Factors Affecting Dough Breakdown During Overmixing

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ABSTRACT

When doughs were overmixed in the farinograph in the presence of linoleic acid, rested for 30 min, and then remixed, dough consistency recovered completely. The remixed rested dough turned white in the presence of linoleic acid and smelled rancid. Adding ferulic acid to the dough increased the rate of dough breakdown and made the dough sticky. Dough consistency in the presence of ferulic acid did not recover even after a resting period. The amounts of linoleic acid and sulfhydryl (SH) groups in dough decreased during overmixing, but the amount of ferulic acid remained constant. Heat treatment of flour reduced the rate of dough breakdown and decreased the level of SH groups. The SH contents of flour decreased by 63% as a result of dialysis against oxygen-free distilled water. Dough made from dialyzed flour did not breakdown during overmixing. Amperometric titration of dough mixed with various amounts of *N*-ethylmaleimide (NEMI) showed that 15 ppm of NEMI was equivalent to the amount of SH involved in the thiol-disulfide interchange reaction. The subunit fraction containing disulfide bonds that react with NEMI in acetic acid insoluble protein and glutenin was identified by the distribution of *S*-succinyl-L-cysteine.

The mixing properties of dough are important factors in breadmaking. The mechanism of dough breakdown during mixing has been studied by many workers (Goldstein 1957, Mecham 1959, Mecham et al 1960, Sokol et al 1960, Sullivan et al 1961). Meredith and Bushuk (1962) assumed that dough breakdown could arise by two different mechanisms: a physical mechanism in which both covalent and other linkages are ruptured by purely physical forces, or a chemical one in which structural cross-linkages are broken by reductive, oxidative, or hydrolytic cleavage. Tsen and Bushuk (1963) suggested that dough breakdown under certain conditions may involve oxidative cleavage of disulfide (SS) bonds. Dronzek and Bushuk (1968) confirmed, using ^{14}C-labeled methacrylate, that free radicals are formed during dough mixing. Tanaka and Bushuk (1973a,b) emphasized the depolymerization mechanism of dough breakdown during mixing. The changes in dough components by overmixing have also been studied (Weak et al 1976; Schroeder and Hoseney 1978; Sidhu et al 1980 a,b; Danno and Hoseney 1982 a,b). It was generally concluded from the above studies that disulfide bonds in gluten are broken during dough mixing to create thiol radicals, which then react with activated doublebond compounds.

The purpose of this study was to identify the sulfhydryl (SH) groups involved in thiol-disulfide interchange reactions, the amounts of reactive SH groups, and the subunit fractions of glutenin containing the reactive SS bonds that react with the activated doublebond compounds.

MATERIALS AND METHODS

Wheat Flour

A commercial, untreated flour milled from No. 1 Canada western red spring wheat was used. Flour protein (N × 5.7) was 13.1% and flour ash was 0.51%. All flour data were expressed on a 14.5% moisture basis. α-Tocopherol (minimum 98% purity) was obtained from Nisshin Chemical Co. Ltd., Tokyo, Japan. All other chemicals used were reagent grade.

Operation of Dough Analysis Equipment

A Brabender farinograph equipped with a large mixing bowl was operated at 30° C using AACC method 54-21 (1982). After resting for 30 min, the dough was remixed. Thirty-gram flour samples were mixed in a mixograph (National Mfg. Co., Lincoln, NE) at 60% absorption (14.5 mb) using AACC method 54-40 (1982).

Linoleic acid or α-tocopherol dissolved in petroleum ether were added to the flour at a level of 0.5% based on flour. *N*-ethylmaleimide (NEMI) was dissolved in water. Ferulic acid solution was neutralized to pH 7.0 with 0.5*N* sodium hydroxide. All experiments were replicated at least twice.

Preparation of Dried Doughs

Mixed doughs were frozen immediately by immersion in liquid nitrogen, lyophilized, and finely ground.

Determination of SH and SS Contents

SH and SS contents of flour were determined by amperometric titration, using the method developed by Sokol et al (1959) and modified by Tsen and Anderson (1963) and Okada and Yonezawa (1967). The reactive SH contents were determined by the amperometric titration method of Tsen and Bushuk (1968) without urea. Determinations were made at least in duplicate.

Determination of Ferulic Acid and Linoleic Acid Contents

Free ferulic acid and free linoleic acid were determined by the method of Maga and Lorenz (1974). The bound ferulic acid and total linoleic acid were determined by the method developed by Faush et al (1963) and modified by Yeh et al (1980). The trimethylsilyl derivatives were prepared by the method of Morita (1972).

