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Impact of Redox Agents on the Extractability of Gluten Proteins during Bread Making

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The gluten proteins gliadin and glutenin are important for dough and bread characteristics. In the present work, redox agents were used to impact gluten properties and to study gliadin−glutenin interactions in bread making. In control bread making, mixing increased the extractability of glutenin. The level of SDS-extractable glutenin decreased during fermentation and then further in the oven. The levels of extractable α- and γ-gliadin also decreased during bread baking due to gliadin−glutenin polymerization. Neither oxidizing nor reducing agents had an impact on glutenin extractabilities after mixing. The redox additives did not affect ω-gliadin extractabilities during bread making due to their lack of cysteine residues. Potassium iodate (0.82−2.47 μmol/g of protein) and potassium bromate (1.07−3.17 μmol/g of protein) increased both α- and γ-gliadin extractabilities during baking. Increasing concentrations of glutathione (1.15−3.45 μmol/g of protein) decreased levels of extractable α- and γ-gliadins during baking. The work not only demonstrated that, during baking, glutenin and gliadin polymerize through heat-induced sulfhydryl−disulfide exchange reactions, but also demonstrated for the first time that oxidizing agents, besides their effect on dough rheology and hence bread volume, hinder gliadin−glutenin linking during baking, while glutathione increases the degree of covalent gliadin to glutenin linking.

KEYWORDS: Bread making; wheat gluten; gliadin−glutenin interaction; protein extractability

INTRODUCTION

Gluten proteins (consisting of monomeric gliadin and polymeric glutenin) have the unique rheological ability to form a dough matrix which determines bread quality. Gliadin represents a heterogeneous mixture of proteins containing α-, γ-, and ω-gliadins. All cysteine residues in α- and γ-type gliadins are involved in intrachain disulfide (SS) bonds. In contrast, ω-gliadins lack cysteine residues. Glutenin consists of glutenin subunits (GSs) of high molecular weight (HMW-GSs) and low molecular weight (LMW-GSs). The LMW-GSs are classified as B, C, and D types. LMW-GSs form both intrachain and interchain disulfide bonds among themselves and with HMW-GSs, leading to glutenin polymers (*1*). During dough mixing, gliadin interacts with glutenin to form a viscoelastic gluten network that retains gas in dough during proofing (*2*).

Dough mixing increases the extractability of protein in different media (*3*). Whether this increase is due to depolymerization (*3*−*5*), conformational rearrangement (*6*), or better dissolution by a changed effective surface area (*7*) is still subject to debate. During fermentation, protein extractability decreases, and again, different hypotheses have been developed to explain the phenomenon (*3*, *7*). However, longer extraction times than those used in previous studies increase protein extractability of flour, and then, depending on the wheat variety, no change or a decrease of protein extractability in 0.5% SDS is observed after mixing and fermentation (*8*). The polymerization of gluten proteins in the baking phase contributes to the final bread structure and quality. Sulfhydryl (SH)−disulfide (SS) interchange reactions induced by heat result in the incorporation of gliadin monomers in the glutenin network (*9*−*13*).

Redox additives affect dough rheological properties and gluten structure during mixing, fermentation, and heating. The main effect of oxidants in dough appears to be the oxidation of free SH groups to SS moieties to yield an increased number of cross-links between proteins and to minimize SH−SS interchange reactions. In contrast, reducing agents react with SS bonds in dough and reduce the average molecular weight of glutenin protein aggregates (*14*). Lagrain et al. (*15*) indicated that redox additives may affect the capacity of gluten proteins to associate during hydrothermal treatment through SH−SS exchange reactions by altering the level of free SH groups. These groups can affect the flexibility of glutenin chains and initiate the polymerization reactions (*15*). Although the overall effects of redox agents in bread making have been described (*16*−*18*), their effects on gliadin and glutenin extractability in bread making have not been elucidated. Moreover, the hypothesis that redox agents affect the capacity of gluten proteins to associate during heating, as put forward by Lagrain et al. (*15*) on the basis of results obtained from hydrothermal treatment of gluten

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proteins by means of a rapid visco analyzer (RVA), has, to the best of our knowledge, never been investigated in the case of the bread making process.

