Characterization of Acetic Acid-Soluble and Insoluble Proteins Isolated from Doughs Mixed in the Presence of *N*-Ethylmaleimide

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Cereal Chem. 65(3):248–252

ABSTRACT

Acetic acid-soluble and insoluble proteins were isolated from doughs mixed in the presence of *N*-ethylmaleimide (NEMI). Reduced-pyridylethylated acetic acid-insoluble protein (AF) and reduced-pyridylethylated acetic acid-soluble protein (BF) were sequentially fractionated into an acetic acid-insoluble fraction (F-0), an acidic 70% ethanol-insoluble fraction (F-I), a neutral 70% ethanol-insoluble fraction (F-II), and a neutral 70% ethanol-soluble fraction (F-III). Only about 30% of AF dissolved in 0.1*N* acetic acid solution, whereas BF dissolved completely. The AF-I fraction was recovered in trace amounts and could not be characterized. The fractions AF-0, AF-II, and AF-III contained higher contents of aspartic acid, alanine, lysine, and arginine and lower contents of glutamic aeid and proline than the BF fractions (BF-I, BF-II, and BF-III). The AF and BF fractions differed in reversed-phase high-performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) subunit patterns. Subunits of AF-0 eluted later by RP-HPLC than those of other fractions, indicating that they have higher surface hydrophobicities. Distribution of *S*-succinyl-L-cysteine in the protein fractions showed that NEMI reacted with AF-0 and BF-I more than with the other protein fractions during dough mixing. *S*-succinyl-L-cysteine was present at similar high levels in peaks separated by RP-HPLC from AF-0, whereas it was present in variable amounts in peaks separated by RP-HPLC from the other protein fractions. We speculate that dough breakdown in the presence of NEMI is caused by partial depolymerization of glutenin and changes in protein surface hydrophobicities, mainly in AF-0 and BF-I, induced by modification of the key disulfide bonds by NEMI. RP-HPLC, SDS-PAGE, and amino acid analysis showed that acetic acid-insoluble protein is composed of different subunits than acetic acid-soluble protein.

The importance of glutenin and/or acetic acid-insoluble protein in breadmaking quality has been discussed by many workers (Shogren et al 1969; Huebner 1970; Orth and Bushuk 1972, 1973; Tanaka and Bushuk 1973a–c; Bietz and Wall 1975; Huebner and Wall 1976; Khan and Bushuk 1979; Chung and Pomeranz 1979). Orth and Bushuk (1972) reported that the proportion of acetic acid-soluble glutenin in the total flour protein was negatively correlated with loaf volume per unit protein, whereas residue protein (acetic acid-insoluble glutenin) and loaf volume per unit protein were positively correlated. Acetic acid-insoluble protein has not been studied extensively in comparison to other wheat flour proteins because of its poor solubility in conventional protein solvents. Bietz and Wall (1975) reported that $HgCl_2$ in acetic acid solubilizes most of the acetic acid-insoluble protein. Khan and Bushuk (1979) studied acetic acid-insoluble protein using two other solvents, AUC (0.1*N* acetic acid/3*M* urea/0.01*M* hexadecyltrimethyl ammonium bromide) and 70% chloroethanol/0.1*N* HCl. They showed that the AUC and chloroethanol extracts of the residue protein contained proportionately more high molecular weight subunits (subunits that will not enter an SDS electrophoresis polyacrylamide gel unless reduced) than the acetic acid-soluble glutenin fraction. Burnouf and Bietz (1984), who studied the separation of the subunits of glutenin by RP-HPLC, concluded that separation by surface hydrophobicity complements methods of separation by molecular weight or charge and reveals new information on the biochemistry of these proteins. They reported that peaks in the early elution time area on RP-HPLC tend to have lower surface hydrophobicities.

In a previous study (Okada et al 1987), we found that *N*-elthylmaleimide (NEMI) reacts more with acidic 70% ethanol-insoluble protein subunits than other protein subunits during dough mixing by determining the distribution of *S*-succinyl-L-cysteine in the subunits of acetic acid-insoluble and soluble proteins. The purpose of this study was to clarify the character of acetic acid-soluble and insoluble proteins isolated from dough mixed in the presence of NEMI, and to identify the subunit fraction containing the key disulfide bonds involved in the mechanism of dough breakdown during mixing for acetic acid-soluble and insoluble proteins by the distribution of *S*-succinyl-L-cysteine.

