

Extrusion Chemistry of Wheat Flour Proteins:

II. Sulfhydryl-Disulfide Content and Protein Structural Changes

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ABSTRACT

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Effects of twin-screw extrusion conditions on wheat flour proteins were studied, using a two-level fractional factorial experimental design (11 and 14% protein content, 160 and 185°C, 16 and 20% moisture, 300 and 500 rpm screw speed, mass flow rate of 225 and 400 g/min). Total protein detectable by solid-phase bicinchoninic acid assay decreased slightly after extrusion, with greatest protein loss at 16% moisture and 160°C. Sulfhydryl content of both flours increased after extrusion at 185°C and 16% moisture with moderate specific mechanical energy (SME = 400–600 kJ/kg) or 160°C and 16% moisture with high SME (SME > 1,000 kJ/kg). Disulfide bonds increased under comparable conditions but with moderate shear (SME = 510–540 kJ/kg). At 20% moisture and either

temperature, sulfhydryl and total thiol contents decreased without corresponding increases in disulfides. Reversed-phase HPLC indicated gliadins were the fractions most affected by extrusion; high molecular weight glutenin subunits also were affected. Changes in gliadins were extensive at 185°C and 16% moisture and were minimal at 160°C and 20% moisture. SDS-PAGE confirmed the disappearance of protein bands and appearance of new material at low and high molecular weights, presumably resulting from polypeptide fragmentation followed by random radical recombination. Both protein fragmentation and cross-linking appeared to involve free radicals.

Extrusion technology plays a central role in modern cereal-based industry, and significant advances in engineering have led to production of a wide range of extruded products. Nevertheless, surprisingly little still is known about the basic molecular phenomena that contribute to texturization and other product characteristics during extrusion of wheat flour products.

Early studies on extrusion of wheat flour suggest that the mechanical and textural properties of extrudates are largely determined by gluten proteins in dough (Hauck 1980). The mechanical strength of extrudates is directly related to the gluten content of flour (Linko et al 1981). Faubion and Hosney (1982a,b) extruded wheat starch with varying amounts (1–16%) of added wheat gluten or soy protein isolate and showed that the presence of protein is critically important for texturization and that the amount and type of protein in wheat flour markedly affects the textural properties of extrudates. Expansion decreased steadily as gluten protein content increased from 1 to 11%; at levels higher than 11%, expansion again increased. Shear and break strengths changed correspondingly. Similar effects were produced when yeast protein concentrate was extruded with wheat starch (Lai et al 1985). However, addition of soy proteins had the opposite effect: increasing expansion with protein levels of up to 8% and decreasing expansion with protein levels higher than 10%. Extrudates containing soy protein showed substantially greater expansion than those containing wheat gluten.

These results indicate that both the type and level of protein exert important effects on the physical properties of extruded cereal products. However, relatively little is known about the specific molecular changes responsible for these properties. Studies on soy flours and concentrates have attributed texturization to cross-linking of soy proteins, involving both main-chain polypeptide and disulfide bonds (Cumming et al 1973, Hansen et al 1975, Burgess and Stanley 1976, Hager 1984, Neumann et al 1984), although the relative contributions of the two types of cross-linking have not been distinguished. In contrast, information about molecular changes in wheat proteins during extrusion is limited.

Electron paramagnetic resonance (EPR) studies have demonstrated the presence of nitrogen- and sulfur-centered radicals during extru-

sion of wheat flour (Koh et al 1996, Schaich and Rebello 1999). The presence of these radicals suggests fragmentation at disulfide bonds and main-chain peptide or side-chain amide bonds, and radical recombinations could be responsible for cross-linking in extruded products. The chemistry potentially associated with free radical production in proteins of extruded wheat flour should be traceable. We report the effects of extrusion on protein thiol-disulfide content and changes in protein molecular weight arising from fragmentation and cross-linking. Correlation of these chemical changes with free radical production in wheat flour extrudates is demonstrated.

MATERIALS AND METHODS

Two commercial wheat flours were obtained from Bay State Milling Co. (Minneapolis, MN): Bouncer (14% protein, 0.52% ash, and 14% moisture) and Boss (11.4% protein, 0.41% ash, and 14% moisture). Both flours were mixtures of hard red wheat cultivars. Bouncer was composed of spring wheats, and Boss was composed of winter wheats containing up to 50% Bouncer. The flours were chosen for evaluation and comparison of extrusion behavior because they are commonly used in breadmaking. Protein levels were selected to bracket (low and high) the useful functional range shown by Faubion and Hosney (1982b) for such flours.

Bicinchoninic acid (BCA) test reagents for determination of protein were purchased from Pierce Chemical Co. (Rockford, IL). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) for free sulfhydryl and total thiol assays were obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-Vinyl pyridine, trichloroacetic acid, and 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) were used in reversed-phase (RP) HPLC of wheat proteins. SDS (BioRad Laboratories, Richmond, CA); ISS-Pro-Blue staining solution, dithioerythritol, Daiichi silver stain (Integrated Separation Systems, Natick, MA); and acrylamide and bisacrylamide (Amresco Corp., Solon, OH) were used in PAGE of wheat flour proteins. All reagents used in extractions and analyses were of the highest grade available.

Extrusion Conditions

Extrusion of the two wheat flours was conducted on a twin-screw extruder (ZSK30, Werner and Pfleiderer, Ramsey, NJ) as described in Schaich and Rebello (1999).

Chemical Analyses

Extrudates were ground in a mill (Micro Wiley, Thomas Scientific, Swedesboro, NJ) to pass through a 40-mesh sieve. Ground extrudates were placed in glass containers, flushed with nitrogen,

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sealed, and stored frozen until analysis, which usually occurred within a few days. Four replications were performed for each analysis.

