Changes in the Gliadin Fraction(s) During Breadmaking: Isolation and Characterization by High-Performance Liquid Chromatography and Polyacrylamide Gel Electrophoresis

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ABSTRACT

Polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) were used to study the stability and interaction of gliadin proteins during mixing, fermentation, and baking. The proteins were stable during dough mixing or fermentation, as evidenced by absence of changes in gliadin patterns. However, gliadin degradations or interactions were found during baking. The PAGE gliadin bands with low relative mobility (RM < 40) were more intensely stained and those with RM > 40 were less intensely stained from the extract of the crumb of bread baked from a composite hard red winter wheat flour when compared to the flour extract. The intensity (concentration) of all gliadin PAGE bands in the bread crust was significantly reduced. HPLC analysis of gliadin crumb extracts indicated that the highly hydrophobic gliadins (longest elution times > 23 min) were more heat labile (and probably interacted more with other flour components) than the less hydrophobic gliadins (shorter elution times < 23 min). That interaction was confirmed by in vitro heat treatments of HPLC-isolated fractions (20–23 min and 23–26 min) and subsequent analysis by PAGE and HPLC.

Breadmaking quality largely depends on the quality and quantity of wheat flour proteins (Pomeranz 1968, 1980, 1985; Finney 1945, 1971; Hoseney and Finney 1971; Finney et al 1982). Finney et al (1982) fractionated the endosperm wheat proteins and interchanged the protein fractions between good- and poor-quality flours to show correlations of protein fraction(s) to breadmaking quality. The biochemical and physical properties of those fractions (including characterization by electrophoresis) were also studied (Jones et al 1983, Finney et al 1982).

Significant improvements in studying proteins have evolved in the past four years. Polyacrylamide gel electrophoresis (PAGE) methods are routinely used to identify wheat cultivars in the United States (Lookhart et al 1982b, 1983; Jones et al 1982), Canada (Bushuk and Zillman 1978), France (Autran 1979), and Australia (Wrigley and Shepherd 1974). The high-performance liquid chromatography (HPLC) technique, which can separate proteins on the basis of hydrophobicity, was introduced by Bietz (1983) and since has been used to study genetic changes in wheat (Bietz 1985), effect of sulfur/nitrogen fertilizer ratios in wheat (Lookhart and Pomeranz 1985b) and other cereals: oats (Lookhart 1985, Lookhart and Pomeranz 1985a); rice (Lookhart et al 1987); barley (Marchylo and Kruger 1984); and corn (Smith and Smith 1986).

This study was made to characterize proteins during processing (including fermentation and baking) of flour to further understand the role of gliadins, the changes they undergo, and at what stage the changes take place.

MATERIALS AND METHODS

Chemicals and Reagents

Acrylamide, *N,N'*-methylene-bisacrylamide, ascorbic acid, Coomassie Brilliant Blue R-250, methyl green, and trichloroacetic acid were from Sigma Chemical Company, St. Louis, MO; trifluoroacetic acid (TFA) was from Sigma or Pierce (Rockford, IL). Lactic acid (USP grade) and ferrous sulfate heptahydrate (AR grade) were obtained from Mallinckrodt Chemicals. St. Louis, MO, and HPLC-grade ethanol and acetonitrile from Burdick and Jackson Laboratories, Muskegon, MI. Hydrogen peroxide (3% practical grade) was purchased from a local pharmacy. Water was purified by passage, in series, through two mixed-bed ion-exchange cartridges, a charcoal bed, and a membrane filter. Aluminum lactate was from Fluka Chemicals, Hauppauge, NY.

Baking Materials

Flour was RBS-78, a composite of flours milled from hard red winter wheats grown throughout the Great Plains during the 1978 crop year. It was stored in the cold after milling in 1978. Yeast was a 50:50 blend of fresh, weekly shipments from Fleishmann Yeast, Nabisco Brands, Inc., St. Louis, MO, and Red Star Yeast, Universal Foods Corp., Milwaukee, WI. Whey (X-L Whey) and nonfat dry milk solids (NFDMS) were from Land O'Lakes Company, Arden Hills, MN; soy flour was from Archer Daniels Midland Co., Decatur, IL.

Breadmaking

Bread was produced by the 100-g straight-dough method (Finney 1984). Nonfermented and fermented doughs (3% yeast) were made from RBS-78 flour, and they were also used as the base for the additives, at the 3% level, of one of the following: whey, soy flour, or milk solids. The usage and properties of whey and NFDMS in breadmaking were described by Pomeranz (1985).

