Effects of Cysteine on Free Radical Production and Protein Modification in Extruded Wheat Flour[1,2]

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ABSTRACT

Addition of cysteine as a free radical scavenger during extrusion markedly affected physical and chemical properties of wheat flour extrudates. Radial expansion at the die decreased in linear extrudates and longitudinal expansion through surfaces cut at the die increased, reflecting weakened dough strength. Cell size decreased, cell walls thinned, and cells became more evenly distributed and densely packed. Cysteine decreased electron paramagnetic resonance (EPR) free radical signal intensity but increased free radical density of the extrudates, preferentially quenching nitrogen-centered radicals. Nonprotein thiols decreased and disulfides increased in samples with added cysteine, whereas the opposite occurred with protein SH-SS groups. Protein solubility in 1.5% sodium dodecyl sulfate (SDS) without β-mercaptoethanol decreased markedly with extrusion but increased with each increment of cysteine. Addition of cysteine slightly increased protein solubility in SDS plus β-mercaptoethanol compared to that of unextruded flour. Polyacrylamide gel electrophoresis (PAGE) patterns of extracted proteins showed increasing amounts of high molecular weight material solubilized from previously insoluble aggregates. Glycoproteins, probably starch-protein complexes, were detected in the highest molecular weight material. PAGE patterns after reduction of disulfide bonds showed shifts in molecular weight distributions from high to low molecular weight products with added cysteine. All together, data indicates that cysteine acts predominantly by free radical quenching. Little or no thiol-disulfide interchange or sulfur radical addition was detected.

Extrusion has become an important method for processing cereal grains into a variety of unique products. The thermal and mechanical energy imparted to the dough during extrusion causes characteristic textural changes of wheat flour dough. Proteins become denatured and the forces that stabilize the tertiary and quaternary structure of proteins are weakened by a combination of increased heat and shear within the extruder (Camire 1991). The functionality of denatured proteins is very critical for the characteristic textural differences between baked and extruded wheat flour doughs (Faubion and Hoseney 1982). However, extrusion remains largely an art. Not enough is yet understood about molecular changes occurring during extrusion for the process to be fully controllable and predictable.

It has been proposed that protein crosslinking during extrusion is responsible for the textural characteristics of extruded products. One obvious source of crosslinks is disulfide bonds. Disulfide crosslinks (-S-S-) have been identified in spun soy protein fibers (Chiang and Sternberg 1974) as well as in heated soy protein systems (Wolf 1970), and a patent has been issued for improving texture of extruded soy granules by addition of elemental sulfur or potassium or sodium sulfides, presumably by increasing disulfide crosslinks (Jenkins 1970).

A second source of protein crosslinks that has received less attention is intermolecular peptide crosslinks. Burgess and Stanley (1976) proposed that during thermoplastic extrusion the major crosslinking occurs via intermolecular peptide bonds, with hydrophobic interactions plus hydrogen and disulfide bonds playing important but secondary roles. Work of Cumming et al (1973) supports this proposed mechanism for soy proteins.

The situation with wheat flour proteins is somewhat different. The role of disulfide crosslinks in wheat flour proteins and bread dough formation has been established for some time (Tsen and Anderson 1963, Tsen and Bushuk 1963, Mauritzen and Stewart 1965, Bloksma 1975, Graveland et al 1980, Ewart 1988, Sarwin et al 1993). Numerous studies have shown that addition of sulfhydryl compounds such as cysteine or glutathione modified dough characteristics during mixing and baking, presumably due to free radical scavenging and thiol-disulfide interchange (Mauritzen 1967, Jones and Carnegie 1971, Kilborn and Tipples 1973, Moss 1975, Kaufman et al 1986, Dreese et al 1988). However, analogous studies of sulfhydryl effects on extruded wheat flour proteins have not been reported.

Recently, Schaich (1992) observed that protein free radicals were produced during extrusion by thermal and mechanical forces, and that reactions and rearrangements of these radicals are critically involved in protein crosslinking and textural characteristics of extruded wheat flour. Free radical production was correlated with protein crosslinking and fragmentation, with SH-SS contents, volatile retention, and puff ratios (Rebello 1993).

In the present study cysteine was added as a free radical scavenger during extrusion to verify and investigate the involvement of free radicals in extrusion chemistry. We report here the interrelationships between free radical formation, protein modifications, and textural characteristics of extruded wheat flour in the presence of cysteine.

MATERIALS AND METHODS

Flour

Untreated straight-grade flour milled from hard red winter wheat (Bay State Milling Co., Winona, MN) was used. The ash and protein (N × 5.7) contents of the flour were 0.52 and 14%, respectively, and moisture content was 14%.

Extrusion Conditions

Wheat flour extrudates were generated by a Werner & Pfleiderer ZSK-30 twin-screw extruder, (Werner & Pfleiderer Corp., Ramsey, NJ). The extruder has two co-rotating, self-wiping screws in a steel barrel with five zones. The temperature of each zone can be controlled independently. Each zone is heated by resistive electric heaters. Water flow through the cooling jackets of the barrel is controlled by solenoids. Each of the screws has an outside diameter (D) of 30.7 mm and total length (L) of 878 mm

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(total L/D = 29.3). The screw configuration (Fig. 1) used in the extrusion experiments consisted of forward conveying elements (L/D = 21.9), two mild mixing elements (L/D = 2.7), six kneading elements (L/D = 3.6), and two reverse elements (L/D = 1.1). Each extruder die had two circular outlets with diameters of 3 mm and land length of 5 mm.

In a ribbon mixer, flour was dry-mixed with cysteine at concentrations of 0, 0.5, 1, and 2% of flour weight for 15 min and fed into the extruder with a K-Tron series 7100 loss-in-weight feeding system (K-Tron Corp., Pitman, NJ). Water was fed into the first extruder zone, immediately after the feed zone, by a metering pump (US Electric Motors, Milford, CT). Temperature and pressure of the extrudate at the die were measured by a TPT 463E-10M transducer (Dynisco, Sharon, MA). Process variables were selected to provide optimum wheat flour expansion, flavor, and textural quality, based on earlier experiments (Rebello 1993). The mass flow rate for all experiments was 225 g/min and the screw speed was 500 rpm. Total process moisture was 16% (w/w) and temperature of the extrudate at the die was constant at 185°C.