To that end, the extractabilities of both glutenin and gliadin at different stages of the process in the presence of redox additives were evaluated. Size-exclusion (SE) high-performance liquid chromatography (HPLC) was used to determine changes in gliadin and glutenin extractabilities during bread making, and reversed phase (RP) HPLC was used to determine more in detail specific changes in α-, γ-, and ω-gliadin extractabilities and in the extractabilities of B-, C-, and D-type LMW-GSs and HMW-GSs after reduction.

EXPERIMENTAL PROCEDURES

Materials. Flour [moisture content 13.9%, protein content ([N] × 5.7) 10.5% on a dry basis] was obtained by milling (extraction rate 70%) the French bread making wheat cultivar Legat (harvest 2004) on a Bühler MLU-202 laboratory mill (Uzwill, Switzerland) according to AACC Method 26-31 (*19*). Protein contents were determined by Dumas analysis as described earlier (*13*). All chemicals and reagents were from Sigma-Aldrich (Steinheim, Germany) and of analytical grade unless otherwise specified.

Methods. *Bread Making*. To monitor protein extractability during bread making, bread was made according to the procedure of Shogren and Finney (*20*) for 10 g of flour. Legat flour (10.0 g), sucrose (0.6 g), NaCl (0.15 g), compressed yeast (0.53 g) (Bruggeman, Ghent, Belgium), and water (5.9 mL) were mixed at 25 °C with a 10 g pin mixer (National Manufacturing, Lincoln, NE) for 4.5 min, which is the optimal mixing time for control dough. Potassium iodate [20−60 ppm range (flour basis), corresponding to 0.82−2.47 μmol/g of protein], potassium bromate (20−60 ppm range, corresponding to 1.07−3.17 μmol/g of protein), or gluthathione (40−120 ppm, corresponding to 1.15−3.45 μmol/g of protein) was added as an aqueous solution immediately before mixing. The dough was fermented at a temperature of 30 °C and relative humidity of 95%. During fermentation, the dough was punched after 52, 77, and 90 min. Fully fermented dough was molded, proofed for 36 min in a baking pan at 30 °C and 95% relative humidity, and baked for 13 min at 232 °C. Samples were withdrawn after mixing, after final proofing, and after 2, 6, 10, and 13 min of baking time. Dough and partially and fully baked bread samples were immediately frozen in liquid nitrogen, freeze-dried, and ground in a laboratory mill (IKA, Staufen, Germany). Loaf volumes were measured by glass bead displacement (*21*).

Size-Exclusion HPLC. Protein extraction and SE-HPLC were conducted as described by Lagrain et al. (*13*). Flour, dough, and (partially baked) bread samples containing 1.0 mg of protein were extracted with 1.0 mL of a 0.05 M sodium phosphate buffer (pH 6.8) containing 2.0% sodium dodecyl sulfate (SDS), hereafter referred to as SDS buffer, and loaded (60 μL) onto a Biosep-SEC-S4000 column (Phenomenex, Torrance, CA). The elution solvent was acetonitrile (ACN)/water (1:1, v/v) and contained 0.05% (v/v) trifluoroacetic acid (TFA). The flow rate was 1.0 mL/min at a temperature of 30 °C, and eluted protein was detected at 214 nm.

The elution profiles were divided into three fractions using the lowest absorbance reading between the peaks as the cutoff point (*8*). The first fraction corresponds to the level of glutenin extractable in the SDS buffer (SDS-extractable glutenin); the second is assigned to the level of SDS-extractable gliadin and the third to the level of SDS-extractable albumin and globulin for wheat flour protein extracts. SDS-extractable protein (total peak area), SDS-extractable glutenin, and SDS-extractable gliadin levels were calculated from the peak areas and expressed as a percentage of the peak area of gluten extracted from the flour or dough with the SDS buffer in the presence of 1.0% dithiothreitol (DTT), which represented the total SDS-extractable protein in the flour or in the dough since reduced gluten proteins are completely extractable.