Total protein content of control wheat flours and 16 extrudates were determined according to the solid-phase BCA colorimetric assay of Chan and Wasserman (1993b). Six samples of each extrudate were analyzed.

Colorimetric quantification of thiol and disulfide groups was conducted according to the method of Thannhauser et al (1987) as modified for solid-phase reactions by Chan and Wasserman (1993a). The assay provides a rapid, convenient method for quantifying the thiol and disulfide contents of a cereal-based sample without prior extraction of protein in the sample. It overcomes the drawbacks of incomplete extraction or solubilization of the extracted protein, as well as sulfhydryl oxidation or protein loss during subsequent handling. Samples (20-mg), ground to 40 mesh and dried in vacuo, were suspended in 1.0 mL of total sulfhydryl reaction buffer consisting of 6M guanidine hydrochloride, 0.1M sodium sulfite, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.2M Tris/HCl (pH 9.5), and 10 mM disodium 2-nitro-5-thiosulfobenzoate (NTSB²⁻) synthesized from DTNB in the presence of sodium sulfite and oxygen (Thannhauser et al (1987)). Samples were incubated for 60–80 min under nitrogen in the dark and centrifuged for 10 min at 13,600 × g in a microcentrifuge to remove particulate material. A 0.1-mL aliquot of supernatant was removed and diluted with 0.9 mL of 6M guanidine hydrochloride, 0.1M sodium sulfite, 3 mM EDTA, and 0.2M Tris/HCl (pH 8.0). The solution was centrifuged in a microcentrifuge at 13,600 × g for 10 min, and its absorbance was read at 412 nm on a spectrophotometer (Hitachi U-3110). Disulfide content was calculated as the difference between thiol content before and after reduction of disulfide bonds with sodium sulfite. The free sulfhydryl and total thiol contents were calculated assuming an extinction coefficient of 13,600 M⁻¹ cm⁻¹ for yellow chromophore 2 nitro-5-thio-benzoate anion (NTB²⁻). Results were expressed as nanomoles/mg of protein, using protein content determined by BCA assay.

Proteins from 300-mg samples of wheat flour and extrudates (ground to 40 mesh) were extracted with a 12 mL of solution of

70% aqueous ethanol, 1% SDS, and 5% β-mercaptoethanol by magnetic stirring for 24 hr at room temperature. This procedure was adapted from Zhen and Mares (1992). The mixture of solvents commonly used with wheat proteins was found in preliminary studies to be more effective in extracting total protein from extrudates than SDS/β-mercaptoethanol alone, and coextraction of starch was minimized. The samples were then centrifuged in a refrigerated centrifuge (Sorvall RC-D5) at 14,500 rpm for 40 min. Protein in the supernatant was precipitated with 25 mL of cold acetone, and the suspension was held at refrigerated temperatures for 30–60 min to ensure complete precipitation of protein. The isolated protein was recovered by centrifugation of suspensions for 40 min at 14,500 rpm, air-dried overnight, and weighed.

To assess the extent of protein fragmentation and cross-linking as well as to determine the class of wheat proteins most susceptible to extrusion-induced changes, RP-HPLC of pyridylethyl (PE) derivatives of extracted wheat proteins was performed according to the method of Burnouf and Bietz (1984). Samples (20 mg) of air-dried protein were suspended in 2,070 μL of a solution containing 0.05M Tris-HCl, 8M urea, and 5% β-mercaptoethanol (pH 7.5) with magnetic stirring for 2 hr at room temperature.

The reduced protein sulfhydryl groups were alkylated with 30 μL of 4-vinyl pyridine for 2 hr at room temperature to form PE derivatives. The reaction was terminated by adding 400 μL of glacial acetic acid.

RP-HPLC of extracted proteins was performed on a chromatograph (BIO-LC, Dionex Corp., Sunnyvale, CA). A 20-μL sample of the solution described above was injected onto an RP-HPLC column (C18, 250 × 4.1 mm i.d.; Vydac 218TP, Separations Group, Hesperia, CA). Proteins were eluted with a linear gradient from 25 to 55% of acetonitrile in water containing 0.1% trifluoroacetic acid over 60 min. Column temperature was maintained at 70 ± 0.5°C by means of a heating tape connected to a temperature controller (CN76130, Omega Engineering, Stamford, CT) to improve the resolution of the separation (Bietz 1986). The eluent was monitored at 210 nm with a diode array detector (1000S,

TABLE I
Experimental Extrusion Design and Relationship Between Extrusion Conditions and Protein Changes in Wheat Flour Extrudates^a

