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Impact of Acid and Alkaline Pretreatments on the Molecular Network of Wheat Gluten and on the Mechanical Properties of Compression-Molded Glassy Wheat Gluten Bioplastics

Koen J. A. Jansens,*[,†,#] Bert Lagrain,[†,#] Kristof Brijs,[†,#] Bart Goderis,[‡,#,⊥] Mario Smet,[‡] and Jan A. Delcour[†,#]

[†]Laboratory of Food Chemistry and Biochemistry, KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

[‡]Polymer Chemistry and Materials Division, Chemistry Department, KU Leuven, Celestijnenlaan 200F, B-3001 Leuven, Belgium

[#]Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, B-3001 Leuven, Belgium

[⊥]Leuven Material Research Centre (Leuven-MRC), KU Leuven, B-3001 Leuven, Belgium

ABSTRACT: Wheat gluten can be converted into rigid biobased materials by high-temperature compression molding at low moisture contents. During molding, a cross-linked protein network is formed. This study investigated the effect of mixing gluten with acid/alkali in 70% ethanol at ambient temperature for 16 h followed by ethanol removal, freeze-drying, and compression molding at 130 and 150 °C on network formation and on types of cross-links formed. Alkaline pretreatment (0−100 mmol/L sodium hydroxide or 25 mmol/L potassium hydroxide) strongly affected gluten cross-linking, whereas acid pretreatment (0−25 mmol/L sulfuric acid or 25 mmol/L hydrochloric acid) had limited effect on the gluten network. Molded alkaline-treated gluten showed enhanced cross-linking but also degradation when treated with high alkali concentrations, whereas acid treatment reduced gluten cross-linking. β-Elimination of cystine and lanthionine formation occurred more pronouncedly at higher alkali concentrations. In contrast, formation of disulfide and nondisulfide cross-links during molding was hindered in acid-pretreated gluten. Bioplastic strength was higher for alkali than for acid-pretreated samples, whereas the flexural modulus was only slightly affected by either alkaline or acid pretreatment. Apparently, the ratio of disulfide to nondisulfide cross-links did not affect the mechanical properties of rigid gluten materials.

KEYWORDS: *acid, alkali, high-temperature compression molding, disulfide bond, lanthionine, mechanical properties*

INTRODUCTION

Gluten proteins show interesting features for nonfood applications. Most researchers in this area focus on the production of gluten-based films by solution casting. In such processes, gluten is dispersed in a solvent, usually based on water and ethanol with acid or alkali.[1] Wheat gluten films can be produced from dispersions above or below the isoelectric point of gluten, but overall alkaline conditions result in stronger films.[2−4] This may be due to the increased gluten cross-linking under alkaline conditions, which not only involves disulfide bonds but also irreversible intermolecular cross-links, such as lysinoalanine (LAL).[3,4] However, faster techniques that require less or no solvent such as thermomolding or, more specifically, high-temperature compression or injection molding are more suited for industrial production and less demanding on the environment.[5]

High-temperature compression molding of gluten at low moisture or plasticizer content results in a stiff, but brittle, material.[6] Plasticizers (e.g., glycerol) are often used to reduce the brittleness of gluten-based materials.[7] When high levels of plasticizer (typically exceeding 15%) are used, the gluten-based material tends to be rubbery rather than glassy at room temperature. The mechanical properties of rubbery materials are quite different from those of rigid materials. Whereas Young's modulus of gluten rubbers depends on gluten cross-linking, the introduction of cross-links does not heavily affect the modulus of glassy gluten materials.[8,9] The strength of gluten rubbers increases with the molding temperature, whereas their elongation at break decreases when the molding temperature increases.[8] In contrast, for rigid gluten materials both strength and failure strain increase with increasing molding temperature, which corresponds to an increased degree of cross-linking.[9] Meijer and Govaert,[10] in their review on the mechanical performance of polymer systems, state that 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), whereas the toughness is governed by the network's strong bonds (ionic bonds and covalent bonds such as peptide and disulfide bonds) and their ability to delocalize local strains.

Different types of protein cross-links can be present in rigid gluten materials depending on the molding conditions. Disulfide bonds are the predominant gluten cross-links, but at molding temperatures exceeding 130 °C also the dehydroalanine (DHA)-derived cross-link lanthionine (LAN) has been detected.[11] Such cross-links are typically formed during heat and alkaline treatment of proteins.[12,13] Alkaline conditions

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affect cross-linking and the mechanical properties of gluten rubbers obtained by compression molding.[14,15] Zhang et al.[14] reported an improved Young's modulus, tensile strength, and strain at break for gluten rubbers molded in the presence of added base and attributed these effects to cross-linking. However, the importance of alkaline treatment and, hence, the resulting nondisulfide bonds relative to that of disulfide bonds for the material properties of glassy gluten bioplastic is not yet known.

Despite recent advances, current gluten-based materials are still outperformed by their synthetic polymer counterparts.[1] Most research efforts in this area focus on the production of gluten rubbers, and literature describing the production of glassy gluten materials is scarce. To improve the properties of the latter materials, it is necessary to understand and control the chemistry behind the formation of different cross-links and to determine their importance for the mechanical properties of rigid gluten-based materials.