Reversed-Phase HPLC. Samples of flour, dough, and bread (500.0 mg) were extracted twice with 5.0 mL of a salt solution (0.4 M NaCl, 0.125 M sodium phosphate, pH 7.6) (yielding the albumin/globulin extract), three times with 3.0 mL of 60% (v/v) ethanol (yielding the gliadin extract), and twice with 5.0 mL of 0.05 M Tris/HCl buffer (pH 7.5) containing 50% propan-1-ol, 2.0 M urea, and 1.0% (w/v) DTT and kept under nitrogen (yielding the reduced glutenin extract). The gliadin and glutenin extracts were loaded (100 μL) onto a Nucleosil 300-5 C8 column (Machery-Nagel, Düren, Germany). The elution medium consisted of deionized water containing 0.1% (v/v) TFA (A) and ACN containing 0.1% TFA (v/v) (B). Proteins were eluted with a flow rate of 1.0 mL/min and a linear gradient from 24% B to 56% B in 50 min and detected at 214 nm.

α-Gliadin, γ-gliadin, ω-gliadin, B/C-LMW-GSs, D-LMW-GSs, and HMW-GSs were distinguished on the basis of absorbance minima between specific peaks as outlined earlier by Wieser (*22*) and Wieser et al. (*23*).

Statistical Analysis. Baking experiments were performed in duplicate except for loaf volume determination, which was performed in triplicate. SE- and RP-HPLC analyses were performed with duplicate samples. Two extracts per sample were used. The means, standard deviations, and errors were calculated using the Microsoft Excel software package. Analysis of variance (ANOVA) was made using the SAS statistics software, version 8e (SAS Institute Inc., Cary, NC). A level of significance of 95% was used in all statistical analysis.

Table 1. Extractabilities (%) of Protein in 2.0% SDS Buffer, As Calculated from SE-HPLC Data, Expressed as a Percentage of Total Protein during Different Stages of Control Bread Making[a]

bread making stage	SDS-extractable protein	SDS-extractable gliadin	SDS-extractable glutenin
flour	74.9 a	58.9 a	13.4 a
mixed dough	91.0 b	58.6 a	28.9 b
fermented dough	83.1 c	57.5 a	22.3 c
dough baked for 2 min	79.0 ac	57.0 a	19.4 c
dough baked for 6 min	39.6 d	34.7 b	2.8 d
dough baked for 10 min	25.6 e	23.3 c	1.0 e
bread	16.6 f	14.8 d	1.0 e

[a] Values are means of four replicates. The maximum relative standard deviation (as a percentage of the mean) observed among replicates was 9.7%. Means followed by different letters in the same column are significantly ($P < 0.05$) different.

RESULTS AND DISCUSSION

Gluten Extractability during Control Bread Making. Table 1 shows protein extractabilities in SDS buffer during control bread making as calculated from SE-HPLC data. Mixing significantly ($P < 0.05$) increased protein SDS extractability. While gliadin extractability in SDS remained constant, mixing doubled the level of SDS-extractable glutenin in control dough. During fermentation, the level of SDS-extractable glutenin decreased. Short (2 min) baking at 232 °C had no significant ($P < 0.05$) effects on protein SDS extractability. However, heating dough for 6 min significantly decreased gliadin and glutenin extractabilities. The levels of SDS-extractable gliadin and glutenin further decreased during the remainder of the baking phase.