Gliadin Extractions

Bread samples were lyophilized, air-dried, and pulverized (by mortar and pestle) before extraction. Samples (250 mg) of flour, bread crumb, and crust were each extracted with 750 μl of 70% ETOH by the method of Lookhart et al (1982b).

PAGE

Two drops of glycerine containing the tracking dye methyl green were added to the 400 μl of gliadin extracts used for electrophoresis. The electrophoresis apparatus, power supply, staining, and destaining procedures have been described (Lookhart et al 1982b, 1986). The gel acrylamide concentration (6%), temperature (20° C), electrophoresis time (2 hr), and voltage (500 V) were those that optimized separations (Lookhart et al 1986).

HPLC

The remaining 100 μl of gliadin extract was analyzed by a modification to the HPLC procedures of Bietz (1983), Lookhart (1985), and Lookhart et al (1987). The HPLC system was

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composed of a Varian Associates (Walnut Creek, CA) model 5060 microprocessor-controlled pumping system, a Waters Associates (Milford, MA) model 710 A autosampler (10 μl), a Tracor (Austin, TX) model 970 variable wavelength detector (210 nm), and a Hewlett-Packard (Avondale, PA) model 3388A printer-plotter automation system. Samples were eluted at 1 ml/min on a SynChrom Inc. (Linden, IN) SynChropak RP-P (C_{18}) 6.5 μm particle column (250 × 4.1 mm i.d.) at 45° C using a series of linear binary gradients. Solvent A was acetonitrile with 0.1% TFA, and solvent B was water containing 0.1% TFA. The multistep gradient conditions were: 25% A at time 0, A held constant for 5 min, then a linear increase of A to 35% at 13 min, to 38% at 17 min, to 50% at 25 min, to 75% at 26 min, to 85% at 27 min, and a return to initial conditions (25% A) at 28 min. Reinjections were made at 45-min intervals.

In Vitro Heat Stability of Gliadins

Ten 50-μl aliquots of RBS-78 flour gliadin extracts were injected onto the HPLC column, and peaks of interest were manually collected in separate vials. The combined samples for each peak contained about 2 ml. Two aliquots (500 μl) of each were placed into separate tightly closed hydrolysis tubes; one was placed in a heating block at 100° C for 15 min and the other in an oven at 200° C for 15 min. Those temperatures were chosen to represent the approximate temperatures that the crumb and crust approached during bread baking (Barackman and Bell 1938, Walden 1955).

All extractions and PAGE and HPLC determinations were made at least in duplicate.

RESULTS AND DISCUSSION

Gliadin extracts of RBS-78 flour, mixed and fermented doughs, and crumb and crust of bread made from RBS-78 flour were analyzed by PAGE (Fig. 1) and HPLC (Fig. 2).

PAGE

Electrophoregrams of gliadins extracted from flour, mixed doughs, and fermented doughs (Fig. 1) were identical, which implied that the gliadins did not undergo changes or that the subtle changes they may have undergone were not as drastic as the denaturation changes occurring during extraction and electrophoresis. However, differences were found among the PAGE patterns of the flour (control) and the crumb and crust extracts (Fig. 1). Only a few very light bands for the crust extract were visible after six times the sample load of the control was put on the gel. The first four crust bands appeared to be similar to the first four crumb bands in number and separation from each other but with major differences in their relative mobilities. Those differences may be attributable to major changes in their apparent molecular weights because breaking intra-S-S bonds cause the gliadins to be more extended and less globular or to heat-mediated reactions with nonprotein molecules that cause polymerization. The crumb bands (Fig. 1D) had the same mobility as the control bands (Fig. 1A), but the intensities were different. All bands in the crumb electrophoregram with relative mobility (RM) less than 40 appeared to have higher intensities than the bands at the same position in the control. Crumb electrophoretic bands with RM > 40 appeared lighter than the corresponding bands in the control. The disappearance of these faster moving (possibly lower molecular weight) bands and the increase in the slower moving bands (higher molecular weight) implies either specific interactions among proteins during baking or that the proteins are binding rather specifically with some other nonprotein components in the dough. This is in agreement with the gliadin sodium dodecyl sulfate-PAGE results of Meier et al (1985). The only bands even faintly visible in the crust electrophoregram were also the slower moving (higher molecular weight) bands.

