STUDIES ON SHORT- AND LONG-MIXING FLOURS

I. Solubility and Electrophoretic Composition of Proteins[1]

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ABSTRACT

A short- and a long-mixing flour were separated into 0.1*M* sodium chloride-soluble, water-soluble, and protein-starch residue fractions. These fractions contained primarily the (a) albumins and globulins, (b) gliadin, and (c) glutenin proteins, respectively. The distribution of protein among the fractions was determined and fractions were analyzed by moving boundary electrophoresis. The separation methods resulted in fractions which, when recombined, had farinograph mixing properties essentially like those of the original flours. Similar amounts of 0.1*M* sodium chloride-soluble nitrogen were obtained from the two flours, but electrophoresis indicated quantitative differences in protein composition. The differences corresponded to reported differences between hard red spring and winter wheats. Electrophoretic comparisons of the glutens indicated similar amounts of alpha, gamma, and omega gluten components. The short-mixing flour had more of the beta gluten component and less acid-insoluble protein. The outstanding difference between the two flours was the greater water solubility of gluten components from the short-mixing flour. Treatment of flour doughs with N-ethylmaleimide did not alter the electrophoretic analyses of the gluten components.

The proteins of wheat flour are chiefly responsible for the rheological properties of dough. Little is known, however, about the relation of specific proteins to rheological differences of flours. Some previous

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work has dealt with rheological differences of specific fractions such as water-soluble proteins (1). Other work has attempted to relate specific fractions to baking quality (2). Interpretation of results was complicated by a lack of quantitative methods to determine the type and distribution of components present in flour fractions. The recent advances in physical-chemical methods for the analysis of wheat proteins (3) now makes it possible to obtain information on the relationship between specific proteins and dough properties.

The work reported here was undertaken to investigate the nature of the protein components in two flours having similar nitrogen contents but markedly different farinograph mixing times and stabilities. Electrophoretic and solubility experiments were designed to study quantitative differences in all of the protein components. This paper describes the separation, analysis, and properties of the protein components. The accompanying paper (4) describes the relation of this information to dough-mixing behavior.

Materials and Methods

Flours. Flours prepared on a Multomat mill from Willet, a weak spring wheat, and Rodco, a strong winter wheat, were used in these studies. The composition and farinograph values of these flours are given in Table I. Rodco contains slightly less protein, but its mixing time and stability are six times greater than that of Willet.

TABLE I
ANALYSES AND FARINOGRAPH VALUES OF FLOURS

Flour	Ash [a]	Protein [a]	Absorption [a]	Maximum	Time to Maximum	Stability
	%	%	%	*B.U.*	*min.*	*min.*
Willet	0.33	14.3	58.4	510	3.5	3.5
Rodco	0.43	13.6	61.0	500	22	22

[a] 14% moisture basis.

Preparation of Flour Fractions. The fractionation procedure shown in Fig. 1 is based on the studies of Baker *et al.* (5), Holme (6), and Mattern and Sandstedt (1). General fractionation conditions included a preliminary extraction of flour with benzene to remove loosely bound fat that might be involved in oxidative changes during fractionation. Benzene extraction had very little effect upon the farinograph mixing-properties of the flours other than increasing the absorption from 1.5 to 2%. All dispersions were done in a N_2 atmosphere. Dispersions and centrifugations were done at 0°–5°C. All solutions contained 0.001*M* ethylenediamine tetraacetic acid (EDTA),

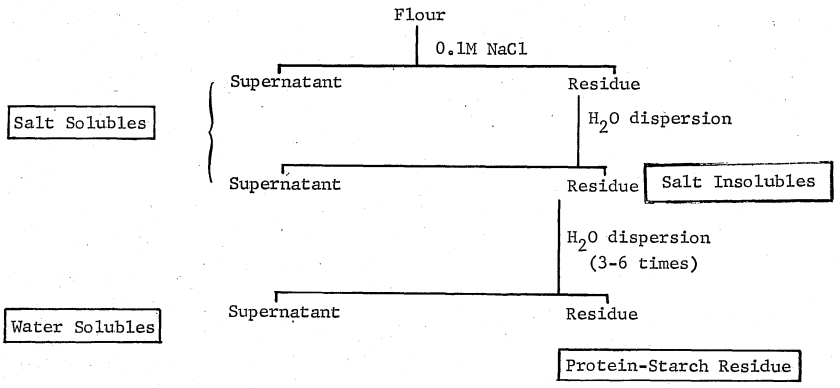

Fig. 1. Flour fractionation procedure.

disodium salt, to minimize metal-catalyzed changes in lipoproteins (7). All fractions were lyophilized and ground to pass a 30-mesh screen.

