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Identification of Isopeptide Bonds in Heat-Treated Wheat Gluten Peptides

Ine Rombouts,*[,†,∥] Bert Lagrain,[†,∥] Markus Brunnbauer,[§] Peter Koehler,[§] Kristof Brijs,[†] and Jan A. Delcour[†]

[†]Laboratory of Food Chemistry and Biochemistry and Leuven Food Science and Nutrition Research Centre (LFoRCe), Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, Box 2463, B-3001 Leuven, Belgium

[§]German Research Center for Food Chemistry, Lise Meitner Strasse 34, D-85354 Freising, Germany.

[∥] These authors contributed equally to this work.

ABSTRACT: Results in this paper confirm heat-induced isopeptide bond formation in wheat gluten. Heating (24 h, 130 °C) of wheat gluten [moisture content 7.4%] decreased its extractability in sodium dodecyl sulfate containing buffer (pH 6.8), even after reduction of disulfide (SS) bonds. Thus, both SS bonds and non-SS bonds were responsible for the extractability loss. Cross-links of the lysinoalanine and lanthionine type were not present in the heated samples, but heat treatment reduced levels of available amino groups. Heating of purified and alkylated high molecular weight glutenin subunits (HMW-GS) under similar conditions also resulted in extractability loss, demonstrating that cross-linking did not solely depend on the availability of cysteine or cystine. These observations indicated that heat treatment had induced isopeptide bond formation, resulting in larger and unextractable molecules. Heating HMW-GS lysine- and glutamine-containing peptides induced the formation of isopeptide bonds, thereby supporting the above hypothesis. The level of isopeptide bond formation increased with heating time.

KEYWORDS: Isopeptide bond, wheat gluten, cross-linking, electron transfer dissociation

INTRODUCTION

Thermal polymerization of wheat gluten proteins, containing monomeric gliadin and polymeric glutenin, is essential for several cereal-based applications and occurs, for instance, during the baking phase of bread-making[1] and the drying step in pasta-making.[2] However, its exact mechanism is not fully understood. High molecular weight and limited solubility of gluten proteins complicate the study of covalent bond formation in and between the proteins. The most studied covalent cross-link in and between gluten proteins is cystine, which links two peptide chains through a disulfide (SS) bridge. This cross-link is present in native wheat gluten proteins, and oxidation of additional free sulfhydryl (SH) groups and SH−SS interchange reactions play an important role in heat-induced gluten cross-linking.[3] Other cysteine/cystine-related cross-links have also been identified in thermally treated wheat gluten. In the case of gliadin and gluten, under alkaline conditions β-elimination of cystine occurs and leads to the intermediate dehydroalanine (DHA), which can then react with cysteine or lysine to form unreducible covalent cross-links such as lanthionine (LAN) or lysinolanine (LAL), respectively.[4] Tilley and co-workers[5] and Koehler and Hanft[6] have studied the potential presence of (iso)dityrosine in wheat gluten and their concentrations. The latter concluded that (iso)dityrosine levels were too low to significantly contribute to the gluten network. Also, other cross-links can be formed. The reactive ε-amino group of lysine can, for example, be involved in the Maillard reaction, which leads to a variety of reaction products, including protein cross-links.[7] Reaction of the ε-amino group of lysine with the side-chain amide group of glutamine or asparagine results in the isopeptide bonds ε-(γ-glutamyl)lysine or ε-(β-aspartyl)lysine, respectively.[8] Glutamine is quantitatively the most important amino acid in gluten,[9] but it remains to be seen whether the formation of isopeptide bonds contributes to the gluten network. Such reaction is catalyzed by the enzyme transglutaminase (TG), but also takes place chemically at high temperatures, neutral pH, and dry conditions.[10] This type of heat-induced modification has been observed in animal proteins after severe heat treatment[11] and in proteins of roasted soybean flour.[12] There are indications for heat-induced isopeptide bonds in bread,[12] and the bond has been suggested in pasta[13] and in wheat gluten films produced for food-packaging applications.[14] However, to the best of our knowledge, ε-(γ-glutamyl)lysine has not been identified as a cross-link in thermally treated wheat gluten.

Isopeptide bonds in wheat gluten have mainly been studied after enzymic treatment with microbial TG. Gluten aggregation in the presence of TG has been demonstrated, and isopeptide bonds have been identified in TG-treated model peptides mimicking amino acid sequences of gluten proteins.[15,16] The location of TG-mediated cross-links in wheat gluten has not been elucidated. Compared to other cross-links, isopeptide bonds cannot be identified by means of conventional amino acid analysis, because the technique involves acid hydrolysis of all peptide bonds, including isopeptide bonds. They can be determined after enzymic hydrolysis, but even exhaustive enzymic hydrolysis of gluten proteins may result in underestimation or apparent absence of isopeptide bonds.[17]

Heat-induced isopeptide bond formation may well be relevant during drying processes, such as in pasta-making[13] or the industrial isolation of wheat gluten itself. Also, the thermal treatment of

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wheat flour to alter its properties and the thermomolding of wheat gluten through dry processing may well generate isopeptide bonds.[14] Nevertheless, the chemistry behind chemically induced isopeptide bond formation has not been widely studied in the context of food in general[7] and even less so in the case of gluten.

Against this background, the main objective of this study was to investigate whether heat treatment induces isopeptide bond formation in wheat gluten. Because the identification of isopeptide bonds in complete wheat gluten is complex, a step-by-step experimental approach was designed. In a first part, gluten [moisture content (mc) 7.4%] was heated for 24 h at 130 °C and isopeptide bond formation was evaluated by comparing extractabilities, amino acid compositions, levels of reactive amino groups, and levels of possible cross-links of unheated and heated gluten. To obtain additional evidence for isopeptide bond formation, high molecular weight glutenin subunits (HMW-GS) were alkylated to prevent SS cross-linking and then subjected to heat treatment. Again, extractability loss and cross-link formation were evaluated. Finally, we aimed to identify isopeptide bonds in heat-treated model peptides containing amino acid sequences naturally occurring in HMW-GS. We here report the outcome of this work.

MATERIALS AND METHODS

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

Microbial TG (Activa WM, 1.2% protein) was purchased from Ajinomoto (Hamburg, Germany). Enzyme activity, determined using Tris-HCl buffer (0.2 M) and the hydroxamate procedure,[18] was 99 units/g of enzyme preparation at pH 6.0 in the presence of 0.1% (w/v) NaCl and 110 units/g of enzyme preparation at pH 7.0. One unit catalyzes the formation of 1.0 μmol of hydroxamate/min from *N*-carbobenzoxy-Gln-Gly and hydroxylamine.