Immediately after extrusion, the extrudates were cooled, packed into glass Ball canning jars, flushed with nitrogen, sealed, and frozen until analysis.

Expansion Ratio

The expansion ratios of the extrudates were determined as the cross-sectional diameters of the extrudates divided by the diameter of the die of the extruder (Alvarez-Martinez et al 1988). The data were obtained by averaging caliper measurements from 20 random samples of each extrudate.

Scanning Electron Microscopy

Individual extrudate samples were broken to expose cross-sectional areas. The cross-sections were mounted fracture face up, coated with 40 nm of gold-palladium and examined in an Awray 1830I scanning electron microscope (SEM). Details of each sample were recorded using Polaroid photography.

Electron Paramagnetic Resonance Analysis

Free radicals in extrudates were detected using a Varian E-12 Electron Paramagnetic Resonance spectrometer equipped with a Q-band (9.5 GHz) microwave bridge and operated at 100 kHz modulation. Samples were analyzed at 77 K using a Varian variable temperature controller cooled by a constant flow of vapor from liquid nitrogen. Low temperature measurements of the extrudates were necessary to detect sulfur-centered radicals that anneal or broaden at room temperature (Schaich 1992).

Data collection and analysis was controlled through a MassComp 5500 minicomputer. Spectra were collected by signal averaging of repetitive 30-sec scans, typically 20 to 30 scans per sample. Signal intensities were calculated by double integration of standard first-derivative presentations of spectra. Spectral *g*-values were determined by comparison with a DPPH (α,α-diphenyl-β-picrylhydrazyl) standard according to standard methods (Bolton et al 1972).

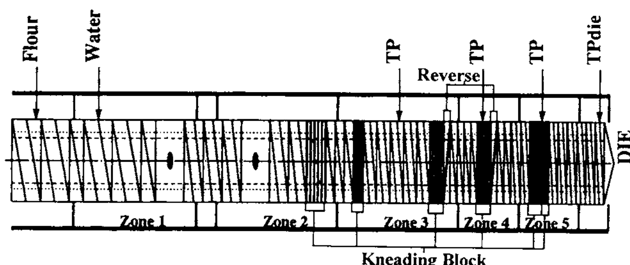


Fig. 1. Screw configuration of ZSK-30 twin-screw extruder from hopper to die. TP = temperature and pressure measured; TPdie = temperature and pressure measured at the die.

Because the physical force of grinding produces free radicals in biological materials (Swartz 1972), extrudate samples were gently ground to visible homogeneity using a mortar and pestle. Comparisons of samples analyzed whole and ground by various methods showed that this gentle hand-grinding caused minimal modification of electron paramagnetic resonance (EPR) signals in the extrudates (Schaich 1994a). Samples were immediately packed into 10-mm i.d. quartz EPR tubes, weighed, frozen by immersion in liquid nitrogen, then transferred to the EPR cavity for EPR analysis. Sample densities (g/cm) calculated from sample weight and packed sample height were used to normalize EPR signal intensities. At least three samples were analyzed for each extrudate.

Chemical Analyses

For all chemical analyses extrudates were ground in a Micro Wiley Mill (Thomas Scientific, Swedesboro, NJ) to pass through an 40-mesh sieve, flushed with nitrogen, sealed, and frozen between analyses. This degree of grinding was sufficient to facilitate extraction of protein without causing molecular damage (Schaich 1994a).

Sulfhydryl and Disulfide Contents

Sulfhydryl and disulfide contents of extrudates were determined according to a modification of the solid-phase colorimetric assay of Chan and Wasserman (1993), using higher levels of the DTNB (5,5'-dithiobis [2-nitrobenzoic acid], Aldrich, Milwaukee, WI) reagent to adjust for the added cysteine. Disulfide contents were calculated from differences between free and total sulfhydryl contents.

To assay nonprotein thiol and disulfide groups that did not react with flour proteins during extrusion, 1-g samples of flour or extrudates were extracted with 25 ml of distilled water, then centrifuged at 23,425 × *g* for 1 hr. Protein in the extract was precipitated with cold ethyl alcohol (1:4, v/v) and removed by centrifugation (23,425 × *g*, 10 min). The solvent in the protein-free supernatant was evaporated by rotary evaporator, and the sulfhydryl-disulfide contents of the residues were assayed with DTNB as described above. Protein thiol and disulfide groups were calculated from difference between total extrudate and nonprotein thiol and disulfide content. At least five samples were analyzed for each extrudate.

Extraction and Determination of Protein

Sodium dodecyl sulfate (SDS) soluble proteins were extracted from 1 g of flour or extrudates with 25 ml of 1.5% SDS solution (Graveland and Henderson 1987). Samples were stirred for 10 min to make a uniform slurry, then shaken at 300 rpm for 24 hr at room temperature in the dark under a N_2 atmosphere to minimize oxidation of protein. This incubation time maximized protein extraction by SDS in previous studies (He et al 1991).

Proteins soluble in SDS and β-mercaptoethanol were extracted from 1 g of flour or extrudates with 25 ml of mixture of 1.5% SDS and 1% β-mercaptoethanol solution for 1 hr under the conditions described above. Extracts were centrifuged at 23,425 × *g* for 1 hr, and each supernatant was diluted to less than 0.1% SDS concentration. Total protein content of the extracts was deter-

TABLE I
Expansion Ratio of Extruded Wheat Flour with Cysteine

Extrudate	Expansion Ratio[a]
+0% cys	3.05 ± 0.14
+0.5% cys	2.75 ± 0.17
+1% cys	2.36 ± 0.18
+2% cys	2.24 ± 0.07

[a] Values are averages of each 20 samples ± standard deviations.

mined by the Bio-Rad protein assay (Bio-Rad Laboratory, Richmond, CA) based on the Bradford dye binding method, using bovine gamma globulin (Bio-Rad) as a standard.