Gas chromatographic analyses were performed with a model 073 gas chromatograph (Hitachi) equipped with a flame-ionization detector. A glass column of 100 × 3 mm was packed with 2% OV-17. The carrier gas was nitrogen at a flow rate of 40 ml/min. The column temperature was programmed from 200 to 320° C at 3° C per minute. The components were identified by gas chromatography-mass spectrometry (Hitachi M-80). Determinations were made in duplicate.

Heat Treatment of Flours

Flours were heated on plates in the oven at 130° C for five, 10, and 15 min.

Preparation of Dialyzed Flour

One part of flour or dried dough was suspended in five parts of oxygen-free distilled water. The suspension was dialyzed for 72 hr against four changes of oxygen-free distilled water at 5° C. Dialyzed flour (or dialyzed dried doughs) and its dialysate were lyophilized.

Amino Acid Analysis

Proteins were hydrolyzed with 6*N* HCl in sealed and evacuated tubes at 110° C for 22 hr. Amino acid analysis was performed by a

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Hitachi 835 analyzer. Authentic *S*-succinyl-L-cysteine was synthesized from maleic acid and cysteine according to the method of Morgan and Friedmann (1938). Determinations were made in duplicate.

Sugar Analysis

The contents of sugars in the dialysates from flours were determined by the method of Okazaki (1983) using high-performance liquid chromatography. Chromatographic conditions were: column, nucleosil-5 NH (4.2 × 250 mm); mobile phase, acetonitril-water (7:3, v/v%); flow rate, 0.8 ml/min; temperature, 40° C. Twenty microliters of the sample solution (5%) was injected. Determinations were made in duplicate.

Gel Filtration on Sephacryl S-300

Gel filtration on a Sephacryl S-300 was performed as described by Okada et al (1986). Experiments were performed in duplicate.

Fractionation of Glutenin and Acetic Acid Insoluble Protein

The reduced and cyanoethylated glutenin or acetic acid insoluble protein was fractionated according to the method of Danno et al (1978) (Fig. 1). Glutenin was prepared by the method of Jones et al (1959). The acetic acid insoluble protein is usually referred to as residue protein (Khan and Bushuk 1979). All experiments were performed in duplicate.

RESULTS AND DISCUSSION

Recovery of Dough Consistency

As shown by the farinograph curves in Figure 2, dough remixed in the presence of linoleic acid completely recovered its consistency. The color of the dough turned white, and the dough smelled rancid. In contrast, dough remixed in the presence of linoleic acid together with α-tocopherol, which is an antioxidant of linoleic acid, showed no change in color or odor, and exhibited no recovery of dough consistency. This observation that the oxidation of an unsaturated fatty acid, such as linoleic acid, affected the rheological properties of dough corroborates previous reports by Bloksma (1964) and Daniels et al (1971). Schroeder and Hoseney (1978) showed that α,β-unsaturated carbonyl compounds, such as fumaric acid, maleic acid, and ferulic acid, affect overmixing in a way similar to that of the thiol-blocking reagent, NEMI. As shown in Figure 2, adding ferulic acid increased the rate of dough breakdown and made the dough sticky. The consistency of remixed dough did not recover, even after resting.

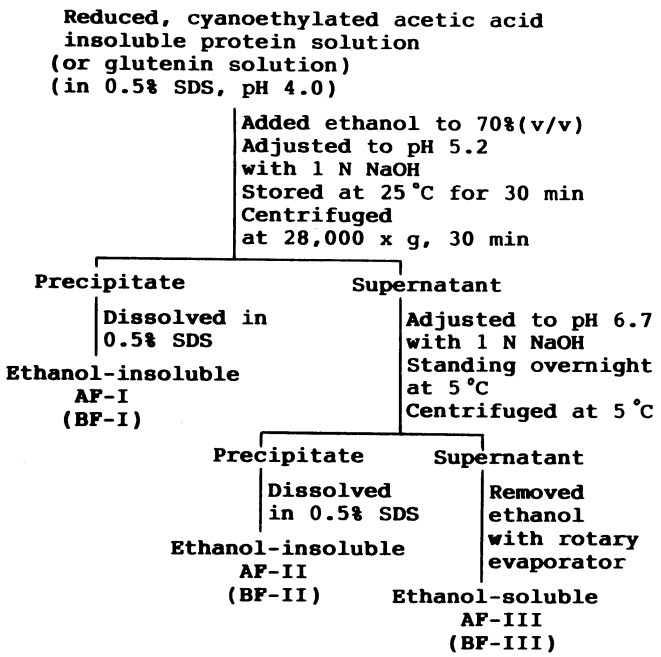

Fig. 1. Fractionation of reduced, cyanoethylated acetic acid insoluble protein and glutenin. Acetic acid insoluble protein: AF-I, AF-II, and AF-III, glutenin: BF-I, BF-II, and BF-III.

