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Effect of Ascorbic Acid in Dough: Reaction of Oxidized Glutathione with Reactive Thiol Groups of Wheat Glutelin

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The reactions of oxidized glutathione generated from endogenous glutathione by the addition of ascorbic acid (AA) prior to dough mixing on free thiol groups of gluten proteins have been investigated. A small amount of ^{35}S-labeled glutathione was added as a tracer to identify the reaction products of GSSG and free protein thiols by radioactivity measurement. First, gluten was isolated from the dough, then the gliadins were extracted, and residual glutenin was partially hydrolyzed with thermolysin. After preseparation by gel permeation chromatography, the fractions with the highest radioactivity were separated by high-performance liquid chromatography. Radioactive peptides were identified, isolated, sequenced, and assigned to amino acid sequences of gluten protein components. The isolated peptides contained exclusively the cysteine residues C^{b*} and C^{x} of low molecular weight subunits of glutenin, which are supposed to be highly reactive in forming intermolecular disulfide bonds. From these results it can be assumed that the cysteine residues C^{b*} and C^{x} of the low molecular weight subunits of glutenin are at least partly present in the thiol form in flour. During dough mixing they are converted to protein−protein disulfides or glutathione−protein mixed disulfides by thiol/disulfide interchange reactions. Oxidized glutathione necessary for this reaction is generated from glutathione by the action of AA. These results are in accordance with the major hypothesis about the mechanism of action of AA.

KEYWORDS: Ascorbic acid; oxidized glutathione; reactive thiol groups; wheat; gluten; localization; thiol/disulfide interchange

INTRODUCTION

Due to its positive effects on dough properties, ascorbic acid (AA) has been used as a flour improver for a long time. For the mechanism of action, a hypothesis shown in **Table 1** has been postulated (*1*−*3*), which has been established experimentally with the exception of one reaction. It has been shown that in the initial phase of mixing, AA is quickly oxidized to dehydroascorbic acid (DHA) by ascorbate oxidase (AOX). DHA oxidizes endogenous glutathione (GSH) to its disulfide, GSSG. In this way a thiol/disulfide interchange of GSH with gluten proteins is inhibited. A depolymerization of gluten proteins corresponding to a weakening of the dough can therefore not take place, because disulfide bonds of gluten proteins are not involved in these reactions.

The enzyme-catalyzed reactions 1 and 2 of the hypothesis (**Table 1**) have already been established (*4*−*9*), and also the ability of GSH to reduce cystine (CSSC; **Table 1**, reaction 5) has been demonstrated (*1*). To get evidence about the reaction of the low molecular weight thiols GSH and CSH with disulfide bonds of gluten proteins (**Table 1**, reactions 4 and 6), doughs that were prepared with and without addition of AA were extracted by Osborne fractionation, and acid soluble and insoluble glutenins were separated (*2*). It has been demonstrated that the concentration of protein-bound glutathione (PSSG) increased during mixing. On addition of AA this effect was more pronounced and the concentration of PSSG was increased, especially in the acid-soluble glutenins. Further evidence for these suggestions was obtained by a study of Huettner and Wieser (*10*), who investigated the incorporation of ^{35}S-labeled GSH into the Osborne fractions. In the presence of AA a significantly higher portion of GSH was bound to the glutenins than in the experiment without addition of AA. This study

Table 1. Proposed Reactions To Explain the Improver Effect of AA According to the Hypothesis of Grosch (*1*–*3*)[a]

reaction	educts		products
1	AA + $^1/_2O_2$	$\xrightarrow{\text{AOX}}$	DHA + H_2O
2	DHA + 2GSH	$\xrightarrow{\text{GSH-DH}}$	AA + GSSG
3	PSH + GSSG	→	PSSG + GSH
4	GSH + PSSP	→	GSSP + PSH
5	GSH + CSSC	→	GSSC + CSH
6	CSH + PSSP	→	CSSP + PSH
7	PSH + CSSC	→	PSSC + CSH

[a] AOX, ascorbate oxidase; GSH-DH, glutathione dehydrogenase.