Figure 1 shows the levels of gliadin subgroups, extractable in 60% ethanol, and glutenin subunits, extractable in 50% propan-1-ol, 2.0 M urea, and 1.0% (w/v) DTT, during control bread making as calculated from RP-HPLC data. No significant ($P < 0.05$) differences were observed in the extractabilities of cysteine-free ω-gliadin during the process, except that ω-gliadin extractability from bread differed significantly from its extractability from flour. The baking step decreased ω-gliadin extractability to 74% of its original value. Mixing increased α-gliadin significantly, but not that of γ-gliadin. The increase in extractable α-gliadin after mixing (**Figure 1a**) was accompanied by a

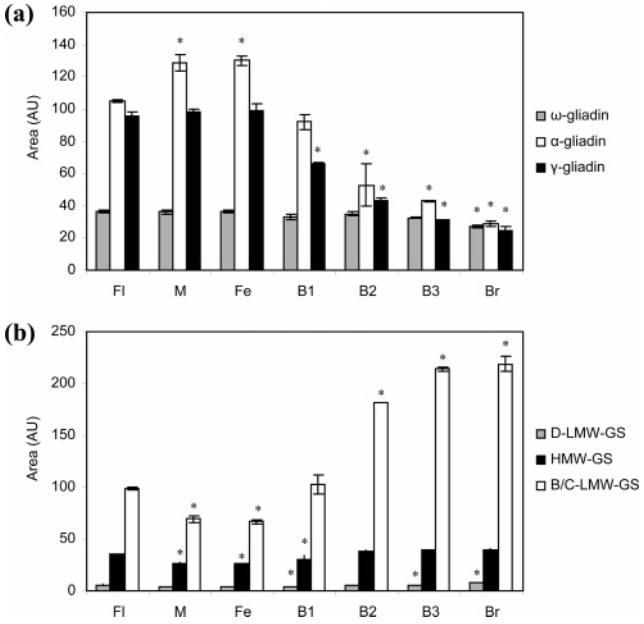

Figure 1. Areas in RP-HPLC chromatograms representing gluten extractabilities with 60% ethanol and 0.05 M Tris/HCl buffer (pH 7.5) with 50% propan-1-ol, 2.0 M urea, and 1% (w/v) DTT during control bread making: (**a**) gliadin fraction, (**b**) reduced glutenin fraction with the apparent levels of glutenin subunits. Key: Fl, flour; M, mixed dough; Fe, fermented dough; B1, dough baked for 2 min; B2, dough baked for 6 min; B3, dough baked for 10 min, Br, bread. AU = arbitrary units. Asterisks indicate values differing significantly ($P < 0.05$) from the respective values for flour.

significant decrease in the apparent levels of HMW-GSs and B/C-LMW-GSs (**Figure 1b**). During fermentation, α- and γ-gliadin extractabilities did not change. The baking step had a strong impact on both α- and γ-gliadin extractabilities. After 2 min in the oven, γ-gliadin extractability was already significantly reduced. Baking for at least 6 min resulted in a further significant decrease of both α- and γ-gliadin extractabilities, and at the end of the baking process, the extractabilities of α- and γ-gliadin were reduced to, respectively, 28% and 25% of their orginal levels in flour (**Figure 1a**). The decrease in extractable gliadin in the baking step (**Figure 1a**) was accompanied by a significant increase in the apparent levels of B/C-LMW-GSs and, to a lesser extent, D-LMW-GSs after 10 min of baking (**Figure 1b**), suggesting that a major portion of gliadins, unextractable in 60% ethanol after heat treatment, became extractable with the B/C-LMW-GSs fraction. The sum of extractable gliadin and reduced glutenin remained constant during bread baking.

From the results of both SE- and RP-HPLC it is clear that mixing increased not only glutenin extractability in SDS buffer, but also glutenin extractability in 60% ethanol. SE-HPLC indicated that the level of SDS-extractable gliadin did not change after mixing; however, RP-HPLC showed a significant increase of α-gliadin extractability in 60% ethanol together with a decrease of the levels of extractable HMW-GSs and B/C-LMW-GSs. Thus, part of glutenin, containing HMW-GSs and B/C-LMW-GSs, became extractable with α-gliadin in 60% ethanol.