Sample	Extrusion Parameter ^b						Extrudate Property ^c						
	Prot.	Moist.	DT	SS	MF	SME	PC ^d	SH ^e	S-S ^{e,f}	Total SH ^e	EPR ^g	S ^h	(S-S) ^h
Bouncer							14.1 ± 0.8	11.5 ± 0.7a	51.1 ± 0.7b	113.7 ± 1.4c	...		
G45	14	20	185	500	400	356	13.2 ± 0.5	6.9 ± 0.4	52.0 ± 0.7b	110.9 ± 1.5c	1,410		
G46	14	20	185	300	225	302	13.6 ± 0.5	7.7 ± 0.8	51.5 ± 0.3b	110.6 ± 0.6c	5,554	++	++
G51	14	20	160	500	225	512	13.0 ± 0.1	<0.1	54.7 ± 0.8	109.4 ± 1.5	1,793		
G50	14	20	160	300	400	356	12.3 ± 0.3	1.0 ± 0.3	52.9 ± 1.1b	106.7 ± 2.3	1,318	+	
G44	14	16	185	500	225	656	13.9 ± 0.5	21.6 ± 0.9	43.1 ± 0.8	107.9 ± 1.8	6,823	++	(+)
G43	14	16	185	300	400	356	12.8 ± 0.4	11.4 ± 0.6a	48.9 ± 0.4	109.3 ± 1.0	8,973	++	++
G40	14	16	160	500	400	518	12.8 ± 0.6	<0.1	56.4 ± 1.3	112.8 ± 2.6c	4,771	+	+
G39	14	16	160	300	225	533	12.2 ± 0.5	3.8 ± 0.3	55.7 ± 0.6	115.3 ± 1.3c	3,460	+	(+)
Boss							11.4 ± 0.1	10.0 ± 1.0d	54.8 ± 0.2e	119.5 ± 0.9f	...		
G42	11.4	20	185	500	400	491	11.3 ± 0.1	15.4 ± 2.3	48.0 ± 1.0	111.4 ± 1.0	4,901	++	
G49	11.4	20	185	300	400	236	11.3 ± 0.7	5.3 ± 0.2	52.8 ± 0.9	110.8 ± 1.8	1,540		
G47	11.4	20	160	500	400	410	11.2 ± 0.3	6.5 ± 0.3	56.0 ± 0.5	119.0 ± 1.0f	1,704		
G38	11.4	16	185	500	225	1,016	11.0 ± 0.4	17.5 ± 0.8	49.6 ± 1.2	116.6 ± 2.6f	4,492	+	(+)
G37	11.4	16	185	300	400	554	10.9 ± 0.7	1.6 ± 0.3	56.9 ± 0.7	115.5 ± 1.4	1,580	(+)	
G48	11.4	16	160	500	225	416	10.3 ± 0.2	9.6 ± 1.0d	50.7 ± 1.5	110.9 ± 3.2	3,108	+	
G41	11.4	16	160	300	225	432	11.5 ± 0.5	14.6 ± 0.6	48.2 ± 0.3	111.0 ± 0.8	4,652	++	++
G52	11.4	20	160	300	225	331	11.2 ± 0.4	3.1 ± 2.1	54.5 ± 0.3e	112.1 ± 2.0			

^a G37–G51 are internal codes for extrusion runs under different conditions and are arranged in sequence to facilitate comparisons, not in the order in which experiments were performed. Details of extrusion are provided in Schaich and Rebello (1999).

^b Prot. = protein (%); Moist. = moisture (%); DT = die temperature (°C); SS = screw speed (rpm); MF = mass flow (g/min); SME = specific mechanical energy (kJ/kg).

^c PC = protein content (%); SH, S-S, and total SH are in nmol/mg of protein. EPR = electron paramagnetic resonance.

^d Mean ± standard deviation of six determinations.

^e Mean ± standard deviation of four determinations. Identical letters indicate extrudate samples that are not significantly different from flour values; all other extrudates are significantly different from flour values at *P* < 0.05.

^f Calculated as total thiol content – free sulfhydryl content.

^g Signal intensity. Data from Schaich and Rebello (1999) (arbitrary units/g of extrudate).

^h Relative intensity of sulfur radical species in EPR signals, estimated by visual inspection. ++ = strong; + = moderate; (+) = present, but weak.

ⁱ Signal too weak to be determined.

Applied Biosystems, Foster City, CA) with the detector set at 0.4 AUFS (absorbance units full scale). Data acquisition, storage, and manipulation were controlled by software (Lab Calc, Galactic Industries Corp., Salem, NH).

Purified components from each main wheat protein class were used to calibrate HPLC chromatograms and provide presumptive identification of the wheat fractions most affected by the extrusion process (Rebello 1993). Wheat flours were extracted sequentially with 0.1*N* NaCl and 70% ethanol to isolate albumins and globulins, and then gliadins, respectively. The remaining material was

treated with 70% ethanol, 1% SDS, and 5% 2-mercaptoethanol to extract glutenins (Zhen and Mares 1992). Proteins in extracts were isolated by precipitation in cold acetone.

To provide additional clarification of fragmentation and cross-linking patterns observed using RP-HPLC, SDS-PAGE of the extracted proteins was performed (SE600, Hoefer Scientific Instruments, San Francisco, CA). A 9–18% gradient gel (14 × 16 cm) was used for the separation. A solution (1 mg/mL) of the extracted protein in 62.5 mM Tris-HCl, 3% SDS, and 5% β-mercaptoethanol was prepared. The loading buffer consisted of 100 mM Tris-HCl, 4% SDS, 4*M* urea, 50 mM dithioerythritol, 2.5% glycerol, and 1% bromophenol blue dye. Samples for electrophoresis were prepared by adding 10 μL of loading buffer to 10 μL of protein solution. Samples were incubated at 45–50°C for 5 min prior to loading on gel. Electrophoresis was performed for 16–18 hr at 7°C at a constant current of 12 mA per gel. Gels initially were stained with ISS-Pro-Blue staining solution according to the procedure provided by the manufacturer (Integrated Separation Systems). Gels were destained with 20% ethanol and subsequently silver-stained with Daiichi, according to the procedure provided by the manufacturer (Integrated Separation Systems).

Statistical Analysis

To determine the significance of the effects of each extrusion variable, the sulfhydryl, disulfide, and total thiol content data were fit to a general linear model, and analysis of variance was performed with statistical software (version 6, SAS Institute, Cary, NC). Main

TABLE II
Statistical Significance (*P*)^a of Main Effects (ME) and Interactions of Extrusion Variables on Free Sulfhydryl and Disulfide Contents of Wheat Flour Extrudates

Variable	Free Sulfhydryls		Disulfides	
	ME	Interaction	ME	Interaction
<i>R</i> ²	0.738	0.781	0.702	0.815
Model	0.010	0.013	0.017	0.007
Protein	0.232	0.219	0.884	0.862
H ₂ O	0.020	0.095	0.099	0.091
Flow	0.103	0.019	0.145	0.058
Speed	0.110	0.102	0.303	0.227
Temp.	0.005	0.005	0.002	0.001
H ₂ O-Temp.		0.218		0.047

^a Significance levels determined by analysis of variance, using main effect with or without two-factor interaction models.