The patterns of gliadins extracted from flour, mixed and fermented doughs, and crumb and crust made from those doughs were identical to gliadin patterns of the same fractions with 3% soy flour, whey, or NFDMS added. The additives (at 3% level) did not alter the electrophoregrams (data not shown). No meaningful PAGE patterns were obtained for the yeast, soy flour, whey, or NFDMS separately. At the 3% level, it would have been nearly impossible to see any additional bands (Lookhart et al 1982a) caused by the presence of an additive, but differences would be visible if interactions did occur.

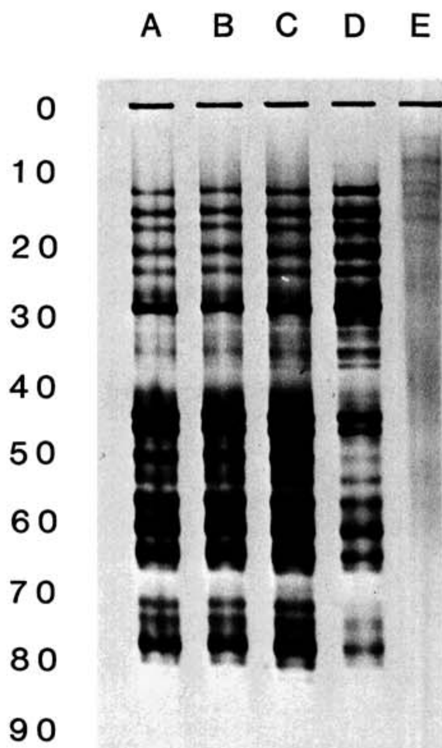

Fig. 1. Polyacrylamide gel eletrophoresis patterns of gliadins extracted from RBS-78 flour (**A**), nonfermented dough (**B**), fermented dough (**C**), bread crumb (**D**), and bread crust (**E**).

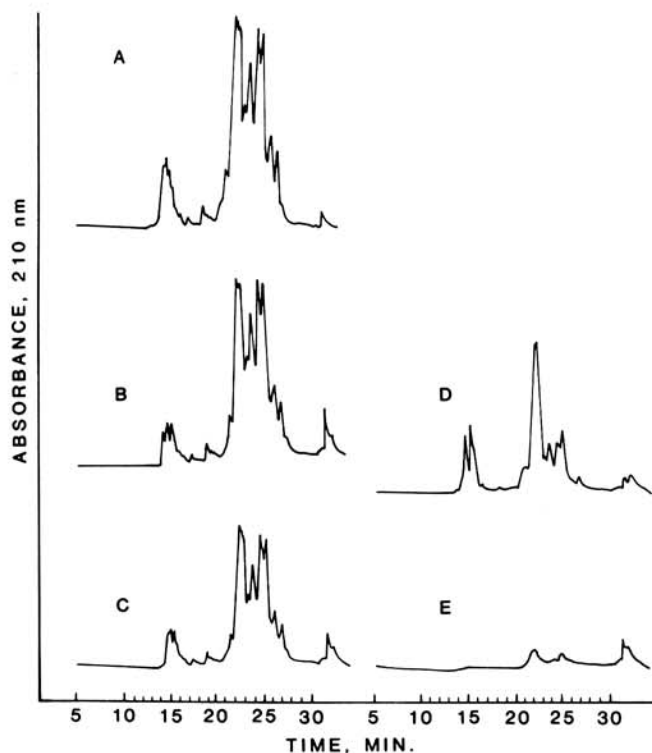

Fig. 2. High-performance liquid chromatography patterns of gliadins extracted from RBS-78 flour (**A**), nonfermented dough (**B**), fermented dough (**C**), bread crumb (**D**), and bread crust (**E**). The absorbance scale was the same for all samples.

HPLC

The gliadin chromatograms of the RBS-78 flour, mixed and fermented doughs, and the crumb and crust of bread baked from the RBS-78 flour (Fig. 2) showed the same differences and similarities as did the PAGE data. The flour gliadin chromatogram (control, Fig. 2A) was similar to those of the mixed and fermented doughs. The peak sizes of the chromatogram of the fermented dough (Fig. 2C) were about one-half as large as those of the control, apparently due to the amount injected onto the column, because the PAGE patterns were of similar intensity, and the PAGE and HPLC extracts analyzed were aliquots of the same total extract. However, the crumb and crust chromatograms differed from each other and the control. Peaks in the crumb chromatogram (Fig. 2D) between 20 and 23.5 min (35–38% acetonitrile) were very similar to those in the same region in the control. Peaks in the 23.5–26 min range (38–42% acetonitrile) were similar in general but substantially reduced in size from those of the control. The crust chromatogram's major peak was at 22.8 min, the same as the control, but all peaks were very small. The reduced peak size of the crusts' HPLC gliadin peaks were in agreement with the reduced intensities of the PAGE results.