SDS-Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed as a modification of the method of Laemmli (1970), using a Hoefer SE600 vertical slab electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA) operated at a constant current of 30 mA. PAGE gels with a length of 16 cm, width of 14 cm, and thickness of 1.5 mm were cast with 12 sample slots. The stacking gel was 5.2% T (total gel concentration) and 2.7% C (crosslinker concentration), whereas the separating gel was 13 to 18% T gradient and 2.6% C. Gels were run under nonreducing conditions.

Protein samples for PAGE were prepared from 1 ml of protein extracts (1.5% SDS extracted protein solution with or without 1% β-mercaptoethanol solution, as described above). Proteins in extracts were precipitated with cold acetone (1:4, v/v), 3 hr at –20°C. The precipitates were centrifuged (10,400 × *g*, 10 min), and the residue proteins were dried under vacuum. Protein pellets (1 mg) were dissolved in 1-ml sample buffers including 8*M* urea for unreduced protein (SDS soluble) or 4% β-mercaptoethanol for reduced protein (SDS and β-mercaptoethanol soluble). Unreduced and reduced proteins were run on the same gel, always leaving two empty slots between samples to prevent partial reduction of unreduced samples. Molecular weight protein markers (Bio-Rad) were run on each gel as standards.

Duplicate gels were run under identical conditions for each set of protein samples. One gel was stained for general protein detection with 0.02% Coomassie Brilliant Blue R250 in 12% trichloroacetic acid and 12% ethanol overnight, following the procedures of MacRitchie et al (1991). The second gel was stained specifically for glycoproteins with a phenol-sulfuric acid mixture according to the method of Racusen (1979). Three sets of gels were run for each extrudate.

Densitometry Scans

To quantitate individual protein bands and regions on the PAGE gels, stained gels were scanned with a computer-controlled Laser Densitometer (Ultroscan XL, Pharmacia LKB Biotechnology) equipped with a Gel Scan XL software program.

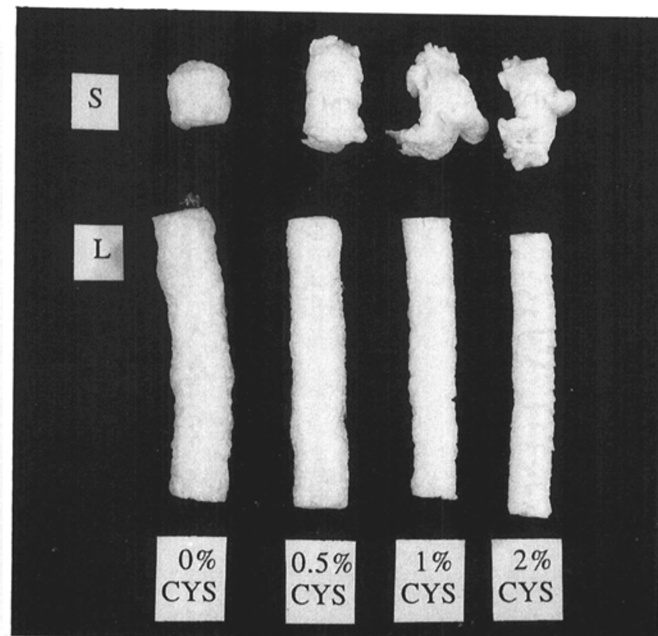


Fig. 2. Physical appearance of wheat flour extrudates extruded with addition of varying amounts of cysteine. S = short extrudate cut into 1-cm pieces at the die, L = long strands from continuous extrusion, % cys = cysteine added during extrusion.

RESULTS

Physical Characteristics of Extrudates

Addition of cysteine during extrusion markedly changed the physical characteristics of extrudates. Two seemingly contradictory effects on puffing or expansion were observed. The expansion ratio of wheat flour samples extruded as long strands decreased as the added cysteine increased, as shown in Figure 2 and Table I. However, when the dough was cut into 1-cm pieces as the dough passed through the die during extrusion, there was puffing through the cut surfaces, so that the extrudates gradually changed from small spheres (little or no cysteine) to irregular jagged particles that were popcorn-like at the higher levels of cysteine (Fig. 2).

SEM micrographs (Fig. 3) of cross-sections of long strand extrudates showed that when cysteine was added, cells were smaller and cell structure of the extrudates was finer and more even than without cysteine. The average cell wall thickness of extrudates became thinner (Fig. 4), decreasing from 1.2–2.3 μm to <0.6 μm (Lei and Lee 1994). It should be noted, however, that cell wall thicknesses were not completely uniform.

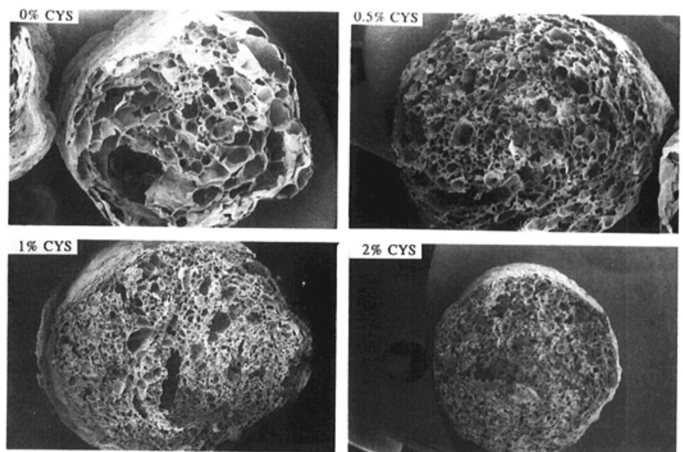


Fig. 3. Scanning electron microscopic images (×7.8) of cross-sections from long strand wheat flour extrudates. % cys = cysteine added during extrusion.