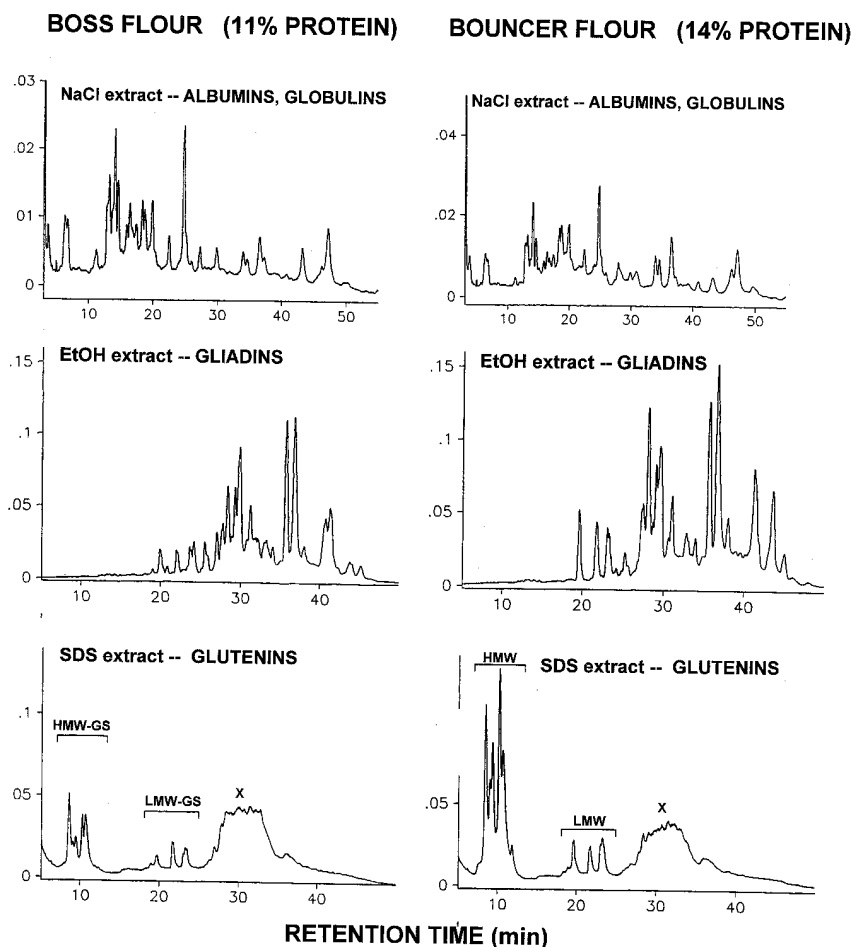


Fig. 1. Reversed-phase HPLC chromatograms of protein fractions extracted sequentially from Boss and Bouncer wheat flours by 0.1*M* NaCl (albumins and globulins), 70% ethanol (EtOH) (gliadins), and finally 70% ethanol plus 1% SDS with 5% β-mercaptoethanol (glutenins). These patterns provided the basis for assignment of peaks in chromatograms of proteins from full flours and extrudates, soluble in 70% ethanol plus 1% SDS with 5% β-mercaptoethanol. X = an unresolved glutenin fraction eluting in the gliadin region in standards and possibly underlying gliadins in flours and extrudates. HMW-GS = high molecular weight glutenin subunits; LMW-GS = low molecular weight glutenin subunits.

effects were evaluated in terms of a main-effect model, with second-order and other higher order interactions pooled with the error. Two-factor interactions between significant main effects (second-order interactions) were determined in a second-order model (Box et al 1978).

RESULTS AND DISCUSSION

Chemical data and extrusion parameters are presented in Table I. The G37–G51 notations are internal codes for extrusion runs performed under the different conditions given in Table I. The data are presented in a sequence that facilitates comparisons, not in the order in which experiments were performed.

Protein Content Determination

Extrusion induced small decreases (at most 14%) in the total protein contents detectable by the BCA reagent compared with the protein contents of the original flours (Table I). Apparent protein loss was greatest in the higher protein flour (Bouncer) and under low moisture and temperature extrusion conditions (16% moisture, 160°C) for both flours.

The solid-state BCA protein assay is a rapid method that has proven useful for quantifying the total protein contents of cornmeal extrudates (Chan and Wasserman 1993b). It is much less time-consuming than Kjeldahl analyses for quantitating both the soluble and insoluble protein fractions simultaneously. In the BCA assay, color formation results specifically from the reactions of cysteine, cystine, tryptophan, and tyrosine (Weichelman et al 1988)

and also is affected by the overall structure of the protein. Consequently, the measured decrease in the protein content of the extrudates may reflect the diminished accessibility of these residues due to cross-linking or aggregation of protein chains, or to destruction or alteration of these amino acids (e.g., by dimerization of cysteine to form disulfide cross-links, oxidation of cysteine to cysteic acid, etc.) rather than loss of intact protein molecules.

Destruction of BCA-reactive amino acids under high heat conditions similar to those found in extrusion have been reported previously (Evans and Butts 1949, Hansen et al 1975, Pinter-Szakacs and Molnar-Perl 1990). The aromatic and sulfur-containing amino acids comprise 10–12% of the total amino acids in wheat proteins (Wrigley and Bietz 1988). Thus, destruction of these amino acids during extrusion could result in significant changes in apparent protein contents determined by the BCA method.