Heat Stability—HPLC Characterization

The HPLC pattern for the crumb extract (Fig. 2D) discussed above showed two areas of interest: fraction I (20–23.5 min region) and fraction II (23.5–26 min region). To determine the relative heat stability of the proteins or the degree of protein interaction with other components during heating, fractions I and II were collected and heated as previously described. The samples were cooled and analyzed by HPLC (Fig. 3) as were the unheated controls I and II. The chromatogram of the isolated gliadin peak I heated at 100° C for 15 min (Fig. 3B) was not appreciably changed from the gliadin pattern of the control (Fig. 3A). However, heating I at 200° C for 15 min (Fig. 3C) caused major changes in the 22-min peak by modifying the proteins into appreciably less hydrophobic materials. The heating of the isolated gliadin fraction II (Fig. 3D) for 15 min at 100° C (Fig. 3E) showed major changes in the peak sizes in their chromatograms, which may be caused by thermal degradation. The fraction II peaks heated at 200° C for 15 min (Fig. 3F) appear changed from the control, with material of lower hydrophobicity being produced and with loss of peak structure on the sides of the main peak. However, the increase in size of the peak was not expected. Possible reasons for that increase include false absorption due to slight turbidity of those proteins eluted between 20 and 25 min on the polymerization of proteins. The peak characteristics of the products appear very complex and polymeric, which is in agreement with a similar study by Meier et al (1985).

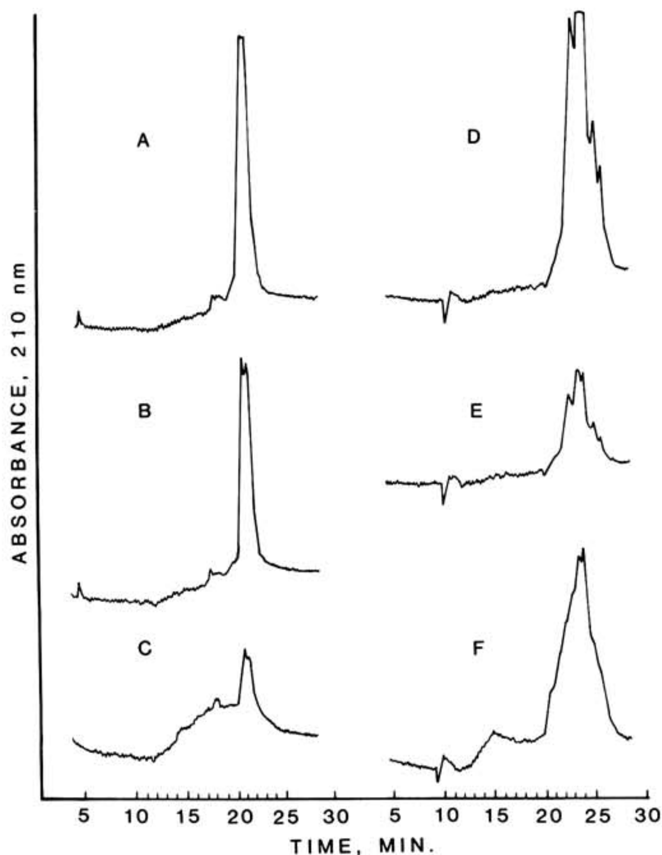

Fig. 3. High-performance liquid chromatography patterns of heated and unheated gliadin fractions I and II collected from control extract at 20–23.5 min and 23.5–26 min, respectively. Fraction I control (**A**), I heated at 100° C for 15 min (**B**), I heated at 200° C for 15 min (**C**), II control (**D**), II heated at 100° C for 15 min (**E**), and II heated at 200° C for 15 min (**F**). The absorbance scale was the same for all samples.