Changes in the Amount of SH, Ferulic Acid, and Linoleic Acid During Mixing in a Farinograph

The level of linoleic acid and SH groups in farinograph dough decreased by the peak time, whereas bound ferulic acid content did not decrease even during overmixing (Table I). Jackson and Hoseney (1986) also found that ferulic acid in the insoluble, bound form did not decrease in concentration during dough overmixing. The above authors showed that free ferulic acid and ferulic acid

TABLE I
Changes in the Amounts of Sulfhydryl, Linoleic Acid, and Ferulic Acid During Mixing in a Farinograph[a]

Dough Stage	Sulfhydryl (μeq/g protein)	Linoleic Acid (ppm)	Ferulic Acid (ppm)
No mixing	16.7	416	26.3
Peak time	12.9	80	27.4
Overmixing	11.7	110	31.0

[a]Values are the average of duplicate determinations. All duplicates were within 5% of the mean.

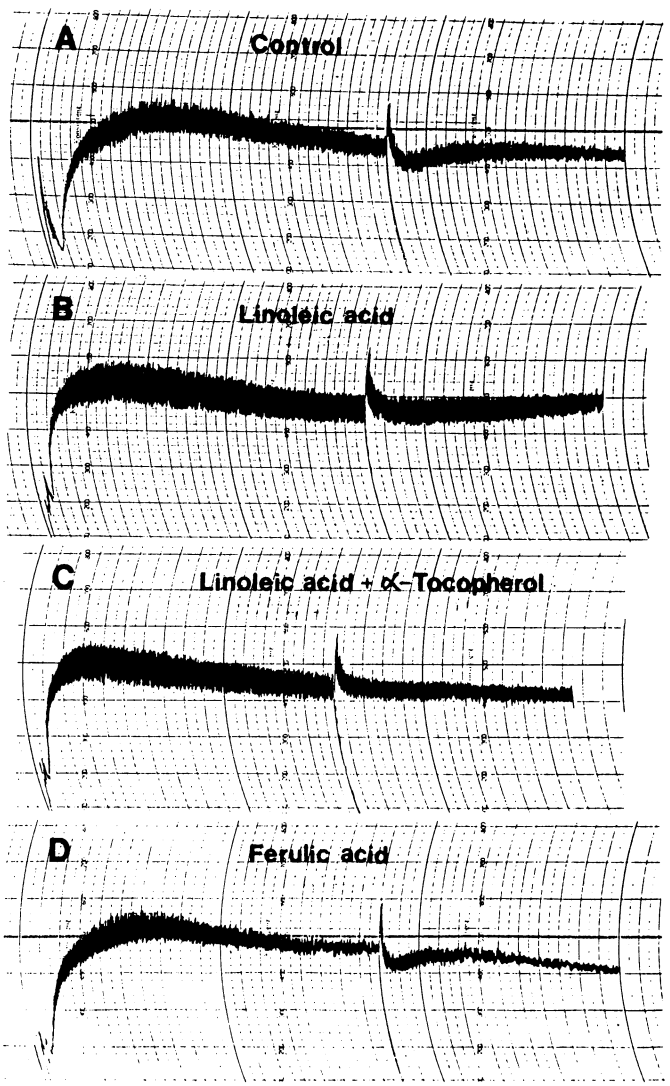

Fig. 2. Farinograms showing effects of linoleic acid, linoleic acid plus α-tocopherol, and ferulic acid on mixing characteristics. **A,** control; **B,** linoleic acid, 0.5%; **C,** linoleic acid, 0.5%; α-tocopherol, 0.5%; **D,** ferulic acid, 250 ppm.

esterified to water-soluble pentosans decreased during mixing. However, in the present work analysis failed to detect free ferulic acid.

It is assumed that the endogenous bound ferulic acid in flour did not relate to the phenomenon of dough breakdown during the extended mixing.

TABLE II
Components of Flour Dialysate[a]

Component	Amount
Cystein[b]	44.31×10^{-7} mol
Reduced glutathione[b]	17.73×10^{-7} mol
Maltose	46.24%
Sucrose	3.85%
Glucose	4.40%
Fructose	1.16%

[a]Values are the average of duplicate determinations. All duplicates were within 5% of the mean
[b]Per gram of dialysate.

TABLE III
Changes of the Amount of Reduced Glutathione in Dialysates from Mixograph Doughs[a]

Dough Stage	$\times 10^{-9}$ mol/N mg
No mixing	71.4
Peak time	68.8
Overmixing	61.8
Peak time with NEMI[b] (50 ppm)	60.5
Overmixing with NEMI[b] (50 ppm)	61.5

[a]Values are the average of duplicate determinations. All duplicates were within 5% of the mean.
[b]*N*-ethylmaleimide.