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This manuscript was prepared for electronic processing.

MATERIALS AND METHODS

Wheat Flour

A commercial, untreated flour milled from no. 1 Canada western red spring wheat was used. Its protein (N × 5.7) was 13.1%, ash was 0.51%, and moisture was 14.5%

Preparation of Acetic Acid-Soluble and Acetic Acid-Insoluble Proteins

Dough of 62% absorption containing 50 ppm NEMI was mixed in the mixograph for 10 min, then hand-kneaded in distilled water to remove starch and water solubles. The wet gluten was lyophilized and finely ground to pass through a 70-mesh sieve. The dry gluten (yield, 17.3%; protein content, 76.0% on a dry basis) was dispersed in 0.01*N* acetic acid in a Waring Blendor at 10,000 rpm for 3 min and centrifuged at 28,000 × *g* for 20 min. The precipitate was designated crude acetic acid-insoluble protein. The yield of acetic acid-insoluble protein was 12.6% of the total proteins of gluten. Acetic acid-soluble protein was separated from the gluten by precipitation in 70% ethanol according to the method of Jones et al (1959); its yield of acetic acid-soluble protein was 45.2% of the total proteins of gluten.

Reduced-pyridylethylated acetic acid-soluble (BF) and acetic acid-insoluble (AF) proteins were prepared according to the method of Burnouf and Bietz (1984). The reaction mixture was dialyzed against 0.1*N* acetic acid. The dialyzed solution was lyophilized and stored at −20°C until required.

Fractionation of Reduced-Pyridylethylated Proteins

Reduced-pyridylethylated proteins were fractionated by the method of Danno et al (1978) except that 0.1*N* acetic acid was used as a solvent (Fig. 1). Only about 30% AF dissolved in 0.1*N* acetic acid. The insoluble fraction was designated AF-0.

RP-HPLC

RP-HPLC was performed at 40°C with a Hitachi 665A-12 liquid chromatograph, using the method of Bietz at al (1984) with modifications of trifluoroacetic acid concentration and gradient conditions. Samples (50 μl) were injected with a Hitachi 665A-40 automatic sample injector onto a 150 × 4.6 mm i.d. Nucleosil 5C18 column. A Hitachi L-5000 LC controller was used to produce a linear 45-min gradient from 80% solvent A (15% acetonitrile and 0.05% trifluoroacetic acid) + 20% solvent B (80% acetonitrile

and 0.05% trifluoroacetic acid) to 45% solvent A + 55% solvent B at 1.0 ml/min, followed by a 10-min hold at final gradient conditions. Eluted components were detected at 210 nm with a Hitachi 655A variable wavelength ultraviolet monitor, and data were recorded by a Hitachi D-2000 chromatointegrator. Experiments were performed in triplicate.

Amino Acid Analysis

Amino acid analysis was performed as described previously (Okada et al 1987). Proteins were hydrolyzed with 6*N* HCl in sealed and evacuated tubes at 110°C for 22 hr. Amino acid hydrolyzates were analyzed by a 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.

SDS-PAGE

The SDS-PAGE procedure described previously (Okada et al 1987) was performed on a column gel (5 mm × 70 mm × 12 tubes) at 3 mA per tube for 2.5–3.0 hr using the buffer system of Orth and Bushuk (1973). The concentration of acrylamide in the gel was 7.5%. The gels were scanned at 590 nm with a dual-wavelength scanner CS-900 (Shimadzu Seisakusho Ltd., Japan). Experiments were performed in duplicate.