The goal of this research was to manufacture glassy gluten-based materials with mechanical properties approaching those of rigid plastics such as polystyrene. To that end, the overall degree of cross-linking and the type of cross-links in compression-molded samples were modified by pretreatments with acid and base. The effect thereof on gluten cross-linking before and after molding and on the mechanical properties of glassy gluten materials was investigated at two molding temperatures. Gluten cross-linking was evaluated using its protein extractability in sodium dodecyl sulfate (SDS)-containing medium and size exclusion high-performance liquid chromatography (SE-HPLC). Gluten cross-linking reactions were further investigated by determining the amino acid composition and the free SH and DHA contents. The mechanical properties of the resulting materials were determined as well.

MATERIALS AND METHODS

Materials. Commercial wheat gluten with a protein content of 77.8% [dry basis (db)] and a moisture content of 5.6% was obtained from Tereos Syral (Aalst, Belgium). The moisture content was determined according to AACC Approved Method 44-19.[16] Protein content 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), using 5.7 as the nitrogen-to-protein conversion factor.[17]

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

Sample Preparation. Gluten was stirred at ambient conditions in 70% ethanol (10% w/v) containing different concentrations of strong acids [sulfuric acid (H_2SO_4), 0.1, 5.0 and 25 mmol/L; hydrochloric acid (HCl), 25 mmol/L] and alkali [sodium hydroxide (NaOH), 0.1, 1.0, 5.0, 10, 25, 50, and 100 mmol/L; potassium hydroxide (KOH), 25 mmol/L]. As a control, gluten was mixed with 70% ethanol (10% w/v). Doing so allowed the gliadins to solubilize and to homogenize the acid/alkali distribution over the gluten. After 16 h of mixing, ethanol was removed by rotary evaporation at 35 °C, and the remaining mixture was freeze-dried.

The samples were then ground in a laboratory mill (IKA, Staufen, Germany) and sieved to pass a 250 μm sieve. The moisture content of each sample was adjusted to 7.0% 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 pestle. Finally, to homogenize it, the sample was shaken overnight with a head-over-head shaker. The obtained samples 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 and 150 °C for 5 min. Before the samples were removed, the mold was allowed to cool to 30−35 °C in about 30−40 min.

Mechanical Property Determination. The compression-molded specimens were cut into five or six bars, 60 mm long and 11 mm wide, stored for 2 days at 50% relative humidity and 20 °C, and tested in a three-point bending test, performed 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, UK) at 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). Flexural modulus, flexural strength, and failure strain were determined from the acquired stress−strain curves. Prior to other analysis, the bioplastic samples were ground and sieved 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.[11] Samples containing 1.0 mg of protein were extracted with 1.0 mL of 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. 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 mol/L urea. All extractions were performed in triplicate. After centrifugation (10 min, 10000*g*) and filtration over polyethersulfone (0.45 μm, Millex-HP, Millipore, Carrigtwohill, Ireland), extracted proteins were separated with SE-HPLC.

SE-HPLC was conducted as described by Lagrain et al.[18] using an LC-2010HT system (Shimadzu, Kyoto, Japan) with automatic injection. The extracts (60 μL) were loaded on a BioSep SEC-S4000 column with separation range from 15000 to 500000 (300 × 7.8 mm, Phenomenex, Torrance, CA, USA) 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.[19] Eluted protein was detected at 214 nm.

The elution profiles of unreduced samples were divided into two fractions using the lowest absorbance value between the two first eluting peaks as the cutoff point. The fractions containing higher and lower MW proteins are further referred to as SDS-extractable glutenin and SDS-extractable gliadin, respectively. SDSEP, SDSEPred, SDS-extractable gliadin, and SDS-extractable glutenin levels were calculated from the corresponding peak areas and expressed as a percentage of the peak area of unmolded gluten extracted under reducing conditions.

Determination of the Free Sulfhydryl Content. Free sulfhydryl (SH) groups were determined colorimetrically following reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Samples (0.8−1.3 mg protein/mL) were first suspended in 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 ethylenediaminetetraacetate]. Mixtures were then shaken (60 min), 100 μL of DTNB reagent [0.1% (w/v) in sample buffer] was added, and the samples were shaken for another 10 min. After filtration over polyethersulfone (0.45 μm) as above, the extinction at 412 nm was read, exactly 45 min after the addition of DTNB reagent. Extinction values were converted to concentrations of free SH using a calibration curve with reduced glutathione.[19] Controls without DTNB or sample were used to correct for background extinction of DTNB and sample. All analyses were performed in triplicate.

Amino Acid Analysis. Amino acid analysis was performed as described by Rombouts et al.[20] Amino acids, including LAN and cystine, were liberated by acid hydrolysis. Samples (16.0 mg protein) were hydrolyzed in 1.0 mL of 6.0 M HCl containing 0.1% (w/v) phenol (VWR, Leuven, Belgium) and 3.0 mM norleucine (as internal standard) at 110 °C for 24 h, after the samples had been flushed with nitrogen to prevent amino acid oxidation. Reaction mixtures were subsequently diluted (200-fold) and filtered over polyethersulfone (0.22 μm, Millex-HP, Millipore). All hydrolyses were performed in triplicate.

Amino acids were separated (injection volume = 10 μL, 30 °C, flow rate = 0.25 mL/min) by high-performance anion-exchange chromatography with integrated pulsed amperometric detection on an AminoPac PA10 analytical column (250 × 2 mm, Dionex, Sunnyvale, CA, USA) using a Dionex BioLC system. Gradient conditions and peak detection are described elsewhere.[20] Amino acid levels were calculated on the basis of the ratio of the peak areas of amino acids to that of the internal standard in sample hydrolysates and in standard solutions and expressed on dry matter protein (μmol/g protein). Because the hydrolysis conditions deamidate asparagine and glutamine, only the sums of asparagine and aspartic acid and of glutamine and glutamic acid were determined. Because the experimental conditions degraded cysteine and tryptophan, their levels could not be determined.

