Use of ^{14}C-Labeled Flour Proteins to Assess the Effect of Dough Mixing on Their Mobility in Gel Electrophoresis[1]

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ABSTRACT

A technique has been developed for using radioactive tracer to study changes in electrophoretic mobility of wheat-flour proteins during dough mixing. ^{14}C-labeled flour was prepared from two varieties of wheat injected with either lysine-^{14}C or leucine-^{14}C. Labeled proteins were isolated by extracting the flour with 0.05M acetic acid. To obtain labeled fractions, the solubilized proteins were subjected to polyacrylamide gel electrophoresis, gel sections containing four of the principal band groups were extracted with 0.05M acetic acid, and the extracts were lyophilized. Changes in electrophoretic mobility of these fractions with increasing dough-mixing time were then studied. The labeled fractions were added to nonradioactive flour which was made into undermixed, optimally mixed, and overmixed flour-water dough. Aluminum lactate extracts of the dough and of the flour before mixing were used for comparative gel electrophoresis. A small change in the amount of the glutenin fraction with mixing was noted for both varieties. Also, dough mixing seemed to lessen the extractability of the labeled material, particularly the less mobile (gluten) material.

It has long been known that the baking value of a particular flour may be correlated with the rheological properties of the dough, particularly its resistance to mixing (1). During dough mixing, development occurs to a point of minimum mobility followed by mechanical breakdown. These changes in rheological properties may be readily observed with a recording dough mixer. Little is known about the relation between rheological properties and chemical and physical structure of dough.

It is generally agreed that gluten forms the framework of dough structure. It has also been observed that those disulfide (SS) groups thought to be most intimately concerned with changes in dough properties during mixing are probably located in the glutenin portion of the gluten complex (2). Sulfhydryl groups have been shown to decrease when dough is mixed in air (3). It has been proposed that flour protein subunits interchange by way of shifts in the equilibria of subunits bound by SS groups, as disulfides are reduced to sulfhydryls and reoxidized (3). It has also been suggested that dough mixing decreases the size of protein aggregates in flour (4), and there have been reports (5,6) of experimental evidence for protein changes during dough mixing.

The hypothesis that protein aggregates decrease in size during dough mixing and that interchange reactions occur suggests that changes in electrical charge might well accompany smaller size of some of the protein molecules. This in turn suggests

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change in mobility of the altered proteins during gel electrophoresis. A method using radioactive tracer technique has been developed to investigate the existence of these changes.

METHODS

Production of Labeled Flour

Flour containing proteins labeled with carbon-14 was obtained by injecting wheat plants at the soft dough stage (7) each with either 25 μc. uniformly labeled L-lysine-^{14}C or L-leucine-^{14}C. Two varieties of wheat were chosen: Federation, grown at Pullman, Wash., and Thatcher, grown at Lind, Wash. The wheat was harvested by hand and milled in a Brabender Quadrumat Junior mill. The material passing an 80-mesh screen was taken as flour. Comparable control was prepared in a similar fashion with wheat from noninjected plants grown near by.

Separation and Isolation of Labeled Protein Fractions

The wheat-flour proteins were solubilized for preparative gel electrophoresis by a 1-hr. dialysis-extraction. A suspension of the radioactive flour in 0.05M acetic acid was placed in a dialysis bag held open by a stiff plastic ring inserted at the top. A one-hole rubber stopper was fitted into the ring. Through it a stirrer was lowered for continuous mixing of the flour suspension. During extraction the dialysis bag was suspended in a beaker of 0.05M acetic acid which was stirred magnetically.

Two volumes of extractant were used for all flours containing less than 8% protein, 2.5 vols. for flours with 8 to 12% protein, and 3 vols. for flours with a protein content greater than 12%. The extracts were clarified by centrifuging at about 100,000 X g (average) for 2 hr. after removal of much of the insolubles in a short preliminary centrifugation. The clear middle layer of supernatant solution was removed with a syringe for electrophoresis.

Four labeled fractions were separated by polyacrylamide-gel electrophoresis, with the preparative mold previously described (8). These fractions were the protein remaining in the slot (defined as glutenin (9)) and protein band groups A, B, and D (10). Groups A and B have been identified as including gliadins (10) and group D corresponds to the base proteins of Gehrke et al. (11). The separation was done in gels made and equilibrated as previously described (10), but with 0.05M acetic acid (pH 3.2) in the buffer chambers for both equilibration and the 5-hr. electrophoretic separation. For isolation of the fractions the slot section was cut out of the gel immediately after electrophoresis and was frozen at once. The guide section (minus slot portion) was dyed with 0.1% amido black in 7% acetic acid and the remainder of the gel was stored in the refrigerator, wrapped in Saran film. As soon as the bands became visible in the guide section when washed with 7% acetic acid, the main undyed portion was fractionated; the guide gel was placed on it, separated by Saran film, and the three other protein band groups were cut out: A and B (gliadin-type protein) and D (primarily albumins). It is convenient to cut both the guide and sample part of the preparative gel down one side with a corrugated cutter before separating them, to facilitate matching the two for final fractionation of protein bands. Also, a notch is needed to distinguish between the two ends. Sections were stored frozen until approximately 30 similar sections had

