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Heat-Induced Cross-Linking and Degradation of Wheat Gluten, Serum Albumin, and Mixtures Thereof

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ABSTRACT: Some wheat-based food systems, such as cakes, cookies, and egg noodles, contain mixtures of animal and plant (gluten) proteins and are processed under (mildly) alkaline conditions. Although changes in these proteins during processing can affect end product quality, they have seldom been studied. This study investigated protein cross-linking and degradation during heating (0−120 min, pH 8.0, 50−130 °C) of (mixtures of) wheat gluten and bovine serum albumin (BSA). The decrease in protein extractabilities in sodium dodecyl sulfate containing buffer under (non)reducing conditions and the levels of (cross-linked) amino acids were measured. No indications for polymerization at 50 °C were found. Below 100 °C, BSA polymerized more readily than wheat gluten. Above 100 °C, the opposite was observed. The kinetics of heat-induced polymerization of a 1:1 gluten−BSA mixture were similar to that of isolated gluten, implying that gluten decelerated BSA denaturation. Severe heating (130 °C, >15 min) induced degradation reactions in gluten but not in BSA. At all conditions used in this study, disulfide (SS) bonds contributed to the extractability loss. In addition, above 110 °C, β-elimination of cystine led to non-SS cross-links. Intramolecular SS bonds more often transformed in intermolecular non-SS bonds in BSA than in gluten.

KEYWORDS: *bovine serum albumin, wheat gluten, aggregation, polymerization, protein network, degradation, disulfide, β-elimination, lanthionine, lysinoalanine, dehydroalanine*

INTRODUCTION

The most important food protein source worldwide is wheat, with an average consumption of 16 g of protein/capita/day.[1,2] Wheat flour proteins are classified into albumin, globulin, gliadin and glutenin fractions by sequential extraction in water, dilute salt solutions, aqueous ethanol, and dilute acid.[3] Albumins and globulins represent about 20% of all wheat flour proteins. Gliadins and glutenins are quantitatively more important and develop into gluten upon mixing flour with water. Gluten proteins are of great technological importance in many food systems.[4] Baking, boiling, or extrusion steps during cereal-based food production induce reshuffling of existing and formation of additional intermolecular disulfide (SS) cross-links, thereby forming a strong gluten network, which greatly determines the structure of, for example, bread[5] and pasta.[6] SS cross-links, which can be cleaved by reduction, are the most studied cross-links with regard to gluten network formation. In addition, at high temperatures and/or alkaline conditions, β-elimination of cystine releases a free sulfhydryl group and the reactive intermediate dehydroalanine (DHA). DHA can then react with cysteine or lysine to form the nonreducible cross-link lanthionine (LAN) or lysinoalanine (LAL), respectively.[7] Such cross-links are usually less abundant, but are important because of their more permanent character. In contrast with most proteins, gluten proteins fail to show a definite denaturation peak in differential scanning calorimetry (DSC) thermograms.[8] Therefore, heat-induced gluten changes are typically evaluated on the basis of gluten extractability loss in sodium dodecyl sulfate (SDS)-containing media,[9−13] which generally follows first-order kinetics.[5,14,15] The impact of temperature on the loss of gluten extractability during heating at mildly alkaline pH has, to the best of our knowledge, not yet been reported. The formation of nonreducible cross-links is accompanied by the loss of extractability in SDS-containing media even if they contain a reducing agent.[7] β-Elimination of cystine during hydrothermal treatment of isolated gliadin also follows first-order kinetics.[16] For the entire gluten protein mixture, the kinetics of β-elimination of cystine during heat treatments at alkaline pH remain to be studied.

In most bread and pasta products, gliadins and glutenins are by far the most abundant proteins. However, some cereal-based foods also contain milk, soy, or egg proteins for structural, nutritional, or taste-related reasons. In, for example, cakes, cookies, pancakes, milk bread, and egg noodles, which are sometimes processed at (mildly) alkaline pH due to the use of stored eggs and/or baking powder, wheat gluten proteins co-occur with albumins from eggs and/or milk. Here, changes in both wheat gluten proteins and albumins during processing can affect end product quality. One of the most important functional properties of albumins is their ability to denature. Denaturation depends on temperature, pH, and protein concentration. It starts with the exposure of hydrophobic groups above the protein unfolding or denaturation temperature and can result in protein aggregation due to interprotein noncovalent bonding (hydrogen and hydrophobic) as well as covalent cross-linking (often through SS bonds).[17] When intermolecular interactions are depressed, the denaturation is reversible.[18] However, more typical is the case of irreversible denaturation,[19] in which large clusters are formed and gel

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formation can occur.[18] Noncovalent, heat-induced albumin aggregation typically follows first-order kinetics and is characterized by high activation energies, for example, 490 kJ/mol for ovalbumin aggregation at pH 7.0.[20] We are not aware of published data on the kinetics of albumin cross-linking, for which the loss of extractability in SDS-containing media is a measure.