Determination of the Dehydroalanine Content. DHA was determined according to the method of Rombouts et al.[21] Samples (80 mg protein) were hydrolyzed in 0.5 mL of 1.5 mol/L HCl at 110 °C (120 min) to liberate DHA as pyruvic acid. After hydrolysis, the reaction mixture was clarified with Carrez I solution [0.5 mL, 0.085 mol/L potassium hexacyanoferrate] and Carrez II solution [0.5 mL, 0.25 mol/L zinc sulfate] and neutralized with 1.0 mol/L NaOH. The mixture was diluted to 10.0 mL and filtered through paper (MN615, diameter = 90 mm, Machery-Nagel, Düren, Germany). Pyruvic acid concentration was then determined enzymatically according to a Megazyme (Bray, Ireland) procedure based on the stoichiometric conversion of pyruvic acid into D-lactic acid by D-lactate dehydrogenase in the presence of reduced nicotinamide-adenine dinucleotide (NADH). The consumption of NADH was determined from the decrease in extinction at 340 nm and used to calculate the concentration of pyruvic acid, applying 6300 $mol^{-1} \cdot L \cdot cm^{-1}$ as the extinction coefficient of NADH at 340 nm.

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

RESULTS AND DISCUSSION

Impact of Acid and Alkaline Pretreatment. *Protein Extractability.* Gluten was mixed at ambient conditions in 70% ethanol or 70% ethanol containing added acid or alkali to obtain a homogeneous dispersion. Table 1 shows the SDSEP and SDSEPred contents of gluten mixed with different concentrations of acid and alkali. The SDSEP content of gluten mixed with 70% ethanol not containing added acid or alkali (the reference sample) was lower than that of untreated gluten due to a decreased glutenin extractability (Figure 1). This indicates that during mixing and/or removal of the solvent, glutenin cross-linking took place. At low concentrations (≤10 mmol/L), NaOH had little impact on the SDSEP content after mixing (Table 1). After mixing with 25 mmol/L NaOH or KOH in 70% ethanol, significantly lower SDSEP contents were observed, indicating more pronounced cross-linking. Both gliadin and glutenin extractabilities were lower than for untreated gluten (Figure 1). Further increase in NaOH concentrations (from 25 to 100 mmol/L) increased the SDSEP content (Table 1). The SE-HPLC profile of gluten mixed with 100 mmol/L NaOH differed from those of the other samples (Figure 1). More protein eluted in the region between the gliadin and the glutenin peaks (respectively around 6 and 9.5 min), and an additional peak with a retention time of around 10.2 min was detected. This peak eluted later than the main gliadin peak, indicating the presence of protein fragments with a MW lower than that of most of the gliadins. This suggests that mixing gluten with 100 mmol/L NaOH induced protein degradation, likely caused by peptide bond hydrolysis, with the formation of low MW protein fragments. Mixing gluten with dilute H_2SO_4 in 70% ethanol had little, if any, impact on the SDSEP content, resembling that of the reference sample at any concentration (Table 1). The SDSEP content after mixing with

Table 1. Levels of Extractable Protein with Sodium Dodecyl Sulfate (SDS) Containing Medium (SDSEP) and SDSEP Level under Reducing Conditions (SDSEPred), both Expressed as Percentages of the Total Protein Extractability of Untreated Gluten in SDS-Containing Medium under Reducing Conditions, for Untreated Gluten and Gluten Pretreated with Different Concentrations of Acid [Sulfuric Acid (H_2SO_4), Hydrochloric Acid (HCl)] and Alkali [Sodium Hydroxide (NaOH), Potassium Hydroxide (KOH)] in 70% Ethanol at Ambient Temperature[a]

	[acid/alkali][b]	SDSEP (%)	SDSEPred (%)
untreated gluten		88.3 (2.4) a	100.0 (0.6) a
NaOH	0	75.2 (0.6) b	100.5 (0.2) a
	0.1	81.3 (0.1) cd	100.4 (2.0) a
	1	77.9 (0.4) bcef	99.3 (0.4) a
	5	79.3 (1.3) cef	100.3 (0.3) a
	10	76.2 (1.4) be	101.2 (1.2) a
	25	55.9 (0.8) h	100.6 (2.0) a
	50	62.4 (1.2) g	84.5 (1.8) b
	100	74.1 (1.7) b	79.4 (1.8) c
KOH	25	56.4 (0.4) h	100.0 (0.6) a
H_2SO_4	0	75.2 (0.6) b	100.5 (0.2) a
	0.1	80.5 (0.5) cf	98.5 (0.3) a
	5	75.2 (0.8) b	100.7 (1.7) a
	25	76.9 (1.0) bef	100.0 (2.0) a
HCl	25	84.6 (0.4) ad	99.2 (1.3) a

[a]Standard deviation of triplicate protein extractability determinations is given in parentheses. Values with the same letter are not significantly different ($P < 0.05$). [b]Acid/alkali concentration during pretreatment in mmol/L.