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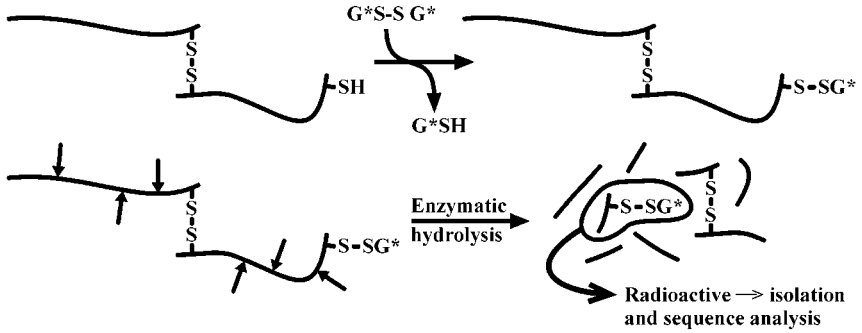

Figure 1. Schematic representation of the approach used for the evaluation of the AA improver action. [^{35}S]GSSG (G*S–SG*) is formed during mixing by reaction of added [^{35}S]GSH with DHA (**Table 1**, reaction 2).

showed that SH groups of gluten proteins that are forming PSSG on addition of AA can be selectively modified by the addition of [^{35}S]GSH (**Table 1**, reaction 3). By means of this modification it should be possible to localize these SH groups of the gluten proteins.

Evidence for the direct cleavage of interprotein disulfide bonds of gluten proteins by endogenous GSH (**Table 1**, reaction 4) was provided by Huettner and Wieser (*11*), who mixed doughs after the addition of [^{35}S]GSH. The results showed that GSH reacted specifically. Intermolecular disulfide bonds originating from low molecular weight (LMW) subunits of glutenin were preferably attacked by GSH and cleaved by thiol/disulfide interchange. Another reaction site was a disulfide bond between high molecular weight (HMW) and LMW subunits of glutenin. Intramolecular disulfide bonds of gluten proteins were, however, attacked only to a small extent (≈5%). The same reactions as for GSH could be assumed for CSH (**Table 1**, reaction 6). These considerations show that all reactions of the hypothesis about the AA improver action have been proven experimentally with the exception of the thiol/dislfide interchange of GSSG or CSSC with free thiol groups of gluten proteins (**Table 1**, reactions 3 and 7).

The present study is the second part of a series of investigations to gain further insight into the mechanism of action of AA. The aim of this part of the study was to detect products of the reaction between GSSG, generated by the addition of AA on dough mixing, and SH groups of gluten proteins and to localize those cysteine residues of the gluten proteins that are accessible for GSSG. Therefore, AA in a concentration necessary to convert a maximum amount of GSH to GSSG and a small amount of [^{35}S]GSH as a tracer were mixed into a dough, glutenin was isolated and partially hydrolyzed, and relevant peptides were identified by their radioactivity, isolated, sequenced by automated Edman degradation, and assigned to amino acid sequences of gluten protein components.

MATERIALS AND METHODS

Chemicals. [^{35}S]GSH was purchased from NEN (Köln, Germany), thermolysin from Fluka (Taufkirchen, Germany), and triethylamine from Sigma (Taufkirchen, Germany); all other chemicals were from Merck (Darmstadt, Germany). The quality was "pro analysi" or better.

Flour. Kernels of the wheat class Canadian Western Red Spring (CWRS) from the 1991 harvest were milled into flour at 14% moisture by means of a Quadrumat Junior mill (Brabender, Duisburg, Germany), sieved (∅ = 0.2 mm), and stored for a minimum of 2 weeks prior to use. The moisture of the flour was determined according to AACC method 44-19 (*12*), the ash content according to ICC method 104/1 (*13*), and the nitrogen content on an FP328 nitrogen analyzer (Leco, Moenchengladbach, Germany). The protein content of the flour was 13.0% (N × 5.7), the moisture content was 13.2%, and the ash content (dry mass) was 0.8%.