been collected. To recover the protein from the gel, the gel sections were partially thawed and homogenized with 0.05M acetic acid. The insoluble material was centrifuged down and washed twice with additional extractant. The extract and washings for similar gel sections were pooled and dialyzed overnight in the cold against two changes of distilled water. Dialyzed extracts were then lyophilized and the resulting dried material was finely ground in a mortar to be added to the nonradioactive flour prior to mixing of the dough. A small amount was reserved for direct analysis of radioactivity (I).

Mixing Experiments with Doughs

The dried, ground fractions representing the isolated radioactive protein groups were incorporated into the unlabeled control flour in a ratio of less than 1 part in 100 (except 1:50 in one case) by tumbling for 0.5 hr. just before the dough was mixed. A 1-g. sample was removed for preparation of an extract (II) of the mixed flour before it was made into a dough. The dough was mixed in a National mixograph after the previously determined amount of water for desirable absorption had been added. Mixing was interrupted and a small sample removed after 1 min. for an undermixed sample (III), at the point of maximum resistance (3 min. for Thatcher; 2 min. for Federation) for an optimally mixed sample (IV), and after 5 min. for an overmixed sample (V). These samples were immediately wrapped in waxed paper and frozen in dry ice, and then put on a lyophilizer to dry. The dried dough was ground in a Wig-l-bug (Crescent Dental Mfg. Co., Chicago, Ill.) and then extracted with 0.1 ionic strength aluminum lactate buffer, pH 3.2 (12). The material not made into dough (II) was similarly extracted. The labeled fraction (I) was completely soluble in the buffer. The distribution of the radioactive protein fractions in the solution of I and the extracts of II to V was determined by gel electrophoresis (10) and compared.

Assay of Mobility Changes in the Doughs

One of the six gels in the mold was used as a blank. The electrophoresis was run no longer than necessary for adequate band separation, so as to keep the band sections small. As in the case of the preparative gel, the slot sections and the area preceding the slot were removed immediately after electrophoresis. The remaining part of each gel was soaked in 12.5% trichloroacetic acid (TCA) for 0.5 hr. to render the bands visible. Half the gel blank was similarly treated. When the bands became visible the gels were laid out on Saran film on black paper and the bands were observed with oblique lighting. The gels were divided into sections cut with a razor blade, comprising bands A, B, C, D, E, and F, and the remaining gel. All sections were prepared for counting by overnight incubation in graduated tubes at 40°C. in 2 vols. of 30% H_2O_2 based on wet gel weight (13,14). The volumes of dissolved gel were recorded, and 2-ml. aliquots were taken for liquid scintillation counting, with the use of 18 ml. of *p*-dioxane scintillator (15) plus Cab-o-sil (0.8 g. per vial) for each sample. Dissolved gel samples that were less than 4 ml. in volume were diluted with the appropriate dissolved gel blank to over 4 ml. to allow for two 2-ml. aliquots for counting. The radioactivity of sections was considered significant only when there was at least 95% statistical probability that the count was greater than that of the blank gel (2 × s.d. mean). Where possible, duplicate samples of the

dissolved gels were counted. At least three 10-min. counts were made on all samples.

DISCUSSION OF METHODS

Lysine-^{14}C and leucine-^{14}C are more efficient than many other amino acids for labeling wheat proteins, since relatively little is metabolized to nonprotein material. When hydrolysates of labeled flours were fractionated in the amino acid analyzer, it was found that 86 to 90% of the carbon-14 from leucine and 89 to 93% of that from lysine was recovered in the amino acids. Injection with these compounds also permits preferential labeling of the flour proteins in different ways. With lysine-^{14}C, the more mobile soluble proteins received relatively more of the radioactivity. This would be expected, since the gluten proteins are known to have a much lower lysine content (16,17) than the soluble proteins, and lysine is deposited in the wheat proteins with little conversion to other amino acids (7). Hence, the soft wheat, Federation, with its lower proportion of gluten protein, was treated with lysine-^{14}C. Leucine is more evenly distributed among the wheat proteins, and its use results in more labeling in the gluten proteins which are present in greater amounts, particularly in the hard wheat variety, Thatcher, which was treated with it in this work. Our more recent work has shown that injection of phenylalanine-^{14}C results in even greater differentiation of label in favor of the gluten proteins, which suggests its usefulness for further work. Since hypotheses to explain changes in dough mixing concern protein subunits as a whole, there is no reason to expect the source of the label to affect the changes investigated.