α-Chymotrypsin (from bovine pancreas, treated with N^{α}-tosyl-lysine-chloromethylketone to inactivate residual trypsin) was from Sigma-Aldrich and had an enzyme activity of 40 kilounits/g. One unit hydrolyzes 1.0 μmol of *N*-benzoyl-L-tyrosine ethyl ester/min at pH 7.8 and 25 °C.

Vital wheat gluten [74.7% protein (N × 5.7) on dry matter basis (db), mc 7.4%] was obtained from Syral (Aalst, Belgium), and HMW-GS were purified therefrom as described by Verbruggen and co-workers.[19] The procedure consisted of defatting gluten with chloroform (Acros Organics, Geel, Belgium), extracting gliadin with 50.0% (v/v) propan-1-ol, resuspending glutenin at 60 °C with 50.0% (v/v) propan-1-ol containing 1.0% dithiothreitol (DTT), and selectively precipitating HMW-GS at 4 °C by adjusting the propan-1-ol concentration of the supernatant to 60.0% (v/v). The purified HMW-GS were freeze-dried and ground in a laboratory mill (250 μm, IKA, Staufen, Germany) and contained 99.1% protein (db).

Protein and Moisture Contents. Protein contents were determined in triplicate, using an adaptation of AOAC Official Method 990.03[20] to an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands). A conversion factor of 5.7 was used to calculate protein from nitrogen content. Moisture contents were determined according to AACC-I AM 44-19.[21]

Alkylation of HMW-GS. Cysteine residues of the purified HMW-GS were alkylated with iodoacetamide (IAM; Amersham Biosciences, Diegem, Belgium) to prevent SS bond formation during heat treatment. HMW-GS (200 mg) were dissolved in 20.0 mL of Tris-HCl buffer (0.5 M, pH 8.5), containing 1.0% (w/v) sodium dodecyl sulfate (SDS; Acros Organics) and 0.1% (w/v) dithioerythritol (DTE; AppliChem, Darmstadt, Germany), and diluted to 50% (v/v) with propan-1-ol. The mixtures were shaken for 60 min at 60 °C under nitrogen atmosphere. Subsequently, 2.0 mL of a 1.5 M IAM solution (1000-fold molar excess over SH groups) was added, also under nitrogen atmosphere, and the mixture was incubated for 30 min in the dark at 37 °C. IAM was chosen as alkylating agent because the formed *S*-carbamidomethyl cysteine residues are not bulky, reducing the chance that they would interfere with cross-linking reactions during heat treatment. After dialysis in 0.1% (v/v) acetic acid, alkylated HMW-GS were freeze-dried. The obtained alkylated HMW-GS contained 99.2% protein (db).

Partial Hydrolysis and MS Analysis of Alkylated HMW-GS. To select model peptides with HMW-GS amino acid sequences, the alkylated HMW-GS were hydrolyzed and the obtained peptides sequenced. Also, control and heat-treated alkylated HMW-GS were hydrolyzed and analyzed by MS for possible isopeptide bond formation. To these ends, alkylated HMW-GS (2.0 mg) were cleaved into soluble peptides by incubating them for 24 h at 37 °C in 0.200 mL Tris-HCl buffer (0.1 M, pH 7.8) containing 8.0 units of α-chymotrypsin/mL, 2.0 M urea, and 10 mM $CaCl_2$. After chymotryptic digestion, 3 μL of TFA was added and the peptide mixture was purified by solid-phase extraction. Strata-X-C standard tubes (33 μm, polymeric strong cation sorbents, 30 mg/3 mL, Phenomenex) were first conditioned with 1.0 mL of methanol and equilibrated with 1.0 mL of 0.1% (v/v) TFA. The peptide mixture was then loaded, and the sorbent was washed with 5.0 mL of 50% (v/v) methanol. Ultimately, the purified peptides were eluted with 1.0 mL of 2.0 M ammonia, freeze-dried, and dissolved in 1.0 mL of 0.1% (v/v) formic acid. Part of the obtained peptide mixture (5 μL) was separated by RP-HPLC (UltiMate 3000, Dionex, Idstein, Germany) on an XBridge BEH 130 C_{18} column (3.5 μm, 1.0−150 mm; Waters, Taunton, MA) at 50 °C, and two elution solvents [A, water containing 0.1% (v/v) formic acid; B, acetonitrile containing 0.1% (v/v) formic acid] were used. Gradient conditions included a linear 0−25% B increase in 80 min. Peptides were subsequently analyzed by ESI-MS/MS using an ion trap MS (HCT ultra PTM Discovery System, Bruker Daltonik, Bremen, Germany). The mass spectrometer was operated in the ESI^+ mode. The parameters for the ion source were 8.0 L/min dry gas (nitrogen) and 325 °C dry temperature. The scan range was m/z 150−2000. For MS/MS collision-induced dissociation (CID) was used (collision gas, helium; fragmentation amplitude, 0.4 V). Fragmentation was executed in the AutoMS/MS mode (target mass, 900). Data were analyzed using Bruker Daltonics DataAnalysis 3.4 and Biotools 3.2 software. HMW subunits and their peptides were identified with the SwissProt 57.8 database using Mascot [taxonomy, *viridiplantae* (green plants); enzyme, chymotrypsin; fixed modification, carbamidomethyl (C); variable modification, oxidation (M)].

Synthesis of HMW-GS-Related Peptides. The glutamine- and lysine-containing peptides QQPGQG and VVPPKGG (referred to by their one-letter codes) were selected for heat treatment. The peptides were automatically assembled by solid-phase Merryfield peptide synthesis on a polymeric support (Wang resin) with protected amino acid derivatives (Fmoc amino acids, obtained from Millipore, Eschborn, Germany) using a synthesizer (433A, Applied Biosystems, Weiterstadt, Germany). The resin and all side-chain protecting groups were cleaved from the peptides (100 μmol) by incubating them with a mixture containing 10.0 mL of trifluoroacetic acid (TFA)/0.5 mL of H_20/0.5 mL of thioanisole/0.75 g of phenol/0.2 mg of DTE for 120 min. The resulting mixture was washed with 5.0 mL of dichloromethane (four times) to separate the resin from the peptide mixture. After rotary evaporation at 20 °C, the necessary volume of water was added to obtain a final peptide concentration of 1.0 mg/mL, and this mixture was extracted with *tert*-butyl methyl ether (4 × 10.0 mL) to remove the protecting groups. The deprotected peptide was then purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC; Jasco, Gross-Umstadt, Germany) on a Nucleosil C_{18} 120-5 column (EC 250/4.6, Machery-Nagel, Dueren, Germany) using the gradient conditions given in Table 1. The identity of the eluted peptides was confirmed by ESI-MS/MS using an ion trap mass spectrometer (LCQ classic, Thermo Fisher Scientific, Dreieich, Germany). The solutions