For both gluten and albumins, cross-linking of the isolated proteins has been reported, but the kinetics of the reactions involved in network formation and degradation during heating at alkaline pH remains to be studied. In addition, little is known about these reactions in mixtures of gluten and albumins. The observation that interactions and reactions between different proteins in multiprotein food systems can lead to fundamental changes in system behavior is referred to as the coprotein effect.[21] In this context, it is of interest to investigate the cross-linking of mixtures of gluten proteins and albumins, as such reactions may affect food structure and nutritional value. Therefore, the present paper will also investigate whether and how albumins and gluten proteins affect each other in establishing a heat-induced protein network.

More specifically, protein cross-linking and degradation reactions in isolated gluten and serum albumin, at pH 8.0 and temperatures ranging from 50 to 130 °C, will be compared to those in their mixture. At pH 8.0, both reducible and nonreducible cross-linking occurs.[7] The extent and type of cross-linking will be evaluated on the basis of the analysis of protein extractability and amino acid analysis following hydrolysis. Bovine serum albumin (BSA), the most abundant blood plasma protein and also present in milk, is chosen as a model albumin. It is structurally related to ovalbumin, the most important egg protein, and is readily available in its native conformational state. Its primary, secondary, and tertiary protein structures are well documented. Moreover, the potential use of blood plasma proteins in pastry has been studied.[22−25]

MATERIALS AND METHODS

Materials. Vital wheat gluten [74.7% protein (N × 5.7) on a dry matter basis (db)] was obtained from Syral (Aalst, Belgium). Purified α-, γ-, ω5-, and ω1,2-gliadin fractions were obtained by preparative RP-HPLC from "Prolamin Working Group" (PWG) gliadin reference material[26] using a Jasco LC-2010 system. PWG reference gliadin (5.0 mg protein/mL) was solubilized (20 min, 60 °C) in 60% ethanol, filtered over polyethersulfone, and loaded (300 μL) on a Nucleosil 300-5 C8 column (Machery-Nagel, Düren, Germany). The elution system consisted of deionized water (A) and acetonitrile (ACN) (B), both containing 0.1% trifluoroacetic acid (TFA) (v/v). The following gradient was used: (i) linear from 24 to 56% B over 50 min; (ii) linear from 56 to 90% B over 0.1 min; (iii) isocratic at 90% B for 9.9 min; (iv) linear from 90 to 24% B in 0.1 min; (v) isocratic at 24% B for 7.9 min. Proteins were eluted (60 °C, flow rate = 1.0 mL/min) and detected at 214 nm. Separated gliadin fractions were collected and, after removal of ACN by rotary evaporation, freeze-dried. BSA (fraction V for biochemistry) was purchased from Acros Organics (Geel, Belgium) and contained 96.2% protein (N × 6.25, db).

Chemicals and Reagents. All used chemicals, solvents, and reagents were of at least analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany) or VWR International (Leuven, Belgium), unless specified otherwise.

Thermal Treatment. To 1.000 g of sample (gluten, BSA, or a gluten−BSA mixture of equal mass proportion) was added 5.0 mL of a 50 mmol/L sodium phosphate buffer (pH 8.0). The reaction tubes (glass, i.d. = 27 mm, o.d. = 34 mm, height = 100 mm) were then hermetically sealed and placed in an oil bath at 50, 70, 90, 110, or 130 °C for 0, 15, 30, 45, 60, or 120 min. Heat-treated samples were immediately cooled on ice, freeze-dried, and ground as above. The heat treatment did not affect the pH of the samples.

Protein Content. Protein contents were determined in triplicate, using an adaptation of AOAC Official Method 990.03[27] to an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands). Conversion factors of 5.7 and 6.25 (AACC Method 46-30.01) were used to calculate protein from nitrogen content for gluten or BSA, respectively.

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). SE-HPLC was performed as described by Lagrain et al.,[28] using an LC-2010 system (Shimadzu, Kyoto, Japan) with automated injection. To evaluate extractability in SDS-containing medium, freeze-dried samples [1.0 mg protein (db)/mL] were extracted (60 min, 20 °C) with 50 mmol/L 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 mol/L urea and 1.0% (w/v) dithiothreitol (DTT). Both extractions were carried out separately, that is, not successively. All analyses were performed in duplicate. After centrifugation (10 min, 11000*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, USA). The elution solvent was ACN/water (1:1, v/v) containing 0.05% (v/v) TFA. 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 nonreducing and reducing conditions) was calculated from the corresponding peak area and expressed as a percentage of total extractability, that is, of the unheated sample under reducing conditions. For all proteins under investigation, the UV absorbance is related to their quantities using a single regression curve.[29] Molecular mass marker proteins [blue dextran (2000K), aldolase (23K), catalase (148K), ovalbumin (43K), chymotrypsinogen A (25K), and ribonuclease A (14K)] and purified α-, γ-, ω5-, and ω1,2-gliadin fractions were injected to determine the molecular weights or identities of extracted proteins on the basis of their elution time.