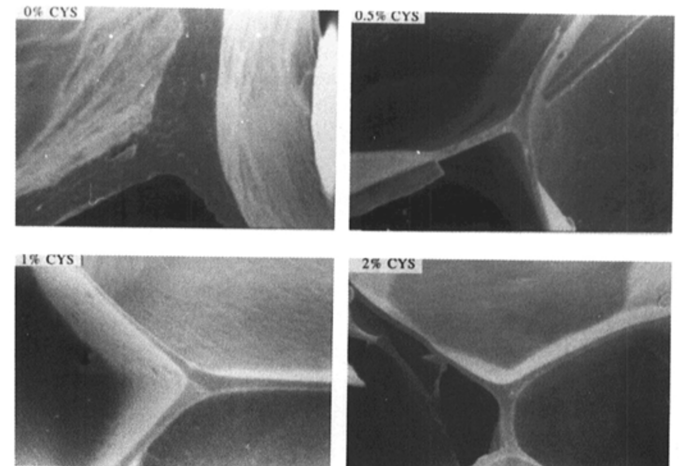


Fig. 4. Scanning electron microscopic images (×1,030) of cell walls in cross-sections of long strand wheat flour extrudates. % cys = cysteine added during extrusion.

EPR Measurements of Free Radicals

Typical EPR spectra from wheat flour extruded with various amounts of cysteine are shown in Figure 5. These spectra are composite spectra typical of proteins with contributions from both nitrogen-centered and sulfur-centered radicals of proteins (Fig. 6) (Schaich 1980a,b).

As the concentration of added cysteine increased, EPR signal intensity appeared to decrease, primarily in the center line attributable to peptide radicals. However, because the samples with added cysteine were very fine and light in texture and density, when signal intensities were normalized to sample density, the free radical concentration per gram of extrudate actually increased with addition of cysteine. Computer simulations indicated that sulfur radicals were responsible for most of the signal intensity.

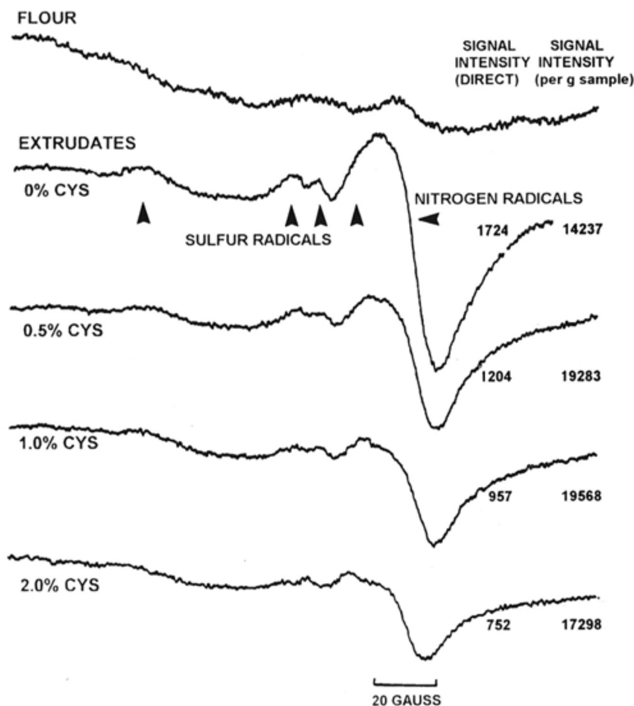


Fig. 5. Electron paramagnetic resonance (EPR) spectra of wheat flour with different amounts of cysteine added during extrusion. % cys = cysteine added during extrusion; EPR signal intensities are given in arbitrary units.

This data suggests that the cysteine donated hydrogens to quench the nitrogen-centered protein radicals, forming cysteine thiyl radicals (cyS•) in the process. Low levels of disulfide radicals $(\text{-S•S-})^+$ were also formed, probably by recombination of cyS• radicals. It was not possible to ascertain from the EPR spectra whether cysteine quenched protein thiyl radicals (PS•).

Sulfhydryl and Disulfide (SH/S-S) Contents

Sulfhydryl and disulfide contents from the flour and extrudates are presented in Table II. Sulfhydryl contents of the flour were consistent with levels previously reported for North American wheat cultivars (Tsen and Anderson 1963, Ewart 1988). Total sulfhydryl contents (SH+S-S) of the extrudates were much lower than would be expected from the amounts of cysteine added, suggesting that a large proportion of cysteine added during extrusion was most likely lost at the die as hydrogen sulfide or volatile organic compounds, such as those formed as flavor compounds in the extrudate.

Total sulfhydryl analyses of the extrudates could not distinguish between free cysteine that was added during extrusion and did not bind to proteins (hereafter referred to as nonprotein sulfhydryls) and cysteine that was bound to proteins (hereafter referred to as protein sulfhydryls). However, when the nonprotein fractions were separated from proteins by aqueous extraction, it became clear that nonprotein free sulfhydryls had changed little, while nonprotein disulfides increased by more than 100-fold in the extrudates. In contrast, protein sulfhydryl contents increased more than tenfold, while protein disulfide contents decreased to about half the original level.

