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Kinetics of Heat-Induced Polymerization of Gliadin

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ABSTRACT: The kinetics of heat-induced polymerization of gliadin, that is, a mixture of monomeric wheat storage proteins, was studied using a model system. Samples were heated at pH 6.0 and 8.0 at 110, 120, and 130 °C for up to 240 min, and their extractabilities were compared under nonreducing and reducing (with 1% dithiothreitol) conditions. Extraction media were sodium dodecyl sulfate (SDS) containing buffer (pH 6.8, SDS buffer) and/or 70% ethanol. Gliadin cross-linking mainly resulted from intermolecular disulfide (SS) bond formation. At higher temperatures and, preferably, alkaline pH, intramolecular SS bonds in gliadin underwent β-elimination reactions, leading to the formation of dehydroalanine (DHA) and free sulfhydryl (SH) groups. The latter interchanged rapidly with SS bonds, leading to intermolecular SS bonds and gliadin extractability loss. When free SH groups had been formed, gliadin extractability in SDS buffer decreased following first-order reaction kinetics, the reaction rate constant of which increased with temperature and pH. Furthermore, the extractabilities of α- and γ-gliadin in 70% ethanol decreased according to first-order reaction kinetics. ω-Gliadin extractability was much less affected. Under the experimental conditions, gliadin polymerization through SH−SS interchange occurred much more rapidly than β-elimination of cystine.

KEYWORDS: gluten, cross-links, β-elimination reaction, sulfhydryl−disulfide interchange reaction

INTRODUCTION

The storage or gluten proteins of wheat consist of monomeric gliadin and polymeric glutenin. Because of its viscoelastic properties, gluten has a key function in the structure and texture of different wheat-based food products, such as bread, pasta, and pastry. Furthermore, wheat gluten and its fractions have a large potential for use in nonfood applications such as adhesives, coatings, and thermoplastic materials.[1−3] In the processing and setting of gluten-containing products, temperature has a very important role. Heat treatment of gluten leads to large protein aggregates as a result of further glutenin polymerization and formation of gliadin−glutenin bonds. Besides high temperatures, also alkaline conditions and/or mechanical energy input strongly contribute to protein cross-linking.[4,5] Gluten network formation has been mainly attributed to the formation of intermolecular disulfide (SS) bonds by oxidation of sulfhydryl (SH) groups of cysteine and/or SH−SS interchange reactions.

Gliadin is mainly a mixture of α-, γ-, and ω-gliadins. Gliadins of the α- and γ-types form three and four intramolecular disulfide (SS) bonds, respectively, whereas ω-gliadin lacks cysteine residues.[6] Evidently, only the former gliadin types become involved in the gluten network following SH−SS interchange reactions initiated by free SH groups in the glutenin fraction.[7] Heat-induced SH−SS interchange reactions within gluten proteins have originally been proposed by Schofield and co-workers.[8] SH−SS interchange between glutenin and gliadin has now been clearly established not only in different model systems[7,9−11] but also in food products.[12] The reaction results in gliadin extractability loss following first-order reaction kinetics.[12] In a nonfood context, it has been demonstrated that the addition of free SH groups to purified gliadin, such as cysteine, initiates gliadin polymerization.[13] This cysteine-polymerized gliadin can be used as biodegradable film with properties similar to those of gluten or glutenin films, but with better processing properties.[13]

A recent given is that subjecting purified gliadin to alkaline pH and high temperatures also initiates gliadin extractability loss even when free SH groups are initially absent.[14] Under such conditions, β-elimination of cystine leads to the formation of dehydroalanine (DHA) and free SH groups in gliadin and in gluten in general.[14] The formed DHA residues and free SH groups can both result in cross-links. DHA residues can react with cysteine to form the DHA cross-link lanthionine (LAN). In gliadin, this occurs both at pH 8.0 and at pH 6.0.[15] At higher pH values, lysinoalanine, a cross-link formed between DHA and lysine, can also be formed in gluten.[5,14] The newly generated free SH groups can participate in oxidation or SH−SS interchange reactions leading to new intermolecular SS bonds in gluten proteins. For gliadin, it has been postulated that these SH−SS interchange reactions are responsible for the transformation from intra- to intermolecular SS bonds, leading to gliadin polymerization.[14]