Differential Scanning Calorimetry. The denaturation temperatures and enthalpies of BSA and gluten−BSA mixtures were determined by DSC using a DSC Q1000 (TA Instruments, New Castle, DE, USA) calibrated with indium. Samples (1.4−1.8 mg of protein) were accurately weighed into coated aluminum pans (Perkin-Elmer, Waltham, MA, USA). Sodium phosphate buffer (50 mmol/L, pH 8.0) was added (5.0 μL/mg sample), and the pans were hermetically sealed. Sample pans and an empty reference pan were equilibrated at 0 °C and heated to 100 °C at a heating rate of 4 °C/min. Peak temperatures and denaturation enthalpies were determined using Universal Analysis 2000 software (TA Instruments). Results are averages of at least two measurements.

Amino Acid Analysis and Determination of Cross-Links. Amino acids and the amino acid cross-links LAL and LAN in gluten proteins and BSA 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.[30] Freeze-dried samples [5.0 mg of protein (db)] were heated (24 h, 110 °C) in 1.0 mL of 6.0 mol/L HCl containing 0.1% phenol and 3.0 mmol/L norleucine (as internal standard). Reaction mixtures were subsequently diluted (200-fold) in deionized 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 × 2 mm; Dionex Benelux, Amsterdam, The Netherlands) using a Dionex BioLC system (Dionex, Sunnyvale, CA, USA) equipped with Chromeleon version 6.70 software (Dionex Benelux). Gradient conditions and detection waveform were as previously described.[30] Amino acid levels were expressed on dry matter protein (μmol/g protein). To calibrate the method, response factors were determined on the basis of injections of standard solutions that contained all amino acids and the internal standard in concentrations ranging from 5 to 15 μmol/L. Response

factors for each amino acid were calculated as the relative concentration of the respective amino acid to that of the internal standard, divided by the relative peak area of this amino acid to that of the internal standard. Correction factors were used to account for incomplete cleavage of peptide bonds involving Val and Ile and losses of Cys due to oxidation during acid hydrolysis. Relative standard deviations did not exceed 10%. LAN and LAL standards were from TCI Europe (Zwijndrecht, Belgium) and Bachem (Weil am Rhein, Germany), respectively.

DHA Determination. Samples (100 mg) were heated in sealed reaction tubes (12 mL) in 0.5 mL of 1.5 N HCl at 110 °C for 120 min to liberate DHA as pyruvic acid, which was quantified colorimetrically.[31]

Data Analysis. Hydrothermal treatment of gluten under conditions relevant for bread baking and pasta cooking decreases the level of gluten extractable in SDS-containing media exponentially toward a minimum.[5,15] One part remains extractable, whereas another part is susceptible to polymerization reactions. The extractability of the fraction that polymerizes (polymerizing gluten, PG) decreases according to pseudo-first-order reaction kinetics

$$-\frac{d[PG]}{dt} = k_P \times [PG] \quad (1)$$

with k_P the reaction rate constant of polymerization (min^{-1}).

Hence, with $[PG]_0$ and $[PG]_t$ the extractability of PG at time zero and time t respectively:

$$\ln[PG]_t = \ln[PG]_0 - k_P \times t \quad (2)$$

or

$$[PG]_t = [PG]_0 \times \exp(-k_P \times t) \quad (3)$$

The total extractability of gluten decreases in time according to the equation

$$[G]_t = \exp(-k_P \times t) \times [PG]_0 + [G]_{minimal} \quad (4)$$

with $[G]_{minimal}$ the extractability of gluten resisting polymerization under the experimental conditions.

at time zero: $[G]_0 = 1 \times [PG]_0 + [G]_{minimal}$

at infinite time: $[G]_{end} = 0 \times [PG]_0 + [G]_{minimal}$

During prolonged heating under severe conditions, gluten extractability increases as a function of heating time due to degradation reactions.[7] In the present study, the data points affected by protein degradation were excluded from the kinetic analysis of gluten polymerization. Polymerization kinetics of gluten and BSA were studied in a similar way. The reaction rate constants (k_P), the minimal extractability levels, and the goodness of fit (R^2) values, including their 95% confidence intervals, were estimated on the basis of nonlinear regression analysis of the total extractable gluten concentration as a function of exposure time. The temperature dependence of the reaction rate constant for polymerization was determined using the Arrhenius model:

$$k_P = k_{P,ref} \times \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (5)$$

E_a is the activation energy (J mol^{-1}) of the polymerization reaction, R is the universal gas constant (8.3143 J K^{-1} mol^{-1}), $k_{P,ref}$ is the reaction rate constant at reference temperature T_{ref} (K), and k_P is the reaction rate constant at temperature T (K). Linearized Arrhenius plots were used to estimate the activation energy.

Earlier research suggested β-elimination of cystine to follow first-order kinetics.[16,32]

$$-\frac{d[cystine]}{dt} = k_E \times [cystine] \quad (6)$$

k_E is the reaction rate constant of β-elimination (min^{-1}).

The reaction rate constants and goodness of fit (R^2) values for β-elimination of cystine during heat treatments of gluten, BSA, and gluten–BSA mixtures were estimated on the basis of nonlinear regression analysis of the cystine concentrations as a function of heating time.