Table 1. Gradient Conditions for Preparative Reversed-Phase High-Performance Liquid Chromatography for Purifying Peptides Derived from HMW-GS[a]

	QQPGQG				VVPPKGG		
time (min)	A (%)	B (%)	C (%)	time (min)	A (%)	B (%)	C (%)
0.0	100			0.0	100		
0.2	100			0.2	100		
0.3	0		100	0.3	0		100
1.3	0		100	1.3	0		100
1.4	100			1.4	100		
5.0	100			13.0	100		
21.0	75	25		17.0	84	16	
22.0	10	90		25.0	68	32	
26.0	10	90		25.1	10	90	
27.0	100	0		27.9	10	90	
37.0	100			28.0	100		
				38.0	100		

[a] A, water containing 0.1% trifluoroacetic acid; B, acetonitrile containing 0.1% trifluoroacetic acid; C, solution containing the deprotected peptide.

containing the purified peptides were evaporated (Rotavapor R210) and freeze-dried.

Thermal and Enzyme Treatment. Thermal treatment of gluten and freeze-dried alkylated HMW-GS was done by incubating samples in sealed reaction tubes at 130 °C for 24 h. Equimolar mixtures of the purified and freeze-dried peptides from HMW-GS were heated in sealed reaction tubes at 130 °C for 1, 6, 12, and 24 h.

Isopeptide bond formation was enzymatically induced by incubating alkylated HMW-GS (50 mg) for 3 h at 37 °C in 4.0 mL of Tris-HCl buffer (0.2 M, pH 7.0) containing 0.93 unit of TG/mL. After the TG treatment, the mixture was heated for 5 min at 90 °C to inactivate the enzyme. A control sample was obtained by following the same procedure but without TG in the buffer. TG treatment of synthetic peptides derived from HMW-GS was done by incubating an equimolar mixture of VVPP-KGG and QQPGQG (5 μmol of both peptides) for 3 h at 37 °C in 300 μL of Tris-HCl buffer (0.2 M, pH 6.0) containing 0.1% (w/v) NaCl and 8.33 units of TG/mL. The enzyme was not inactivated after the treatment of the synthetic peptides to avoid side reactions.

Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). To determine the molecular weight (MW) and the extractability of gluten proteins and alkylated HMW-GS in SDS-containing media, SE-HPLC was performed as described by Lagrain and co-workers,[22] using an LC-2010 system (Shimadzu, Kyoto, Japan) with automatic injection. Protein extractability was estimated by extracting (60 min, 20 °C) freeze-dried samples [1.0 mg of protein (db)/mL] with a 50 mM sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS (further referred to as SDS buffer). The extractability of proteins in SDS-containing media under reducing conditions was determined under nitrogen atmosphere. In this case, 2.0 M urea and 1.0% (w/v) DTT were added to the SDS buffer. All analyses were performed in duplicate. After centrifugation (10 min, 11000*g*) and filtration over polyethersulfone (Millex-HP, 0.45 μm, Millipore), 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) TFA. The flow rate was 1.0 mL/min, and the column temperature was 30 °C. Protein elution was monitored at 214 nm and 30 °C. Extractability in SDS-containing media (under non-reducing and reducing conditions) of any given sample was calculated from the corresponding peak area and expressed as percentage of total extractability. The latter was taken as the peak area of the corresponding unheated sample, extracted in SDS -containing media under reducing conditions.

Amino Acid Analysis and Quantification of Cross-Links. Amino acids, including LAL and LAN, which can be formed during heat treatment, were analyzed after acid hydrolysis of gluten and HMW-GS. Separation was performed by high-performance anion-exchange chromatography with integrated pulsed amperometric detection (HPAEC-IPAD) as described by Rombouts et al.[9] Freeze-dried samples (5.0 mg of protein, db) were heated (24 h, 110 °C) in 1.0 mL of 6.0 M HCl, containing 0.1% (w/v) phenol and 1.5 mM norleucine as internal standard. Reaction mixtures were subsequently diluted (200-fold) in deionized water and filtered over polyethersulfone (Millex-GP, 0.22 μm, Millipore). Amino acids, LAL, and LAN were separated on an AminoPac PA10 column (250 × 2 mm; Dionex Benelux, Amsterdam, The Netherlands), using a Dionex BioLC system (Dionex, Sunnyvale, CA). Their levels were expressed as dry matter protein (μmol/g of protein) based on the relative peak areas of standard solutions. The LAN and LAL standards were from TCI Europe (Zwijndrecht, Belgium) and Bachem (Weil am Rhein, Germany), respectively.

Determination of Free Amino Groups. Free amino groups were determined as described by Tropini and co-workers.[23] The analysis consisted of the reaction of 2,4,6-trinitrobenzenesulfonic acid with the free amino groups, followed by hydrolysis of the peptide with 7.5 M sulfuric acid and quantification of the derivatized amino groups by measuring the absorbance at 345 nm. Free amino group levels were calculated using the molar extinction coefficient of trinitrophenyl lysine (14600 M^{-1} cm^{-1}).

MS Analysis of HMW-GS-Related Peptides. Unheated, heated, and TG-treated HMW-GS related peptides were analyzed by LC-ESI-MS/MS using (i) an ion trap MS [LCQ Classic; LC conditions: injection volume, 100 μL; column, Aqua C_{18} (5 μm, 4.6−250 mm, Phenomenex); gradient, 0−5 min, 0% B, 5−20 min, 0−100% B] and (ii) an ion trap MS with ETD technology [HCT ultra PTM Discovery System; LC conditions: injection volume, 5 μL; column, XBridge BEH 130 C_{18} (3.5 μm, 1.0−150 mm, Waters); column temperature, 50 °C; gradient, 0−30 min, 0−30% B]. The MS parameters were the same as those used for the identification of HMW subunits except for the MS/MS mode: alternating CID/electron transfer dissociation (ETD) was used [ETD parameters: reagent gas (nCI source), methane; reagent ion, fluoranthene with an ion charge control for the ion trap of 600000 (accumulation time, 8−12 ms)].