Given the importance of gluten cross-linking reactions in different food and nonfood processes, it is important to know the rate and extent of the polymerization reactions. It has already been shown that β-elimination of cystine and formation of LAN occurs in conditions relevant for soft wheat food production or gluten-based bioplastics production.[15] However, the impact of the generation of free SH groups on polymerization reactions remains unclear. Purified gliadin is a good model to investigate these SH−SS related reactions. In contrast to glutenin, it contains only intramolecular SS bonds and no free SH groups. Hence, gliadin polymerization is a direct consequence of the formation of free SH groups through β-elimination of cystine.

Therefore, we set out to study the reaction kinetics of gliadin cross-linking through intermolecular SS bond formation. To that end, we monitored the extractabilities of all gliadins in SDS-containing buffer and of α-, γ-, and ω-gliadins in 70% ethanol following hydrothermal treatment of gliadin at pH 6.0 and 8.0.

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MATERIALS AND METHODS

Materials. Vital wheat gluten [77.6% protein on dry matter basis (db)] was obtained from Syral (Aalst, Belgium). Gliadin was extracted (60 min) from gluten (20.0 g) with 70% (v/v) ethanol (250 mL). After centrifugation (10 min, 10000*g*), ethanol was removed by rotary evaporation at 30 °C. The remaining mixture was freeze-dried and the gliadin material ground in a laboratory mill (250 μm, IKA, Staufen, Germany). It contained 78.0% protein on db.

Sodium dodecyl sulfate (SDS) was from Acros Organics (Geel, Belgium), dithiothreitol (DTT) was from AppliChem (Darmstadt, Germany), tetrasodium ethylenediaminetetraacetate (EDTA) was from VWR International (Leuven, Belgium), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was from Sigma-Aldrich (Steinheim, Germany). All other solvents, chemicals, and reagents were at least of analytical reagent grade and purchased from VWR International or Sigma-Aldrich.

Protein Content. Protein contents (N × 5.7) were determined in triplicate, using an adaptation of AOAC Official Method 990.03[16] to an automated Dumas protein analysis system (EAS variomax N/CN, Elt, Gouda, The Netherlands).

Hydrothermal Treatment of Gliadin. Gliadin samples (500 mg) were heated without homogenization in 5.0 mL of sodium phosphate buffer (pH 6.0 or 8.0, 50 mmol/L) in hermetically sealed reaction tubes (glass, inner Ø = 27 mm, outer Ø = 34 mm, height =100 mm) in an oil bath at 110, 120, or 130 °C. Samples were withdrawn after different heating times. To that end, the reaction tubes were immediately cooled in an ice bath to stop any further reaction. The pH, measured after heating and cooling, was unchanged. The mixtures were then freeze-dried and ground in a laboratory mill (250 μm, IKA). Their protein content ranged from 72.0 to 78.6% (db).

Size Exclusion High-Performance Liquid Chromatography (SE-HPLC). SE-HPLC was performed as described by Lagrain et al.,[17] using an LC-2010 system (Shimadzu, Kyoto, Japan) with automatic injection. To evaluate extractability in SDS buffer, gliadin samples [1.0 mg of protein (db)/mL] were shaken [60 min, room temperature (RT)] with a 50 mmol/L sodium phosphate buffer (pH 6.8) containing 2.0% SDS, further referred to as SDS buffer. To evaluate extractability in SDS buffer under reducing conditions, gliadin samples were extracted (60 min, RT) with the SDS buffer, containing 2.0 mol/L urea and 1.0% DTT. All extractions were performed in duplicate, and the resultant extracts were analyzed two times. After centrifugation (10 min, 11000*g*, 20 °C) and filtration over polyethersulfone (Millex-HP, 0.45 μm, Millipore, Carrigtwohill, Ireland), supernatants were loaded (60 μL) on a Biosep-SEC-S4000 column (Phenomenex, Torrance, CA). The eluent (flow rate = 1.0 mL/min, column temperature = 30 °C) was acetonitrile (ACN)/water (1:1, v/v) containing 0.05% (v/v) trifluoroacetic acid (TFA). Protein elution was monitored at 214 nm.[18] Gliadin extractability in SDS buffer was calculated from the peak area and expressed as percentage of the peak area of unheated gliadin, extracted under reducing conditions as outlined above.