RESULTS AND DISCUSSION

Heat-Induced Changes in Wheat Gluten. Figure 1 shows the changes in extractability of gluten as a result of hydrothermal treatment for up to 120 min at pH 8.0 and temperatures from 50 to 130 °C. Under nonreducing conditions (Figure 1A), 75% of gluten was extractable before heating. Treatment at 50 °C for 120 min did not affect the extractability. At 70, 90, and 110 °C, the extractability decreased exponentially to reach a plateau level. At 130 °C, the extractability only decreased for 15 min and then increased with heating time.

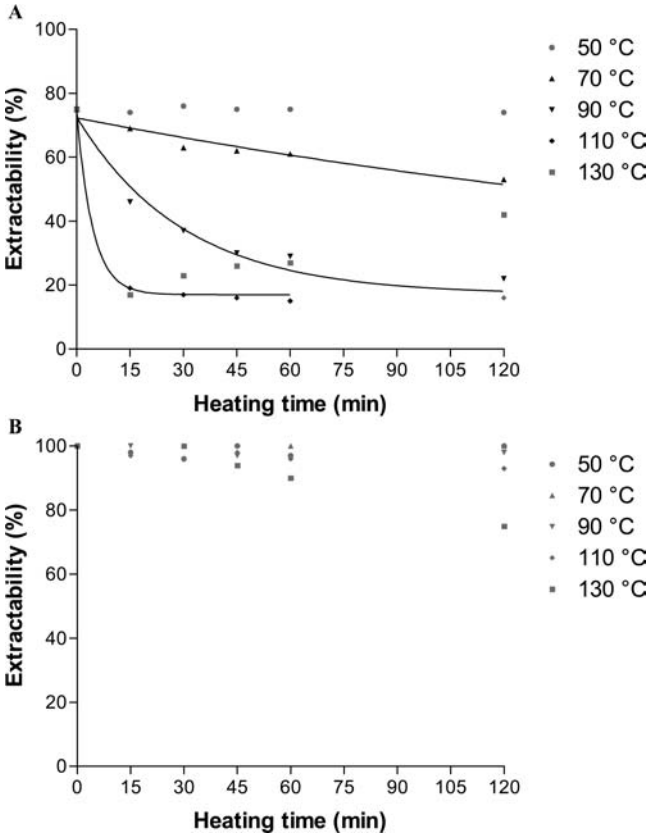

Figure 1. Gluten extractability in SDS-containing media under nonreducing (A) and reducing (B) conditions after heating at pH 8.0 and different temperatures for 0–120 min. Data points fitted according to eq 4 are shown in black; the others are shown in gray. Relative standard deviations did not exceed 5%.

The heat-induced decrease of the level of extractable gluten proteins was fitted using eq 4 (Table 1). Reaction rate constants increased with temperature. The activation energy (110 ± 0 kJ/mol) was in line with an earlier value for gliadin.[5] Whereas the main part of the heterogeneous mixture of gluten proteins was transformed into large unextractable aggregates according to (pseudo-) first-order kinetics, 17 ± 3% remained extractable. The SE-HPLC profiles allowed us to conclude that, under the experimental conditions, glutenins already polymerize during 15 min of heating at pH 8.0 and 90 °C. Most of the

Table 1. Reaction Rate Constants (k_p), Goodness of Fit (R^2) Values, and Activation Energies (E_a) for Extractability Loss during Heating at pH 8.0[a]

sample	T (°C)	k_p (min^{-1})	R^2	E_a (kJ/mol)
gluten	70	0.00	0.924	110
	90	0.03	0.965	
	110	0.22	0.997	
BSA	70	0.07	0.990	22
	90	0.10	0.998	
	110	0.16	0.998	
	130	0.21	0.999	
gluten−BSA, exptl	70	0.00	0.920	121
	90	0.04	0.990	
	110	0.27	0.999	
	130	0.34	1.000	
gluten−BSA, calcd	70	0.02	0.787	48
	90	0.06	0.979	
	110	0.18	0.999	
	130	0.22	0.990	

[a]The extractabilities of gluten, bovine serum albumin (BSA), and gluten−BSA mixtures were determined in SDS-containing media under nonreducing conditions and fitted according to eq 4. The calculated extractabilities of gluten−BSA mixtures were based on the averages of the extractabilities of isolated gluten and BSA. The minimal extractability levels for wheat gluten, BSA, and gluten−BSA mixtures were 17, 0, and 7, respectively.

gliadin became involved in the gluten network during heating for longer times and/or at higher temperatures, yet a relative increase of compounds eluting around 9.5 (ω5-gliadins) and 10 min (ω1,2-gliadins) indicates that most ω-gliadins remained extractable after 120 min of heating at pH 8.0 and 130 °C (Figure 2), which can be ascribed to their lack of Cys residues.