Isolation of [^{35}S]GSH for Dough Preparation. To remove dithiothreitol, which was present as an antioxidant, a solution of [^{35}S]GSH (50 μL; 3.7 × 10^6 Bq; 0.84 nmol) was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) (Beckman, Muenchen, Germany) by using the following conditions: HPLC software, Beckman System Gold; column, ODS-Hypersil (C_{18}), 5 μm, 10 nm, 250 × 4.6 mm; flow rate, 0.5 mL/min; temperature, 16 °C; detection, UV absorbance at 200 nm; solvent, 0.01 mol/L triethylamine, pH 3.5, with formic acid. The peak of [^{35}S]GSH (retention time = 10.5 min) was pooled and made up to 10 mL. Ten microliters of the solution was used for the determination of the radioactivity.

Preparation of Glutenin. Flour (9.91 g; 8.6 g of dry mass) and 0.2 g of sodium chloride were mixed with [^{35}S]GSH solution (5 mL; 1.85 × 10^6 Bq), a solution of AA (1 mL; 1.25 g of AA/L), and water (0.45 mL) in a microfarinograph (Brabender, Duisburg, Germany) at 22 °C and 60 rpm for 7.5 min to a peak consistency of 550 BU. Then the dough was removed from the farinograph and washed with 540 mL of distilled water on a Glutomatic (Perten Instruments, Huddinge, Sweden). The washing water was collected, and 400 μL was used for the determination of the radioactivity. Wet gluten was frozen with liquid nitrogen and ground in a laboratory grinder. The resulting powder was extracted three times with 60% (v/v) aqueous ethanol (14 mL, respectively; pH 5.5, with acetic acid) by means of a magnetic stirrer for 30 min at 22 °C and centrifuged (45 min, 4 °C, 17000*g*). The combined extracts were evaporated and lyophilized. The residue (glutenin) was divided into small pieces, lyophilized, and powdered in a mortar.

Enzymatic Hydrolysis of Glutenin. Glutenin was partially hydrolyzed with thermolysin as previously described (*11*). The volume of the peptide solution was reduced to 10 mL by evaporation, and 400 μL was taken for the determination of the radioactivity.

Gel Permeation Chromatography (GPC). Preseparation according to molecular size was carried out as previously described (*11*). Seven fractions (F1−F7) as outlined in **Figure 1** were collected and lyophilized.

RP-HPLC. Lyophilized fractions 2−5 of the GPC were dissolved in acetic acid (0.1 mol/L) to give concentrations of 10 mg/mL. The solutions were filtered (∅ = 0.45 μm), an aliquot (400 μL) was taken for the determination of the radioactivity, and the solutions were first separated analytically (50 μL) and then preparatively (400−500 μL, respectively) on the following HPLC system: column, ODS-Hypersil (C_{18}), 5 μm, 10 nm, 250 × 4.6 mm; flow rate, 0.8 mL/min; temperature, 60 °C; detection, UV absorbance at 220 nm; solvent A, 0.01 mol/L triethylamine, pH 3.5, with formic acid in 1% (v/v) acetonitrile; solvent B, 0.009 mol/L triethylamine, pH 3.5, with formic acid in 40% (v/v) acetonitrile; elution, isocratic 0−1 min 0% solvent B, then linear 1−51 min 0−40% solvent B and 51−91 min 40−100% solvent B. Eluted peaks were collected by a fraction collector. For each fraction of the GPC 20 subfractions were collected by HPLC (F2-1 to F2-20, F3-1 to F3-20, F4-1 to F4-20, and F5-1 to F5-20), lyophilized, and dissolved in water (400 μL), and an aliquot (400 μL) was taken for the determination of the radioactivity. The residual solutions were used for rechromatography.

The solutions of the subfractions collected in the first HPLC step were further purified by RP-HPLC. Therefore, the solvents were changed in comparison to the first HPLC: solvent A, 0.01 mol/L triethylamine, pH 6.0, with formic acid in 0.1% (v/v) acetonitrile; solvent B, 0.009 mol/L triethylamine, pH 6.0, with formic acid in 40% (v/v) acetonitrile. Several linear gradients were applied (e.g., 0−30 min, 0−30% B; and 0−30 min, 25−40% B) and adapted to give maximum resolution for the relevant peaks. Eluted peaks were collected manually, evaporated to dryness, and dissolved in water (1 mL); an aliquot was taken for the determination of the radioactivity, and the residual solutions were used for the determination of the amino acid sequence.