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). Gliadin samples [5.0 mg of protein (db)/mL] were shaken (20 min, RT) with 70% ethanol in duplicate, and RP-HPLC was conducted using a Shimadzu LC-2010 system. After centrifugation (10 min, 11000*g*, 20 °C) and filtration over polyethersulfone as above, supernatants were loaded (80 μL) on a Nucleosil 300-5 C8 column (Machery-Nagel, Düren, Germany). The elution system consisted of deionized water with 0.1% (v/v) TFA (A) and ACN with 0.1% TFA (v/v) (B). Proteins were eluted (flow rate = 1.0 mL/min, column temperature = 50 °C) with a linear gradient of solvent B from 24 to 56% in 50 min and detected at 214 nm.

Gliadin types (α, γ, and ω) were distinguished on the basis of absorbance minima between specific peaks as outlined by Wieser et al.[19] Extractabilities of gliadin fractions in 70% ethanol were calculated from the corresponding peak areas and expressed as percentage of the extractabilities of the fractions of unheated gliadin.

Free SH Determination. Free SH groups were determined colorimetrically according to the method of Ellman.[20] Gliadin (1.0 mg) was suspended in 900 μL of sodium phosphate buffer (pH 6.5, 50 mM) containing 2.0% (v/v) SDS, 3.0 mol/L urea, and 1.0 mM EDTA. Mixtures were shaken (60 min, RT), DTNB reagent (0.1% w/v in sample buffer, 100 μL) was added, and the mixtures were further shaken. Exactly 45 min after the addition of DTNB reagent, the absorbance of centrifuged (5 min, 11000*g*, 20 °C) samples was determined at 412 nm. Absorbance values of a sample containing gliadin without DTNB and a sample containing DTNB without gliadin were used to correct for background absorbance of gliadin and DTNB. Absorbance values were converted to levels of free SH groups using a calibration curve with glutathione.[21]

Data Analysis. The first reaction of gliadin can be written as

$$\text{Glia} \xrightarrow{k_1} \text{Glia}' \tag{1}$$

In this reaction, the (70% ethanol or SDS buffer) extractable gliadin (Glia) undergoes β-elimination, leading to extractable gliadin, which now contains a free SH group (Glia′). The reactive SH group of Glia′ may react through a SH−SS interchange reaction with an intramolecular SS bond of Glia to form a dimer with an intermolecular disulfide bond and a new reactive SH group. This polymerization step can be repeated i times in which Glia reacts with the free SH from polymerized gliadin (Glia'_i) entities and becomes unextractable (Figure 1).

$$\text{Glia} + \text{Glia}'_i \xrightarrow{k_2} \text{Glia}'_i + 1 \quad i \geq 1 \tag{2}$$

The extractable gliadin concentration then decreases according to

$$-\frac{\mathrm{d}[\text{Glia}]}{\mathrm{d}t} = k_1[\text{Glia}] + k_2[\text{Glia}][\text{Glia}'_i] \tag{3}$$

Assuming that $k_2 \gg k_1$ as reported by Volkin and Klibanov for lysozyme [22] and that the level of reactive Glia'_i remains constant, the loss of Glia follows simple (pseudo) first-order kinetics, and eq 3 can be simplified to

$$-\frac{\mathrm{d}[\text{Glia}]}{\mathrm{d}t} = k[\text{Glia}] \tag{4}$$

and hence

$$\ln[\text{Glia}]_t = -kt + \ln[\text{Glia}]_0 \tag{5}$$

$[\text{Glia}]_0$ is the initial concentration of soluble gliadin. $[\text{Glia}]t$ is the concentration at time t, and k is the pseudo-first-order reaction rate constant (min^{-1}).