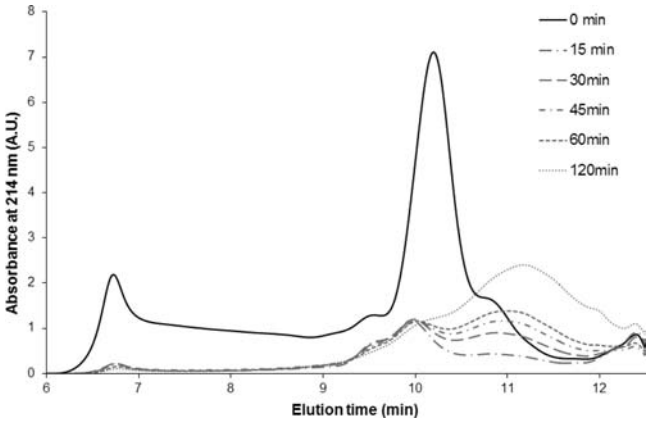

Figure 2. SE-HPLC chromatograms of gluten before and after heat treatment at pH 8.0 and 130 °C. Extraction under nonreducing conditions. A.U., arbitrary units.

However, as 17% of gluten proteins remained extractable, whereas ω-gliadins account for only 7−13% of wheat gluten proteins,[33] it is assumed that, besides ω-gliadins, also other gliadin types failed to incorporate in the protein network. Possibly the absence of gluten denaturation holds back exposure of reactive amino acid side chains, preventing some proteins from incorporation in the protein network.

The increase in the levels of extractable gluten proteins during heating at pH 8.0 and 130 °C was due to an increased amount of small molecules in the peptide mixture, which eluted around 11.2 min (Figure 2). A limited amount of small molecules was also detected in SE-HPLC chromatograms of gluten that had been heated for 120 min at pH 8.0 and 110 °C. These compounds were most likely formed in degradation reactions. β-Elimination of cystine can decrease the molecular weight of proteins by cleaving intermolecular SS bonds.[7] Other important degradation reactions are the formation of succinimide (at aspartate and asparagine residues) and glutarimide (at glutamate and glutamine residues), which can be followed by peptide bond cleavage.[34]

The increased concenration of extractable gluten proteins as a result of heat treatment at 130 °C appeared to follow zero-order kinetics. Hustinx et al.[35] noted a linear increase in the levels of soluble sodium caseinate peptides during heating at pH 7.0 and 140 °C, whereas Saidi et al.[36] noted a linear increase in total non-protein nitrogen content in milk during heating at 80, 100, and 120 °C. The lowest extractability (17%) of isolated gluten was observed after 15 min of heating at pH 8.0 and 130 °C. Extended heating caused the extractability to increase by 0.23 ± 0.02% per min (R^2 = 0.981).

Thermal treatments (120 min, pH 8.0) of gluten samples at 70, 90, and 110 °C decreased their extractability under nonreducing conditions. When the extraction was carried out under reducing conditions, they did not affect gluten extractability (Figure 1B). β-Elimination of cystine leads to DHA, which can react with a free sulfhydryl group of cysteine or an ε-amino group of lysine to form LAN or LAL, respectively.[7] SS bonds are reducible and hence can be distinguished from inter alia DHA-derived cross-links. As reduction of SS bonds entirely reversed the extractability loss caused by heating, it is concluded that polymerization up to 110 °C was mainly the result of SS cross-linking. However, heating at 110 °C increased the level of high molecular mass compounds (300−2000K), extractable under reducing conditions and eluting at 5.5 and 6.5 min (Figure 3). This suggested that non-SS-linked oligomers, consisting of gliadins and/or glutenin subunits, were formed during heating at 110 °C. Thermal treatment for 120 min at 130 °C decreased the

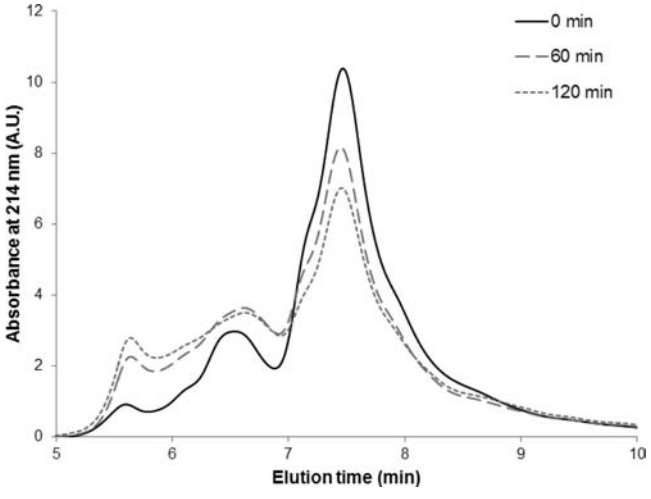

Figure 3. SE-HPLC chromatograms of gluten before and after heat treatment at pH 8.0 and 110 °C. Extraction under reducing conditions. A.U., arbitrary units.

extractability of gluten under reducing conditions from 100 to 75% (Figure 1B). It is concluded that, under these conditions, non-SS cross-links contribute to network formation.