RESULTS AND DISCUSSION

Fractionation of Reduced-Pyridylethylated Proteins

In the present work, a 0.1*N* acetic acid solution was used instead of a 0.5% SDS solution for fractionation of reduced-pyridylethylated proteins to yield protein fractions free of SDS. Only about 30% of AF dissolved in 0.1*N* acetic acid. The insoluble fraction was designated AF-0. The soluble fractions were fractionated by sequential precipitation with 70% ethanol at increasing pH and designated AF-I, AF-II, and AF-III, respectively. The AF-0, AF-I, AF-II, and AF-III contained 68.7, 0.3, 11.2, and 19.8% of AF protein, respectively. BF completely dissolved in 0.1*N* acetic acid. BF-I, BF-II, and BF-III contained 4.5, 43.0, and 52.5% of BF protein, respectively.

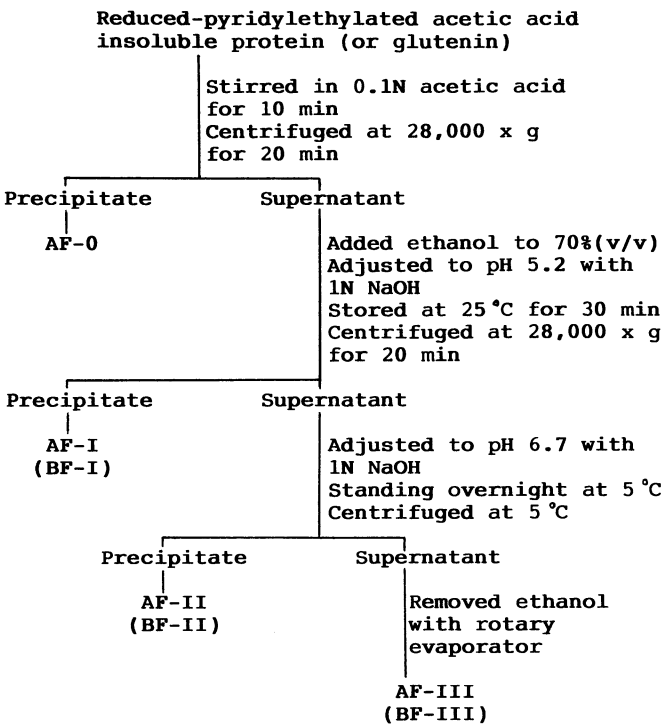

Fig. 1. Fractionation of reduced-pyridylethylated acetic acid-insoluble protein and glutenin.

RP-HPLC of AF and BF Protein Fractions

Figure 2 shows the RP-HPLC elution patterns of AF (AF-0, AF-II, AF-III) and BF (BF-I, BF-II, BF-III) groups. Because AF-0 and BF-I solutions could not be filtered (0.45 μm), both solutions were centrifuged (28,000 × *g*, 20 min), and the clear supernatants were injected for RP-HPLC. As shown in Figure 2A, no distinct early eluting peaks were separated for AF-0. AF-0 had the greatest proportion of protein in the late-eluting region of the HPLC profile of all the protein fractions. The main peaks (c,d,f, and g) of AF-II eluted at early and intermediate times, and only minor peaks were identified in the late elution time area (Fig. 2B). In contrast, AF-III exhibited no main peaks in either the early or the late elution time areas (Fig. 2C). As shown in Figure 2D, BF-I has main peaks (f,g) in the intermediate elution time region, a major peak (j) in the late elution time area, and many minor peaks in the early elution time area indicating that BF-I exhibited strong surface hydrophobicity similar to AF-0. Several main peaks in the early elution time area were resolved from BF-II (Fig. 2E) in contrast to the other protein fractions. This elution pattern clearly shows that BF-II had the lowest surface hydrophobicities of all the protein fractions. The elution pattern of BF-III (Fig. 2F) was similar to that of AF-III (Fig. 2C).