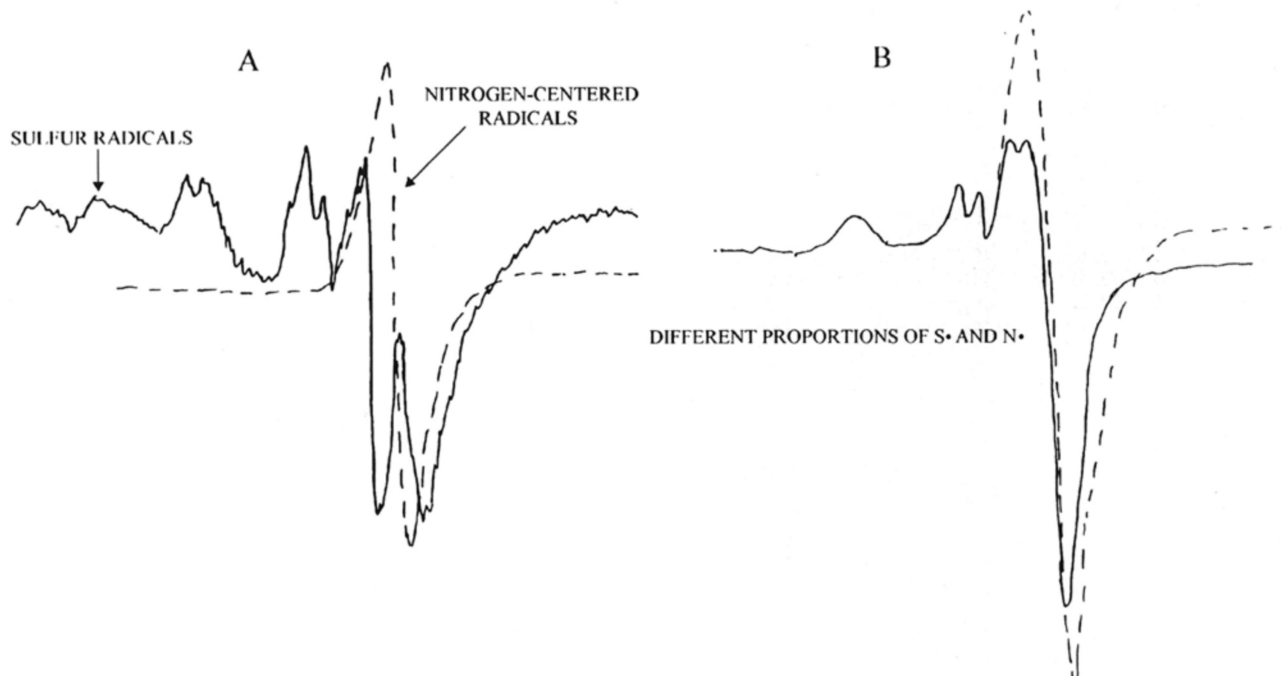


Fig. 6. Components of electron paramagnetic resonance (EPR) spectra from wheat flour extruded with added cysteine. **A,** Superimposed spectra of protein spectrum with only nitrogen-centered radicals (generated by heating dry wheat flour) and sulfur radical spectrum generated from oxidation of cysteine. **B,** Spectra from extruded samples with different proportions of nitrogen-centered and sulfur-centered radicals. Computer addition of the two spectra in A gives a spectrum nearly identical to those in B. Computer subtraction of the nitrogen radical spectrum in A from either extrudate spectrum in B gives a spectrum comparable to the cysteine sulfur radical spectrum in A.

Protein Solubility

Solubilization of proteins in extrudates by 1.5% SDS with and without β-mercaptoethanol is shown in Figure 7. Without reduction of disulfide bonds, extraction of proteins is dramatically decreased by extrusion when compared to the parent flour. However, each addition of cysteine increased protein solubility to the point that the protein solubilization at 2.0% added cysteine was slightly greater than that of the unextruded flour.

When β-mercaptoethanol was included to reduce S-S bonds during extraction, protein extractability in the various extrudates increased slightly with addition of cysteine, and protein solubilization in all the extrudates was greater than in the unextruded flour. However, the differences between the various sample groups were relatively small.

Changes in Protein Molecular Fragmentation

PAGE patterns of flour and extrudate proteins extracted in 1.5% SDS with or without β-mercaptoethanol and separated on nonreducing gradient gels are shown in Figure 8. Without β-mercaptoethanol during extraction (left set of gels), much of the distinction between protein bands was lost after extrusion, and increased smearing of bands was visibly apparent in both high molecular weight and low molecular weight regions of the gels, particularly in samples with cysteine added during extrusion. Some of high molecular weight fractions of extrudates with or without cysteine were glycoproteins or starch-protein complexes, as detected by phenol sulfuric acid staining (Fig. 9). All of these changes arose from protein fragmentation.

After reduction of disulfide bonds with β-mercaptoethanol (right set of gels, Fig. 8), strong bands appeared or increased between 66 and ≈100 kDa in both flour and the extrusion control without any cysteine. The extrusion control also showed marked increases in diffusely distributed high molecular weight material. As the amount of cysteine added to extrudates increased, the bands between 66 and ≈100 kDa decreased, and there appeared to be a shift from high molecular weight material to low molecular weight fractions (<30 kDa).

Densitometer scans of PAGE gels with mercaptoethanol verified increases in high and low molecular weight fractions (Fig. 10).

TABLE II
Thiol and Disulfide Contents[a] of Wheat Flour Extrudates with Cysteine

	SH[b]	S-S[b]	SH + S-S[b]
Total			
Flour	4.32 ± 1.12	26.27 ± 6.94	56.86 ± 13.84
Extrudate[c]			
+0% cys	17.73 ± 1.89	25.29 ± 7.08	68.32 ± 14.03
+5% cys	73.00 ± 11.50	59.75 ± 8.83	192.50 ± 13.39
+1% cys	103.86 ± 22.21	69.66 ± 12.35	243.18 ± 10.80
+2 % cys	192.58 ± 20.81	144.78 ± 32.61	482.15 ± 61.82
Nonprotein			
Flour	0.34 ± 0.01	4.02 ± 0.99	8.38 ± 1.98
Extrudate			
+0% cys	0.37 ± 0.03	0.92 ± 0.41	2.21 ± 0.82
+5% cys	0.88 ± 0.02	36.17 ± 2.78	73.22 ± 5.64
+1% cys	1.07 ± 0.01	47.62 ± 4.98	96.31 ± 9.95
+2 % cys	1.15 ± 0.03	131.63 ± 6.27	264.40 ± 12.54
Protein[d]			
Flour	3.98 ± 0.56	22.25 ± 3.51	48.48 ± 6.94
Extrudate			
+0% cys	17.36 ± 0.95	24.38 ± 3.55	66.11 ± 7.02
+5% cys	72.12 ± 5.75	23.58 ± 4.63	119.28 ± 6.84
+1% cys	102.79 ± 11.11	22.04 ± 6.66	146.87 ± 5.95
+2 % cys	191.43 ± 10.41	13.16 ± 16.61	217.75 ± 31.07

[a] All values presented are expressed as nmole/mg of protein. Values are averages of five experiments ± standard deviations.
[b] SH = free sulfhydryl; S-S = disulfide (disulfide contents were calculated as [total sulfhydryl − free sulfhydryl content]/2. SH + S-S = total sulfhydryl.
[c] Cysteine (nmole) added per mg of protein during extrusion = 294.8 (+5% cys); 589.5 (+1% cys); 1,179.1 (+2 % cys).