The significant increase in glutenin extractability during mixing is most likely to be ascribed to mechanical disruption of glutenin and formation of protein aggregates from partially disrupted glutenin linked through hydrogen bonds and hydrophobic interactions. It seems plausible that the unfolding, alignment, and partial depolymerization of proteins during mixing rendered them more susceptible for extraction with the SDS buffer and with 60% ethanol. During fermentation, these protein aggregates became less extractable in SDS buffer, but

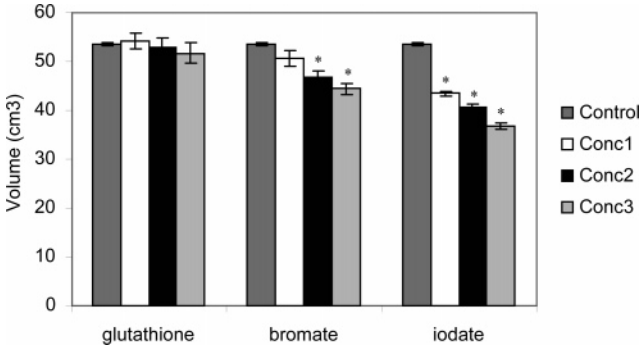

Figure 2. Volumes of breads baked on a 10 g scale in the presence of increasing concentrations of redox agents: Conc1, 1.15, 1.07, or 0.82 μmol of glutathione, bromate, or iodate/g of protein, respectively; Conc2, 1.73, 1.59, or 1.24 μmol/g of protein; Conc3, 3.45, 3.17, or 2.47 μmol/g of protein. Asterisks indicate values differing significantly ($P < 0.05$) from the value for control bread.

not in 60% ethanol. Bread baking significantly reduced α- and γ-gliadin extractabilities and simultaneously increased the apparent amounts of reduced glutenin, suggesting formation of gliadin−glutenin disulfide bond linking in the process. γ-Gliadins were more affected during baking than α-gliadins.

Reduction of SS bonds with DTT rendered all proteins in bread extractable, suggesting a major proportion of SS covalent linkages between gluten proteins in the product.

Effect of Redox Agents on Gluten Extractability during Bread Making. **Figure 2** shows the volumes of breads prepared on a 10 g scale with different concentrations of glutathione, potassium bromate, or potassium iodate. Increasing concentrations of glutathione did not change the bread volume significantly, while potassium bromate and especially potassium iodate strongly decreased the bread volume with increasing concentrations of additive. The negative effect of oxidants on the bread volume can be explained by the strong bread making characteristics of the particular flour used. The too high dough strength obtained by use of oxidizing agents resulted in lower bread volumes. **Figure 3** shows the gliadin extractabilities (in 60% ethanol) of the breads. Neither of the additives significanty affected ω-gliadin extractability. The effect of glutathione in the bread making recipe on α- and γ-gliadin extractabilities was small, but with 3.45 μmol of gluthathione/g of protein, the level of extractable γ-gliadin decreased significantly (**Figure 3a**). In contrast, increasing levels of added potassium bromate significantly increased α-gliadin extractability (**Figure 3b**), and 2.47 μmol of potassium iodate/g of protein increased significantly both α- and γ-gliadin extractabilities after baking (**Figure 3c**). The effects of increasing levels of redox agents on glutenin SDS extractability of bread were not significant even at the highest concentration used in this study (**Table 2**).

To evaluate the effects of the redox agents during the different stages of bread making on gliadin and glutenin extractabilities, the highest level of each additive which still allowed correct dough handling was chosen.