Amino Acid Composition of AF and BF Protein Fractions

The amino acid compositions of AF and BF groups are shown in Table I. The ratio of mole percent hydrophobic amino acids to mole percent hydrophilic amino acids was used to compute a hydrophobicity index. In this study, alanine, valine, methionine, isoleucine, leucine, phenylalanine, and cystine were considered

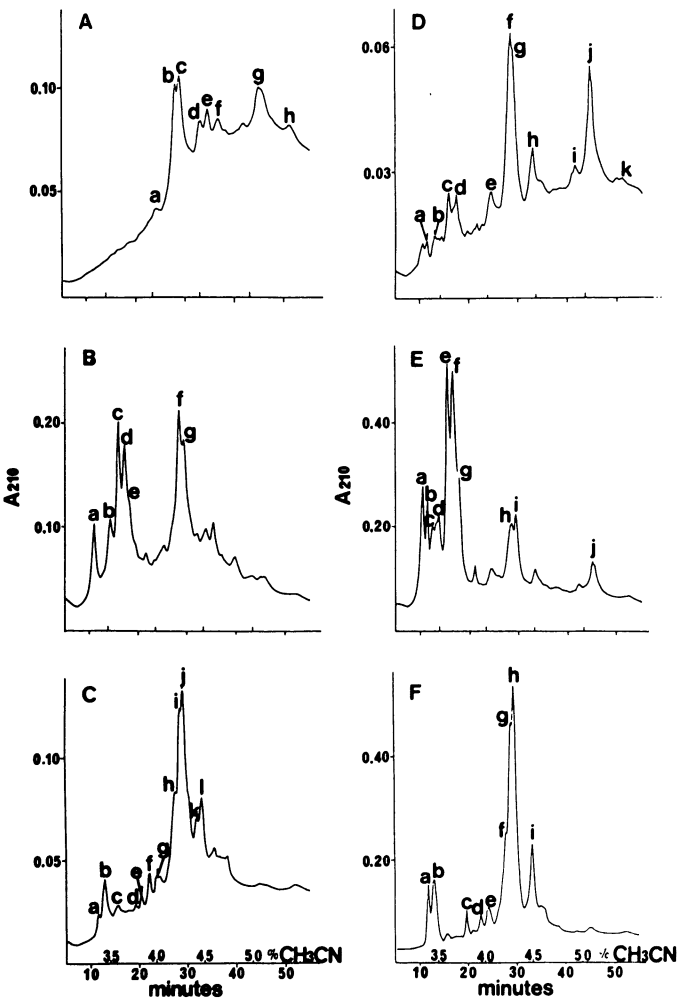

Fig. 2. Reversed-phase high-performance liquid chromatographic separation of reduced-pyridylethylated acetic acid-insoluble (AF) and soluble (BF) protein groups. **A**, AF-0; **B**, AF-II; **C**, AF-III; **D**, BF-I; **E**, BF-II; **F**, BF-III.

TABLE I
Amino Acid Composition (mol %) of the AF and BF Groups[a]

Amino Acid	AF-0	AF-II	AF-III	BF-I	BF-II	BF-III
Aspartic acid	9.33	6.54	2.57	7.57	3.76	1.65
Threonine	4.66	4.26	2.76	4.26	3.61	2.57
Serine	6.83	6.68	6.55	6.23	6.00	6.78
Glutamic acid	13.61	19.80	34.62	18.07	28.75	34.96
Proline	6.96	9.24	18.42	9.94	12.73	20.54
Glycine	8.60	11.06	3.89	8.98	12.87	3.08
Alanine	8.38	7.49	3.37	7.16	4.66	2.46
Valine	5.93	4.65	3.91	5.91	3.69	4.05
Methionine	2.04	1.53	1.11	1.90	1.19	1.41
Isoleucine	3.99	2.74	3.26	3.74	2.43	3.82
Leucine	8.62	6.52	7.22	8.06	5.88	7.31
Tyrosine	3.00	3.21	1.70	3.06	4.25	1.52
Phenylalanine	4.13	2.84	4.61	3.74	2.54	5.17
Lysine	5.59	5.34	1.53	4.13	2.31	0.69
Histidine	2.53	2.24	1.86	2.42	1.71	1.78
Arginine	5.78	5.78	2.57	4.69	3.13	2.16
Cysteine	0.07	0.08	0.10	0.19	0.48	0.09
Hydrophobicity index value	0.90	0.65	0.55	0.83	0.53	0.59

[a]Values are the average of duplicate determinations. Standard error is within 5%.