Effects of Heat Treatment on Dough Properties

To clarify the phenomenon of dough breakdown during extended mixing, mixograph curves were obtained for heat-treated flour. Mixing time increased and the rate of dough breakdown decreased with progressive heat treatments (Fig. 3). As shown in Figure 4, the SH content of flour decreased with prolonged heat treatment. It was also shown that addition of reduced glutathione equivalent to the decrease in SH content caused by heat treatment, reduced mixing time and increased the rate of dough breakdown of heat-treated flour dough (Fig. 3). The results clearly demonstrated that SH groups play an important role in dough rheology.

Effects of Low Molecular SH Compounds on Dough Properties

Flour was dialyzed to clarify the types of SH compounds involved in the thiol-disulfide interchange reaction. The yield of dialysate from flour was 5.4%. Figure 5 shows a flour amino acids chromatogram. Dialysate components from flour are summarized in Table II. Cysteine and reduced glutathione accounted for 2.39×10^{-7} mol/g of flour and 0.96×10^{-7} mol/g of flour of SH, respectively. Maltose was a main component in the flour dialysate.

As shown in Figure 6, dough made from dialyzed flour did not break down during overmixing. The addition of cysteine (7.17×10^{-6} mol) or reduced glutathione (2.88×10^{-6} mol) equivalent to the values in the dialysate had little effect on the rate of dough breakdown. The addition of reduced glutathione (24.10×10^{-6} mol) equivalent to the amount lost by dialysis reduced mixing time and also greatly increased the rate of dough breakdown. The SH content in the dialysate was found to be not equivalent to that which was lost from flour during dialysis. The reason for this is not clear at this stage. It is, however, clear that the low molecular SH compounds contribute to the phenomenon of dough breakdown.

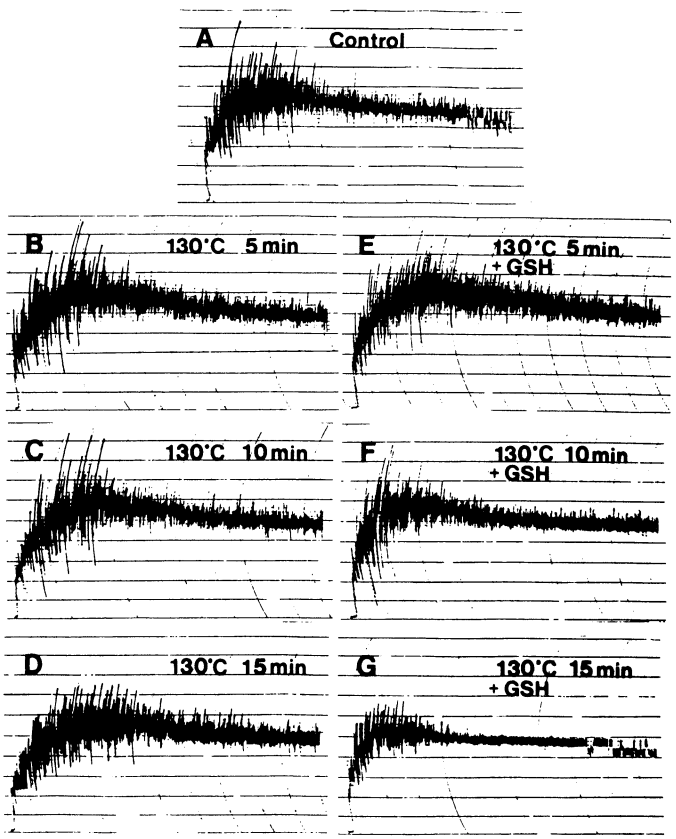

Fig. 3. Mixograms showing effects of heat treatment and reduced glutathione (**GSH**) on mixing characteristics. **E,** GSH, 1.05×10^{-6} mol; **F,** GSH, 4.91×10^{-6} mol; **G,** GSH, 10.49×10^{-6} mol.