Flour and Extrudate Free Sulfhydryl, Disulfide, and Total Thiol Contents

The sulfhydryl and disulfide contents of Boss and Bouncer flours, measured by solid-phase assay, were comparable to those reported for other wheat flours, indicating the validity of the assay for determining protein SH-SS contents. Boss flour (11% protein) had a free sulfhydryl content of 1.26 ± 0.26 mmol/kg and a disulfide content of 12.66 ± 0.12 mmol/kg, whereas the sulfhydryl and disulfide contents for Bouncer flour (14% protein) were 1.66 ± 0.10 mmol/kg and 14.60 ± 0.02 mmol/kg, respectively (Table I). Bloksma (1972) reported values of 0.71 ± 0.02 mmol/kg and 11.0 ± 0.18 mmol/kg

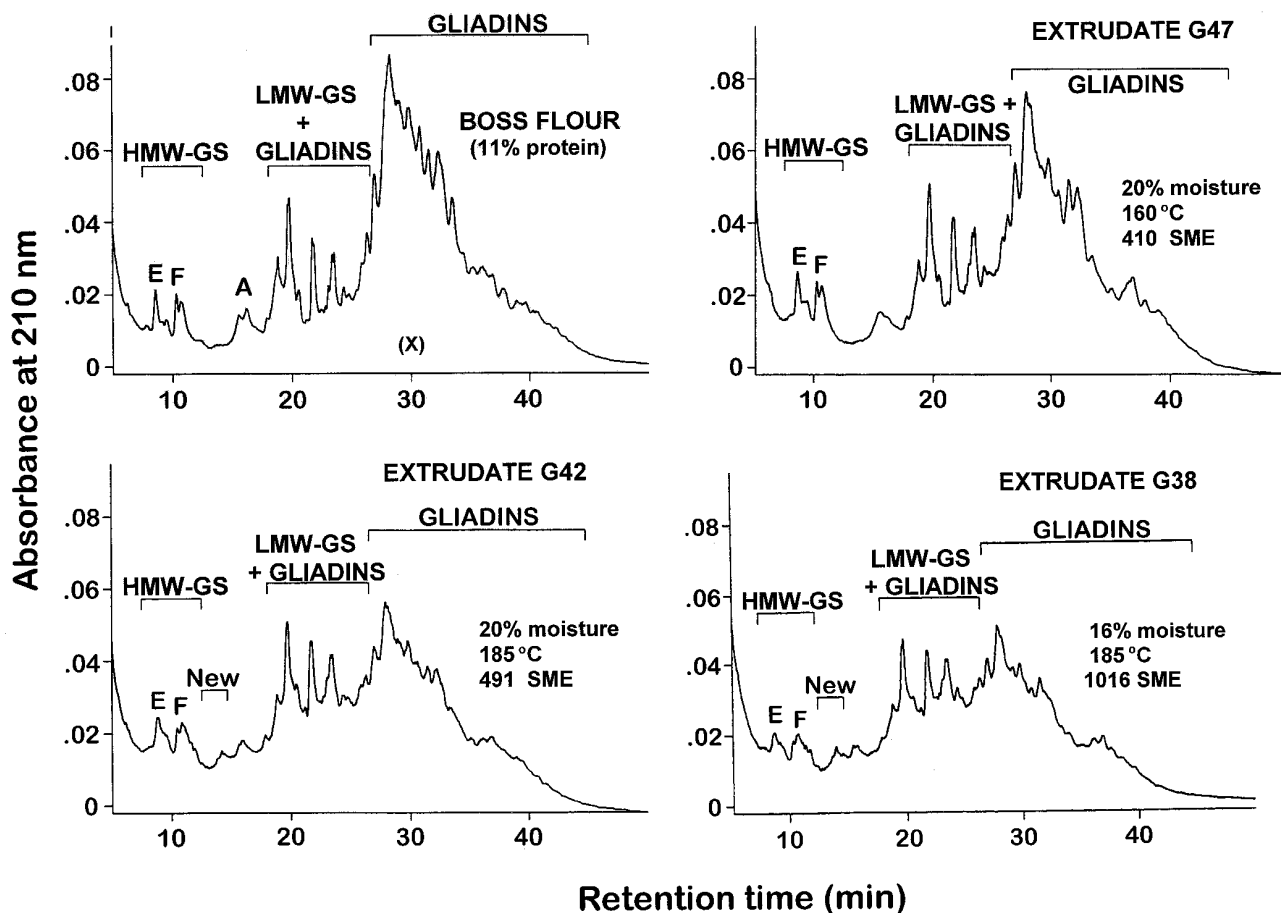


Fig. 2. Reversed-phase HPLC chromatograms of proteins extracted from Boss wheat flour and extrudates (11.4% protein). Upper left: unextruded flour control. Other chromatograms are typical of minor (G47), moderate (G42), and extensive (G38) changes in protein elution patterns. G47, G42, and G38 are extrusion run numbers. A = predominantly an albumin and globulin fraction. HMW-GS = the region in the chromatogram where the high molecular weight glutenin subunits elute. E and F are the two major peaks of this fraction. LMW-GS + gliadins = the region where the low molecular weight glutenin subunits together with the least hydrophobic gliadins elute. New = fractions found only in extrudates, resulting from fragmentation of gliadins or glutenins. SME = specific mechanical energy. Complete extrusion conditions are given in Table I.

for the free sulfhydryl and disulfide contents, respectively, of an 11% protein wheat flour. Tsen and Bushuk (1963) reported free sulfhydryl and disulfide contents of 1.11 mmol/kg and 16.05 mmol/kg, respectively, for a 13.2% protein wheat flour.

Three ranges of extrusion effects on free sulfhydryl contents in wheat flour were found: increased SH, decreased SH, and near or total elimination of free sulfhydryls. In addition to changes in free sulfhydryl content, there were accompanying, although not proportional, increases or decreases in disulfide content and slight decreases in total thiol content in extrudates (Table I).