Determination of the Amino Acid Sequence. Peptide (10−50 pmol) was dissolved in 50% (v/v) methanol (30 μL) and applied onto a polyprene-treated glass fiber membrane, and the solvent was removed under a stream of argon. Sequence analysis was carried out by automated Edman degradation on a protein sequencer Procise 492 (Applied Biosystems, Darmstadt, Germany) running in the pulsed-liquid mode.

Nanospray Mass Spectrometry (MS). Peptide (10−50 pmol) was dissolved in 50% (v/v) methanol/1% (v/v) formic acid (10 μL) and filled into a metal-coated nanospay capillary (Protana, Odense, Denmark). The capillary was positioned in front of the heated capillary of an ion-trap mass spectrometer LQC (Finnigan MAT, Egelsbach, Germany). The capillary was opened, and a mass spectrum was recorded until the capillary was empty (~5 min) by using the following conditions: full MS mode, measurement of positively charged ions, no nebulizing gas flow, capillary voltage = 5 V, capillary temperature = 180 °C, and mass range = 150−2000.

Other Analytical Methods. Radioactivity was measured by the external standard method according to Rauschenbach and Simon (*14*). Sample solution (0.01−0.4 mL) was mixed with a scintillation cocktail [15 mL; 90 mL of 2-phenylethylamine, 125 mL of methanol, 285 mL of toluol, 2.75 g of permablend (= 2,5-diphenyloxazole/1,4-di(5′-phenyloxazol-2-yl)-benzene 91:9 (w/w)] and measured on a liquid scintillation counter LS 7800 (Beckman, Muenchen, Germany). The maximum time of measurement was 20 min to obtain a variation (2Σ) of individual values of <2%. Solid samples that did not dissolve in water (10 mg) were heated to 90 °C in hydrochloric acid (0.4 mL; 10 mol/L) for 3 h under magnetic stirring. The acid was removed under a stream of nitrogen, and the residue was dissolved in water (0.01−0.4 mL) and used for the measurement of the radioactivity. Amino acid analysis was carried out without and with performic acid oxidation as described previously (*15*) by means of an amino acid analyzer LC 3000 (Onken, Gründau, Germany).

RESULTS AND DISCUSSSION

Previous studies on the mechanism of the effect of AA revealed oxidation of GSH to GSSG on addition of AA and reaction of GSSG with free SH groups of gluten proteins (*2*, *16*, *17*). To identify the products of this reaction, a tracer experiment was performed by adding a small amount of [^{35}S]-GSH for dough mixing. A schematic representation of the approach is presented in **Figure 1**. AA was added during mixing in a concentration just high enough to convert a maximum amount of the GSH to GSSG. As determined in the first part of the study, this concentration was 125 mg of AA/kg of flour. The concentration of [^{35}S]GSH was 0.043 nmol/g of flour, whereas the concentration of endogenous GSH was 80.9 nmol/g. This means that only 0.05% of the total GSH in the dough were [^{35}S]GSH so that the rheological properties of the dough were not changed in comparison to a dough without the addition of [^{35}S]GSH. However, the amount was high enough to be detected by scintillation analysis. After the isolation of gluten with distilled water, all operations were carried out at pH values that were not higher than 6.5 to minimize thiol/disulfide interchange reactions.

Isolation of Glutenin. Starch and solubles were removed from the dough by washing on a Glutomatic. A magnetic stirrer was used for the extraction of residual gluten to inhibit the formation of artifacts by high intake of energy, for example, by an Ultraturrax. The results of the isolation of glutenin are presented in **Table 2**. Extraction of gluten gave an excess of the glutenin fraction over the gliadin fraction. Magnetic stirring during extraction was obviously not intensive enough to remove all of the gliadins from the glutenin. The protein content of the glutenin (64.3%) indicated that starch had not completely been removed during dough washing by the Glutomatic. The major portion of the radioactivity was in the soluble/starch fraction. This has already been demonstrated by Huettner and Wieser (*10*), who showed that most of the labeled GS was not covalently bound to proteins.