Concentrations of monomeric gliadins during hydrothermal treatment are directly proportional to their HPLC absorbance areas. Hence, the percentage of gliadin extractable in SDS buffer (see above) is used to represent extractable gliadin concentration. Concentrations of α-, γ-, and ω-gliadins are represented by their percentages of extractability in 70% ethanol as described above.[19] The rate constants for losses of gliadin extractability, their 95% confidence intervals, and the goodness of fit (R^2 value) were estimated on the basis of a linear regression analysis of the natural logarithm of the residual gliadin concentration as a function of exposure time. The temperature dependence of the reaction rate constants was determined using the Arrhenius model:

$$k = k_{\text{ref}} \exp\left[\frac{E_a}{R}\left(\frac{1}{T_{\text{ref}}} - \frac{1}{T}\right)\right] \tag{6}$$

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

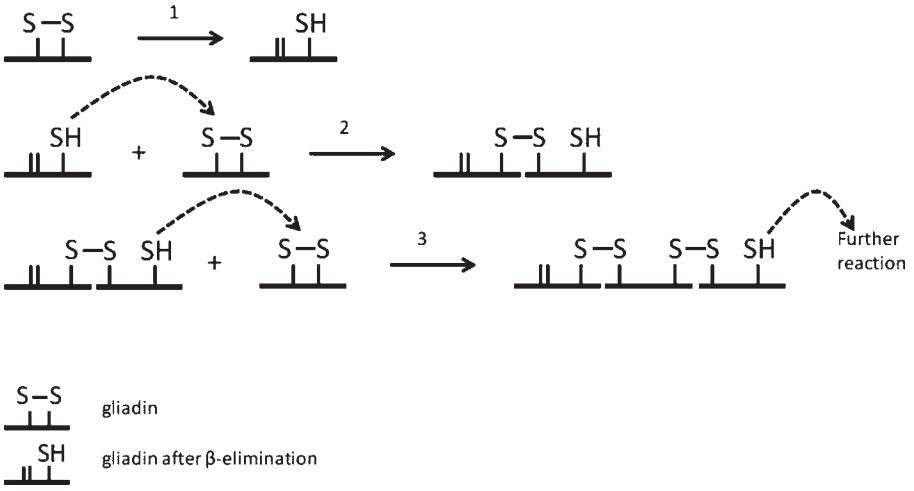

Figure 1. Model for gliadin polymerization through SH−SS interchange reactions after β-elimination of cystine. (1) Gliadin undergoes β-elimination, leading to gliadin, which now contains dehydroalanine (DHA) and a free SH group. (2) The reactive free SH group can react through a SH−SS interchange reaction with an intramolecular SS bond of another gliadin molecule to form a dimer with an intermolecular disulfide bond and a new reactive SH group. (3) This polymerization step can be repeated many times in which a gliadin molecule reacts with the free SH from polymerized gliadin and becomes attached to the gliadin polymer. In this model, the reactions of DHA are considered to be neglible when compared to SH−SS exchange reactions.

(E_a) was calculated by linear regression analysis by plotting the natural logarithm of k values as a function of the reciprocal of the absolute temperature.

RESULTS AND DISCUSSION

Gliadin SDS Extractability after Hydrothermal Treatment. Gliadin suspensions were heated at 110, 120, and 130 °C at pH 6.0 and 8.0, extracted with SDS buffer, and analyzed with SE-HPLC. Figure 2 shows the course of gliadin SDS extractabilities under these conditions. The extractability of gliadin under nonreducing conditions exponentially decreased to a minimum of about 10% during hydrothermal treatment, but then increased to >25% at the highest temperature. The observed drop of extractability in SDS buffer suggests formation of intermolecular cross-links during hydrothermal treatment, leading to unextractable gliadin polymers. The extractability increase during prolonged heating is in line with the earlier observation in gliadin elution patterns that protein hydrolysis occurred.[14] Indeed, longer heating times resulted in elution of a number of molecules at high retention times (10−12 min, results not shown).