To evaluate the importance of DHA-derived cross-links for heat-induced gluten network formation, amino acid compositions of unheated and heated gluten were determined. Heating experiments at pH 8.0 and 110 or 130 °C resulted in cystine losses and LAN formation, but no LAL was detected in any of the heated gluten samples. Table 2 shows the levels of cystine, DHA, LAN, and LAL in gluten during heat treatment at pH 8.0 and 130 °C. The reaction rate constant for β-elimination of cystine during such treatment was $(18 \pm 2) \times 10^{-3}$ min^{-1} ($R^2 > 0.976$). It is of note that β-elimination of cystine cleaves both intramolecular and intermolecular SS bonds. In a similar way, LAN can be formed in or between gluten proteins. On the basis of the extractability loss under reducing conditions, we hypothesize that at least some intramolecular SS bonds are cleaved by β-elimination and subsequently transformed into intermolecular non-SS bonds.

Table 2. Formation of Dehydroalanine (DHA), Lanthionine (LAN), and Lysinoalanine (LAL) during Thermal Treatment of Gluten, Bovine Serum Albumin (BSA), and 1:1 Gluten–BSA Mixtures at pH 8.0 and 130 °C

heating time (min)	gluten	BSA	gluten–BSA
	Cystine Level (μmol/g protein)		
0	79	258	169
15	69	242	168
30	62	200	154
45	52	195	159
60	47	178	110
120	32	155	87
	DHA Level (μmol/g protein)		
0	0	0	0
15	5	7	2
30	9	8	3
45	10	7	3
60	10	8	5
120	10	13	7
	LAN Level (μmol/g protein)		
0	0	0	0
15	7	13	6
30	19	56	11
45	27	62	8
60	32	64	47
120	48	80	57
	LAL Level (μmol/g protein)		
0	0	0	0
15	0	2	0
30	0	4	0
45	0	4	0
60	0	3	0
120	0	6	0

Heat-Induced Changes in Bovine Serum Albumin. Before heat treatment, BSA was completely extractable even under nonreducing conditions. It remained extractable during 120 min of heating at pH 8.0 and 50 °C (Figure 4A). Heat treatment at higher temperatures resulted in a strong

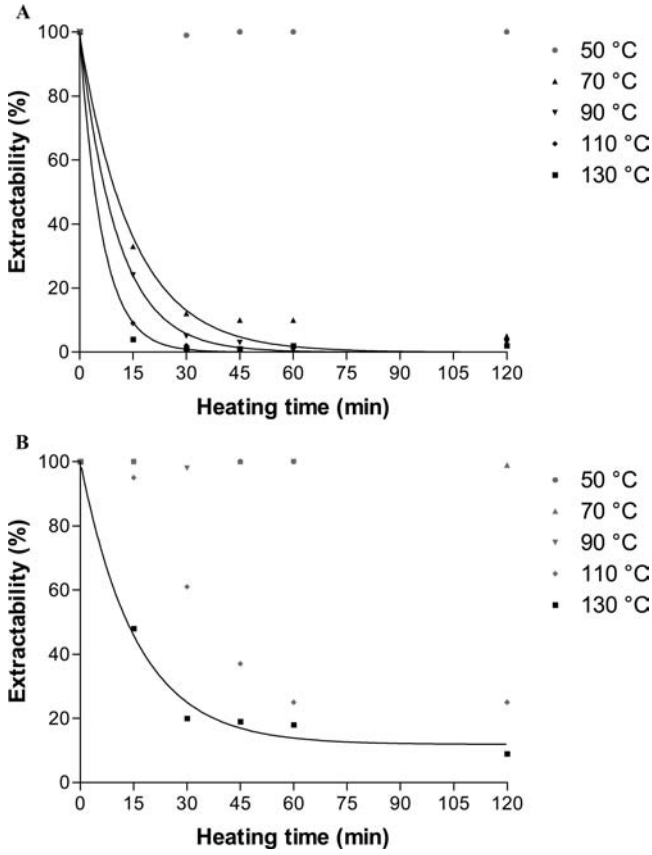

Figure 4. Bovine serum albumin (BSA) extractability in SDS-containing media under nonreducing (A) and reducing (B) conditions after heating at pH 8.0 and different temperatures for 0–120 min. Data points fitted according to first-order kinetics are shown in black; the others are shown in gray. Relative standard deviations did not exceed 5%.

exponential decrease in extractability. The denaturation temperature of BSA, measured with DSC, was about 57 °C for 20% (w/w) aqueous solutions at pH 8.0. An extractability decrease was observed at all temperatures exceeding the denaturation temperature. Higher temperatures induced a faster decrease of extractability, as reflected by the reaction rate constants that increased with temperature (Table 1). However, the activation energy for BSA (22 ± 2 kJ/mol) was lower than that for gluten (110 ± 0 kJ/mol). Thus, gluten extractability loss was more sensitive to temperature increase than that of BSA. Up to 103 °C, the extractability of BSA decreased more rapidly than that of gluten, whereas, above 103 °C, the opposite was observed. In contrast to the extractability of gluten, that of BSA did not tend toward a plateau. Indeed, the decrease in protein extractability could be fitted by first-order kinetics, that is, according to eq 4 in which the minimal extractability level equaled zero. That the extractability of BSA did not tend toward a plateau, whereas that of gluten did, can be ascribed to the presence of Cys residues in all BSA molecules and the exposure of reactive amino acid side chains during BSA denaturation.