The salt-soluble fraction was prepared by stirring 1 part flour with 3 parts 0.1*M* NaCl for 5 min. The slurry was centrifuged at 13,200 × *g*. The insoluble residue was redispersed in sufficient water to give a 2:1 solvent-to-solids ratio. Redispersion was done in a 1-gal. Waring Blendor at low speed for 3 min. Following centrifugation, the supernatant solution was combined with the initial 0.1*M* sodium chloride extract. The sodium chloride present in the initial slurry, and that remaining upon subsequent water-dispersion of the residue, solubilizes albumin and globulin proteins as well as some of the pentosans. The salt depresses solubility of gluten proteins.

After the salt content is reduced by the first water-dispersion, a water-soluble gliadin fraction can be separated by repeated water-dispersions in a Waring Blendor. The term "water-soluble" is used to describe this fraction even though a portion of the material may be only dispersed in the water. Two methods were used to prepare water-solubles. In method 1, four water-dispersions were made with each flour. In method 2, three and six water-dispersions were made with Willet and Rodco, respectively. With method 2, water-extracts were combined as follows: fraction A, first; B, second and third; and C, fourth to sixth.

The residue remaining after repeated water-dispersions contains starch and water-insoluble gums as well as protein. It will be designated protein-starch residue in subsequent discussion. It is rich in glutenin, the most insoluble wheat protein fraction.

Electrophoresis. Moving boundary electrophoresis was performed at 1°C. in a Spinco Model H electrophoresis-diffusion apparatus. Salt-

soluble fractions were analyzed in veronal buffer ($\mu = 0.1$, pH = 8.6) as described by Kelley and Koenig (8). Water-solubles and protein-starch residues were analyzed in aluminum lactate-lactic acid buffer (pH = 3.1, $\mu = 0.1$) (9) at a concentration of approximately 0.5% and field strength of 9.8 v./cm. Photographs were taken at 81 and 161 min. and relative amounts of components were determined by counting squares on enlarged tracings.

The water-solubles were dissolved directly in the buffer. The proteins in the protein-starch residues were solubilized by a modification of the method of Cunningham *et al.* (10). Five grams of material was dispersed in 40 ml. of 0.02*M* formic acid in a 250-ml. Waring Blendor bowl for 3 min. at top speed. After 1 drop of octanol was added, the dispersion was centrifuged at 38,000 × *g* at 1°C. The dispersion process was repeated two more times with 25 ml. of solvent. The three extracts were combined, diluted to 100 ml., and aliquots taken for Kjeldahl and electrophoretic analyses. Decreased solubilities were obtained in the absence of shearing forces such as were produced in the Waring Blendor.

Starch-gel electrophoresis was carried out in trays constructed according to diagrams of Smithies (11). Gels were prepared by heating 18 g. starch (Connaught Medical Research Labs, Toronto, Can.) with 100 ml. aluminum lactate buffer (pH 3.1, $\mu = 0.1$) containing 3*M* urea (12). After the solution came to a boil it was allowed to stand for 3 min. and was poured into the tray containing a slot-former. Parafilm-covered gels were kept at 5°C. for 3 hr. before use.

The protein samples were heated in a boiling-water bath for 5 min. to destroy enzymatic activity, and rapidly cooled. The slot was loaded with 0.05 ml. of approximately 5% protein solution and covered with molten petroleum jelly. The remainder of the gel was covered with parafilm. An electric field of approximately 7 v./cm. (120 volts, 6 ma.) was applied for 17 hr. Sliced gels were stained in Nigrosine (13) and photographed with a red filter.

Quantities and Protein Contents of Fractions. Analyses of the materials obtained by method 1 are given in Table II. These values

TABLE II

DISTRIBUTION OF PROTEIN BY FRACTIONATION METHOD 1

FRACTION	PERCENT OF TOTAL PROTEIN	
	Willet	Rodco
	%	%
Salt-solubles	10	10
Water-solubles	49	23
Protein-starch residues	41	67

are average results of two fractionations using 750 g. of defatted flour for each.

Similar amounts of salt-soluble protein were obtained from the two flours. A marked difference was observed, however, in the amount of water-soluble protein. Over twice as much of the Willet protein was soluble in water. This left only 41% of the Willet protein not soluble in water under these conditions, as compared to 67% of the Rodco protein. The Willet protein-starch residue contained only 8% protein; the Rodco residue contained 12%. Qualitative differences in composition of these fractions are considered in the discussion of electrophoretic results.