Table 2. Isolation of Glutenin from a Dough That Contained AA (125 mg/kg of Flour) and [^{35}S]GSH (1.85×10^6 Bq): Characterization of the Resulting Fractions

	starch/solubles	gluten	gliadin	glutenin
mass distribution (%)	76.3	11.4	4.1	8.0
protein content[a] (%)	2.2	81.7	80.0	64.3
protein distribution[b] (%)	14.3	78.2	27.7	42.9
radioactivity distribution[c] (%)	37.9	nd[d]	5.9	11.1

[a] Determined by amino acid analysis. [b] On a flour basis (protein content 11.9% as determined by amino acid analysis). [c] Based on initial activity (1.85×10^6 Bq). [d] nd, not determined.

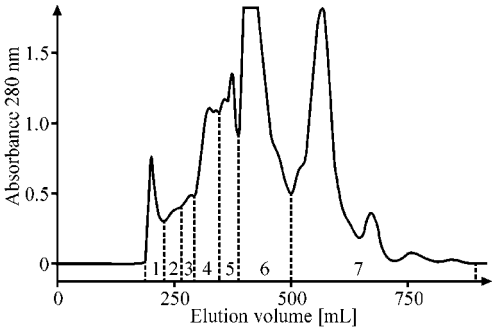

Figure 2. Preseparation of a thermolytic digest of glutenin by GPC on Sephadex G25 fine. Fractions F1–F7 are marked 1–7.

Partial Hydrolysis of Glutenin. Studies on disulfide bonds of gluten proteins have shown that thermolysin is well suited for partial hydrolysis of glutenin (*18*). In contrast to many other proteases, the crucial property of thermolysin is its ability to hydrolyze a high percentage of the theoretical cleavage sites even in highly bridged or insoluble proteins, for example, glutenin (*11*, *20*). An enzyme concentration of 1% (w/w) corresponding to 0.94 unit/mg of protein was used for hydrolysis. A high percentage of the glutenin (83.3%) was converted to soluble peptides on digestion with thermolysin, and 16.7% of the glutenin remained nondissolved. However, the partial hydrolysate represented the glutenin to a very high extent. The peptides generated in this step contained the reaction products of protein thiols and GSSG as mixed disulfides. The ^{35}S tracer, which was present in the mixed disulfides, enabled identification by scintillation analysis.

GPC. Due to the extremely complex composition of the thermolytic digest, preseparation by GPC on Sephadex G25 (separation range of 1000−5000) had to be performed. A typical chromatogram is presented in **Figure 2**. The eluate was divided into seven fractions. The yield of the GPC was 82.5% on a mass basis and 89.2% on a protein basis. The results of the separation are shown in **Table 3**. The major portion of protein

Table 3. Results of the GPC Separation of the Thermolytic Digest of Glutenin on Sephadex G25[a]

distribution based on	GPC fraction							
	glutenin	F1	F2	F3	F4	F5	F6	F7
protein	100.0	4.7	5.7	9.0	28.5	26.3	14.3	0.6
cysteine	100.0	6.7	7.3	17.1	46.1	13.8	8.8	0.2
radioactivity	100.0	0.8	7.4	5.2	45.8	37.0	3.5	0.3
mass	100.0	4.8	6.7	7.9	31.2	38.1	8.7	2.6

[a] Distribution of the material among fractions F1–F7 (**Figure 2**) in percent.

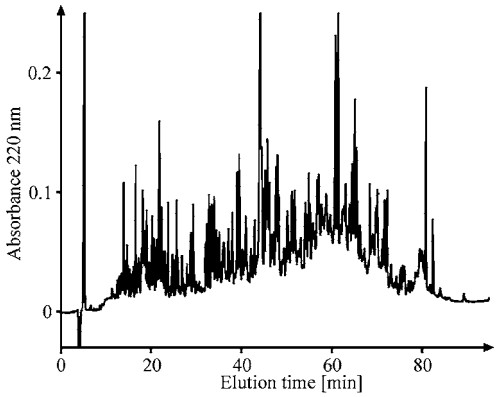

Figure 3. RP-HPLC of fraction F3 on C18 silica gel (first HPLC step).