However, some native intermolecular disulfide crosslinks remained unaltered by extrusion or the addition of cysteine, because protein solubility and regions of PAGE patterns after S-S reduction with β-mercaptoethanol were nearly the same in the flour and all the extrudates.

DISCUSSION

This study was part of a long-term research program seeking to understand the molecular mechanisms that control product structures and sensory characteristics during extrusion processing. Following up previous observations of free radical formation during extrusion, cysteine was added to wheat flour extrudates to test the involvement of free radicals in macromolecular crosslinking reactions responsible for formation of matrix structures during extrusion.

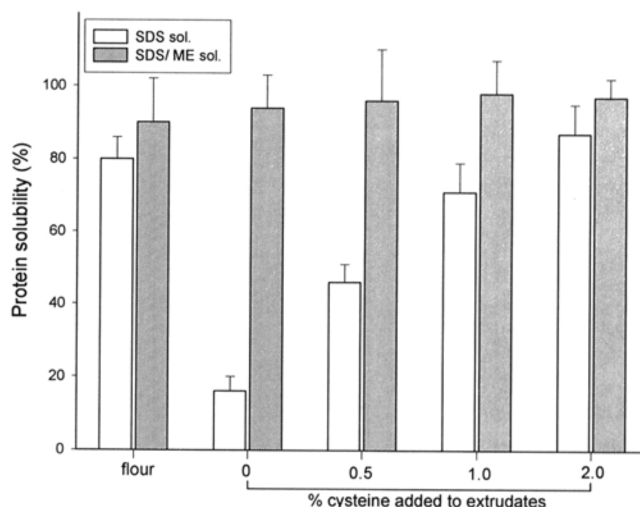


Fig. 7. Solubility of flour and extrudate proteins in 1.5% sodium dodecyl sulfate (SDS) and 1.5% SDS + 1% β-mercaptoethanol (ME). Values are presented as percent of total protein in unextruded flour (nitrogen basis).

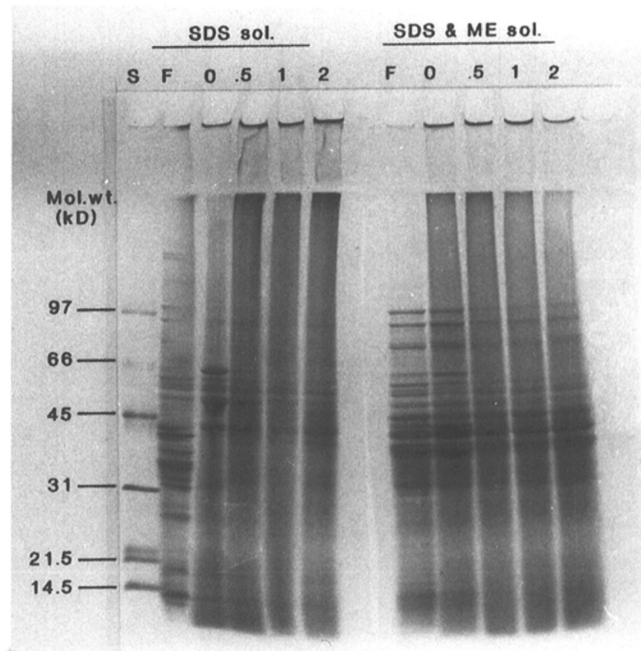


Fig. 8. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) patterns of Coomassie blue stained SDS-soluble and SDS + β-mercaptoethanol (ME) soluble proteins from flour and extrudates. S = standard molecular weight marker, F = unextruded flour. Numbers over gel lanes refer to % cysteine added to flour during extrusion.

Cysteine can have multiple chemical effects in dough systems. Free radical scavenging and thiol-disulfide interchange actions of cysteine are well known in wheat protein chemistry (Stewart and Mauritzen 1966, Kilborn and Tipples 1973), but a third reaction, radical recombination, may also be expected. These three reactions along with their distinguishing characteristics are shown in Figure 11.

These characteristics provide markers by which the occurrence of each reaction can be distinguished under different processing conditions. In the present study, for example, scavenging of protein nitrogen-centered radicals by cysteine was demonstrated by the decrease in the protein nitrogen EPR signal, the increase in the sulfur EPR signal, and the increase in nonprotein disulfides formed in the added cysteine (Rx. 1, Fig. 11). The marked increase in protein -SH content in the presence of cysteine suggests that quenching of protein thiyl radicals (PS•) by cysteine also may have occurred. Increased PS• would be expected if macromolecular fragmentation in the dough matrix (see below) made previously buried disulfide bonds more accessible to scission by the shear forces of extrusion and by chemical reactions. The resulting PSH groups also become more accessible for detection by DTNB reaction.

The increase in protein thiol content observed when cysteine was added during extrusion is a marker for thiol-disulfide interchange (Rx. 2, Fig. 11) and for quenching of protein-S• free radicals by cysteine. Increased macromolecular exposure due exclusively to diminished peptide crosslinking (not involving S-S or -S•) could also make more protein -SH groups available for detection. How can contributions from these three possibilities be distinguished?