In the fractionation by method 2, the salt-solubles were removed and the salt-insolubles dried and analyzed before additional separations were made. Fractionation of dry salt-insolubles was then made by the slurry and centrifugation method to retain about the same amount of protein in each protein-starch residue. This required three water-dispersions with the Willet and six dispersions with the Rodco flour. Distribution of protein upon fractionation is shown in Table III.

TABLE III
DISTRIBUTION OF PROTEIN BY FRACTIONATION METHOD 2

FRACTION	PERCENT OF TOTAL PROTEIN	
	Willet	Rodco
	%	%
Salt-solubles	10	10
Water-solubles A	4	2
Water-solubles B	30	7
Water-solubles C	..	16
Protein-starch residues	56	65

The proportions of salt-soluble protein were similar for the two flours. The proportions of water-soluble protein were, however, markedly different. The greater water-solubility of the Willet protein is evident. Three extractions (water-solubles A and B) solubilized 34% of the Willet protein but only 9% of the Rodco protein. The Rodco fraction was subjected to three more extractions to obtain a protein-starch residue of approximately the same protein content as the Willet. This additional extraction still did not lower the protein content of the Rodco residue below that of the Willet.

Typical Electrophoretic Patterns. A commercial bread flour was used in development of the fractionation methods. Figure 2 depicts the electrophoretic composition of this flour and fractions prepared

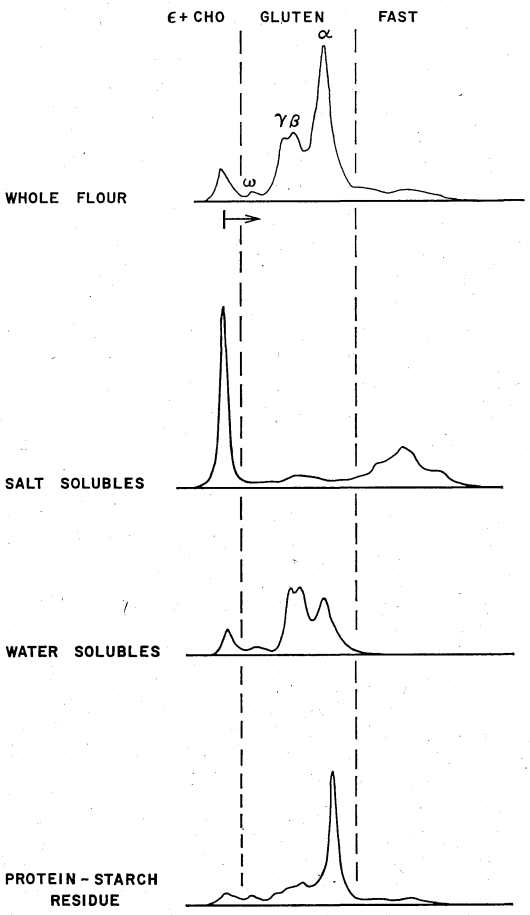

Fig. 2. Descending electrophoretic patterns of commercial bread flour and fractions in aluminum lactate buffer (pH = 3.1, $\mu = 0.1$) after 81.0 min. at field strength of 9.8 v./cm.

therefrom. Corresponding Willet and Rodco preparations had patterns with these general contours.

The components are designated as originally proposed by Jones *et al.* (9). As depicted by the dashed lines in Fig. 2, each pattern in aluminum lactate buffer shows three main regions. The fastest-moving region contains material with mobility of the albumin-globulin fraction. The middle area represents gluten components which are further resolved by longer electrophoresis. The stationary area is a composite of the salt boundary and soluble carbohydrate material. The stationary area is not included in calculations of protein composition.

Although the fast region has been assumed to contain most of the albumins and globulins, some always remains in the water-solubles and protein-starch residues after salt and multiple water-extractions. Other workers (14,15) have assumed that this residual fast material represents albumins and globulins trapped within the gluten mass.

As shown in Fig. 2, the salt-soluble fraction has a high proportion of material with mobility of albumin-globulins. This fraction was also analyzed in veronal buffer for optimum resolution (6,8), and typical patterns are shown in Fig. 3.

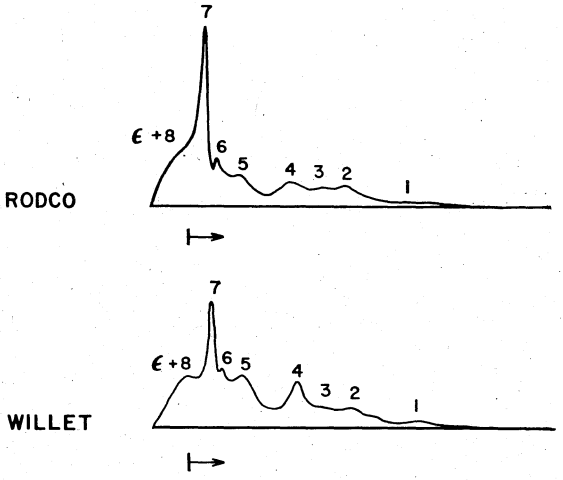

Fig. 3. Descending electrophoretic patterns of salt-solubles in veronal buffer (pH = 8.6, $\mu = 0.1$) after 211.0 min. at field strength of 6.5 v./cm.