Table 2 shows protein extractabilities in SDS buffer during bread making in the presence of redox agents, as calculated from SE-HPLC data. In each stage of the bread making process SDS extractabilities of samples with additives are compared to the corresponding extractabilities of the control samples. No significant differences in SDS-extractable protein after mixing were observed in the presence of redox agents. Redox agents did not impact significantly the level of SDS-extractable gliadin after fermentation. However, glutenin extractability was significantly higher in dough supplemented with glutathione or

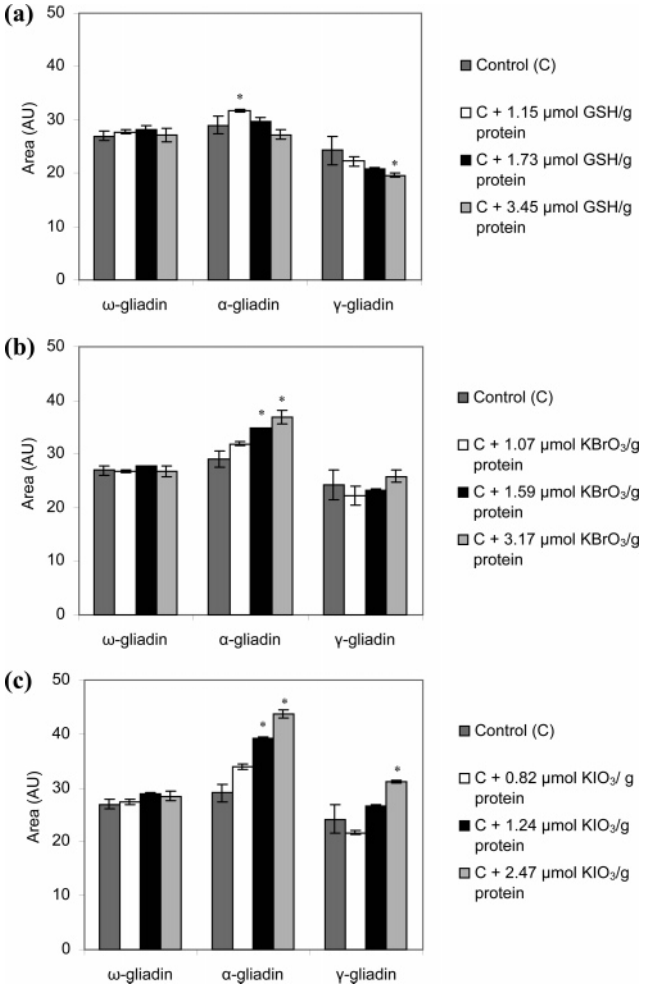

Figure 3. Areas in RP-HPLC chromatograms representing gliadin extractabilities in 60% ethanol of breads supplemented with increasing concentrations of (**a**) glutathione (GSH), (**b**) potassium bromate ($KBrO_3$), and (**c**) potassium iodate (KIO_3). AU = arbitrary units. Asterisks indicate values differing significantly ($P < 0.05$) from the values for control bread.

potassium iodate than in control dough and in dough with potassium bromate. Protein extractability decreased after 2 min in the oven, except for breads with potassium iodate. Baking for 6 and 10 min yielded levels of both SDS-extractable gliadin and glutenin which were significantly higher in breads with potassium bromate and iodate than in control bread and bread with glutathione. The level of SDS-extractable gliadin in bread with glutathione was significantly lower than that of the control bread after 10 min of baking (**Table 2**).

Figure 4 shows gliadin extractabilities in 60% ethanol in the presence of potassium iodate (60 ppm, 2.47 μmol/g of protein), potassium bromate (60 ppm, 3.17 μmol/g of protein), or glutathione (120 ppm, 3.45 μmol/g of protein) during bread making. Redox agents did not impact ω-gliadin extractabilities significantly during bread making. After only 2 min of baking, ω-gliadin extractability of dough with potassium iodate differed significantly from that of the control dough (**Figure 4a**). This corresponds with the significant increase in SDS-extractable gliadin in dough containing iodate after 2 min in the oven (**Table 2**). Mixing lowered α-gliadin extractability significantly more for dough containing potassium iodate or potassium bromate than for control dough and dough containing added glutathione. The extractability of α-gliadin after 2 min of baking with added glutathione did not differ significantly from that of the control, but for bread with potassium iodate the level of extractable

