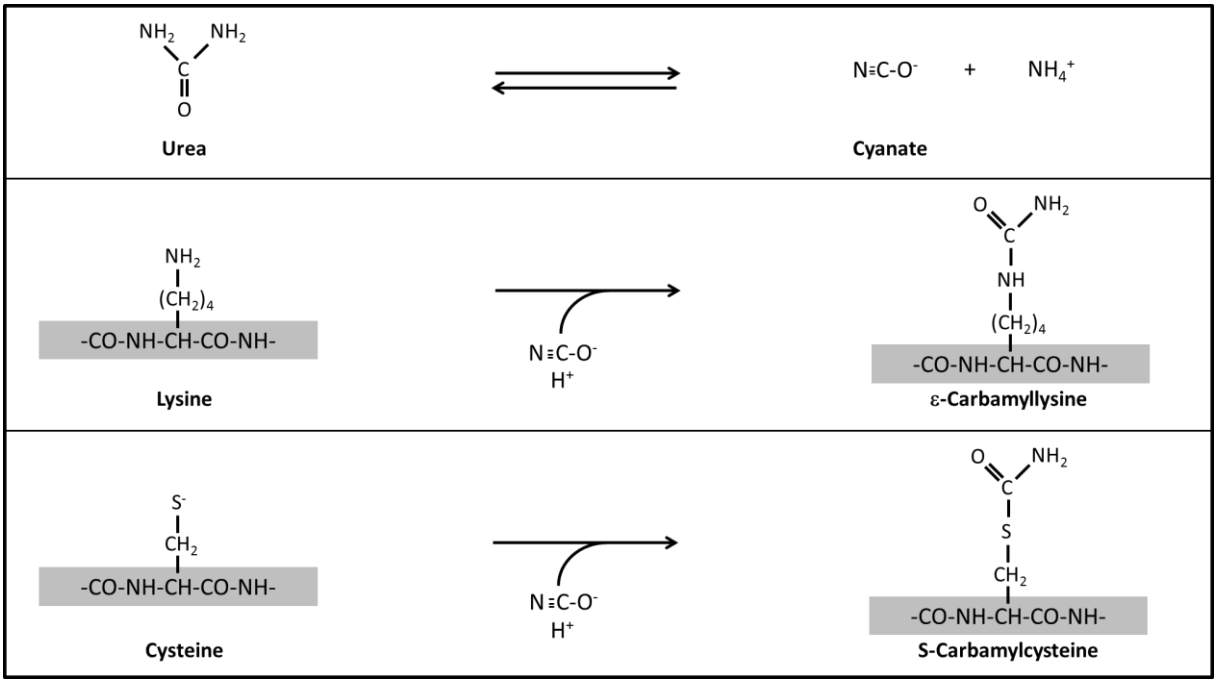
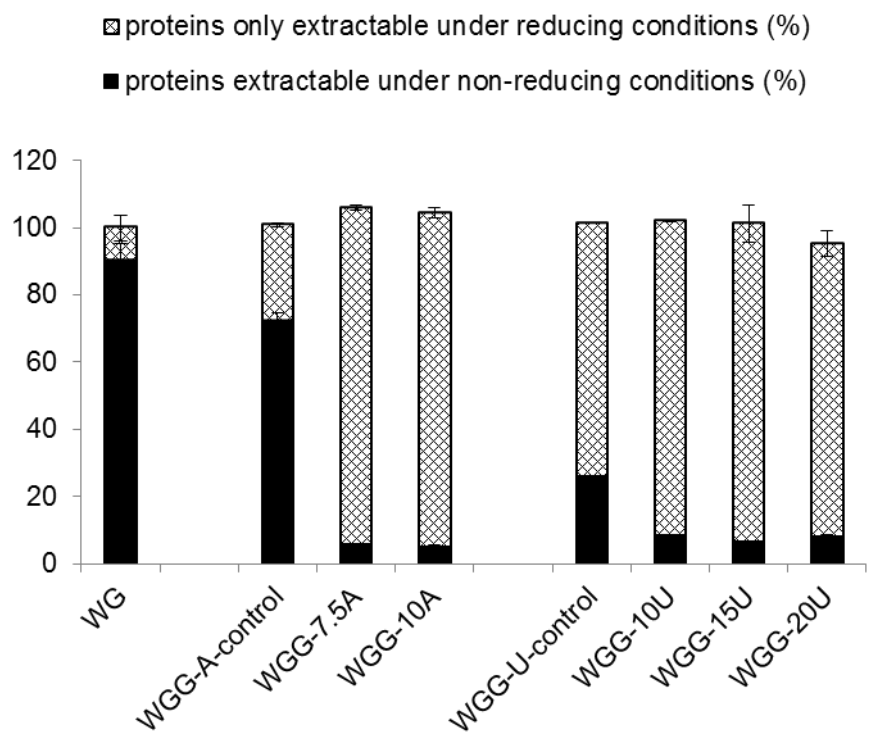