No degradation compounds were detected in the chromatograms of heated BSA. That BSA is less susceptible to protein degradation than gluten is in line with findings by Mohammed et al.[37] In general, proteins with a small and compact structure, such as BSA, are more resistant to protein degradation. More specifically, proteins with an open random coil type structure,

which at least in part can be ascribed to high levels of proline and hydroxyproline (residues that are incompatible with an α-helix) and a low level of SS bonds, are most susceptible to degradation during severe heating. Gluten contains higher levels of proline and lower levels of SS bonds (1080 μmol proline and 79 μmol cystine/g protein) than BSA (421 μmol proline and 258 μmol cystine/g protein). In addition, deamidation and subsequent succinimide (from asparagine) and glutarimide (from glutamine, gluten's most abundant amino acid) formation strongly depend on the availability and conformational freedom of the susceptible amino acid residues.

Heat treatment at temperatures exceeding 100 °C decreased the extractability of BSA under both nonreducing and reducing conditions (Figure 4). As a result of such treatment, both SS and non-SS cross-links contribute to network formation. The loss of extractability under reducing conditions of BSA was greater than that of gluten or its isolated fractions. Either more non-SS bonds were formed in BSA than in gluten or the non-SS cross-links in gluten were rather formed in than between gluten proteins. We here evaluate both possibilities.

Treatments above 100 °C resulted in losses of cystine and formation of DHA, LAN, and LAL (Table 2). The reaction rate constant $[(16 \pm 5) \times 10^{-3}\ (\text{min})^{-1}]$ for β-elimination of cystine in BSA at 130 °C ($R^2 = 0.989$) was not significantly different from that in gluten. However, as native BSA contains relatively more cystine (258 μmol/g) than gluten, greater cystine losses were noted in BSA than in gluten. Heating for 120 min at pH 8.0 and 130 °C induced more LAN formation (80 μmol/g protein) in BSA than in gluten (Table 2). In addition, the treatment produced 6 μmol LAL/g BSA. LAL is not easily formed at pH 8.0, because the reaction of lysine with DHA involves nonprotonated lysine residues, which hardly are present at pH 8.0, the p*K* of lysine being 10.3.[1] However, native BSA contains a high level of lysine (887 μmol/g protein). This explains why, even at pH 8.0, detectable amounts of lysine could be involved in LAL formation. That no LAL was found in any of the heated gluten samples can be ascribed to the low lysine levels in gluten (110 μmol lysine/g protein) in combination with the relatively mild alkaline pH. In conclusion, more non-SS cross-links were formed in BSA than in gluten.

In addition, it seems likely that the structure of gluten proteins allows less intermolecular cross-linking than that of BSA. The extractability of gluten under reducing conditions did not decrease in an exponential way. That non-SS cross-linking in gluten does not follow first-order kinetics may be due to steric hindrance. In contrast, the extractability of BSA under reducing conditions decreased according to first-order kinetics (at 130 °C: $k_p = 0.04$, $R^2 = 0.952$). Even though the shape of BSA changes relatively little during denaturation, internal hydrophobic groups "flip" to the outside of the molecule.[38] Possibly, because of these small structural changes, DHA residues formed by β-elimination of cystine can be easily approached by free SH groups of cysteine and amino groups of lysine of neighboring BSA molecules. Also, on the basis of the structure of gluten proteins and the position of disulfide bonds,[33] it is estimated that approximately 90 and 10% of the SS bonds are intra- and intermolecular, respectively. Thus, β-elimination in gluten only occasionally involves an intermolecular SS bond. Note that when β-elimination of an intermolecular SS bond is followed by formation of an intermolecular non-SS bond, no additional cross-link has been formed. In contrast, native BSA lacks intermolecular SS bonds, but contains 17 intramolecular SS bonds (equivalent to 258 μmol/g protein). Hence, β-elimination of cystine in BSA consistently cleaves an intramolecular SS bond. For the two reasons given above, it is hypothesized that β-elimination of cystine in BSA more often results in the transformation of an intramolecular SS bond to intermolecular LAN or LAL than in gluten.

Heat-Induced Changes in Gluten–BSA Mixtures. The extractable proteins in a gluten–BSA mixture (weight ratio 1:1) included polymeric glutenin (eluting before 7.5 min), a small portion of dimeric BSA (eluting between 7 and 7.5 min), monomeric BSA (eluting around 7.8 min), and gliadins (eluting between 8.5 and 10.5 min) (Figure 5). The extractability of a gluten–BSA mixture was 86% before heating and remained constant during heating at 50 °C. Heating at higher temperatures resulted in an exponential decrease of extractability toward a plateau, as noted for isolated gluten but not for isolated BSA. Reaction rate constants and activation energy (94 ± 18 kJ/mol) for polymerization of the gluten–BSA mixture were not significantly different from those of isolated gluten (Table 1). Thus, the kinetics of heat-induced polymerization of a mixture of gluten and BSA appear to be similar to that of isolated gluten. The dominating role of gluten implies that addition of gluten to BSA decelerates cross-linking at temperatures below 100 °C. This was also shown in the fitting of the decrease of the average extractabilities of isolated gluten and BSA as a result of heating (Table 1). Assuming that gluten and BSA would not affect each other's cross-linking behavior, the experimental reaction rate constants for gluten–BSA mixtures would approximate the calculated ones. However, at 90 °C and especially at 70 °C, the extractability of a gluten–BSA mixture decreased at a slower rate than expected on the basis of the decreases in extractability of isolated gluten and BSA.