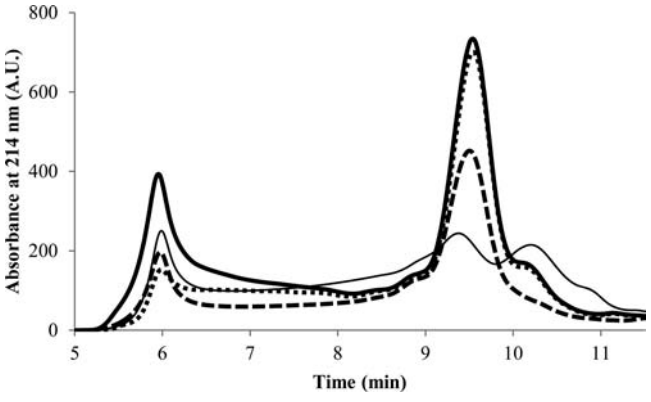

Figure 1. Size-exclusion HPLC profiles of the extracts in SDS containing medium of control gluten (——), gluten mixed in 70% ethanol (- - -), gluten mixed with 25 mmol/L (— —), and gluten mixed with 100 mmol/L (—) NaOH in 70% ethanol for 16 h at ambient temperature. Following mixing, ethanol was removed by rotary evaporation, and the gluten was recovered by freeze-drying. The peak around 6 min of elution time corresponds to extractable glutenin, whereas extractable gliadins elute around 9.5 min. The peak at 10.2 min represents protein degradation products. A.U., arbitrary units.

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dilute HCl in 70% ethanol seemed higher than that of the reference sample.

The SDSEPred content provides an indication of the presence of nondisulfide cross-links,[11] as disulfide bonds are reduced by DTT. However, even when all proteins are extractable under reducing conditions, low levels of intermolecular nondisulfide bonds may still be present. This can be derived from the MW distribution of the reduced samples. The SDSEPred content of gluten, gluten mixed with acid, and gluten mixed with low levels of NaOH (≤10 mmol/L in 70% ethanol) was 100%, and the reduced protein samples all had similar MW distributions (data not shown), indicating that few, if any, intermolecular nondisulfide bonds were present. When using 25 mmol/L of NaOH or KOH in 70% ethanol, the SDSEPred content was also 100%, but relatively more proteins with higher MW were observed in the corresponding SE-HPLC profiles (data not shown), indicating the presence of low levels of intermolecular nondisulfide bonding. Higher levels of NaOH (50 and 100 mmol/L in 70% ethanol) led to both a further enrichment in protein aggregates with a high MW and decreasing SDSEPred readings, indicating higher levels of intermolecular nondisulfide bonding.

Free SH Content, Amino Acid Composition, and DHA Content. The free SH content of untreated gluten was 2.4 μmol/g protein. It was unaffected by mixing in 70% ethanol (Figure 2), decreased in the presence of low levels of NaOH

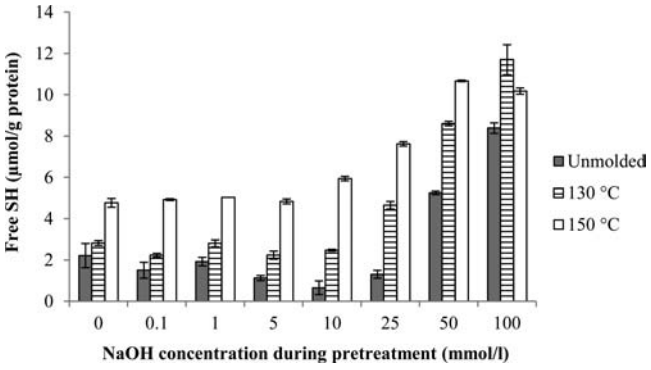

Figure 2. Free sulfhydryl (SH) content of gluten pretreated with different concentrations of NaOH before and after molding at 130 and 150 °C.

(≤10 mmol/L), but increased with increasing NaOH concentrations. The decrease in free SH content can be explained by oxidation reactions, whereas the increase in free SH content was likely caused by cystine β-elimination reactions. β-Elimination of cystine residues yields cysteine and DHA residues.[22] This does not necessarily decrease the average MW of gluten aggregates. Gluten contains many intramolecular cystine residues. β-Elimination of these residues hardly affects their MW. In contrast, β-elimination of intermolecular cystine residues initially decreases the MW. However, both cysteine and DHA, formed by β-elimination of cystine, can react further. Cysteine can be involved in oxidation and SH−disulfide interchange reactions, whereas DHA can react with a number of amino acids with formation of covalent bonds in or between protein chains, which can contribute to the gluten network. Reaction of DHA with cysteine and lysine, respectively, yields LAN and LAL.[13,22] Furthermore, because the reactivity of free SH groups is directly related to their pK_a value (about 8.5), an increased reactivity of such groups can be expected under alkaline conditions.[1,23] The overall effect of mixing gluten with 25 mmol/L alkali was an increased degree of cross-linking as evidenced by the decreased SDSEP contents. The free SH content was not significantly affected by mixing gluten with acid (Table 3). Furthermore, the reactivity of these free SH groups can be expected to be decreased under acidic conditions.[1,23]

To further verify that cystine elimination reactions had occurred, DHA contents and amino acid compositions were determined (Table 2). With NaOH concentrations exceeding 25 mmol/L in 70% ethanol, the cystine content strongly decreased, whereas DHA and LAN contents increased with increasing NaOH concentrations. These results confirm the occurrence of β-elimination reactions and the formation of DHA-derived cross-links during alkaline pretreatment. After mixing gluten with 50 mmol/L NaOH in 70% ethanol, similar levels of LAN and cystine residues were observed. Mixing with 100 mmol/L NaOH in 70% ethanol resulted in a loss of >90% of the original cystine content (Table 2).