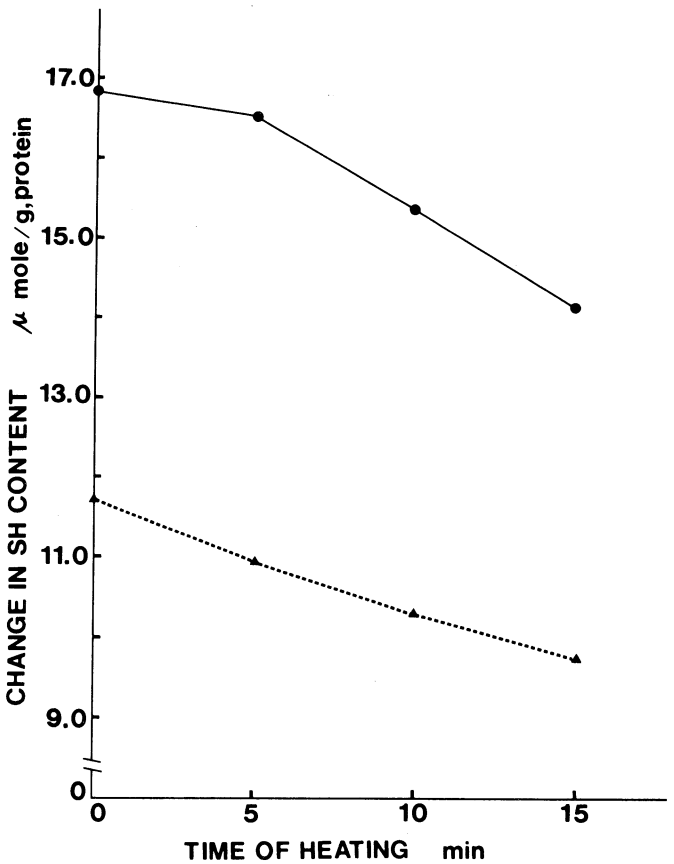

Fig. 4. Changes in sulfhydryl (SH) contents with heat treatment. •—•, SH; ▲- - -▲, reactive SH.

TABLE IV
Changes of the Proportion of Amino Acid Compositions in Dialysates from Mixograph Doughs[a]

Amino Acid	No Mixing Flour (%)	% Change			
		Peak Time Dough	Overmixed Dough	Peak Time Dough (× NEMI[b] 50 ppm)	Overmixed Dough (× NEMI[b] 50 ppm)
Aspartic	26.51	− 29.16	−13.01	− 28.03	− 7.13
Threonine	10.21	− 41.82	−2.63	− 59.35	− 14.59
Serine	4.79	− 55.32	−17.33	− 56.58	− 12.53
Glutamic acid	6.50	+141.54	+81.69	+102.92	+ 83.54
Proline	4.86	+132.72	+22.63	+196.91	+106.79
Glycine	6.89	+ 14.22	−16.84	− 11.76	− 13.06
Alanine	11.47	+ 40.45	+16.13	+ 30.34	+ 33.91
Valine	4.80	− 2.50	+11.67	+ 71.88	+ 37.29
Methionine	1.72	− 42.44	−20.93	− 41.28	− 27.33
Isoleucine	2.30	− 58.26	−13.04	− 47.39	− 20.87
Leucine	3.64	− 31.59	+ 6.59	− 22.80	− 10.16
Tyrosine	2.02	− 1.98	− 0.50	− 5.94	+ 11.39
Phenylalanine	3.14	+ 11.78	+ 7.01	+ 18.15	+ 28.03
Lysine	2.73	− 61.17	−22.71	− 47.62	− 20.51
Histidine	1.44	− 12.50	−18.75	− 34.03	− 20.14
Arginine	5.53	− 29.48	−13.02	− 34.18	− 1.63
Cysteine	1.46	− 10.27	−19.86	− 21.92	− 16.44

[a] Values are the average of duplicate determinations. Standard error is within 5%.
[b] *N*-ethylmaleimide.

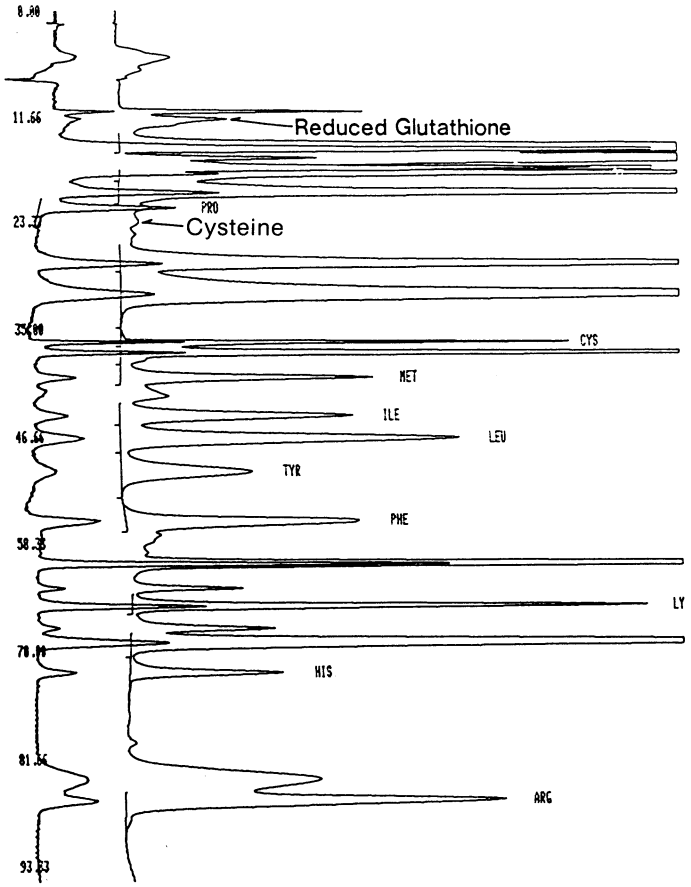

Fig. 5. Chromatographic analysis of amino acid.