RESULTS AND DISCUSSION

Heat Treatment of Dry Gluten. Unheated and heated (130 °C, 24 h) gluten samples were extracted with SDS-containing media under nonreducing and reducing conditions and analyzed by SE-HPLC (Figure 1). Heat treatment reduced the extractability in SDS-containing media under nonreducing conditions from 90.0 to 16.2% (Figure 1a), whether it decreased the extractability under reducing conditions from 100.0 to 59.5% (Figure 1b). Reduction of SS bonds increased the extractability of unheated gluten, but even more that of heated gluten, indicating that SS bonds contributed to the extractability loss during heating. However, reduction of SS bonds did not completely restore the extractability of heated gluten, so heat treatment was assumed to also induce the formation of non-SS bonds, which also contributed to the extractability loss during heating.

To detect the nature of the non-SS cross-links in heated gluten, amino acid compositions of unheated and heated gluten samples were compared following hydrolysis. Under such conditions, heat treatment significantly affected only lysine. The lysine level was reduced to 72% of the initial value, that is, from 136 (±5) to 98 (±4) μmol/g. It should be noted that the amino acid analysis consisted of acid hydrolysis, which cleaves all peptide, including isopeptide, bonds, followed by chromatographic

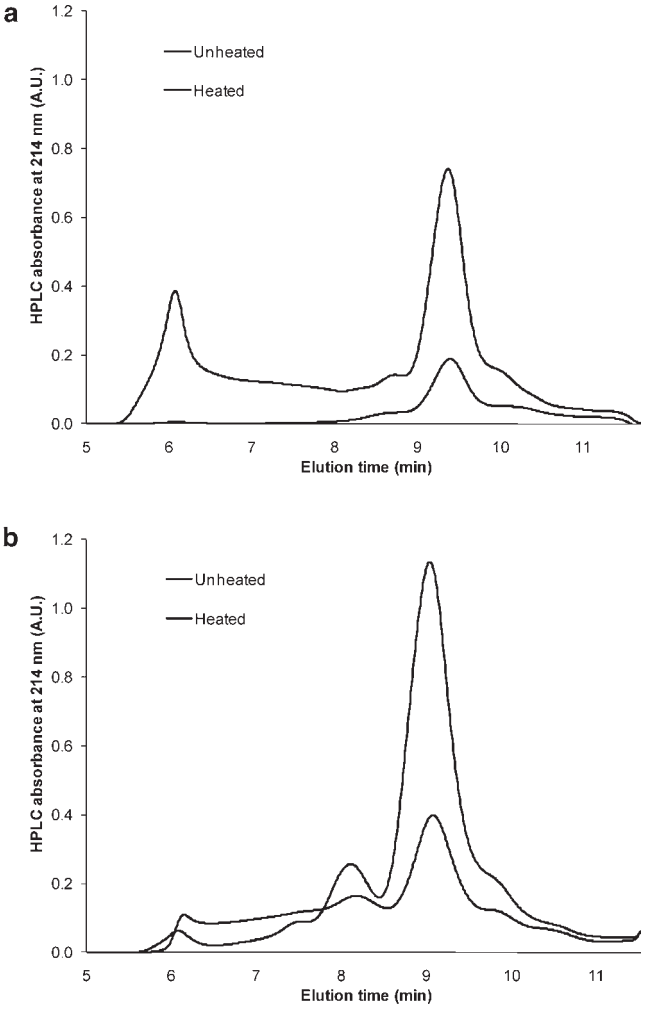

Figure 1. SE-HPLC profiles of unheated and heated (130 °C, 24 h) gluten samples, extracted with SDS buffer under (a) nonreducing conditions and (b) reducing conditions. A.U., arbitrary units.

quantification of the liberated amino acids. Thus, the decreasing lysine level cannot be explained by isopeptide bond formation and must have resulted from other heat-induced reactions. Although the presence of (iso)dityrosine bonds in heated gluten has been reported,[5] the tyrosine level did not significantly decrease (paired *t* test, two tailed, $P > 0.05$). It was also checked whether the heated gluten sample contained cross-links, such as LAN and LAL, because previous studies demonstrated their formation during hydrothermal treatment of gliadin and gluten.[4] No such cross-links were detected, possibly because the experimental conditions, more specifically the low water availability, do not favor the formation of LAN and LAL. Unheated gluten contained 95 (±8) μmol/g available amino groups, mainly consisting of ε-amino groups of lysine, and, to a lesser extent, N-terminal α-amino groups. Heat treatment reduced the level of available amino groups to 19% of the initial value, that is, to 18 (±1) μmol/g. Thus, the reactive ε-amino groups were involved in heat-induced reactions. As the loss of available amino groups (77 μmol/g of protein) was greater than that of lysine estimated following acid hydrolysis (38 μmol/g of protein), decreasing levels of amino groups were most likely the result of isopeptide bond formation in combination with Maillard and/or other heat-induced reactions. During 24 h of heat treatment of gluten at 130 °C, 39 μmol of lysine/g of protein is consumed but can be recovered by acid hydrolysis, so it is estimated that 39 μmol of isopeptide/g of protein is formed.

Table 2. Levels (Micromoles per Gram of Protein) of Amino Acids, *S*-Carboxymethyl Cysteine (S-CMC), Lanthionine (LAN), and Lysinoalanine (LAL) in Unheated and Heated (24 h, 130 °C) Alkyated High Molecular Weight Glutenin Subunits

	unheated	heated
Ala	235 ± 8	244 ± 8
S-CMC	47 ± 3	46 ± 4
Cys	0	0
His	124 ± 4	121 ± 1
Ile	121 ± 5	122 ± 4
LAL	0	0
LAN	0	0
Leu	389 ± 17	389 ± 2
Lys	110 ± 6	105 ± 4
Met	35 ± 3	35 ± 0
Phe	93 ± 5	93 ± 3
Pro	975 ± 31	1022 ± 34
Ser	466 ± 19	470 ± 8
Thr	258 ± 15	262 ± 1
Tyr	303 ± 2	314 ± 10
Val	180 ± 7	183 ± 2

Although all of the above experiments supported the hypothesis of isopeptide bond formation during heat treatment, by their own nature they were not able to directly detect this type of cross-link. To establish the nature of the non-SS bonds and to verify or deny the hypothesis of isopeptide bond formation, a model approach based on HMW-GS, which are generally richer in lysine than other gluten protein types, was used.[24]