Mixing decreased the lysine content to a degree depending on the NaOH concentration used (Table 2). After mixing, LAL (15 μmol/g protein) was detected only at the highest concentration of base. Because the lysine loss in all samples exceeded the LAL formed, lysine is most likely also consumed in other reactions in an alkaline environment.

The cystine and lysine contents remained unaffected by mixing gluten with acid, and no DHA, LAN, or LAL was detected (Table 3).

Impact of Compression Molding. *Protein Extractability.* Table 4 shows the SDSEP and SDSEPred contents of the pretreated samples after compression molding at 130 and 150 °C. Compression molding decreased the SDSEP content of all samples, indicating gluten protein cross-linking (see also Table 1). The SDSEP content of alkaline-pretreated gluten molded at 130 °C decreased with increasing NaOH concentration (≤50 mmol/L), indicating an enhanced overall degree of cross-linking. To reach an SDSEP content similar to that of gluten pretreated with 50 mmol/L NaOH in 70% ethanol and molded at 130 °C without alkaline pretreatment, a molding time of about 25 min instead of 5 min at the same molding temperature was required. Sample pretreated with 100 mmol/L NaOH and molded at 130 °C had a higher SDSEP content than gluten pretreated with 50 mmol/L NaOH and molded at the same temperature. This could be ascribed to degradation reactions. Gluten pretreated with 25 mmol/L KOH and molded at 130 °C had a lower SDSEP content than gluten pretreated with the same concentration of NaOH and molded at the same temperature. Molding at 150 °C resulted in an extractability of only about 6% for the samples pretreated with low levels of NaOH (≤25 mmol/L in 70% ethanol). Molding at 150 °C induced (further) protein degradation in samples pretreated with at least 50 mmol/L NaOH in 70% ethanol (Table 4).

With increasing acid concentrations, the molding-induced (both at 130 and 150 °C) decrease in SDSEP content was less than for the reference, suggesting that acid pretreatment hindered, but not completely inhibited, cross-linking during the subsequent compression molding process.

The high SDSEPred content of the reference sample molded at 130 °C indicated that disulfide bonds were the dominant protein cross-links. Compression molding at 130 °C of gluten pretreated with NaOH (10−100 mmol/L in 70% ethanol) decreased its SDSEPred content, indicating an increased level of nondisulfide cross-link formation as a result of molding. The effect depended on the NaOH concentration and was most

Table 2. Cystine, Lysine, Dehydroalanine (DHA), and Lanthionine (LAN) Contents (Micromoles per Gram Protein) of Gluten Pretreated with Different Concentrations of Alkali before and after Molding at 130 and 150 °C[a]

	[alkali][b]	cystine	lysine	DHA	LAN
			Unmolded		
NaOH	0	77.8 (1.2) abc	148.7 (4.1) a	nd[c]	nd
	0.1	76.0 (0.1) abcd	148.6 (4.3) a	nd	nd
	1	77.0 (1.4) abcd	147.4 (1.6) ab	nd	nd
	5	77.6 (0.9) abc	150.8 (2.0) a	nd	nd
	10	76.6 (2.1) abcd	135.5 (3.2) bcd	nd	nd
	25	72.5 (1.2) de	143.2 (5.1) abc	2.7 (0.1) a	1.5 (0.2) ab
	50	37.3 (0.9) f	113.8 (0.9) ef	11.5 (0.2) b	38.2 (0.5) c
	100	5.3 (0.1) g	91.2 (3.5) g	20.0 (0.1) c	59.9 (0.3) d
KOH	25	73.6 (1.3) cd	131.7 (2.4) cd	1.6 (0.2) d	0.7 (0.2) a
			Molded at 130 °C		
NaOH	0	75.8 (1.6) abcd	139.0 (3.9) abc	4.3 (0.2) e	nd
	0.1	79.8 (2.3) a	147.9 (0.9) ab	4.5 (0.3) e	nd
	1	79.2 (1.3) ab	149.8 (4.0) a	4.0 (0.2) e	nd
	5	75.8 (2.2) abcd	140.5 (4.9) abc	4.6 (0.4) e	nd
	10	74.4 (0.6) bcd	141.5 (5.0) abc	4.3 (0.5) e	nd
	25	62.4 (2.5) h	112.0 (4.4) fh	8.1 (0.2) f	7.7 (0.4) e
	50	28.0 (1.3) i	99.5 (2.5) ghij	13.8 (0.4) g	42.9 (0.5) f
	100	4.6 (0.1) g	96.2 (2.3) gij	22.7 (0.8) h	45.5 (2.0) gh
KOH	25	61.6 (0.2) h	106.0 (4.8) fhij	6.1 (0.2) i	9.1 (0.9) e
			Molded at 150 °C		
NaOH	0	74.6 (2.1) bcd	125.7 (4.3) de	6.8 (0.2) ijk	1.0 (0.1) a
	0.1	75.5 (1.5) abcd	132.5 (5.2) cd	7.0 (0.1) ijkl	1.2 (0.0) ab
	1	76.0 (2.2) abcd	132.6 (5.5) cd	7.0 (0.1) jkl	1.2 (0.1) ab
	5	73.0 (1.3) cd	130.9 (3.5) cd	7.3 (0.2) fkl	1.9 (0.1) ab
	10	68.0 (1.7) e	116.0 (4.2) ef	7.8 (0.2) fl	3.6 (0.1) b
	25	54.3 (0.5) j	107.3 (4.9) fhj	9.8 (0.2) m	14.3 (0.2) i
	50	17.6 (0.4) k	97.8 (3.8) gij	13.3 (0.3) g	48.0 (1.1) g
	100	4.5 (0.2) g	96.9 (0.9) gij	20.0 (0.1) c	43.8 (1.3) fh
KOH	25	51.1 (0.9) j	94.0 (2.7) gi	7.7 (0.2) fl	19.4 (0.4) j

[a]Standard deviation of triplicate amino acid and DHA determinations is given in parentheses. Values with the same letter are not significantly different ($P < 0.05$). [b]Alkali concentration during pretreatment in mmol/L. [c]nd, not detected.