In general, increased free sulfhydryl and decreased disulfide contents resulted from high temperature (185°C) and low moisture (16%) extrusion conditions. In contrast, extrusion at the lower temperature (160°C) and low-to-moderate shear led to near or total destruction of free sulfhydryl groups, especially when low temperature (160°C) was combined with high moisture (20%). The dominant factor affecting thiol and disulfide contents was temperature ($P \leq 0.005$ for both SH and SS; Table II); moisture effects also were significant ($P \leq 0.02$ for SH; $P \leq 0.10$ for SS). Temperature-moisture interactions were important for disulfides ($P \leq 0.05$) but not for SH (Table II).

Samples showing the greatest increase in free sulfhydryls also showed the greatest decrease in disulfides, although the changes were not proportional. Certainly part of the free sulfhydryl loss could be accounted for by increased disulfide cross-linking under milder extrusion conditions with more moisture to increase protein mobility and intermolecular contact. However, it also is likely that some of the thiol groups released by fragmentation of disulfide bonds were oxidized to sulfoxyl compounds, as evidenced in the

EPR spectra of the extrudates (Schaich and Rebello 1999), or formed sulfur-containing volatiles released at the extruder die and during storage (Riha et al 1996). Destruction of sulfur amino acids previously has been observed during thermal treatment (Evans and Butts 1949) and heating of wheat flours at 174°C and >20% moisture (Hansen et al 1975).

Shear stress had less influence on thiol-disulfide content than did temperature or moisture. The two samples with greatly increased free sulfhydryls (Bouncer G44 and Boss G38) were extruded with the highest specific mechanical energy (SME) (656 and 1,016 kJ/Kg); the relationship was significant at $P \leq 0.10$ (Table II). The effects of shear force (determined by screw speed and flow rate) on disulfide content in extrudates were minor.

Chromatograms of Boss and Bouncer Flours and Extrudates

Historically, structural changes in wheat proteins during heating or dough mixing have been monitored using physical techniques, such as viscosity and turbidity tests (Bietz 1985). More recently, specialized methods of electrophoresis and chromatography have been developed for use in analysis and characterization of cereal endosperm proteins (Bietz 1986). RP-HPLC has been particularly useful for analyzing the complicated heterogeneous mixture of proteins found in wheat (Bietz 1986, Lookhart et al 1986, Lookhart et al 1989, Marchylo et al 1989). Because RP-HPLC offers the potential of better resolution of complex protein mixtures than conventional PAGE and requires smaller protein quantities, we used it to monitor structural changes in extruded wheat proteins. Our main objectives were to determine whether correlations exist between protein structural changes, free radical production, and SH-SS content.