Heat Treatment of Alkylated HMW-GS. To prevent SS bond formation during heat treatment, cysteine side chains in HMW-GS, isolated under reducing conditions, were alkylated to *S*-carbamidomethyl cysteine. Amino acid analysis of alkylated HMW-GS yielded neither cysteine nor *S*-carbamidomethyl cysteine but 47 (±3) μmol of *S*-carboxymethyl cysteine/g of protein (Table 2). Indeed, during acid hydrolysis, *S*-carbamidomethyl cysteine is converted into *S*-carboxymethyl cysteine.[25]

Unheated and heated alkylated HMW-GS were extracted with SDS buffer and separated on the basis of molecular size using SE-HPLC (Figure 2a). Identical chromatograms of the alkylated HMW-GS, both before and after heating, were obtained under nonreducing and reducing conditions, which confirms the absence of SS bonds in the alkylated HMW-GS. Heat treatment reduced SDS extractability of the alkylated HMW-GS to 51.5% of the initial value. This indicated the formation of non-SS bonds. Following acid hydrolysis, all amino acid levels appeared to remain constant during the heat treatment and no cross-links such as LAN and LAL were detected in the heat-treated alkylated HMW-GS (Table 2), which, considered together with the reduced extractability in SDS containing media, supports the hypothesis of isopeptide bond formation. Also, similar observations were made for the TG-treated alkylated HMW-GS: the extractability decreased as a result of the enzyme treatment (Figure 2b), whereas, following acid hydrolysis, an unchanged amino acid composition was noted (results not shown). In a next step, unheated,

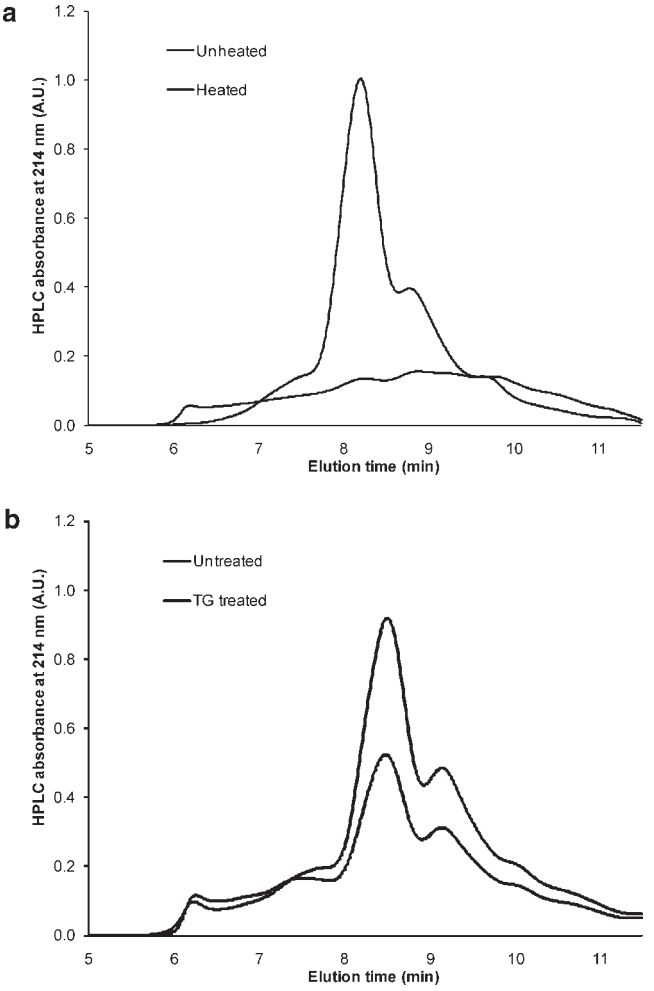

Figure 2. SE-HPLC profiles of unheated, heated (130 °C, 24 h) (a), and TG-treated (b) alkylated HMW-GS samples, extracted with SDS buffer. A.U., arbitrary units.

TG-treated, and heated alkylated HMW-GS were enzymatically digested with α-chymotrypsin, and peptides were separated by RP-HPLC and analyzed by MS/MS. However, the obtained peptide mixtures were very complex, so the MS/MS results did not provide solid proof for isopeptide bond formation.

In general, similar observations were made for heat-treated gluten and HMW-GS, except for the following. In purified HMW-GS samples, lysine levels following acid hydrolysis were unaffected by heat treatment, but in gluten, they decreased as a result of the treatment. This can be explained by the fact that the gluten samples contain more nonprotein material than the HMW-GS sample, so lysine in gluten can be involved in several reactions with nonprotein material, which is hardly the case for alkylated HMW-GS.

Heat Treatment of HMW-GS Related Peptides. Several glutamine- and lysine-containing peptides were successfully identified after comparison of the MS data of the alkylated HMW-GS with the SwissProt 57.8 database. A very common glutamine-containing sequence from the repetitive domain (QQPGQG) and a lysine-containing peptide present in the 1Dx5, 1Dy10, and 1Dy12 HMW-GS (VVPPKGG) were selected as target sequences to study isopeptide formation during thermal and enzyme treatment within amino acid sequences naturally occurring in HMW-GS. The untreated, heated, and TG-treated peptide mixtures