Electrophoretic Analyses of Salt-Solubles. Electrophoretic results obtained in veronal buffer are given in Table IV.

The values are the average of data on two separate analyses of each fraction. The precision of these analyses has been reported (8). Components 4, 5, and 6 are higher in the Willet, whereas component

TABLE IV

ELECTROPHORETIC COMPOSITION OF SALT-SOLUBLE FRACTIONS

	Component No. and Mobility Range[a]							
	1 4.4–5.8	2 3.8–3.9	3 3.2–3.4	4 2.5–2.7	5 1.0–2.1	6 0.7–0.9	7 0.4–0.6	8 0
	%[b]	%	%	%	%	%	%	%
Willet	5	7	6	14	14	14	14	26
Rodco	5	7	6	10	10	10	22	30

[a] 10^{-5} cm.^{2}/volt/sec.
[b] Percent of pattern area.

7 is higher in the Rodco salt-solubles. Component 7 contains pentosan material (6). These differences correspond to the previously reported (16) differences between hard red spring (HRS) and hard red winter (HRW) wheat varieties.

Electrophoretic Analyses of Water-Solubles and Protein-Starch Residues. The electrophoretic composition of the gluten fractions prepared by method 2 are given in Table V. The two salt-insoluble fractions are not markedly different, but a significant difference is

TABLE V

ELECTROPHORETIC COMPOSITION OF FLOUR FRACTIONS PREPARED BY METHOD 2

FRACTION	COMPONENTS AS PERCENT OF TOTAL PROTEIN IN FRACTION					
	Alpha	Beta	Gamma	Omega	Fast	Insoluble
	%	%	%	%	%	%
Willet salt-insolubles	48	18	11	4	10	9
Rodco salt-insolubles	47	12	12	4	8	17
Willet						
Water-solubles A	32	28	17	4	19	..
Water-solubles B	46	25	20	3	6	..
Protein-starch residue	53	13	8	5	15	6
Rodco						
Water-solubles A	15	19	27	8	31	..
Water-solubles B	32	30	25	4	9	..
Water-solubles C	39	26	27	3	5	..
Protein-starch residue	52	7	10	3	16	12

apparent in the level of the beta components and acid-insoluble matter. The acid-insoluble protein can be rendered soluble by oxidative cleavage of disulfide bonds (17). Thus, this material appears to have intermolecular disulfide bonds, as is characteristic of acid-soluble glutenin (18).

Results in Table V indicate how the protein composition changes with successive water-dispersions. With each flour, the proportion of the alpha component increases as the number of extractions increases. Solubles obtained from the first Willet dispersion (solubles A) had only 32% alpha, whereas material from the second and third dispersions (solubles B) had 46% alpha. The Rodco alpha component is much less readily solubilized by water-dispersion. Only 15% alpha was present in the solubles obtained from the first dispersion. This proportion was increased to 32 and 39% with successive dispersions. Thus, Rodco and Willet glutens differ markedly in ease of solubilization when subjected to shearing forces as produced by the Waring Blendor.

The higher level of beta in Willet and acid-insoluble material in Rodco are the major differences in the two protein-starch residues.

Such differences were also evident in the salt-insoluble fractions.

Data from Tables III and V on the water-soluble and protein-starch fractions have been recalculated to determine the quantitative distribution of each gluten component among the fractions. These data are given in Table VI.

TABLE VI

DISTRIBUTION OF GLUTEN COMPONENTS OBTAINED BY METHOD 2

FRACTION	PERCENT OF TOTAL RECOVERED COMPONENT IN FRACTION							
	Alpha		Beta		Gamma		Omega	
	W[a]	R[a]	W	R	W	R	W	R
Water-solubles A	3	1	7	3	6	4	5	4
Water-solubles B	31	5	47	20	54	14	23	11
Water-solubles C	..	15	..	38	..	33	..	18
Protein-starch residue	66	79	46	39	40	49	72	67

[a] W = Willet; R = Rodco.