hydrophobic amino acids, and aspartic acid, glutamic acid, lysine, histidine, and arginine were considered hydrophilic amino acids. AF-0 contained the highest content of aspartic acid and alanine and the lowest content of glutamic acid and proline of all the protein fractions. The hydrophobicity index value was 0.90 for AF-0. BF-I contained the highest content of lysine and aspartic acid and the lowest content of glutamic acid and proline of the BF protein fractions. This result is similar to published data (Hamauzu et al 1975, Danno et al 1978, Zawistowska et al 1985). The hydrophobicity index for BF-I was 0.83. AF-II had significantly lower proportions of glutamic acid and proline, and higher proportions of aspartic acid, alanine, lysine, and arginine than BF-II. The hydrophobicity indexes were 0.65 and 0.53 for AF-II and BF-II, respectively. Amino acid composition of BF-III was similar to that of the ethanol-soluble fraction reported by Danno et al (1978). To further characterize the differences between AF and BF groups, the amino acid compositions of peaks separated by RP-HPLC were analyzed.

Tables II and III show amino acid compositions of peaks separated by RP-HPLC from AF-0 and BF-I, respectively. Peaks with comparable elution times from AF-0 (Fig. 2A) and BF-I (Fig. 2D) are as follows: a(AF-0) and e(BF-I), b(AF-0) and f(BF-I), c(AF-0) and g(BF-I), d(AF-0) and h(BF-I), g(AF-0) and j(BF-I), h(AF-0) and k(BF-I). Peak a(AF-0) had higher proportions of

TABLE II
Amino Acid Composition (mol %) of Peaks from Reversed-Phase High-Performance Liquid Chromatography of AF-0[a]

Amino Acid	Peak a	b	c	d	e	f	g	h
Aspartic acid	6.89	7.59	6.84	8.06	9.12	7.34	7.49	8.27
Threonine	4.54	4.25	4.40	4.66	4.47	4.75	4.91	5.09
Serine	11.27	8.58	8.48	9.03	7.88	9.22	9.36	10.59
Glutamic acid	15.31	18.87	17.95	15.39	13.44	12.75	13.07	15.78
Proline	6.25	7.61	8.37	8.11	5.31	8.68	8.06	5.13
Glycine	14.83	11.29	9.68	10.12	10.35	10.81	11.98	16.46
Alanine	6.64	6.46	6.35	7.16	7.44	7.30	7.24	6.71
Valine	4.85	4.82	5.35	5.50	6.35	6.53	6.34	5.68
Methionine	0.30	0.38	1.24	0.72	1.04	1.13	0.76	0.49
Isoleucine	2.95	2.96	3.47	4.08	5.08	4.18	3.78	3.83
Leucine	5.72	6.17	6.85	7.20	7.52	7.40	7.38	7.64
Tyrosine	2.83	2.63	2.69	2.67	2.73	2.70	2.67	2.56
Phenylalanine	3.51	3.85	3.92	3.97	3.92	3.59	3.11	3.35
Lysine	4.76	4.44	4.27	4.62	5.65	4.62	4.61	4.50
Histidine	2.99	3.37	3.02	2.59	3.24	2.03	1.99	1.93
Arginine	4.99	6.26	6.36	5.39	5.79	5.13	5.47	5.68
Cysteine	1.39	0.47	0.75	0.73	0.66	1.84	1.77	1.45
Hydrophobicity index value	0.73	0.62	0.73	0.81	0.86	1.00	0.93	0.81

[a]Standard error is within 5%.

TABLE III
Amino Acid Composition (mol %) of Peaks from Reversed-Phase High-Performance Liquid Chromatography of BF-I[a]