Figure 3 shows the extractabilities of heated and reduced gliadin samples in SDS buffer with 1% DTT. DTT increased the extractabilities, which indicated the presence of intermolecular SS bonds. However, even under reducing conditions, some of the heated samples were no longer completely extractable. The extractability of gliadin, heated for 120 min at pH 8.0 and 130 °C, was only 77% after reduction with DTT. Likewise, at pH 6.0 a loss of gliadin extractability of 15% upon DTT treatment was observed at 130°C after 240 min (Figure 3A). In these cases, the presence of nonreducible covalent cross-links affected gliadin extractability even when at least all accessible SS bonds had been reduced, suggesting the formation of other than intermolecular SS bonds.

Hence, three phenomena affected gliadin extractability. First, β-elimination reactions of intramolecular cystine, which themselves did not affect gliadin extractability, led to dehydroalanine (DHA) and free SH groups. The generated free SH groups rapidly interchanged with SS bonds, causing gliadin polymerization and a drop in gliadin SDS extractability. Second, protein degradation reactions

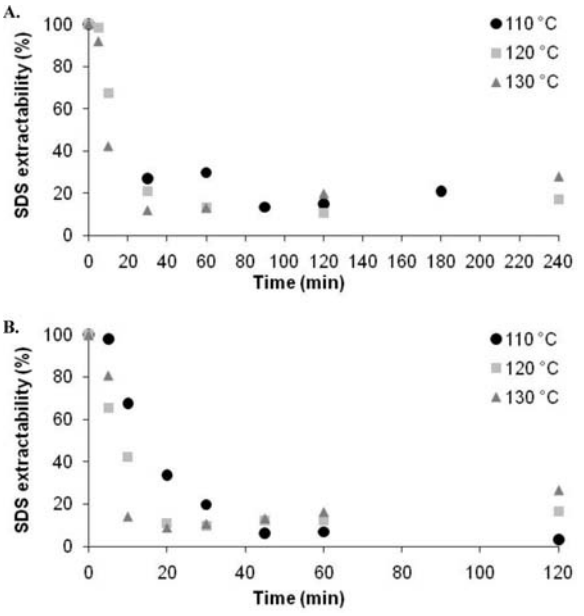

Figure 2. Gliadin extractabilities (expressed as percentage of the extractability of unheated gliadin) in SDS buffer (nonreducing conditions) after hydrothermal treatment of a gliadin model system [sodium phosphate buffer (50 mM)] at pH 6.0 (A) and pH 8.0 (B). Coefficients of variation were <10% for all data points.

during prolonged heating increased the extractability. Finally, DHA could react with cysteine to form (among other components) intermolecular LAN residues, which resulted in a loss of gliadin extractability under reducing conditions.

To determine the reaction kinetics of gliadin extractability loss through intermolecular SS bond formation, it was important that the protein extractabilities were not affected by other reactions. To that end, the calculations were performed with gliadin samples treated under conditions in which both protein degradation reactions and the formation of bonds other than SS bonds relatively occurred to a minimal degree and, hence, affected the mathematics as little as possible. We, therefore, modeled the