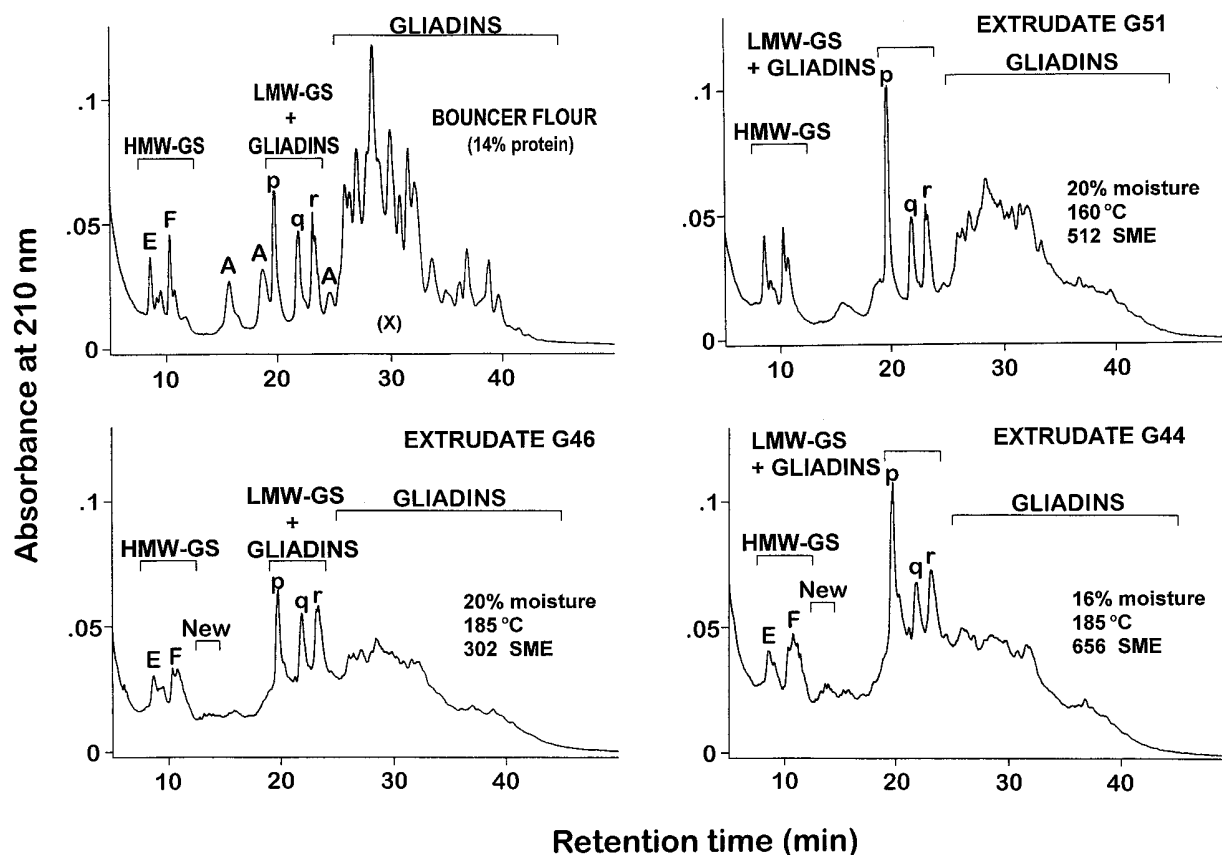


Fig. 3. Reversed-phase chromatograms of proteins extracted from Bouncer wheat flour and extrudates (14% protein). Upper left chromatogram: unextruded flour control. Other chromatograms are typical of minor (G51), moderate (G46), and extensive (G44) changes in protein elution patterns. G51, G46, and G44 are extrusion run numbers. A = predominantly an albumin and globulin fraction, the peaks of which predominate in the mid region of the chromatogram for the control. LMW-GS = the region in the chromatogram where the high molecular weight glutenin subunits elute. E and F are the two major peaks of this fraction. HMW-GS + gliadins = the region where the low molecular weight glutenin subunits together with the least hydrophobic gliadins elute. Peaks p, q, and r indicate well-resolved peaks in this fraction. New = fractions found only in extrudates, resulting from fragmentation of gliadins or glutenins. SME = specific mechanical energy. Complete extrusion conditions are given in Table I.

Samples of each wheat protein class were extracted from the control wheat flours by selective solvents to provide controls for assigning peak groups in chromatograms. Control fractions were analyzed by RP-HPLC under conditions identical to those used for the protein mixtures extracted from the unextruded flours and extrudates. Peaks in control chromatograms (Fig. 1) provided the basis for tentative identification of components in flour and extrudate chromatograms (Figs. 2 and 3).

Representative chromatograms of extrudates and their control flours are shown in Fig. 2 (Boss, 11% protein) and Fig. 3 (Bouncer, 14% protein). The chromatograms selected show typical minimal, moderate, and extensive extrusion-induced changes in extrudate proteins.

Proteins in the region marked "X" on the figures were not soluble in 70% ethanol, were not disassociated by SDS or β -mercaptoethanol and must be covalently cross-linked. A likely source of cross-linking may be radicals produced during grinding and reacting during flour storage. It is unknown whether the proteins involved are glutenins, gliadins, or a mixture of the two.

One of the difficulties in interpreting changes in RP-HPLC patterns is that elution is controlled by complex interactions involving both hydrophobicity and molecular weight. The most hydrophobic and highest molecular weight materials are retained most strongly, but there may be overlaps, or even reversals, in elution sequence of large polar molecules with smaller, more hydrophobic molecules. Gliadins (MW = 25,000 to \approx 75,000) are smaller than glutenin subunits (MW > 100,000) but have more nonpolar functional groups and fewer charged amino acids than glutenin (Wrigley and Bietz 1988). Thus, as can be seen in chromatograms of the control flours, native gliadins are retained longer than other protein fractions. Despite their large size, glutenins elute early because their charged amino acids interact with trifluoroacetic acid in the eluting buffer. Albumins and globulins, which are relatively small, polar, but less charged proteins, elute interspersed with the glutenins.

Gliadins appear to be most affected by extrusion, as judged by the marked change in or loss of gliadin fractions in the RP-HPLC chromatograms. This observation is consistent with the results of Hansen et al (1975), who reported that gliadins were the major

fractions damaged by heat. They observed initial aggregation of the gliadins at 174°C, followed by breakdown of aggregates when wheat flours were heated for longer times. Breakdown of aggregates was proposed to occur both at disulfide and main chain polypeptide bonds. Comparable heat-induced breakdown of gliadins during extrusion could account for the increase in background absorption underlying major peaks and shift of this underlying absorbance toward shorter retention times (decreased hydrophobicity or lower molecular weight). The background absorption results from overlapping peaks of proteins having a broad range of molecular weights and not forming any discrete fractions. The dispersion of protein molecular weights results from random fragmentation of proteins (yielding lower molecular weight fractions), in some cases followed by recombination to polymeric fractions.

Other changes apparent in the chromatograms were decreased distinction of protein peaks, loss of albumin and globulin peaks, and increase in material with very long retention times (>40 min; e.g., G51 and G46 in Fig. 3). Increased retention times could result from increases in either hydrophobicity or molecular weight. Increased hydrophobicity could result from deamidation or decarboxylation of polar side chains, as occurs with formation of flavor compounds during extrusion (Izzo et al 1993, Riha et al 1996). Increased protein molecular weight may be expected to result from cross-linking of intact proteins or recombination of protein fragments to form higher molecular weight fractions. Protein cross-linking reflected in the chromatograms necessarily involved peptide or other nonsulfur cross-links because protein extractions and RP-HPLC analyses were run under conditions that reduced disulfide bonds.

Samples extruded at the lower temperature (160°C), at either protein or moisture content, showed the least change in protein HPLC patterns. Modifications of proteins were most pronounced at the higher extrusion temperature (185°C), particularly in combination with lower moisture. Low-to-moderate shear stress (SME) had no distinguishable effect on the protein patterns at either temperature. However, very high shear (e.g., 656 kJ/kg in G44 and 1,016 kJ/kg in G38) induced marked diminution of gliadin fractions and increases in the "New" (Fig. 2, G42 and G38; Fig. 3, G46 and G44) peaks, over and above the effects of temperature and moisture. This suggests that a high mechanical energy input can bring about changes in wheat proteins similar to those induced by high temperature (e.g., 185°C).