Table 2. Extractabilities (%) of Protein in 2.0% SDS Buffer, As Calculated from SE-HPLC Data, Expressed as a Percentage of Total Protein during Different Stages of Bread Making with Addition of Glutathione (3.45 μmol/g of protein), KIO_3 (2.47 μmol/g of protein), and $KBrO_3$ (3.17 μmol/g of protein)[a]

type	bread making stage	control (C)	C + glutathione	C + KIO_3	C + $KBrO_3$
protein	flour	91.0 a	94.5 a	93.6 a	93.3 a
	mixed dough	83.1 a	93.3 b	92.7 b	79.8 a
	fermented dough	79.0 a	79.9 a	94.7 b	82.0 a
	dough baked for 2 min	39.6 a	40.1 a	55.7 b	49.2 ab
	dough baked for 6 min	25.6 a	23.9 b	36.1 c	31.2 d
	dough baked for 10 min	16.6 a	16.1 a	24.7 b	21.7 ab
gliadin	flour	58.6 a	60.3 a	59.8 a	60.4 a
	mixed dough	57.5 ab	61.6 a	58.1 ab	55.3 b
	fermented dough	57.0 a	58.0 a	64.9 b	59.6 a
	dough baked for 2 min	34.7 a	35.6 a	46.5 b	42.3 c
	dough baked for 6 min	23.2 a	21.8 b	32.3 c	28.3 d
	dough baked for 10 min	14.8 a	14.5 a	22.2 b	19.6 ab
glutenin	flour	28.9 a	30.9 a	30.4 a	29.3 a
	mixed dough	22.2 a	28.1 b	31.2 c	21.3 a
	fermented dough	19.4 a	19.0 a	26.6 c	19.6 a
	dough baked for 2 min	2.8 a	2.5 a	6.6 b	4.7 c
	dough baked for 6 min	1.0 a	0.6 b	2.1 c	1.4 d
	dough baked for 10 min	1.0 a	0.9 a	1.3 a	1.2 a

[a] Values are means of four replicates. The maximum relative standard deviation (as a percentage of the mean) observed among replicates was 15%. Means followed by different letters in the same row are significantly ($P < 0.05$) different.

α-gliadin was significantly higher than that of the control. Baking for 10 min significantly lowered α-gliadin extractability increasingly in the order iodate-treated bread, bromate-treated bread, control bread, and glutathione-treated bread (**Figure 4b**). The γ-gliadin extractabilities showed more or less the same trends as the α-gliadin extractabilities (**Figure 4c**).

The observations on glutenin extractability in the presence of redox agents after dough mixing and fermentation are generally in agreement with the results of Veraverbeke et al. (*17*) for potassium iodate and those of Hamer and Lichtendonck (*24*) for potassium bromate. Mixing and dough rest during fermentation affected protein extractabilities, which in turn can be influenced by redox agents. The effects on gliadin extractability were small.

The different effects of potassium bromate and iodate on glutenin extractabilities after fermentation can be explained by their different reaction mechanisms. Bromate is less active during mixing and more during and after fermentation due to the lower pH (*14*), while iodate already reacts during mixing by oxidizing free SH groups. Iodate complicates further oxidation and covalent aggregation during fermentation, while bromate can stimulate both. The addition of glutathione also prevented a decrease in SDS-extractable glutenin after fermentation. These results suggest covalent reactions during fermentation with SH groups involved.

Heating strongly influenced protein extractabilities with large decreases in glutenin extractabilities (**Tables 1** and **2**) due to polymerization through SH−SS exchange reactions and oxidation of free SH (*17*). The effects were less for breads with oxidizing agent during baking, indicating a more complicated aggregation of glutenin. The higher gliadin extractabilities than in the control samples, during and after baking in the presence of potassium bromate and iodate (**Table 2** and **Figures 2** and **4**), are in agreement with results noted for gluten−water suspensions during analysis with an RVA (*15*) and confirm the hypothesis that redox agents affect the capacity of gluten proteins to associate during heating as put forward by Lagrain