-CO-NH-CH-CO-NH-
-CO-NH-CH-CO-NH-
CH2
CH2
S
S
Cystine
β-elimination
-CO-NH-C-CO-NH-
-CO-NH-CH-CO-NH-
CH2
CH2
SH
Dehydroalanine
Cysteine
S-
CH2
-CO-NH-CH-CO-NH-
Cysteine
-CO-NH-CH-CO-NH-
-CO-NH-CH-CO-NH-
CH2
S
CH2
CH2
SH
-CO-NH-CH-CO-NH-
Lanthionine
Cysteine
-CO-NH-CH-CO-NH-
-CO-NH-CH-CO-NH-
CH2
CH2
S
S
Cystine
β-elimination
-CO-NH-C-CO-NH-
-CO-NH-CH-CO-NH-
CH2
CH2
SH
Dehydroalanine
Cysteine
NH2
(CH2)4
-CO-NH-CH-CO-NH-
Lysine
-CO-NH-CH-CO-NH-
-CO-NH-CH-CO-NH-
CH2
HN
(CH2)4
CH2
SH
-CO-NH-CH-CO-NH-
Lysinoalanine
Cysteine

Figure 3


Urea
Cyanate
Lysine
ε-Carbamyllysine
Cysteine
S-Carbamylcysteine

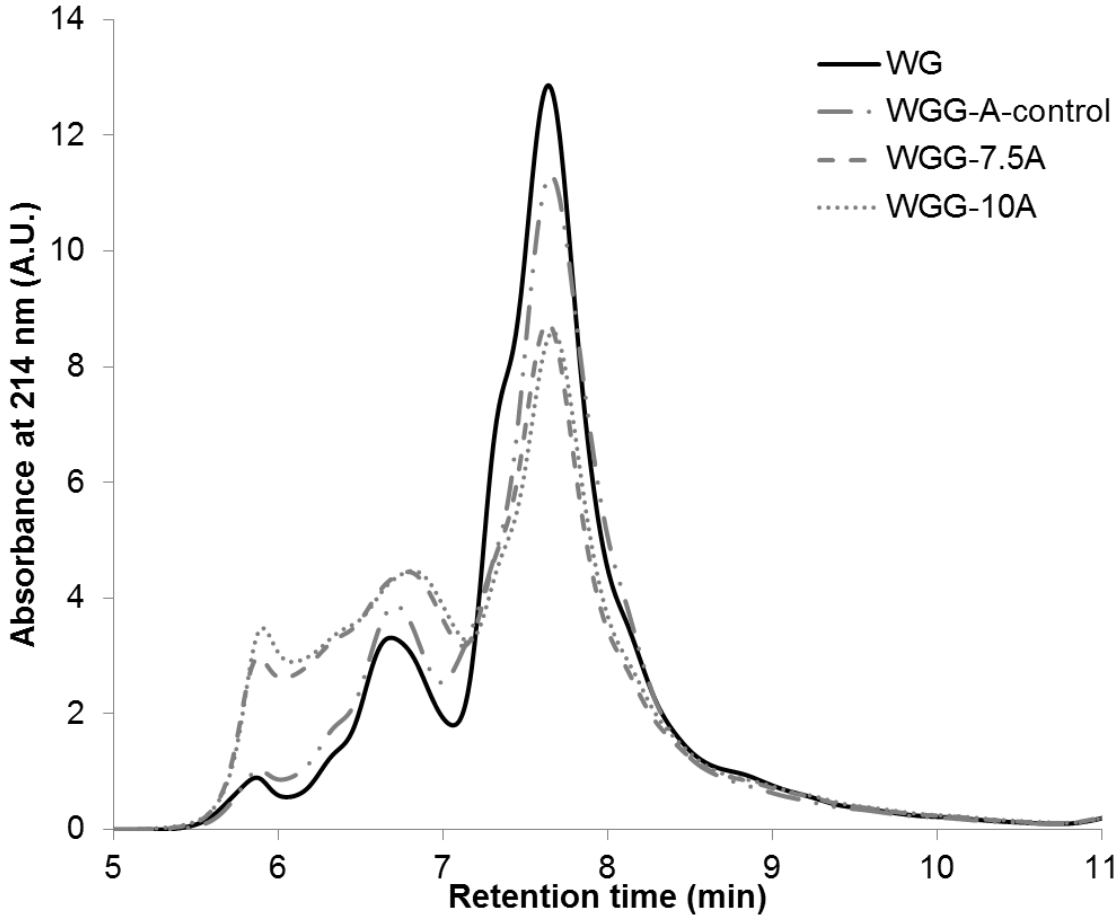
447 **Figure 4**



448

449

Figure 5


14
12
10
8
6
4
2
0
Absorbance at 214 nm (A.U.)
5
6
7
8
9
10
11
Retention time (min)
WG
WGG-A-control
WGG-7.5A
WGG-10A