SDS-PAGE of Extracted Proteins

To determine whether changes in the RP-HPLC patterns of various gliadins and glutenin subunits were due to changes in molecular weight or decreases in hydrophobicity, extracted proteins were analyzed by SDS-PAGE on a 9–18% gradient gel. SDS-PAGE patterns of proteins from the control wheat flours and 10 extrudates are shown in Fig. 4. The appearance of new bands indicates the formation of discrete polypeptides with molecular weight different from those of control flours. However, instead of new protein bands, there was an overall disappearance or weakening of several bands of intermediate and high molecular weight and increased smearing of protein fractions (i.e., enhanced background intensity of staining over a broad range of higher molecular weight without the appearance of discrete bands). Because the intermediate molecular weight polypeptides are mainly gliadins, the weakening of this fraction that is evident in the SDS-PAGE results is consistent with similar results obtained by RP-HPLC analysis. SDS-PAGE patterns directly corresponded to observed shifts in RP-HPLC elution patterns and suggest molecular weight is randomized due to random fragmentation and cross-linking or insolubilization of protein to a point at which it is rendered insoluble. Thus, surface hydrophobicity changes leading to differences in elution profiles of proteins on the RP-HPLC column result primarily from changes in the large-scale redistribution of the molecular weight of gliadins rather than localized modifications in hydrophobic amino acid residues.

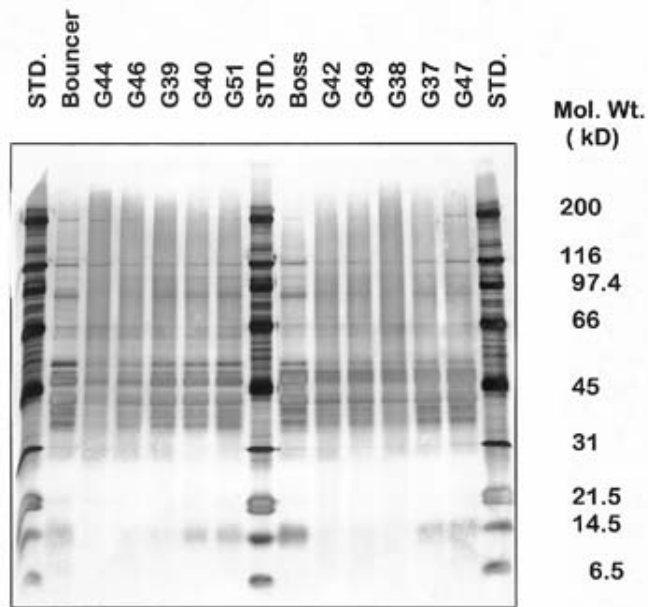


Fig. 4. SDS- β -mercaptoethanol PAGE patterns of proteins extracted from Boss and Bouncer wheat flours and their extrudates, separated on a 9–18% gradient and visualized with silver staining. G37–G51 are extrusion run numbers. Complete extrusion conditions are given in Table I.

Relationships Between Free Radicals and Protein Changes

There was a strong correlation between free radical contents (Schaich and Rebello 1999) and protein sulfhydryl-disulfide and structural changes in wheat flour extrudates (Table I). However, there was little correlation between free sulfhydryl or disulfide contents and structural changes evident in RP-HPLC. Some samples showing moderate-to-extensive RP-HPLC changes had minimal changes in disulfide contents, and extrudates that had widely varying HPLC patterns differed little in disulfide content.

In general, most extensive HPLC and PAGE changes were observed in extrudates having the highest stable free radical content (Schaich and Rebello 1999). These extrudates were extruded at high temperature and low moisture and showed dominant nitrogen-centered radicals. Various sulfur radical species were present to a lesser extent. Under these conditions, the highest concentrations of radicals were generated, and radicals had the least mobility for recombination. However, unless shear stress in these samples was at least moderate, little change in disulfide content occurred.

Conversely, under low temperature-high moisture conditions, less peptide backbone scission occurs, so fewer peptide radicals are generated. Furthermore, high moisture quenches radicals, facilitates radical recombination, and reduces SME by reducing friction in the dough. All of these factors tended to protect the disulfide content of extrudates. The result was a less extensive change in proteins. One exception to this pattern occurs when high shear stress is present to increase fragmentation of disulfides and intermixing of protein chains. This leads to more disulfide scission, a large increase in free sulfhydryls, and marked changes in RP-HPLC patterns.

These observations are consistent with a mechanism in which disulfide bonds between glutenin subunits remain largely intact during extrusion, whether in their initial configuration or randomized intermolecularly, while molecular scission at peptide points along the protein chains dominates. This supports the findings of Koh et al (1996). Formation of high molecular weight protein fractions appears to involve primarily intermolecular cross-linking of wheat proteins, particularly gliadins, through recombination of peptide radicals as the dominant reaction. Disulfide bonds appear to contribute in a minor way, except perhaps under conditions of high shear, when they act by linking cross-linked masses of proteins.

CONCLUSIONS

Sulfhydryl-disulfide contents and protein fragmentation and cross-linking were measured to investigate the chemistry potentially associated with free radical production in proteins of extruded wheat flour and to determine associated effects of extrusion conditions (protein content, moisture content, extrusion temperature, and the SME derived from mass flow rate and screw speed).

These results support the accompanying EPR study (Schaich and Rebello 1999), which found nitrogen- and sulfur-centered radicals in extruded wheat flours, providing evidence that free radicals are important mediators of protein changes during extrusion. Changes in the wheat protein free sulfhydryl contents with accompanying decreases or increases in disulfide contents, as well as fragmentation and cross-linking of proteins, reflect the free radical production observed in these extrudates and are closely linked to die temperature and moisture contents during extrusion. The lack of correspondence between sulfhydryl-disulfide contents and RP-HPLC patterns suggests that sulfur linkages may be less involved than peptide cross-links in overall protein cross-linking and associated product texturization. Studies are underway to identify more specifically the protein fractions involved in fragmentation and cross-linking, the type of cross-linking, and the relationships of these changes to product textures.

Understanding the changes that take place in wheat proteins during extrusion can help guide future design of extrusion-processed foods containing wheat flour or gluten.

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