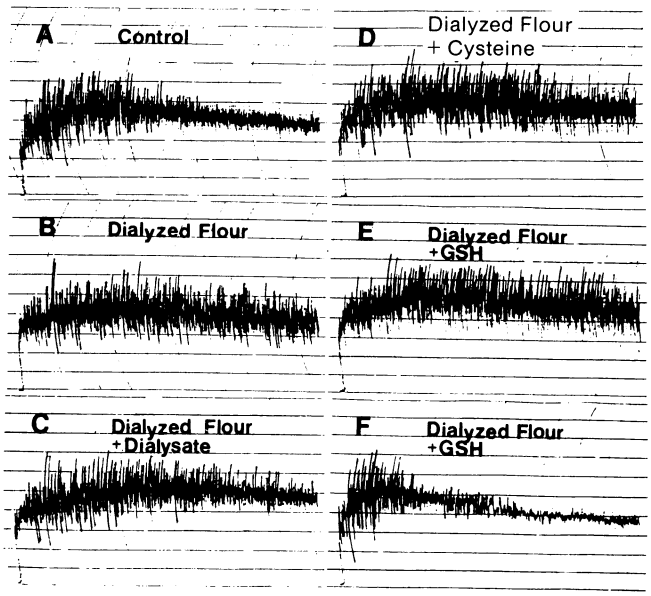

Fig. 6. Mixograms for dialyzed flour. **D,** cysteine, 7.17×10^{-6} mol; **E,** reduced glutathione (**GSH**), 2.88×10^{-6} mol; **F,** GSH 24.10×10^{-6} mol.

Changes in Dialysate Components During Dough Mixing in a Mixograph

Reduced glutathione in the dialysate decreased during dough mixing in a mixograph (Table III). In the presence of NEMI (50 ppm), reduced glutathione decreased markedly at the peak time. The results clearly demonstrate that NEMI and reduced glutathione interact during dough mixing.

The amino acid compositions of the dialysate changed during dough mixing (Table IV). Except for glutamic acid, hydrophilic amino acids in the dialysates decreased during dough mixing. The dialysate from overmixed dough with NEMI contained higher contents of aspartic acid, proline, alanine, valine, tyrosine, phenylalanine, and arginine, and less contents of methionine, isoleucine, and leucine than did that of the control overmixed dough. These differences may relate to the rheological properties of dough. However, more work is required to confirm the above speculation.

Amounts of SH Involved in the Thiol-Disulfide Interchange Reaction

The SH contents in doughs mixed in a mixograph with various amounts of NEMI were determined by amperometric titration. As shown in Table V, the peak time of the mixograph curve increased to a maximum at 15 ppm NEMI, and then decreased with further

addition of NEMI. On the other hand, the reactive SH content of doughs dramatically decreased at 15 ppm NEMI, and then remained constant with further addition of NEMI (Fig. 7). At the value of 15 ppm NEMI, reactive SH decrease coincided with the level of NEMI that gave the longest peak time during dough mixing. This is strong evidence that 15 ppm of NEMI is equivalent to the amounts of SH involved in the thiol-disulfide interchange reaction.

Jones et al (1974) reported that less than 4% of the total disulfide in dough was involved in determining development time. Schroeder and Hoseney (1978) found that the presence of compounds with activated double bonds did not cause a significant decrease in dough SS groups. The present study agrees closely with these reports. Only about 3% of SS bonds were broken during dough mixing with 50 ppm NEMI (results not shown). Schroeder and Hoseney (1978) suggested that dough breakdown occurs because of the reaction of activated double bond compounds with thiol-free radicals created during dough mixing.

Figure 8 shows Sephracyl S-300 gel filtration profiles of proteins extracted from undermixed dough (20 sec), overmixed dough (10 min), and overmixed dough with NEMI (50 ppm, 10 min). The elution profiles of proteins extracted from overmixed dough and overmixed dough with NEMI showed an increase in the high molecular weight fraction compared with the proteins extracted from the undermixed control dough. There were no changes in the medium and low molecular weight fractions. On the basis of these data we speculate that dough breakdown is not caused by complete depolymerization of glutenin with NEMI treatment, but by the partial conformational change on glutenin induced by modification of the key disulfide bonds by the reagent.