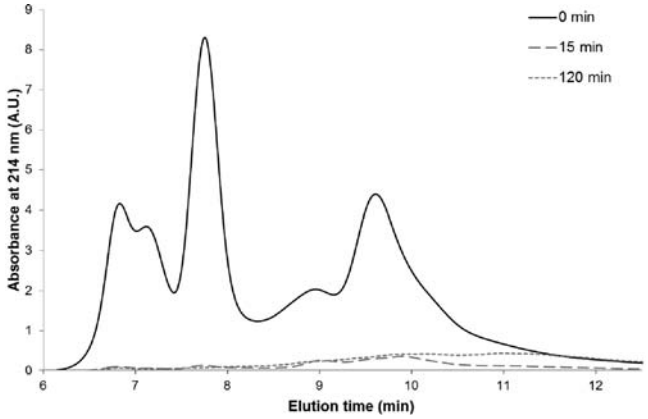

Figure 5. SE-HPLC chromatograms of a gluten–BSA mixture before and after heat treatment at pH 8.0 and 130 °C. Extraction under nonreducing conditions. A.U., arbitrary units.

The origin of the stabilizing effect that gluten has on BSA denaturation was investigated by DSC measurements. Whereas at pH 8.0 isolated BSA denatures at 56.64 ± 0.11 °C, a gluten–BSA mixture denatures at 83.69 ± 0.45 °C. Apparently, the presence of gluten delayed the denaturation of BSA, and, as a result, also its cross-linking. Stabilization of heat-induced protein denaturation by protein–protein association has been noted for interaction between substrates, enzymes, coenzymes, and inhibitors.[39−41] Minton et al.[42] demonstrated that addition of proteins resistant to denaturation by heat can stabilize other

proteins that are highly susceptible to inactivation. In the present case, gluten stabilized BSA at temperatures below 100 °C. The extractability of gluten−BSA mixtures tended toward 7 ± 3% after heating at pH 8.0 and temperatures from 70 to 130 °C. Thus, the minimal extractability level of a 1:1 (w/w) gluten−BSA mixture was about the average of those of isolated gluten and BSA. It is reasonable to assume that the protein fraction not involved in polymerization contained no BSA but only gluten proteins.

Degradation compounds were detected in SE chromatograms of gluten−BSA mixtures that had been heated for 120 min at 110 °C, or for at least 60 min at 130 °C (Figure 5). At 130 °C, the extractability of the gluten−BSA mixture increased with 0.07 ± 0.04%/min (R^2 = 0.750), which corresponds to about 4%/h. Degradation reactions in gluten−BSA mixtures occurred later (start after 45 min) than in gluten. Thus, whereas gluten affects the denaturation and cross-linking of BSA, BSA affects the degradation of gluten during severe heating.

Above 100 °C also non-SS cross-links contributed to network formation in gluten−BSA mixtures. On the basis of the results, the coexistence of BSA and gluten did not change the decrease of extractability under reducing conditions. The reaction rate constant [$(15 \pm 4) \times 10^{-3}$ $(\text{min})^{-1}$; R^2 = 0.911] for β-elimination of cystine in the gluten−BSA mixture at 130 °C was not significantly different from that in gluten or BSA. Treatment for 120 min at 130 °C produced 57 μmol LAN/g gluten−BSA. No LAL was detected (Table 2). The level of non-SS cross-links in a heated gluten−BSA mixture was not significantly different from the average of the levels of non-SS cross-links in corresponding samples of heated isolated gluten and BSA. Hence, with regard to non-SS cross-linking, no effect of gluten on BSA or vice versa was noted.

The present study of protein cross-linking and degradation during heat treatment demonstrates that gluten delays BSA denaturation and that BSA limits gluten degradation. Both observations are relevant for cereal-based foods with extra albumins such as pastry and noodles. In general, it would be interesting to investigate for a great variety of food products the impact of wheat proteins and albumins on the resulting protein network. In addition, better insight into the factors that contribute to the heat-induced network of protein mixtures would be helpful to explore new food recipes.

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Notes

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ABBREVIATIONS USED

ACN, acetonitrile; BSA, bovine serum albumin; db, dry matter basis; DHA, dehydroalanine; DSC, differential scanning calorimetry; DTT, dithiothreitol; HPAEC-IPAD, high-performance anion-exchange chromatography with integrated pulsed amperometric detection; LAL, lysinoalanine; LAN, lanthionine; PG, polymerizable gluten; SDS, sodium dodecyl sulfate; SE-HPLC, size exclusion high-performance chromatography; SS, disulfide; TFA, trifluoroacetic acid.

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