This tabulation emphasizes that all Willet gluten components are more readily solubilized in water. The difference is particularly marked with the alpha component; 31% of this component was dispersed in extract B with Willet but only 5% with Rodco. After six dispersions there was still a greater proportion of the total alpha remaining in the Rodco protein-starch residue than remained in the Willet residue after three dispersions.

Starch-Gel Electrophoresis. Starch-gel electrophoresis was employed as a qualitative tool to detect differences in Willet and Rodco gluten fractions. Unfractionated gluten proteins, as found in the salt-insoluble fractions, possessed characteristic starch-gel patterns. These characteristics are pointed out in Fig. 4 where the nomenclature adopted by Woychik *et al.* (12) is used. The main alpha band for Rodco stains more intensely. In the beta region, four Rodco bands are closer together, whereas the Willet bands are more spread out and deeper-staining. The gamma region follows the alpha in staining intensity and two bands are resolved. Woychik *et al.* originally indicated one band in this region when staining was done with Amido Black 10-B. The two Rodco bands marked with an arrow are not apparent in the Willet gluten.

A thin, dense stain is present at the origin. This material represents high molecular weight glutenin which is unable to penetrate the pores of the starch gel (12). It is significant that water-soluble fractions do not show this heavy staining at the site of sample application, whereas an intense stain occurs here when the protein-starch

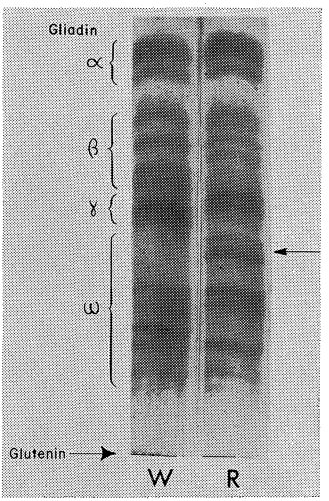

Fig. 4. Starch-gel-electrophoretic patterns of Willet (W) and Rodco (R) gluten.

residues are analyzed. This is evidence that the water-soluble fraction is essentially all gliadin, whereas the protein-starch residue fraction is rich in high molecular weight glutenin.

Differences in starch-gel-electrophoretic patterns of glutens from different wheat varieties have been observed by others. Woychik *et al.* (12) reported seven major gluten components in two HRW wheats having good baking qualities, but the electrophoretic distribution differed in the beta and gamma regions. Elton and Ewart (19) reported largest variations in the alpha region upon comparison of eight different wheat varieties. However, their differences may arise partly from differences in solubility of the glutens. With their single-extract procedure, only about 60–65% of the flour protein was solubilized for analysis. The alpha component is the least soluble gluten component (9) and the present work (Table VI) illustrates that alpha components of different varieties may have twofold differences in ease of dispersibility. Graham (20) and Bourdet *et al.* (21) have also pointed out solubility and electrophoretic differences in glutens and gliadins from different wheat varieties.

Effect of N-Ethylmaleimide (NEMI) upon Solubility and Electrophoretic Composition of Proteins. The addition of NEMI to doughs markedly shortens the mixing requirements and decreases the stability. Mecham *et al.* (22) showed that NEMI increased the amount of protein extractable upon mild agitation with dilute acid, but no study was made to determine whether NEMI altered the electrophoretic distribution of gluten components.

Doughs from Willet and Rodco were prepared with and without NEMI (4). They were extracted by the Waring Blendor procedure used for protein-starch residues and analyzed by moving boundary electrophoresis. Duplicate analyses of each flour indicated that neither the amount of acid-soluble nitrogen nor the electrophoretic composition was significantly altered by the presence of 7 mg. of NEMI per 80 g. dough.

Discussion

The results reported herein describe fractionation methods which result in concentration of three major wheat protein classes: albumin-globulin (salt-solubles), gliadin (water-solubles), and glutenin (protein-starch residues). These methods are useful for reconstitution studies with the farinograph because (a) the large amount of material necessary for farinograph testing can be obtained and (b) fractionation does not significantly alter the mixing properties of the recombined material. Thus, the fractions obtained from short- and long-mixing flours could be reconstituted and interchanged to specify the fractions accounting for the mixing differences.

The factors accounting for the large rheological differences between Willet and Rodco flours are found in the gluten-starch fraction (4). Moving boundary electrophoretic comparison of the acid-soluble glutens showed similar levels of alpha, gamma, and omega components and some difference in levels of beta components. All gluten components of the short-mixing flour are much more easily dispersed in water than those from the long-mixer. Also, a greater proportion of the total protein from the short-mixer can be solubilized in dilute acid. These solubility differences appear to be the most significant properties distinguishing these two flours. The accompanying paper further discusses the relation between gluten solubility and mixing properties (4).

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