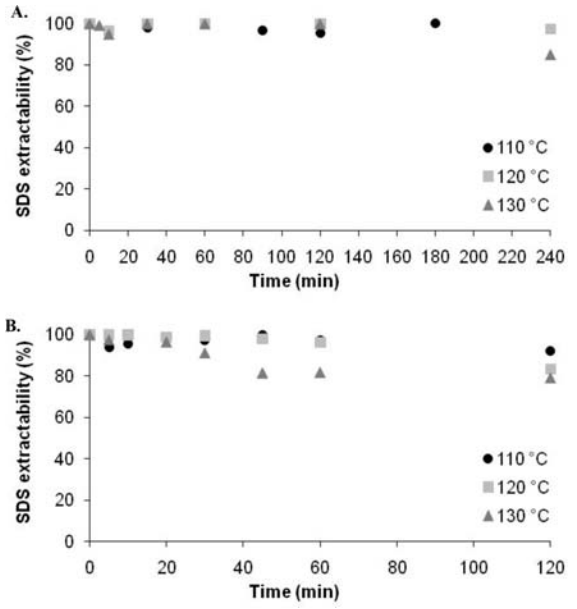

Figure 3. Gliadin extractabilities (expressed as percentage of the extractability of unheated gliadin) in SDS buffer [reducing conditions, DTT] after hydrothermal treatment of a gliadin model system [sodium phosphate buffer (50 mM)] at pH 6.0 (A) and pH 8.0 (B). Coefficients of variation were <10% for all data points.

exponential function only up to the point where the extractability in SDS buffer reached its minimum. At this point, the gliadins were still fully extractable in SDS under reducing conditions.

Figure 4 shows that the selected experimental points indeed could be described using first-order kinetics assumptions for gliadin extractability loss at pH 8.0 ($R^2 > 0.97$) and 6.0 at 110 °C ($R^2 > 0.99$). At higher temperatures and pH 6.0, the experimental data were less in line with the theoretically expected values based on first-order kinetics, leading to lower R^2 values. This discrepancy may have been due to other reactions affecting protein extractability as indicated by color changes of the gliadins, which were most obvious for the samples heated at 130 °C. Table 1 lists the estimated first-order rate constants and half-lives at 110, 120, and 130 °C. At pH 6.0, the extractability decreased less rapidly than at pH 8.0. Indeed, SH−SS interchange reactions can be initiated by a nucleophilic attack of the thiolate anion on a SS bond. Because the pK_a of cysteinyl SH groups in proteins ranges between 8.5 and 9.5 depending on the location in the structure, the reaction is most rapid at alkaline pH.[23] Figure 5 shows that E_a was lower at pH 8.0 than at pH 6.0, indicating that the polymerization reaction is more temperature sensitive at pH 6.0.

Effects on Different Gliadin Types. Figure 6 shows the extractabilities of α- and γ-gliadin in 70% ethanol following hydrothermal treatment at 110, 120, and 130 °C at pH 8.0. ω-Gliadin maximally lost half of its original extractability (results not shown). Whether this is due to conformational changes leading to a lower absorbance and/or reactions involving amino acids other than cysteine remains to be investigated. For α- and γ-gliadin extractability losses, the experimental data were elegantly fitted by first-order kinetics ($R^2 > 0.98$). Table 2 lists the estimated first-order rate constants and half-lives at the experimental temperatures. The 70% ethanol extractability losses of the individual α- and γ-gliadin fractions proceeded at a higher rate than the SDS buffer extractability loss of the entire gliadin fraction due to the slower extractability loss of ω-gliadin and/or a different extraction medium. E_a values of the