Heat Stability—PAGE Characterization

The isolated gliadin fractions I and II and aliquots that had undergone the heating regimen discussed above were subjected to PAGE. The gliadin electrophoregrams of control fraction I (Fig. 4B) and fraction I heated at 100° C for 15 min (Fig. 4C) were similar (contained most of the bands) to the pattern of the parent flour (Fig. 4A). The intensities of the bands of the 100° C heated I fraction were lower than those of either the parent flour or the control fraction I peak. This is in agreement with the HPLC data discussed earlier. Also in agreement with the HPLC data is the absence of PAGE bands produced by heating fraction I material at 200° C for 15 min (Fig. 4D). The fraction II material's electrophoretic band patterns had mobilities $>$ 45 RM, and their intensities were darkest where fraction I bands were very light or nonexistent. The gliadin PAGE patterns of fraction II control (Fig. 4E) and the fraction II heated at 100° C for 15 min (Fig. 4F) appeared similar. This is in contrast to the HPLC data shown in Figure 3. The electrophoregram of the pooled fraction II heated for 15 min at 200° C was blank (Fig. 4G), implying degradation of the proteins as described above.

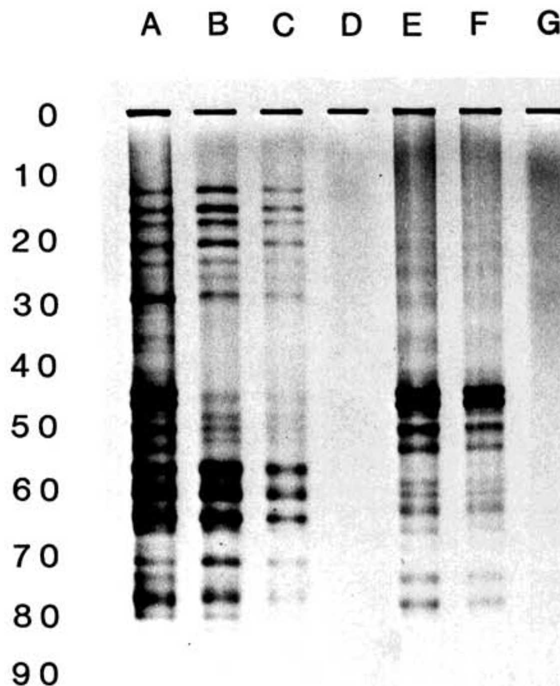

Fig. 4. Polyacrylamide gel eletrophoresis patterns of the heated and unheated gliadin peaks I and II collected from an RBS-78 flour extract in the ranges of 20–23.5 min and 23.5–26 min, respectively. RBS-78 flour extract (**A**), fraction I control (**B**), I heated at 100° C for 15 min (**C**), I heated at 200° C for 15 min (**D**), fraction II control (**E**), II heated at 100° C for 15 min (**F**), and II heated at 200° C for 15 min (**G**).

CONCLUSION

HPLC and PAGE patterns of gliadin proteins demonstrated an interaction of gliadin proteins during bread baking but not during mixing or fermentation. Mixing or fermentation or both may affect the gliadin proteins, but those changes might have been so weak that the severe denaturation of extraction and separation of electrophoresis might not allow determination of it. The more hydrophobic gliadin proteins appeared to be more heat labile than the less hydrophobic gliadin proteins.

Meier et al (1985) reported in a similar study that gliadin HPLC peak 35 (eluting at 34% acetonitrile) increased in intensity as a result of heating doughs above 90° C for 40 min. Our data do not support that finding. However, the gliadins in the two studies were not treated or extracted by the same procedures, and the length of heating was different: 40 versus 15 min. The gliadin peak in this study (elution time of 22 min) that may correlate with the peak 35 of Meier et al was relatively constant in size in the flour, dough, and crumb extracts. It also was eluted with about 34% acetonitrile.

The higher temperature study of Meier et al (1985) at 150° C is in agreement with our in vitro 200° C and crust studies in that all peaks were changed and a polymeric pattern was produced. The sodium dodecyl sulfate-PAGE data of Meier et al (1985), which showed increases in molecular weights of the gliadin proteins on heating, correlated well with our PAGE and HPLC data at elevated temperatures, which also showed major protein changes on heating.

The importance of using HPLC and PAGE together in attacking complex problems was shown clearly. Whereas HPLC was more sensitive (detected smaller amounts of protein) and easier to use in collecting fractions, the PAGE procedure separated the protein fraction into numerous bands. The two HPLC peaks of fraction I were separated by PAGE into about 25 bands, and the four fraction II HPLC peaks were separated by PAGE into 14 bands. Because different and important data can be achieved by both methods, they are truly complementary.

Both the study of Meier et al (1985) (which was brought to our attention after the completion of our work) and the results reported in this paper were conducted on single flours. PAGE and HPLC patterns of gliadin proteins in the crumb of bread from wheats varying widely in breadmaking potential will be reported elsewhere.

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