Amino Acid	Peak a	b	c	d	e	f	g	h	i	j	k
Aspartic acid	4.26	6.26	4.75	6.40	6.97	5.73	7.03	3.76	6.08	6.06	6.85
Threonine	3.39	3.01	3.74	4.30	5.19	3.67	3.47	2.87	3.84	4.04	3.79
Serine	7.60	8.85	7.39	9.77	11.17	8.65	10.13	7.23	10.23	13.02	9.81
Glutamic acid	25.27	21.95	20.26	17.55	14.88	26.36	19.47	30.72	16.20	20.06	19.38
Proline	12.31	7.62	···	8.78	7.72	10.55	···	16.61	9.57	8.60	8.33
Glycine	13.76	17.03	29.02	13.15	12.68	10.94	18.77	7.67	13.49	13.86	14.89
Alanine	···	4.91	6.82	5.52	5.93	4.81	5.95	4.20	7.72	6.17	5.03
Valine	6.21	4.43	5.69	5.13	5.99	5.05	5.97	4.18	6.25	4.39	4.29
Methionine	···	0.96	···	0.77	0.37	0.62	···	···	···	0.54	0.69
Isoleucine	2.99	2.58	1.91	3.08	3.35	3.05	2.92	3.07	2.47	2.62	3.34
Leucine	6.29	5.50	5.71	6.86	7.24	5.96	6.31	6.06	5.77	5.24	6.78
Tyrosine	···	···	···	2.28	1.82	1.19	···	···	···	0.72	1.99
Phenylalanine	7.35	4.86	2.34	4.18	4.63	3.87	5.59	5.88	4.29	3.35	4.03
Lysine	1.87	3.89	2.17	3.13	2.90	2.34	3.83	1.68	4.22	3.12	3.36
Histidine	···	2.10	1.66	2.62	1.51	2.91	2.61	2.10	2.16	4.66	1.66
Arginine	2.36	3.11	3.01	4.00	4.72	2.91	4.28	2.50	4.47	2.60	4.25
Cysteine	6.33	2.91	5.51	2.50	2.91	1.39	3.65	1.45	3.23	0.94	1.51
Hydrophobicity index value	0.86	0.70	0.88	0.83	0.98	0.61	0.82	0.61	0.90	0.64	0.72

[a]Standard error is within 5%.

glycine, lysine, and histidine, and lower proportions of leucine and phenylalanine than e(BF-I). Peak b(AF-0) had lower proportions of glutamic acid and proline, and higher proportions of aspartic acid, alanine, tyrosine, and arginine than f(BF-I). Peak c(AF-0) had a much lower content of glycine than g(BF-I). Peak d(AF-0) had higher contents of aspartic acid, threonine, serine, glycine, alanine, valine, isoleucine, leucine, lysine, and arginine, and lower contents of glutamic acid and proline than h(BF-I). Peak g(AF-0) and j(BF-I) are main peaks in the late eluting area. Peak g(AF-0) had higher proportions of aspartic acid, alanine, valine, isoleucine, leucine, tyrosine, lysine, and arginine, and lower proportions of serine, glutamic acid, glycine, and histidine than j(BF-I). Although retention times of both peaks were similar, the hydrophobicity index values were 0.93 and 0.64 for g(AF-0) and j(BF-I), respectively.

Amino acid compositions of peaks from AF-II (Fig. 2B) and BF-II (Fig. 2E), and from AF-III (Fig. 2C) and BF-III(Fig. 2F) with similar retention times were compared to each other as follows: a(AF-II) and a(BF-II), b(AF-II) and d(BF-II), c(AF-II) and e(BF-II), d(AF-II) and f(BF-II), e(AF-II) and g(BF-II), f(AF-II) and h(BF-II), g(AF-II) and i(BF-II), a(AF-III) and a(BF-III), b(AF-III) and b(BF-III), d(AF-III) and c(BF-III), f(AF-III) and d(BF-III), g(AF-III) and e(BF-III), h(AF-III) and f(BF-III), i(AF-III) and g(BF-III), j(AF-III) and h(BF-III), l(AF-III) and i(BF-III). These comparisons (not shown) showed similar trends to the comparisons between AF-0 and BF-I. It was found that generally the AF group of proteins exhibited higher contents of aspartic acid, alanine, lysine, and arginine, and lower contents of glutamic acid and proline than the BF group of proteins.