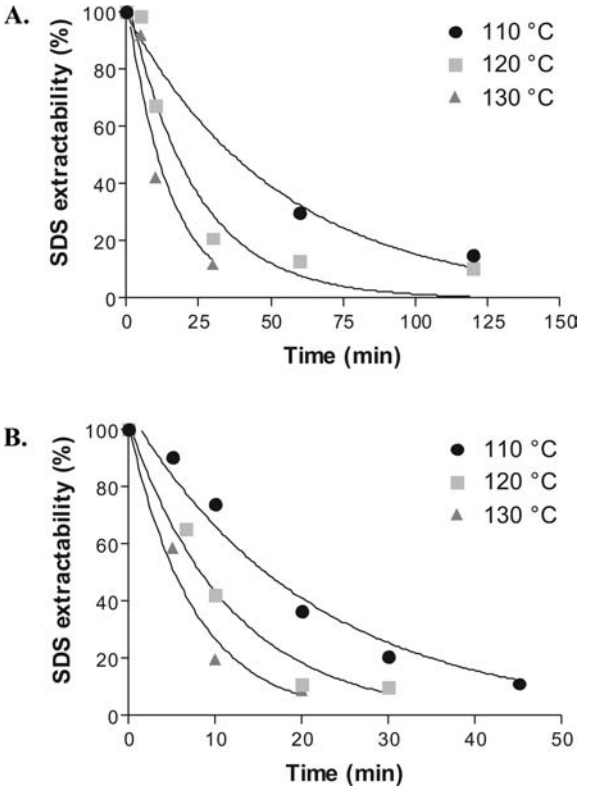

Figure 4. Time course of gliadin extractabilities (expressed as percentage of the extractability of unheated gliadin) in SDS buffer after hydrothermal treatment of a gliadin model system [sodium phosphate buffer (50 mM)] at pH 6.0 (A) and pH 8.0 (B). The full lines represent the fit that was calculated with a first-order exponential rate law, whereas the experimental data are represented by the symbols.

Table 1. Estimated Rate Constants of Loss of Gliadin Extractability in SDS Buffer with Their Respective Half-Lives and Activation Energies (E_a) for a Gliadin Model System in Sodium Phosphate Buffer (50 mM) at pH 6.0 and 8.0 for Treatments at 110, 120, and 130 °C

	pH 6.0			pH 8.0		
T (°C)	k (min^{-1})	half-life (min)	R^2	k (min^{-1})	half-life (min)	R^2
110	0.019	37.2	0.99	0.048	14.5	0.97
120	0.043	16.0	0.96	0.086	8.09	0.98
130	0.070	10.0	0.92	0.130	5.20	0.98
E_a (kJ/mol)		95.7			74.2	

polymerization of α- and γ-gliadin through SH−SS interchange reactions did not significantly differ ($P < 0.05$). Overall, it seems that α- and γ-gliadin randomly polymerized at a similar rate.

Importance of SH−SS Interchange Reactions versus β-Elimination Reactions. On the basis of reaction rate constants of gliadin extractability loss (Table 1) and of β-elimination of cystine in gliadin as calculated by Lagrain and co-workers,[15] we concluded that, under the experimental conditions, SH−SS exchange reactions proceed much more quickly than β-elimination. The latter reaction and concomitant formation of free SH are prerequisites for gliadin polymerization through SH−SS interchange. Gliadin polymerization occurred 45 times more rapidly than cystine

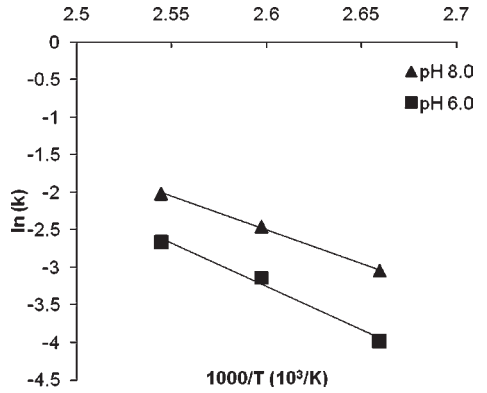

Figure 5. Effect of temperature (in K) on the rate constants (k in min^{-1}) for loss of extractability of gliadin in SDS buffer in a gliadin model system in sodium phosphate buffer (50 mM) at pH 6.0 (■) and pH 8.0 (▲).