The decrease in protein disulfide contents and the distinctive differences between PAGE patterns with and without β-mercaptoethanol (Fig. 8) are inconsistent with thiol-disulfide interchange characteristics (Rx. 2), so this possibility may be excluded. Possible reasons for the apparent absence of thiol-disulfide interchange will be discussed later.

It is well established that both intra- and intermolecular S-S bonds are present in wheat proteins (Graveland et al 1980). If increased molecular exposure due to decreased peptide crosslinking was the sole factor controlling SH-S-S contents, then protein S-S should have increased along with protein -SH. On the other hand, if increased exposure resulted from scission of S-S bonds, net protein S-S could have decreased. However, protein S-S remained nearly constant up to the highest levels of added cysteine (Table II). Similarly, if radical quenching alone, without increased exposure, accounted for the increase in protein -SH, there should be a stoichiometric correspondence between decreased S-S and increases -SH. This did not occur.

A more likely explanation is that the net SH-S-S changes reflect a complex balance between exposure arising from S-S and peptide scissions followed by quenching of -N• and -S• by cysteine, crosslinking involving both -S• and -N•, and other reactions as yet unidentified. From this and other work (K. M. Schaich, *unpublished*), it appears that there is a population of wheat proteins and S-S bonds that are not affected except under extreme conditions, some S-S bonds that are moderately affected, and some which are readily affected by extrusion. This behavior probably reflects, in part, the hydration of protein granules that occurs in situ during extrusion. The protein molecules on the granule surface are the first to hydrate, expand, denature, and undergo cleavage to form radicals. The SH-S-S groups on these molecules should show the highest reactivity, while protein molecules buried most deeply on the protein granule remain relatively unaffected. During extrusion without modifiers, a competition develops between crosslinking of exposed proteins and hydration of deeper layers as protein molecules closer to the granule surface are modified, so a straightforward progressive exposure of protein molecules and their -SH groups during extrusion does not occur.

When lower concentrations of added cysteine quench radicals and inhibit crosslinking in the outer layers, exposure of the middle layers of proteins and shear-mediated scission of S-S bonds in

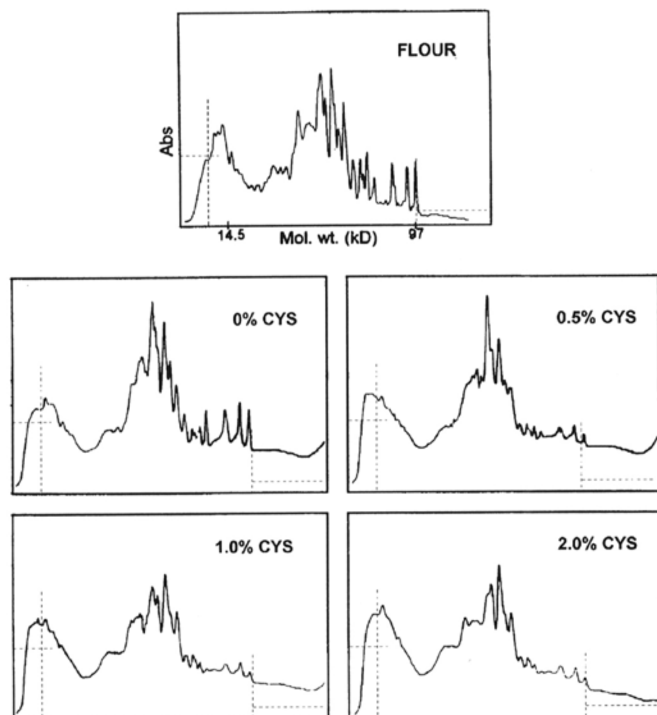


Fig. 9. Densitometry scan of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gels of SDS + β-mercaptoethanol (ME) soluble proteins extracted from flour and extrudates. PAGE gels were stained with Coomassie blue before scanning. % cys = cysteine added during extrusion.

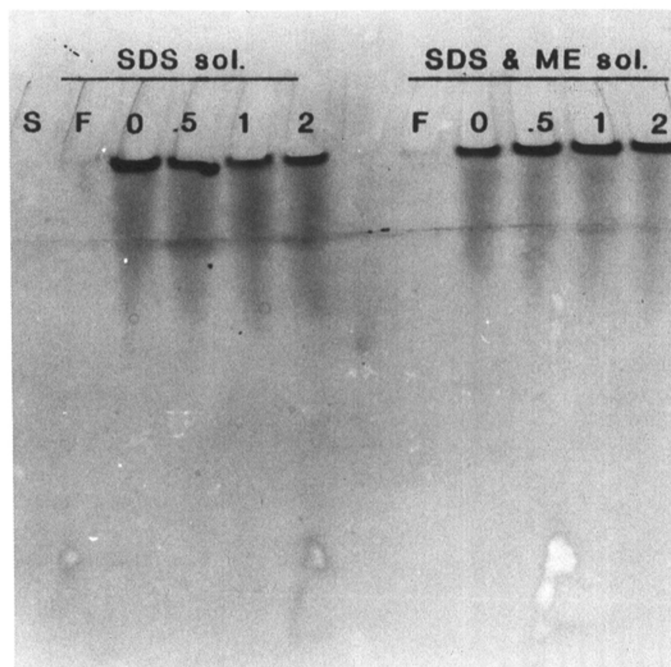


Fig. 10. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) patterns of SDS-soluble and SDS + β-mercaptoethanol (ME) soluble proteins from flour and extrudates, stained with phenol sulfuric acid for detection of glycoproteins. S = standard molecular weight marker, F = unextruded flour. Numbers over gel lanes refer to % cysteine added to flour during extrusion.

those proteins increases, yielding higher -SH concentrations. The innermost proteins still remain unaffected. However, as higher concentrations of cysteine expose protein molecules in the inner layer to S-S scission by shear forces, total S-S content eventually begins to decrease, and -SH continues to increase both from new exposure of previously buried -SH and from S-S scissions followed by radical quenching.