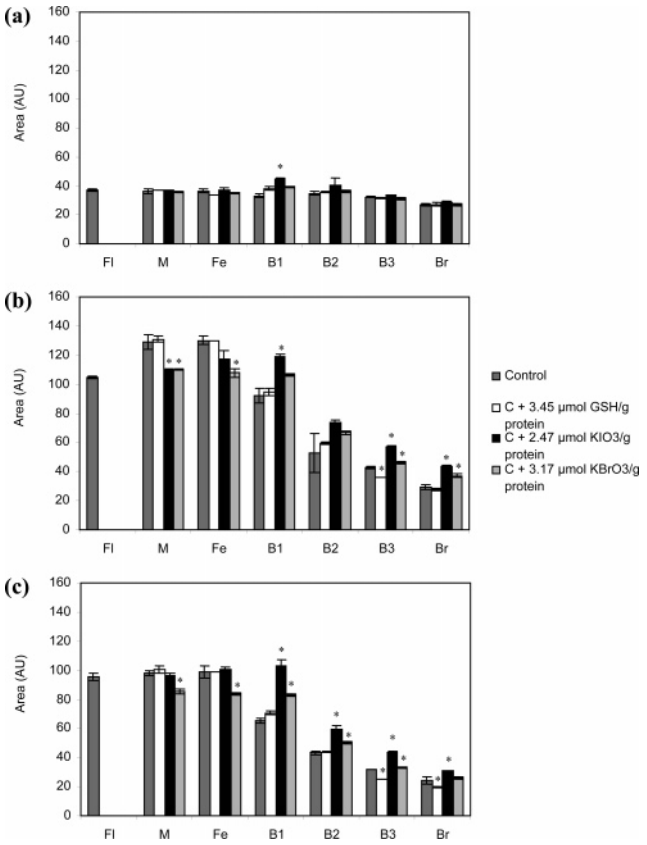

Figure 4. Areas in RP-HPLC chromatograms representing gliadin extractabilities with 60% ethanol during bread making with the ω-gliadin (**a**), the α-gliadin (**b**), and the γ-gliadin (**c**) extractabilities of dough and bread supplemented with GSH, KIO_3, and $KBrO_3$. Key: Fl, flour; M, mixed dough; Fe, fermented dough; B1, dough baked for 2 min; B2, dough baked for 6 min; B3, dough baked for 10 min, Br, bread. AU = arbitrary units. Asterisks indicate values differing significantly ($P < 0.05$) from the values for the corresponding control sample in each stage of bread making.

et al. (*15*) in the case of bread making as well. This indicates less gliadin−glutenin cross-linking during baking in breads with potassium iodate and bromate. Oxidants lower the level of free SH groups prior to heating and decrease glutenin flexibility. This may hinder SH−SS exchange reactions between gliadin and glutenin that normally occur at temperatures of at least 90 °C (*13*). Since ω-gliadins lack cysteine, they are not involved in redox or SH−SS exchange reactions, and hence, they do not link to glutenin and their extractability is not influenced by redox additives.

These results support the theory that gliadin−glutenin linking occurs during baking through a heat-induced SH−SS exchange mechanism and that this association mainly involves covalent association between SH groups. Moreover, besides the well-known impact of redox agents at room temperature on polymeric glutenin and, hence, on dough rheology, resulting in different bread volumes, oxidizing and reducing agents also have an impact on gliadin−glutenin linking during baking by changing the levels of free SH groups, which initiate SH−SS exchange reactions at temperatures exceeding 90 °C.

ABBREVIATIONS USED

DTT, dithiothreitol; HMW-GSs, high molecular weight glutenin subunits; HPLC, high-performance liquid chromatography; LMW-GSs, low molecular weight glutenin subunits; RP, reversed phase; RVA, rapid visco analyzer; SDS, sodium dodecyl sulfate; SE, size exclusion; SH, sulfhydryl; SS, disulfide, TFA, trifluoroacetic acid.

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