Table 3. Cystine, Lysine, Free SH, DHA, and LAN Contents (Micromoles per Gram Protein) of Gluten Pretreated with Different Concentrations of Acid before and after Molding at 130 and 150 °C[a]

	[acid][b]	cystine	lysine	free SH	DHA	LAN
			Unmolded			
H_2SO_4	0	77.8 (1.2) abc	148.7 (4.1) ab	2.2 (0.4) a	nd[c]	nd
	0.1	78.5 (0.7) ab	152.0 (3.8) a	1.8 (0.0) a	nd	nd
	5	77.1 (0.9) abc	151.9 (2.9) a	1.8 (0.1) a	nd	nd
	25	75.3 (1.8) bc	148.7 (4.6) ab	1.7 (0.0) a	nd	nd
HCl	25	76.9 (1.5) abc	153.1 (3.8) a	2.0 (0.1) a	nd	nd
			Molded at 130 °C			
H_2SO_4	0	75.8 (1.6) bc	139.0 (3.9) bcd	2.8 (0.1) b	4.3 (0.2) a	nd
	0.1	77.7 (2.1) abc	142.3 (5.0) abc	2.1 (0.0) a	4.0 (0.2) a	nd
	5	81.2 (1.4) a	144.3 (5.0) abc	1.8 (0.1) a	3.1 (0.1) b	nd
	25	78.1 (1.3) ab	144.4 (2.0) abc	1.7 (0.1) a	0.9 (0.0) c	nd
HCl	25	77.9 (0.7) abc	145.2 (0.1) ab	2.1 (0.1) a	2.2 (0.1) d	nd
			Molded at 150 °C			
H_2SO_4	0	74.6 (2.1) bc	125.7 (4.3) e	4.8 (0.2) c	6.8 (0.2) e	1.0 (0.1) a
	0.1	73.3 (1.1) c	127.7 (2.2) de	5.1 (0.2) c	6.4 (0.4) e	1.2 (0.0) a
	5	77.0 (2.5) abc	133.2 (4.8) cde	3.3 (0.1) d	5.3 (0.2) f	nd
	25	78.5 (0.3) ab	122.6 (3.5) e	2.2 (0.1) a	4.0 (0.1) a	nd
HCl	25	76.9 (1.5) abcd	124.7 (2.5) e	2.0 (0.0) a	4.3 (0.1) a	nd

[a]Standard deviation of triplicate amino acid and DHA determinations is given in parentheses. Values with the same letter are not significantly different ($P < 0.05$). [b]Acid concentration during pretreatment in mmol/L. [c]nd, not detected.

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Table 4. SDSEP and SDSEPred Contents of Gluten Pretreated with Different Concentrations of Acid and Alkali and Molded at 130 and 150 °C[a]

		SDSEP (%)		SDSEPred (%)	
	[acid/alkali][b]	130 °C	150 °C	130 °C	150 °C
NaOH	0	33.5 (0.6) a	6.4 (0.3) a	99.0 (1.3) a	80.1 (1.1) a
	0.1	31.9 (1.8) ab	5.6 (0.2) a	97.6 (0.8) a	78.0 (1.3) ab
	1	31.4 (1.0) ab	5.8 (0.4) a	100.4 (0.5) a	81.7 (1.8) a
	5	28.9 (0.8) bc	5.4 (0.3) a	97.5 (2.4) a	76.2 (0.8) b
	10	25.9 (1.2) c	5.2 (0.1) a	92.5 (2.2) b	66.5 (1.8) c
	25	21.0 (1.2) d	6.4 (0.5) a	60.3 (2.0) c	37.7 (0.3) d
	50	9.8 (0.2) e	19.0 (1.0) b	32.5 (1.0) d	31.4 (0.7) e
	100	17.2 (0.1) f	51.7 (0.7) c	45.3 (0.7) e	55.3 (1.2) f
KOH	25	6.1 (0.4) g	6.0 (0.3) a	62.1 (1.4) c	33.8 (0.7) e
H_2SO_4	0	33.5 (0.6) a	6.4 (0.3) a	99.0 (1.3) a	80.1 (1.1) a
	0.1	30.5 (1.0) ab	5.9 (0.3) a	97.1 (0.8) a	81.5 (1.4) a
	5	46.3 (1.4) h	8.2 (0.2) d	100.0 (1.6) a	87.1 (0.3) g
	25	59.7 (1.4) i	28.4 (0.3) e	100.3 (1.1) a	89.7 (2.5) g
HCl	25	54.1 (0.2) j	16.1 (0.6) f	100.7 (0.4) a	87.5 (1.2) g

[a]Standard deviation of triplicate protein extractability determinations is given in parentheses. Values with the same letter within a molding conditions are not significantly different ($P < 0.05$). [b]Acid/alkali concentration during pretreatment in mmol/L.

pronounced for gluten pretreated with 50 mM NaOH. The higher SDSEPred content of sample pretreated with 100 mmol/L NaOH in 70% ethanol indicated protein degradation. The reference sample molded at 150 °C also contained nondisulfide covalent bonds.[11]

The SDSEPred content of acid-treated samples molded at 130 °C was almost 100%. That of samples molded at 150 °C was higher for the acid-treated samples (≥5 mmol/L in 70% ethanol) than for the reference, indicating that acid pretreatment hindered the formation of nondisulfide cross-links during compression molding.