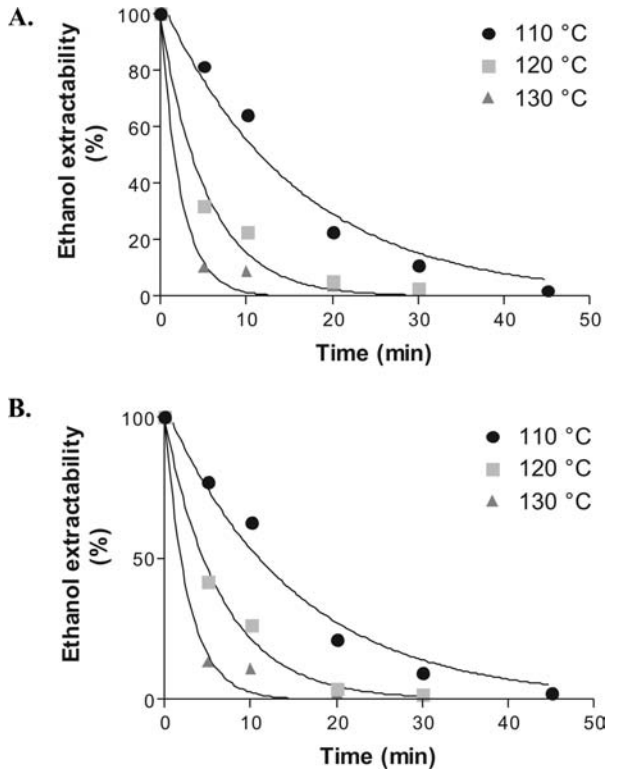

Figure 6. Time course of gliadin extractabilities in 70% ethanol (expressed as percentage of 70% ethanol extractable protein of unheated gliadin) of (A) α-gliadin and (B) γ-gliadin in a gliadin model system in sodium phosphate buffer (50 mM, pH 8.0). The full lines represent the fit calculated with a first-order exponential rate law, whereas the symbols represent the experimental data.

elimination at pH 6.0 and about 15 times more rapidly at pH 8.0. Because SH−SS interchange reactions happen quickly, they play a key role in gliadin and, more in general, in gluten cross-linking. Only at longer heating times or higher pH values did nonreducible covalent cross-links from DHA became increasingly important. It appears that some time was needed before gliadin lost its SDS extractability. This could be observed, for example, when heating gliadin at pH 8.0 and 110 °C (Figure 2B). The exponential decrease started after 5 min of heating. At this point gliadin contained 0.2 μmol of free SH groups per gram of protein. With an estimated

Table 2. Estimated Rate Constants of Loss of α- and γ-Gliadin Extractability in 70% Ethanol with Their Respective Half-Lives and Activation Energies (E_a) for a Gliadin Model System in Sodium Phosphate Buffer (50 mM) at pH 6.0 and 8.0 for Treatments at 110, 120, and 130 °C

	α-gliadin			γ-gliadin		
T (°C)	k (min^{-1})	half-life (min)	R^2	k (min^{-1})	half-life (min)	R^2
110	0.064	10.8	0.98	0.067	10.4	0.98
120	0.190	3.74	0.98	0.150	4.55	0.99
130	0.420	1.65	0.99	0.360	1.91	0.99
E_a (kJ/mol)	136			122		

average molecular weight of gliadin of 30000,[24] this means that maximally 0.6% of the gliadin molecules could contain free SH groups. A similar observation could be made when gliadin was heated at pH 6.0 and 120 °C (Figure 2A). After 5 min of heating and a level of free SH of 0.1 μmol/g protein, gliadin extractability decreased following first-order kinetics. At higher temperatures, the level of SH groups quickly reached sufficient levels to induce SH−SS interchange, apparently resulting in an immediate gliadin polymerization according to first-order reaction kinetics.

In conclusion, with free SH groups available, gliadin polymerized according to first-order reaction kinetics. The generation of free SH groups by β-elimination from cystine is crucial, because they serve as "catalysts" inducing and/or accelerating gliadin and gluten polymerization through SH−SS interchange. As such, the generation of SH groups may well be important in food systems at more alkaline pH. This could be the case in the production of, for example, pretzels or cake. The spontaneous polymerization of gliadin under certain conditions may also be important in nonfood applications.

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ABBREVIATIONS USED

ACN, acetonitrile; db, dry matter basis; DHA, dehydroalanine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, tetrasodium ethylenediamine tetraacetate disodium salt; HPLC, high-performance liquid chromatography; LAN, lanthionine; RP, reversed phase; SDS, sodium dodecyl sulfate; SE, size exclusion; SH, sulfhydryl; SS, disulfide; TFA, trifluoroacetic acid.

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