(peptide) and mass was present in fractions F4−F6, whereas cysteine and radioactivity had a distinct maximum in fraction F4. Because the radioactivity of the fractions was most important for the detection of labeled peptides, fractions F2−F5, which contained >95% of the radioactivity, were selected for the further separations steps by RP-HPLC.

RP-HPLC. Numerous peptides distributed over the whole elution range were detected. As an example, the chromatogram of fraction F3 is shown in **Figure 3**. For fraction F5 all peptides were eluted during the first minutes of the run and the radioactivity was located almost exclusively in the peak representing nonretained material. Therefore, fraction F5 was not used any further for the isolation of radioactively labeled peptides. For fractions F2−F4 numerous HPLC runs were carried out and the eluates were divided and collected in 1−2 min intervals into 20 fractions (F2-1 to F2-20, F3-1 to F3-20, and F4-1 to F4-20), respectively. The subfractions were checked for radioactivity (**Table 4**) and then further subfractionated until pure radioactively labeled peptides were obtained. For this step, the same column but modified solvents and gradients were used. In most of the cases a two-step rechromatography had to be performed to get pure peptides. In **Figure 4** an example of the first rechromatography of peptide F2-10-2 is shown. One additional chromatographic separation had to be performed to obtain peptides F2-10-2a and F2-10-2b that were pure enough for peptide sequencing.

Amino Acid Sequence and Origin of Peptides Containing GS. Altogether, 19 peptides derived from gluten proteins and

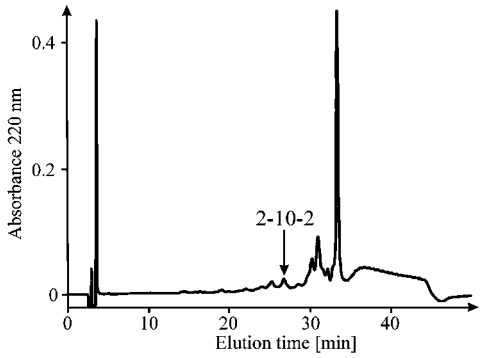

Figure 4. RP-HPLC of fraction F2-10 on C18 silica gel (second HPLC step). The peak 2-10-2 consisted of the two peptides P2-10-2a and P2-10-2b.

containing GS were isolated and sequenced by automated Edman degradation. Only phenylhydantoin derivatives of amino acids from the larger peptide fragment could be determined as the GS fragment was not accessible to the Edman degradation because it contained a γ-glutamyl peptide bond. The cysteine residue involved in the disulfide bond could not be detected by the sequencing system and was therefore represented by a gap in the amino acid sequence. As all isolated peptides were radioactive, it was therefore assumed that they all contained GS. For two peptides (P2-8-6 and P4-2-6), which were present in higher amounts than the other peptides, the presence of GS as well as of the cysteine residue in the large peptide portion was proved by the determination of the molecular mass by MS in the nanospray mode [*m*/*z* (P2-8-6) = 1949, *m*/*z* (P4-2-6) = 778]. The signals of the ^{35}S-labeled isotopes (*m*/*z* + 3) were weaker by >2 orders of magnitude, because of the low amount of labeled GSH that had been added. Sequence analysis revealed that many of the peptides had homologous or identical sequences. This may be due to overlaps of neighboring fractions from GPC or HPLC or to partial cleavage of the GS portion of the peptide by enzymatic action during mixing or partial hydrolysis by thermolysin. The peptides were therefore classified into five groups, which are shown in **Table 5**.

The chain lengths of the peptides ranged from 4 to 21 amino acids. Comparison of the sequences with known sequences of gluten protein components showed that all peptides were derived from LMW subunits of glutenin and contained the cysteine residues C^{b*} and C^{x} according to the nomenclature of Koehler et al. (*18*). The peptides P2-10-2a, P2-10-2b, and P2-8-6 contained the cysteine residue C^{b*} as part of the characteristic tripeptide motif PCS, which is present in the s-type of LMW subunits of glutenin (*19*). Cystine peptides containing this tripeptide as one part of the molecule have already been identified in thermolytic glutenin digests (*18*, *20*). On addition of [^{35}S]GSH during mixing of a wheat dough without AA, peptides of the same type containing GS have been identified by Huettner and Wieser (*11*). The same is true for the cysteine residue C^{x}, which was identified in the two peptides P4-5-3a and P4-2-6.