Distribution of *S*-Succinyl-L-Cysteine

Table IV shows the proportion of *S*-succinyl-L-cysteine in the AF and BF groups of proteins, and selected peaks separated by RP-HPLC. AF-0 contained the greatest proportion of *S*-succinyl-L-cysteine of all the AF and BF protein groups. BF-I ranked second in the proportion of *S*-succinyl-L-cysteine. Peaks c, f, and g separated by RP-HPLC from AF-0 contained comparable levels of *S*-succinyl-L-cysteine. Peak e had the greatest proportion of *S*-succinyl-L-cysteine in the peaks of AF-0. In the case of BF-I, peak a had a greater proportion of *S*-succinyl-L-cysteine than the other peaks. *S*-succinyl-L-cysteine was present in low levels in peaks b, c, d, f, and g from AF-II. In the case of BF-II, peak j (which exhibited stronger surface hydrophobicities than the other peaks) contained a greater proportion of *S*-succinyl-L-cysteine. For AF-III and BF-III, peaks a, b, and i and peak d had higher proportions of *S*-succinyl-L-cysteine than the other peaks. As described above, whereas *S*-succinyl-L-cysteine was distributed at about the same high level in peaks separated by RP-HPLC from AF-0, it was present in variable amounts in peaks from other fractions.

TABLE IV
Proportion of *S*-Succininyl-L-Cysteine in Each Fraction[a]

Fraction/ Peak	Proportion
AF-0	1.00
c	1.39
e	2.01
f	1.23
g	1.39
AF-II	0.53
b	0.80
c	0.83
d	0.87
f	0.87
g	0.72
AF-III	0.56
a	1.61
b	4.47
i	2.03
BF-I	0.90
a	8.78
BF-II	0.61
a	0.72
f	0.48
h	0.61
i	0.54
j	2.28
BF-III	0.40
b	0.69
d	1.64
g	0.22
h	0.32

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

SDS-PAGE

Figure 3 shows densitometer tracings of SDS-PAGE patterns for the AF and BF protein groups. All samples were dissolved in 0.5% SDS solution prior to examination by SDS-PAGE. AF and AF-0 were dissolved incompletely by the 0.5% SDS solution. The