Table VI shows the changes in the proportions of protein fractions during dough mixing with NEMI. In the case of the acetic acid insoluble protein fraction, incorporating NEMI during mixing caused AF-I to increase, and AF-II and AF-III to decrease compared to dough mixed in the absence of NEMI. This was consistent with exposure of hydrophobic groups brought about by modification of acetic acid insoluble protein during overmixing with NEMI. In contrast, the proportion of each fraction of glutenin in dough was not as dramatically influenced by the

TABLE V
Changes of Peak Time During Dough Mixing with NEMI[a] in the Mixograph

NEMI (ppm)	Peak Time (min)
0[b]	2.43 ± 0.04[d]
13[b]	2.51 ± 0.05
15[b]	2.85 ± 0.07
18[c]	2.58 ± 0.11
20[c]	2.55 ± 0.04
50[b]	2.38 ± 0.02

[a] *N*-ethylmaleimide.
[b] Experiments were performed in quintuplicate.
[c] Experiments were performed in quadrupticate.
[d] Standard deviation.

TABLE VI
Changes in the Proportion of Protein Fractions During Dough Mixing in a Mixograph with and Without NEMI[a]

Fraction	Without NEMI (%)	With NEMI (50 ppm) (%)
Insoluble protein		
AF-I	48.1	68.8
AF-II	15.3	7.3
AF-III	36.6	23.9
Glutenin		
BF-I	19.6	16.8
BF-II	30.0	35.1
BF-III	50.4	48.1

[a] *N*-ethylmaleimide. Values are the average of duplicate determinations. All duplicates were with 5% of the mean.

TABLE VII
Proportions of *S*-Succinyl-L-Cysteine in Each Dough Protein Fraction[a]

Fraction	Proportion
AF-I	1.00
AF-II	0.50
AF-III	0.53
BF-I	0.73
BF-II	0.36
BF-III	0.44

[a] Values are the average of duplicate determinations. All duplicates were within 5% of the mean.

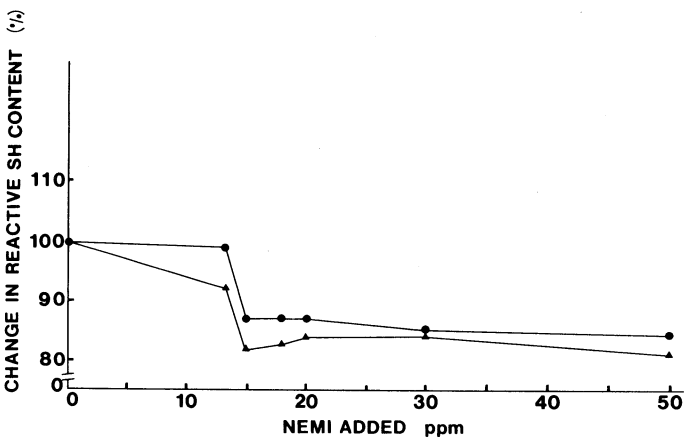

Fig. 7. Changes in reactive sulfhydryl (**SH**) contents during dough mixing with *N*-ethylmaleimide (**NEMI**). ▲—▲, peak time; ●—●, overmixing.

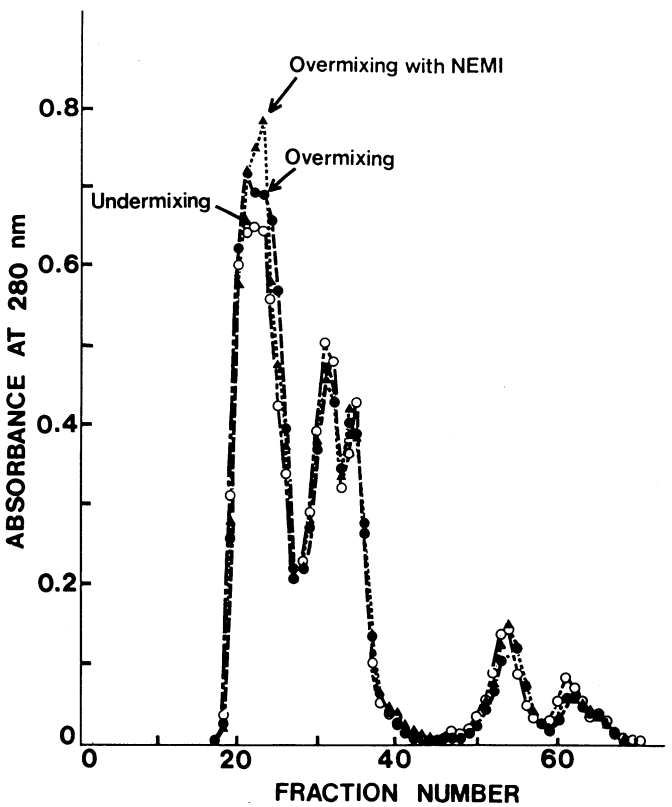

Fig. 8. Elution profiles from gel filtration chromatography on Sephacryl S-300. o----o, undermixing; ●---●, overmixing; ▲----▲, overmixing with *N*-ethylmaleimide (**NEMI**) 50 ppm.