Free SH, Amino Acid Composition, and DHA Content. Molding at 130 °C increased the free SH content of gluten pretreated with at least 5 mmol/L NaOH in 70% ethanol. For all alkali-pretreated samples, except for gluten pretreated with 100 mmol/L NaOH in 70% ethanol, the free SH content further increased with the molding temperature (Figure 2). The increase in free SH content may well result from β-elimination of cystine. The free SH content was higher after molding at 150 °C for gluten pretreated at low acid concentrations (0.1 mmol/L in 70% ethanol) than after mixing and molding at 130 °C and decreased with increasing acid concentration when molding at 150 °C (Table 3).

Molding resulted in the formation of low levels of DHA in the reference sample (Table 2), whereas no significant change in cystine content was observed for this sample. DHA can also be formed by β-elimination of amino acids other than cystine (e.g., serine). However, if β-elimination of serine had taken place, this would have been to only a limited extent, because no significant differences in serine concentrations were observed. With NaOH concentrations exceeding 5 mmol/L in 70% ethanol, molding at 150 °C decreased the cystine content for all samples except for gluten pretreated with 100 mmol/L NaOH in 70% ethanol. For this sample, a low cystine content was already attained after mixing. For all alkaline-pretreated samples, DHA could be detected at both molding temperatures (Table 2).

The LAN contents of gluten pretreated with 25 and 50 mmol/L NaOH in 70% ethanol and molded at 130 °C and of gluten pretreated with 0−50 mmol/L in 70% ethanol and molded at 150 °C were higher than after mixing. Molding thus induced the formation of additional nondisulfide bonds. Gluten pretreated with NaOH concentrations exceeding 25 mmol/L NaOH in 70% ethanol and molded at both 130 and 150 °C even contained higher levels of LAN than of cystine. Interestingly, the LAN content of gluten pretreated with 100 mmol/L NaOH in 70% ethanol decreased as a result of molding at 130 and 150 °C. It is difficult to predict the exact impact of high LAN levels on the gluten network, because both cystine and LAN can be present as either intramolecular or intermolecular cross-links. However, the decreased SDSEPred contents of these samples (Table 2) suggest that at least some of the LAN residues were present as intermolecular cross-links.

The cystine contents of acid-pretreated samples were not significantly affected by molding, indicating that few if any β-elimination reactions of cystine occurred during this unit operation. DHA was detected in the molded samples. Its content decreased with increasing acid concentration. LAN was detected only after molding at 150 °C at the lowest acid concentration. These results show that in gluten pretreated with a low acid concentration (0.1 mmol/L in 70% ethanol) some β-elimination still occurred during molding, but that this reaction was progressively restricted when more acid was used. Not much DHA and free SH was formed after limited β-elimination and, as a result, not much LAN could be detected either. The decrease in LAN content corresponds with an increase in the SDSEPred content. However, even though no LAN was detected at the highest acid concentration, the SDSEPred content was still below 100%, indicating that other types of nondisulfide cross-links, possibly isopeptide bonds and/or ester bonds, were still present.

The decreases in cystine contents corresponded reasonably well to the combined increases in free SH, DHA, and LAN contents, indicating that the above-mentioned reactions indeed explained the cystine losses. For samples pretreated with 100

Table 5. Flexural Modulus, Strength, and Failure Strain of Gluten Pretreated with Different Concentrations of Acid and Alkali and Molded at 130 and 150 °C[a]

		flexural modulus (GPa)		strength (MPa)		failure strain (%)	
	[acid/alkali][b]	130 °C	150 °C	130 °C	150 °C	130 °C	150 °C
NaOH	0	3.5 (0.2) abcd	3.8 (0.1) ef	31.2 (2.0) ab	44.7 (1.7) fgi	0.9 (0.0) ab	1.2 (0.1) fgh
	0.1	3.8 (0.2) ef	3.8 (0.0) efg	37.1 (1.4) cde	51.7 (2.5) jk	1.0 (0.1) ac	1.4 (0.1) ijk
	1	3.7 (0.2) abefg	3.5 (0.0) abdg	37.5 (2.7) cde	49.5 (2.2) ij	1.0 (0.1) acde	1.4 (0.1) ikl
	5	3.7 (0.3) abefg	3.5 (0.1) abcd	40.5 (2.6) cdfg	50.8 (2.3) jk	1.1 (0.0) cdef	1.5 (0.1) kl
	10	3.7 (0.1) abefg	3.9 (0.1) ef	36.4 (1.3) ceh	55.1 (2.8) kl	1.0 (0.0) ac	1.4 (0.1) ikl
	25	3.6 (0.1) abeg	3.5 (0.1) abcd	40.4 (2.7) cdfg	54.2 (2.4) jkl	1.1 (0.1) fgh	1.7 (0.1) m
	50	3.9 (0.1) f	3.6 (0.0) abeg	42.1 (2.1) dfg	52.9 (1.8) jkl	1.1 (0.0) efg	1.5 (0.0) l
	100	4.3 (0.2) h	3.5 (0.1) abcdg	40.6 (2.7) cdfg	41.1 (2.3) cdf	0.9 (0.0) abc	1.1 (0.0) defg
KOH	25	3.7 (0.1) befg	4.0 (0.2) f	45.2 (1.7) gi	58.2 (1.3) l	1.3 (0.1) hij	1.5 (0.0) l
H_2SO_4	0	3.5 (0.2) abcd	3.8 (0.1) ef	31.2 (2.0) ab	44.7 (1.7) fgi	0.9 (0.0) ab	1.2 (0.1) fgh
	0.1	3.3 (0.1) cd	3.4 (0.1) acd	29.3 (0.7) ab	39.2 (3.9) cdf	1.0 (0.0) acd	1.2 (0.1) ghj
	5	3.3 (0.0) cd	3.5 (0.1) abcdg	27.8 (1.0) ab	41.0 (1.8) dfg	0.9 (0.0) ab	1.3 (0.1) hij
	25	3.2 (0.2) c	3.5 (0.1) abcd	32.6 (3.4) aeh	40.6 (2.1) cdfg	1.1 (0.1) cdef	1.2 (0.0) fgh
HCl	25	3.5 (0.1) acd	3.4 (0.1) acd	26.8 (2.0) b	30.7 (2.1) ab	0.8 (0.0) b	0.9 (0.1) ab