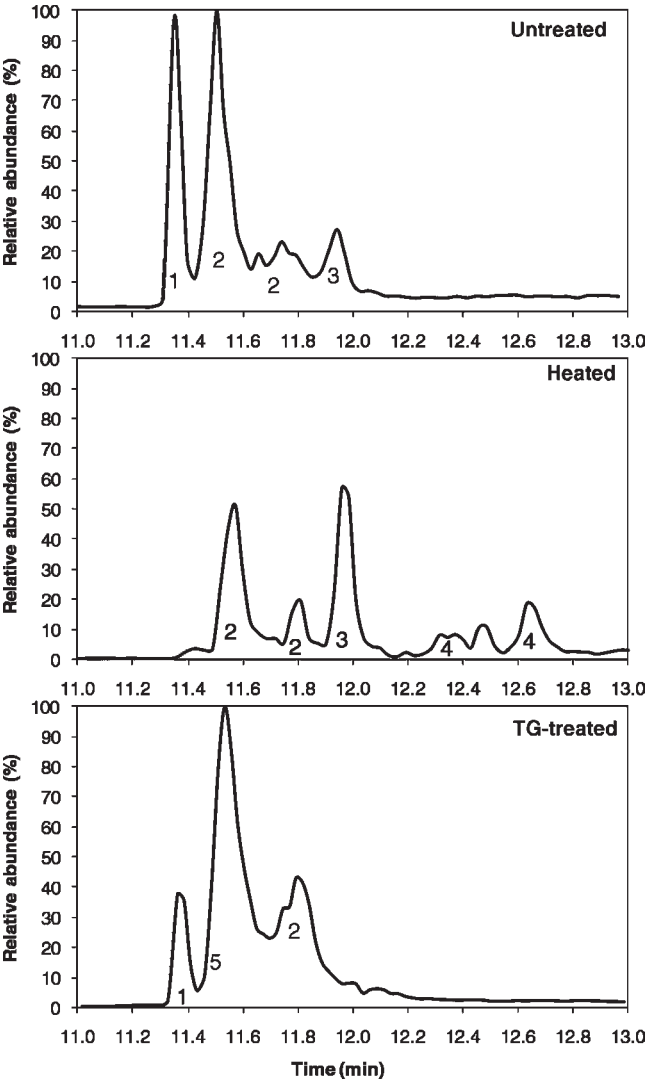

Figure 3. Total ion chromatogram (TIC) of an equimolar mixture of untreated, heated (24 h, 130 °C), and TG-treated (3 h, 37 °C, 0.5 unit of TG/μmol) peptides. The identified compounds are illustrated by numbers 1−5: 1, QQPGQG (m/z 614); 2, VVPPKGG (m/z 653); 3, pEQPGQG (m/z 597); 4, isopeptide-linked dipeptide 2 + 3 (m/z 1232); 5, isopeptide-linked dipeptide 1 + 2 (m/z 1249).

were separated by RP-HPLC and analyzed by ESI-MS/MS using an LCQ Classic MS (Figure 3). In the unheated peptide mixture, both QQPGQG [mass-to-charge ratio (m/z) 614] and VVPPKGG (m/z 653) were identified. Additionally, a compound with m/z 597 was found, which appeared to be QQPGQG after cyclization of the N-terminal glutamine residue to pyroglutamic acid (pE, pyrrolidone carboxylic acid). N-Terminal glutamine or glutamic acid can cyclize to pyroglutamic acid during the later stages of biosynthesis (at the final phases of translation) or as a post-translational event.[26] In the heated peptide mixture, pEQPGQG but no QQPGQG was found, so dry heating (130 °C, 24 h) resulted in complete cyclization of the N-terminal glutamine to pyroglutamic acid. Studies on the formation of pyroglutamic acid in monoclonal antibodies have shown the cyclization reaction to highly depend on temperature.[27,28] Also, an extra compound (m/z 1232) was found in the heated peptide mixture, with a MW corresponding to the sum of the masses of pEQPGQG and VVPPKGG, minus that of one NH_3 group. That one

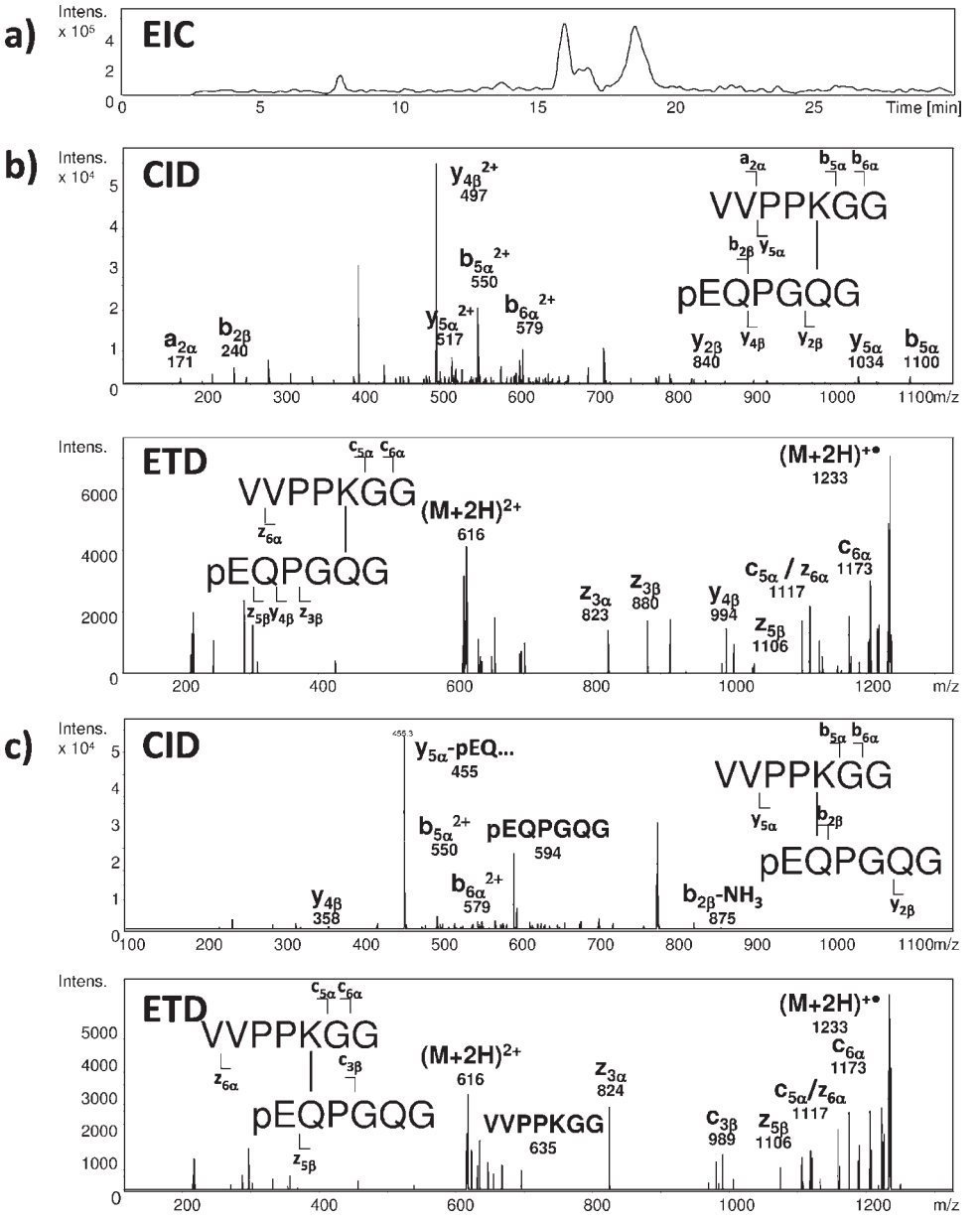

Figure 4. LC-MS/MS profile of an equimolar mixture of peptides derived from HMW-GS (VVPPKGG and QQPGQG) after heat treatment (24 h, 130 °C): extracted ion chromatogram (EIC) of m/z 616 (a); MS/MS profiles (CID, collision-induced dissociation; ETD, electron transfer dissociation) of the compounds eluting at 15.9 min (b) and 18.7 min (c).

isopeptide linkage between both peptides had been formed was confirmed by analyzing the MS/MS fragmentation pattern (data not shown), because the MW of different fragments were in agreement with the theoretically expected MW. In the TG-treated peptide mixture, three compounds were identified: QQPGQG, VVPPKGG, and both peptides linked by an isopeptide bond (m/z 1249). No cross-linked homodimeric peptides were found in any mixture.