As discussed previously, the changes in PAGE patterns and protein disulfide contents suggest that thiol-disulfide interchange had only minor effects, if any, under the extrusion conditions of this study. Why would thiol-disulfide interchange not play a role in extrusion chemistry when it is so important in dough chemistry? Studies in doughs have shown that thiol-disulfide interchange is a rather slow reaction, requiring tens of minutes of mixing of hydrated doughs before mixed disulfides accumulated (Stewart and Mauritzen 1966, Jones and Carnegie 1971). The exchange also requires water. Thus, detectable thiol-disulfide interchange may be inhibited by the low water contents and very short reaction times (≈2 min) of extrusion. The possible role of water and cysteine hydration in thiol-disulfide interchange during extrusion is presently being studied.

Do sulfur radicals recombine with other radicals or add to proteins during extrusion? Cysteine decreased protein crosslinking, but all three of the cysteine reactions outlined in Figure 11 result in decreased crosslinking, so secondary markers are necessary to distinguish individual reaction mechanisms. Preliminary investigations have revealed no new kinds of sulfur bonds in proteins isolated from extrudates. Also, as noted above, there was no clear evidence of mixed disulfide formation. Thus, present results do not support radical recombination action by cysteine in wheat flour extrusion.

Some questions remain to be answered for the actions of cysteine to be understood fully. It is possible that some mixed disulfide formation occurs and is not distinguishable from normal reduction of unmodified disulfides in PAGE patterns by protein size alone. Use of ^{35}S-cysteine could detect mixed disulfides on gels, but it is not practicable in extrusion experiments. However, methods using ultrasonication to extract flour proteins without reduction of S-S bonds (Singh et al 1990) followed by PAGE under reducing and nonreducing conditions may improve detection of mixed disulfides without labeled cysteine. It is also possible that products of cysteine addition to flour proteins account for some of the loss of measurable cysteine during extrusion, but they are not detectable by the analytical methods used in this study. Amino acid and elemental analyses of isolated proteins are being conducted to provide definitive quantitative evidence for (lack of) mixed disulfide formation and addition of new sulfur groups into flour proteins during extrusion in the presence of cysteine.

The principal chemical effect of free radical scavenging of cysteine was inhibited crosslinking of protein fragments into polymeric networks, but this action also had several practical secondary effects on physical properties and on dough chemistry. It is interesting that, while cysteine was added to test the involvement of free radical mechanisms in protein crosslinking during extrusion rather than to condition extruded doughs, the conditioning effects of cysteine on product texture and cell structure in extrudates were comparable to cysteine action in baked doughs: finer texture with smaller cells, more fragile structure, and more tender product. This resulted because the strength of the dough matrix decreased as cysteine interfered with crosslinking and decreased molecular weights of constituent protein fractions. The weaker dough with shorter protein fragments formed smaller cells with thinner cell walls. This fragile structure collapsed more readily where the dough was in contact with the extruder wall, forming a thin surface skin that prevented expansion at the die. However, at cut surfaces, the weakened structure yielded more easily to the force of gases escaping at the die, allowing irregular puffing through the cross-sectional surface (Schaich 1994a,b).

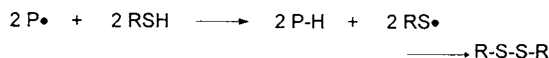
Inhibition of protein crosslinking into polymeric networks dramatically affected protein solubility. SDS disrupts hydrogen and hydrophobic bonding, but not covalent bonds. Extrusion itself produced many intermolecular covalent bonds, potentially both interpeptide bonds and intermolecular disulfide bonds, and thus SDS solubility decreased. However, protein solubility increased as cysteine quenched free radicals and prevented radical recombination and formation of intermolecular crosslinks. The solubility changes were reflected in rehydration characteristics of the extrudates in solution and in the mouth.

PAGE patterns and SH-SS content changes suggest that total protein solubilization was increased both by generalized intramolecular fragmentation of proteins leading to low molecular weight soluble fractions and by decreased intermolecular crosslinking of large protein aggregates involving both peptide and disulfide crosslinks. Disruption of very large polymeric aggregates yielded soluble high molecular weight protein fractions from material previously insoluble in SDS. Some of these very high molecular weight fractions, including those still too large to enter the polyacrylamide gel, may involve protein-starch crosslinks because glycoproteins were detected in those regions of the PAGE gels. This is intriguing evidence that free radical crosslinking of starches and proteins may be very important in forming the thick-walled portions of the solid matrix that provides consistency and crunchiness in extruded wheat flour products.

SUMMARY AND CONCLUSIONS

The dominant reaction mechanism of cysteine added during extrusion of wheat flour appears to be scavenging of protein nitrogen-centered and thiyl free radicals. There is little evidence for thiol-disulfide interchange involving cysteine or recombination of cysteine radicals (RS•) with nonsulfur protein radicals (P•) at the present time. This action of cysteine results in dimin-

1. Radical scavenging or quenching by hydrogen donation:
(P = protein, RSH = thiol compound)

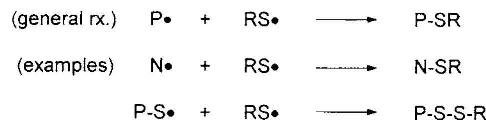


Characteristic: net ↓ RSH, ↑ RSSR (non-protein disulfide)
↓ non-sulfur protein radicals

2. Thiol-disulfide interchange:

Characteristic: net ↑ protein SH, net ↓ crosslinking
formation of protein mixed disulfide:
no ↑ or ↓ in -S-S-
β-mercaptoethanol -//→ change in PAGE

3. Radical recombination:



Characteristic: formation of new functional groups
↑ protein mixed disulfide
net ↓ crosslinking

Fig. 11. Possible reactions of cysteine with protein during wheat flour extrusion.

ished protein crosslinking, increased protein solubility, and refinement and weakening of extrudate textures.

These results provide new evidence that free radical mechanisms play key roles in extrusion chemistry and in determination of product characteristics. This information provides a molecular basis for changes of proteins during extrusion processing, and should help design new approaches for controlling and predicting qualities and characteristics of extruded food products.

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