[a]Standard deviation of 5-fold mechanical property determinations on single samples is given in parentheses. Values with the same letter are not significantly different ($P < 0.05$). [b]Acid/alkali concentration during pretreatment in mmol/L.

mM NaOH in 70% ethanol, the decrease in cystine content exceeded the combined increase in free SH, DHA, and LAN contents, suggesting that cystine degradation at this NaOH concentration did not exclusively occur via β-elimination reactions. LAL was also detected in this sample, but its content was lower than after mixing (data not shown).

Mechanical Properties. Table 5 shows the flexural modulus, strength, and failure strain data of the compression-molded samples. The flexural modulus showed little if any dependence on the molding temperature and on the type of pretreatment (Table 5). These results are in line with earlier findings that the flexural modulus of rigid gluten-based materials does not depend strongly on covalent cross-linking.[9] In their review on the mechanical performance of polymer systems, Meijer and Govaert[10] stated that the modulus of nonoriented polymers in the glassy state is determined by the polymer's weak bonds and free volume kinetics. It seems that neither the acid nor the alkaline pretreatments affect the protein's weak bonds to such an extent that they affect the flexural modulus of the resulting bioplastic.

Molding the reference sample at 150 °C resulted in a higher strength than molding at 130 °C (Table 5), pointing to an increase in strength with increasing degree of cross-linking.[9] The strength was higher after alkaline pretreatment of gluten, but little effect of the NaOH concentration during pretreatment on material strength was noted. When the sample pretreated with the lowest NaOH concentration was compared with that pretreated with the highest NaOH concentration, strong differences in the gluten network were observed. The lowest NaOH concentration resulted in disulfide bonds being the most predominant cross-links, whereas the highest NaOH concentration led to much more LAN than disulfide bonding (Table 2). Nevertheless, similar material properties were obtained. Furthermore, also the degree of cross-linking after molding at 130 °C increased with increasing alkali concentration from 0.1 to 50 mmol/L during pretreatment. However, this too did not significantly affect the strength. This may be related to the effect of alkali on the other components of commercial gluten. In addition to proteins, commercial gluten typically contains 7.0−16.0% starch and nonstarch carbohydrates and 3.5−6.8% lipids.[24] Protein degradation affected only the strength at relatively high levels of degradation, which was the case for the samples pretreated with 100 mmol/L NaOH in 70% ethanol and molded at 150 °C. Alkaline-pretreated gluten required milder molding conditions to achieve a strength similar to that of untreated gluten. For example, whereas untreated gluten needed to be molded for 25 min at 130 °C to reach a strength of about 38 MPa,[9] only 5 min was required for alkali-pretreated gluten at this temperature. To reach a strength of about 55 MPa, gluten needed to be molded at 170 °C for 5 min,[9] whereas a molding temperature of 150 °C for 5 min was sufficient for alkali-pretreated gluten.

The failure strain was slightly affected by alkaline pretreatment and molding at 130 °C, whereas the failure strain was higher as a result of molding at 150 °C. When gluten was pretreated with sulfuric acid, the failure strain of the resultant material was not significantly affected when molding at either 130 or 150 °C.

The present study shows that acid and alkali can affect the degree of gluten cross-linking and the types of cross-links formed during bioplastic production. Apparently, replacement of disulfide bonds with other covalent bonds does not necessarily affect the material properties of the rigid, glassy end-products. In addition, alkaline pretreatment allows processing at lower molding temperatures and/or shorter molding times to bring about strengths similar to those without pretreatment. Future work should investigate whether the beneficial effects of alkali can also be obtained when a setup having better economic viability is used.

AUTHOR INFORMATION

Corresponding Author

*(K.J.A.J.) Phone: + 32 16321634. Fax: + 32 16321997. E-mail: Koen.Jansens@biw.kuleuven.be.

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■ ABBREVIATIONS USED

db, dry basis; DHA, dehydroalanine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HCl, hydrochloric acid; H_2SO_4, sulfuric acid; KOH, potassium hydroxide; LAL, lysinoalanine; MW, molecular weight; NADH, reduced nicotinamine-adenine dinucleotide; NaOH, sodium hydroxide; SDS, sodium dodecyl sulfate; SDSEP, extractable protein with SDS-containing medium; SDSEPred, extractable protein with SDS-containing medium under reducing conditions; SE-HPLC, size exclusion high-performance liquid chromatography; SH, sulfhydryl

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