presence NEMI. However, in the presence of NEMI BF-II increased, and BF-I and BF-III decreased compared to the absence of NEMI. Kanazawa and Yonezawa (1974) reported that aggregative polypeptides may contribute to the association of glutenin molecules and influence rheological properties of wheat flour dough. Huebner and Wall (1974) and Khan and Bushuk (1977) reported that glutenin is composed of three distinct groups of sununits. Peak I subunits from gel filtration on Sephadex G-200 exhibited a strong tendency to aggregate, a phenomenon that might be important in the functionality of glutenin in breadmaking. It is assumed that changes in protein subunits relate to the phenomenon of dough breakdown during overmixing with NEMI in the present work.

Determination of Subunits Containing Key Disulfide Bonds Involved in Dough Properties

The subunit fraction containing SS bonds that react with NEMI in acetic acid insoluble protein and glutenin was determined by the distribution of *S*-succinyl-L-cysteine. The elution position of *S*-succinyl-L-cysteine on the ion-exchange column was essentially the same as that reported by Tkachuk and Hlynka (1963) and Sidhu et al (1980a,b) (Fig. 9). Total *S*-succinyl-L-cysteine contents of each fraction from acetic acid insoluble protein and from glutenin were very small, contents of AF-I were about 0.08% of the total components (excluding ammonia) separated by the amino acid analyzer. NEMI reacted with AF-I and BF-I more easily than with other fractions during dough mixing (Table VII). This result suggests that those aggregative subunits play an important role in the rheological properties of dough.

CONCLUSIONS

From the results of this study, we conclude that the low molecular weight SH compounds, such as glutathione and cysteine were involved in thiol-disulfide interchange reactions. The amount of SH involved in thiol-disulfide interchange was shown to be equivalent to 15 ppm NEMI.

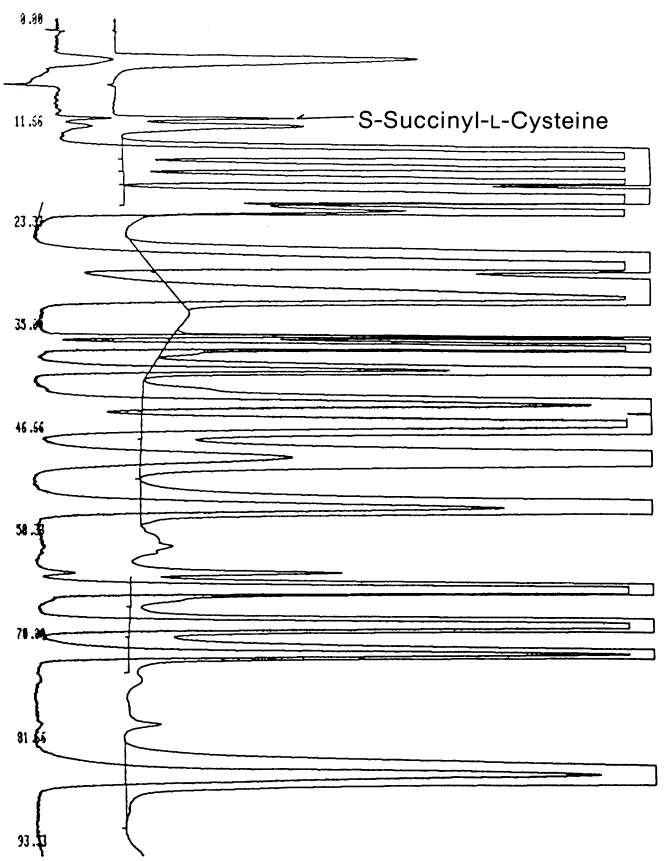

Fig. 9. Chromatographic analysis of *S*-succinyl-L-cysteine.

The level of linoleic acid and SH groups in dough at peak time was less than that of flour. Bound ferulic acid contents did not decrease even during overmixing. It is assumed that SH groups were oxidized by a peroxidized linoleic acid, and the endogenous bound ferulic acid in flour was not related to dough breakdown during overmixing.

AF-I was present in greater amounts in doughs mixed in the presence of NEMI than in doughs mixed in the absence of NEMI. We suggest that this was due to exposure of hydrophobic groups brought about by modification of acetic acid insoluble protein during overmixing with NEMI. NEMI reacted with AF-I and BF-I more easily than other protein fractions during dough mixing, suggesting that the aggregative subunits (AF-I and BF-I) played an important role in the rheological properties of dough.

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