To identify the structure of the heat-induced isopeptide bonds, the unheated and heated peptide mixtures were also analyzed by LC-ESI-MS/MS using an HCT ultra PTM Discovery System. Two compounds not appearing in the unheated sample and eluting after 15.9 and 18.7 min were found. For both compounds, the full-scan MS spectra provided m/z 616 with the ions being double charged (Figure 4a). The precursor ions were subjected to fragmentation by MS/MS, and by means of alternating fragmentation by CID and ETD, isopeptide cross-links between pEQPGQG and VVPPKGG could be identified. CID and ETD provided complementary information, with CID yielding mainly b- and y-fragments and ETD yielding mainly c- and z-fragments. For the compound eluting at 15.9 min, the $z_{3\beta}$-ion (m/z 880) in the ETD spectrum was most characteristic (Figure 4b) because it represented the isopeptide-linked fragment containing the Q residue in position 5 of the sequence pEQPGQG. This means that the peptide that eluted at 15.9 min was a heterodimer of the starting peptides pEQPGQG and VVPPKGG linked via an isopeptide bond between the Q residue in position 5 of the first peptide and the K residue in position 5 of the second peptide. The compound that eluted after 18.7 min was also a heterodimer. However, the isopeptide bond involved the Q residue in position

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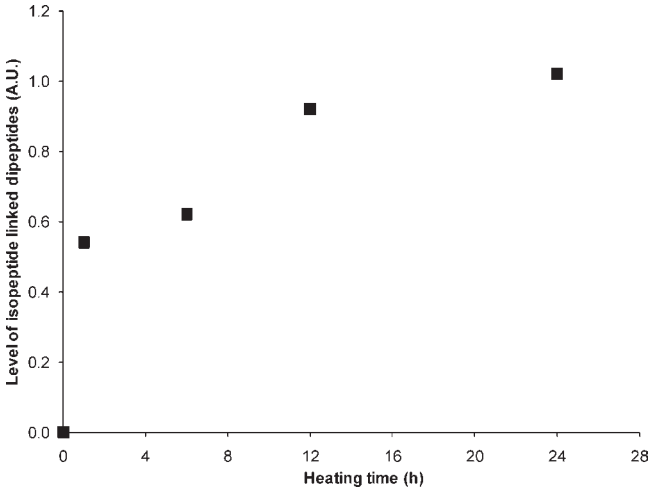

Figure 5. Levels of isopeptide-linked dipeptides in arbitrary units (A.U.) as a function of heating time at 130 °C.

2 of the first peptide and the K residue in position 5 of the second peptide. In this case, the characteristic $c_{3\beta}$-fragment (m/z 989) provided the information to unambiguously locate the cross-link (Figure 4c). Not only did fragmentation yield fragments containing the intact isopeptide bond, but CID allowed identification of two fragments, which were formed by cleavage of the isopeptide bond (m/z 455 and 595) present in the heterodimeric precursor ion with m/z 616 (double charged). It is thus concluded that during dry heating at 130 °C for 24 h both glutamine residues in pEQPGQG react with the lysine residue of VVPPKGG to form an isopeptide-linked heterodimeric peptide.

To study formation of the isopeptide cross-link as a function of the heating time, the peptide mixture was additionally heated for shorter times (1, 6, and 12 h). Again, peptide mixtures were separated by RP-HPLC, and the peak extinction areas of both heterodimers at 214 nm were recorded. The areas of the peaks of the isopeptide-linked peptides as determined by detection at 214 nm increased with heating time (Figure 5).

This paper supports the hypothesis of heat-induced isopeptide bond formation in wheat gluten and is the first to establish isopeptide bonds in heated wheat gluten peptides. Gluten cross-linking can thus occur without involvement of cysteine or cystine residues. The experimental setup included severe heating conditions (130 °C, 24 h). However, isopeptide bonds were also identified in peptides from HMW-GS after heating at 130 °C for 1 h. It remains open whether isopeptide bond formation occurs at lower temperatures. Isopeptide bond formation has been suggested to occur during the drying step of pasta[13] and the production of biodegradable packaging materials, for example, in the casting of wheat gluten films from acidic and basic solutions.[14] However, a clear view of the impact of isopeptide bond formation for such applications requires the development of methods for accurately quantifying isopeptide bonds in wheat gluten. Hence, further research is necessary to evaluate the importance of isopeptide bonds for gluten-based applications.

ABBREVIATIONS USED

CID, collision-induced dissociation; db, dry matter basis; DHA, dehydroalanine; DTE, dithioerythritol; DTT, dithiothreitol; ETD, electron transfer dissociation; HMW-GS, high molecular weight glutenin subunits; HPAEC-IPAD, high-performance anion-exchange chromatography with integrated pulsed amperometric detection; IAM, iodoacetamide; LAL, lysinolanine; LAN, lanthionine; LC-ESI-MS, liquid chromatography−electrospray ionization mass spectrometry; m/z, mass-to-charge ratio; mc, moisture content; MW, molecular weight; pE, pyrrolidone carboxylic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; S-CMC, *S*-carboxymethyl cysteine; SDS, sodium dodecyl sulfate; SE-HPLC, size-exclusion high-performance liquid chromatography; SH, free sulfhydryl; SS, disulfide; TFA, trifluoroacetic acid; TG, transglutaminase.

AUTHOR INFORMATION

Corresponding Author

*Phone: (+32)-16-321634; fax: (+32)-16-321997; e-mail: Ine.Rombouts@biw.kuleuven.be.

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REFERENCES

(1) Lagrain, B.; Thewissen, B. G.; Brijs, K.; Delcour, J. A. Impact of redox agents on the extractability of gluten proteins during bread making. *J. Agric. Food Chem.* **2007**, *55*, 5320–5325.

(2) Cubadda, R. E.; Carcea, M.; Marconi, E.; Trivisonno, M. C. Influence of gluten proteins and drying temperature on the cooking quality of durum wheat pasta. *Cereal Chem.* **2007**, *84*, 48–55.