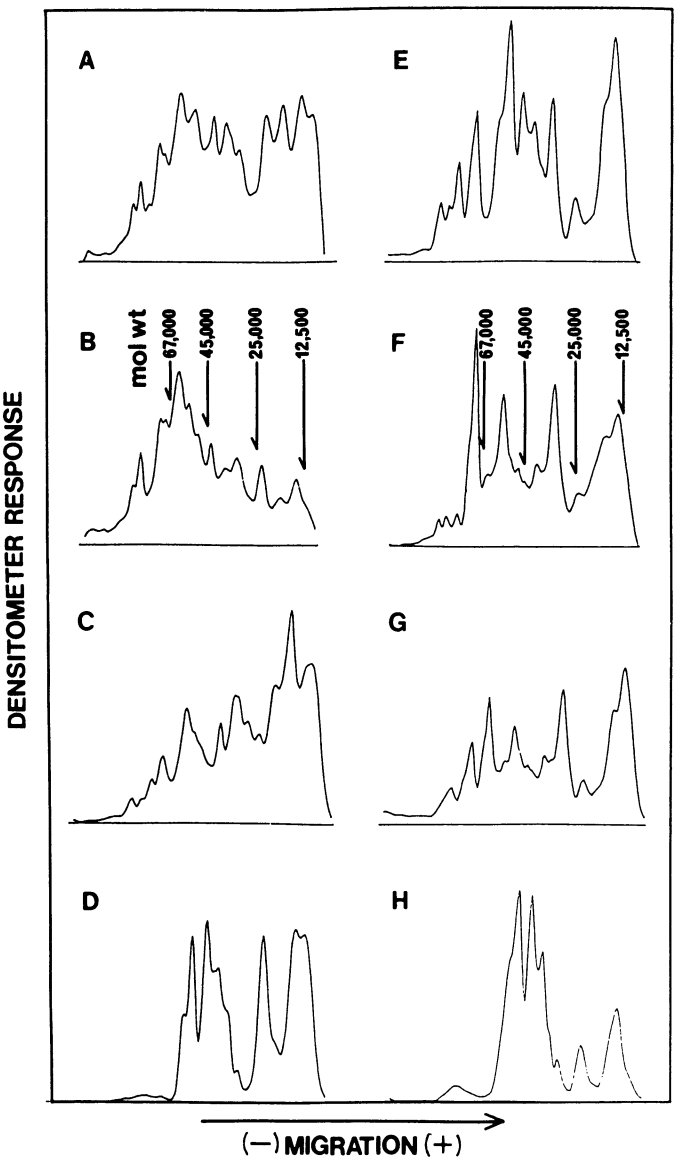

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic scan pattern for reduced-pyridylethylated acetic acid-insoluble (AF) and soluble (BF) protein groups. **A**, AF; **B**, AF-0; **C**, AF-II; **D**, AF-III; **E**, BF; **F**, BF-I; **G**, BF-II; **H**, BF-III.

pattern for the 0.5% SDS-soluble AF-0 shows a main peak at about 60,000 M_r and is composed of subunits from about 100,000 to 14,000 M_r (Fig. 3 B). Comparison of SDS-PAGE patterns from comparable fractions (AF and BF, AF-0 and BF-I, AF-II and BF-II, AF-III and BF-III) revealed that the AF and BF protein groups were composed of different protein subunits.

CONCLUSIONS

It is well known that when wheat flour dough is overmixed in the presence of NEMI, dough consistency decreases. Tanaka and Bushuk (1973a-c) emphasized the depolymerization mechanism of dough breakdown during mixing with NEMI. The changes in dough properties during overmixing have been studied by many workers (Weak et al 1976; Schroeder and Hoseney 1978; Sidhu et al 1980a,b; Danno and Hoseney 1982a,b). The general concensus is that disulfide bonds in gluten break during dough mixing, creating thiol radicals that react with compounds containing activated double bonds.

The subunit fractions containing the most disulfide bonds that react with NEMI in the AF and BF protein groups were determined by the distribution of *S*-succinyl-L-cysteine. It was found that NEMI reacts with AF-0 and BF-I more than with the other protein fractions during mixing. *S*-succinyl-L-cysteine was present at about the same high level in peaks separated from AF-0 by RP-HPLC, whereas it was present in variable levels in RP-HPLC peaks from other protein fractions.

On the basis of these data, we propose that the reaction with NEMI of the key disulfide bonds in AF-0 contributes to dough breakdown. This fraction has a higher content of hydrophobic amino acids than the other protein fractions. RP-HPLC revealed that subunits of AF-0 elute later than those of the other protein fractions, indicating that AF-0 subunits have higher surface hydrophobicities. AF-0 is not dissolved in 0.1 *N* acetic acid solution and is incompletely dissolved in 0.5% SDS solution. SDS-PAGE showed that the AF-0 fraction consists of high molecular weight subunits.

Kobrehel and Bushuk (1977) suggested that virtually all glutenin comprises aggregates in which highly specific hydrophobic interactions hold subunits together. Chung and Pomeranz (1979) reported that glutenin from a poor baking quality flour interacted less with hydrophobic Sepharose gel than glutenin from a good baking quality flour. They showed that the contribution of glutenin to dough properties may involve hydrophobic interactions. We reported that dough overmixed in the presence of NEMI contained more of the high molecular weight fraction compared to that of an undermixed control dough, and there was no change in the medium and low molecular weight proteins (Okada et al 1987).

We speculate that dough breakdown in the presence of NEMI is caused by partial depolymerization of glutenin and changes in protein surface hydrophobicities, mainly in AF-0 and BF-I, induced by modification of the key disulfide bonds by NEMI. However, more work is required to confirm this speculation.

Results of RP-HPLC, SDS-PAGE, and amino acid analysis showed that acetic acid-insoluble protein is composed of different subunits than acetic acid-soluble protein.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of H. Hara and T. Katsuta; the helpful advice of K. Maeda, Biochemistry Department, Fine Chemical and Pharmaceutical Research Laboratory, Research Center, Nisshin Flour Milling Co., Ltd.; and the kind advice of J. E. Dexter, Research Scientist, Grain Research Laboratory Division, Canada, in the preparation of this manuscript.

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[Received August 17, 1987. Revision received December 21, 1987. Accepted December 29, 1987.]