Table 4. Results of the RP-HPLC Separation of Fractions F2–F4[a]

		RP-HPLC fraction																			
	total	–1	–2	–3	–4	–5	–6	–7	–8	–9	–10	–11	–12	–13	–14	–15	–16	–17	–18	–19	–20
F2	100	1.2	0.6	0.9	8.7	1.5	1.8	3.6	24.2	14.2	18.6	5.0	9.8	2.2	1.5	0.8	0.7	0.7	0.7	0.7	2.7
F3	100	3.3	6.3	6.7	6.2	6.3	4.1	5.0	12.2	9.1	11.5	8.4	4.8	4.9	3.1	1.3	1.0	0.6	0.9	0.9	3.3
F4	100	12.4	8.8	3.0	10.0	10.7	8.5	3.0	3.4	8.1	5.1	7.5	5.2	2.3	2.4	3.0	2.6	1.2	1.1	0.6	1.0

[a] Distribution of the radioactivity among the fractions in percent.

Table 5. Amino Acid Sequences and Origin of Isolated Peptides Containing GS

Peptide	Position of the amino acid in the peptide	Origin	Cys[a]
	1 . . . 5 . . . . 10 . . . . 15 . . . . 20 25		
P2-10-2°	I Q Q Q P Q P F P Q Q P P C[b] S Q Q Q Q P P	LMW	C[b*]
P2-10-2b	V Q Q Q P Q P F P Q Q P P C[b] S Q Q Q Q P P	LMW	C[b*]
P2-8-6	F S Q Q Q P C[b] S Q Q Q Q Q P	LMW	C[b*]
P4-5-3a	L G Q C[b] V	LMW	C[x]
P4-2-6	L G Q C[b]	LMW	C[x]

[a] Designation of the cysteine residues according to Koehler et al. (*18*). [b] Gaps in the amino acid sequences were assigned to cysteine residues (C).

The cysteine residues C[b]* and C[x] of LMW subunits of glutenin have been postulated to form intermolecular disulfide bonds and are thought to be more reactive than other cysteine residues of gluten proteins. Evidence for this hypothesis has been provided by the addition of GSH during mixing (*11*), which reacted almost exclusively with these two cysteine residues. The results of the present study and also the findings of Antes and Wieser (*21*) lead to the assumption that these cysteine residues are at least partly present in flour in the thiol form and are quickly converted to disulfides by thiol/disulfide interchange reactions and oxidation during mixing, corresponding to an increase of the molecular mass of the glutenin polymer. In case of the addition of AA during mixing, GSSG formed of GSH by the reaction sequence shown in **Table 1** (reaction 2) is a potential reaction participant in the thiol/disulfide interchange reaction with the free cysteine residues C[b]* and C[x] of LMW subunits of glutenin. This reaction seems to compete with the exchange reactions caused by other disulfides present in the dough system, for example, protein disulfide groups. Another possibility is the reaction of GSSG with high molecular weight aggregates containing LMW subunits of glutenin that still have one free thiol group.

Concluding Remarks. The results of this study give evidence that AA acts as an improver according to the hypothesis of Grosch and Wieser (*3*) because mixed disulfides formed by reaction of GSSG and gluten proteins with free thiol groups were identified. This reaction was possible only after conversion of endogenous GSH to GSSG by DHA formed after oxidation of added AA. Direct oxidation of protein thiols by DHA as proposed by Every et al. (*22*, *23*) would not have yielded peptides containing radioactively labeled GS. The peptides contained exclusively the cysteine residues C[b]* and C[x] present in LMW subunits of glutenin, which are proposed to form intermolecular disulfide bonds. These cysteine residues seem to be at least partly present in the thiol form in flour. During dough mixing they are converted to protein−protein disulfides; however, a small portion remains in the reduced state and is able to react with GSSG to form glutathione−protein mixed disulfides.

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