(3) Lagrain, B.; Thewissen, B. G.; Brijs, K.; Delcour, J. A. Mechanism of gliadin−glutenin cross-linking during hydrothermal treatment. *Food Chem.* **2008**, *107*, 753–760.

(4) Rombouts, I.; Lagrain, B.; Brijs, K.; Delcour, J. A. β-Elimination reactions and formation of covalent cross-links in gliadin during heating at alkaline pH. *J. Cereal Sci.* **2010**, *52*, 362–367.

(5) Tilley, K. A.; Benjamin, R. E.; Bagorogoza, K. E.; Okot-Kotber, B. M.; Prakash, O.; Kwen, H. Tyrosine cross-links: molecular basis of gluten structure and function. *J. Agric. Food Chem.* **2001**, *49*, 2627–2632.

(6) Hanft, F.; Koehler, P. Quantitation of dityrosine in wheat flour and dough by liquid chromatography−tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 2418–2423.

(7) Gerrard, J. A. Protein-protein crosslinking in food: methods, consequences, applications. *Trends Food Sci. Technol.* **2002**, *13*, 391–399.

(8) Asquith, R. S.; Otterbur, M.; Sinclair, W. J. Isopeptide crosslinks − their occurrence and importance in protein structure. *Angew. Chem. Int. Ed. Engl.* **1974**, *13*, 514–520.

(9) Rombouts, I.; Lamberts, L.; Celus, I.; Lagrain, B.; Brijs, K.; Delcour, J. A. Wheat gluten amino acid composition analysis by high-performance anion-exchange chromatography with integrated pulsed amperometric detection. *J. Chromatogr., A* **2009**, *1216*, 5557–5562.

(10) Belitz, H. D.; Grosch, W.; Schieberle, P. Amino acids, peptides, proteins. In *Food Chemistry*, 4th ed.; Belitz, H. D., Grosch, W., Schieberle, P., Eds.; Springer-Verlag: Berlin, Germany, 2009; pp 8−92.

(11) Hurrell, R. F.; Carpenter, K. J.; Sinclair, W. J.; Otterburn, M. S.; Asquith, R. S. Mechanisms of heat damage in proteins. 7. Significance

of lysine-containing isopeptides and of lanthionine in heated proteins. *Br. J. Nutr.* **1976**, *35*, 383–395.

(12) Sakamoto, H.; Kumazawa, Y.; Kawajiri, H.; Motoki, M. ε-(γ-Glutamyl)lysine cross-link distribution in foods as determined by improved method. *J. Food Sci.* **1995**, *60*, 416–419.

(13) Petitot, M.; Brossard, C.; Barron, C.; Larre, C.; Morel, M. H.; Micard, V. Modification of pasta structure induced by high drying temperatures. Effects on the in vitro digestibility of protein and starch fractions and the potential allergenicity of protein hydrolysates. *Food Chem.* **2009**, *116*, 401–412.

(14) Olabarrieta, I.; Cho, S. W.; Gallstedt, M.; Sarasu, J. R.; Johansson, E.; Hedenqvist, M. S. Aging properties of films of plasticized vital wheat gluten cast from acidic and basic solutions. *Biomacromolecules* **2006**, *7*, 1657–1664.

(15) Bauer, N.; Koehler, P.; Wieser, H.; Schieberle, P. Studies on effects of microbial transglutaminase on gluten proteins of wheat. I. Biochemical analysis. *Cereal Chem.* **2003**, *80*, 781–786.

(16) Bauer, N.; Koehler, P.; Wieser, H.; Schieberle, P. Studies on effects of microbial transglutaminase on gluten proteins of wheat. II. Rheological properties. *Cereal Chem.* **2003**, *80*, 787–790.

(17) Schafer, C.; Schott, M.; Brandl, F.; Neidhart, S.; Carle, R. Identification and quantification of ε-(γ-glutamyl)lysine in digests of enzymatically cross-linked leguminous proteins by high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS). *J. Agric. Food Chem.* **2005**, *53*, 2830–2837.

(18) Folk, J. E.; Cole, P. W. Mechanism of action of guinea pig liver transglutaminase. I. Purification and properties of enzyme − identification of a functional cysteine essential for activity. *J. Biol. Chem.* **1966**, *241*, 5518.

(19) Verbruggen, I. M.; Veraverbeke, W. S.; Vandamme, A.; Delcour, J. A. Simultaneous isolation of wheat high molecular weight and low molecular weight glutenin subunits. *J. Cereal Sci.* **1998**, *28*, 25–32.

(20) AOAC. *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Washington, DC, 1995.

(21) AACC. *Approved Methods of the American Association of Cereal Chemists*, 10th ed.; AACC International:: St. Paul, MN, 2000.

(22) Lagrain, B.; Brijs, K.; Veraverbeke, W. S.; Delcour, J. A. The impact of heating and cooling on the physico-chemical properties of wheat gluten−water suspensions. *J. Cereal Sci.* **2005**, *42*, 327–333.

(23) Tropini, V.; Lens, J. P.; Mulder, W. J.; Silvestre, F. Wheat gluten films cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide. *Ind. Crop Prod.* **2004**, *20*, 281–289.

(24) Belitz, H. D.; Grosch, W.; Schieberle, P. Cereals and cereal products. In *Food Chemistry*, 4th ed.; Belitz, H. D., Grosch, W., Schieberle, P., Eds.; Springer-Verlag: Berlin, Germany, 2009; pp 670−745.

(25) Lees, M. B.; Leston, J. A.; Marfey, P. Carboxymethylation of sulphydryl groups in proteolipids. *J. Neurochem.* **1969**, *16*, 1025.

(26) Abraham, G. N.; Podell, D. N. Pyroglutamic acid. *Mol. Cell. Biochem.* **1981**, *38*, 181–190.

(27) Chelius, D.; Jing, K.; Lueras, A.; Rehder, D. S.; Dillon, T. M.; Vizel, A.; Rajan, R. S.; Li, T. S.; Treuheit, M. J.; Bondarenko, P. V. Formation of pyroglutamic acid from N-terminal glutamic acid in immunoglobulin γ antibodies. *Anal. Chem.* **2006**, *78*, 2370–2376.

(28) Dick, L. W.; Kim, C.; Qiu, D. F.; Cheng, K. C. Determination of the origin of the N-terminal pyro-glutamate variation in monoclonal antibodies using model peptides. *Biotechnol. Bioeng.* **2007**, *97*, 544–553.

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