Acetic acid was used for all extractions during preparation of the labeled flour-protein fractions, since, in contrast to aluminum lactate buffer, it can be removed by lyophilization leaving a presumably unchanged protein fraction. That this may not actually be the case is suggested by the work of Candlish and Tristram (18) on the binding of acetic acid by proteins. However, they report relatively little binding by proteins with low helix content, among which the main wheat proteins would be classed, on the basis of determinations of 14% for glutenins and 23% for gliadins (19).

All radioactive flours and the resulting dough fractions were extracted overnight with aluminum lactate to recover as much radioactivity as possible. The possibility of changes in the proteins during such an extended extraction was investigated. Gel electrophoresis was used to separate the proteins from a 1-hr. extract and from an overnight extract of Federation flour. Densitometry revealed no significant differences between the extracts.

In the final analysis of distribution of activity, TCA was used to reveal the location of the bands rather than amido black dye, so that washing out of background dye and probable loss of radioactivity was avoided. In Thatcher flour and dough, the separation point between groups A and B was not clear-cut. The results indicate that in the series where group B material was used as tracer, the division point was misjudged, and a considerable proportion of the group B bands were included in group A. However, all cuts within that series were made alike and results should be comparable.

RESULTS AND DISCUSSION

Only the most recent experimental results incorporating latest improvements in method are presented, with some reference to earlier work. Possible additional experimentation is suggested. In general, there was no significant change due to dough mixing in the gel-electrophoresis band pattern, and little change in the radioactivity distribution, but in a few cases changes seem to have significance.

There appear to have been changes in the extractability of doughs as compared with flour. When equal amounts of similarly prepared extracts of these materials were counted, the results shown in Table I were obtained. In nearly all cases the dough extracts show less radioactivity than the flour, indicating reduced extractability. Other workers (5,6,20,21,22) have shown that protein extractability increases with mixing time of dough, but have worked with mixing times exceeding 5 min. Mamaril and Pomeranz (6) report results of a 3M urea extraction of doughs mixed 1, 3, 5, 10,and 20 min. The amount of protein extracted is reported as 71.4, 70.4, 74.7, 76.9, and 78.8%, respectively. These figures suggest a drop in extractability of proteins during the early stages of mixing; ours showed a drop between undermixed and optimally mixed (1 and 3 min. respectively, for Thatcher). The extractability decreased more when the radioactivity was incorporated from fractions predominantly from material remaining in the slot during the preparative run, or from proteins mainly of group A. These are the larger, less mobile proteins. Mixing beyond that time to give overmixed dough resulted in some increase in extractability for all but the flours labeled primarily in group D proteins. Data of Mamaril and Pomeranz (6) deal with all protein extractable with 3M urea; hence, one would expect less of an over-all change.

Mecham et al. (20) have proposed that any purification of crude gluten, including acetic acid extraction, results in a disaggregation, possibly involving disruption of protein bodies, which perhaps anticipates the changes which might occur in our first phase of dough mixing. If this were the whole picture, one would not expect any change in the radioactivity pattern due to short-term mixing in our experiments, since our radioactive fractions were obtained by extraction with acetic acid.

It should be pointed out (without tabulation of the data) that there appeared to be a difference in the distribution of carbon-14 in the electrophoretic bands from the isolated labeled protein fractions (I), and the flour to which these fractions had been added (II). Relatively less of material II remained in the starting slot, with consequent larger percentages in the various gel bands. The explanation is probably related to the fact that the isolated fractions I could be completely dissolved in

TABLE I. EXTRACTION OF RADIOACTIVITY FROM LABELED THATCHER FLOUR AND DOUGHS

	Radioactive Fraction Added to Flour			
	Glutenin cpm.	Group A cpm.	Group B cpm.	Group D cpm.
Labeled flour	378	5,741	4,436	462
Undermixed dough	387	5,824	3,793	457
Optimally mixed dough	281	4,431	3,926	431
Overmixed dough	309	4,480	4,125	404

TABLE II. EFFECT OF DOUGH MIXING ON FLOUR PROTEIN ELECTROPHORETIC MOBILITY

Labeled Fraction Added	Amount of Mixing	Radioactivity in Band Groups					
		Slot (Glutenin) %	Thatcher A %	B %	C %	D %	More Mobile %
Glutenin	None	73	14	4	4	3	
	Under	79	19	2			
	Optimum	74	22	4			
	Over	72	26	2			
Group A	None	7	83	9	1		
	Under	4	84	9	4	0.2	
	Optimum	3	87	8	2		
	Over	2	87	8	2	0.1	
Group B	None	2	62	34	2		
	Under	2	56	38	4		
	Optimum	2	59	37	3		
	Over	2	56	38	3		
Group D	None	4	20	8	28	34	5
	Under	2	8	6	33	45	6
	Optimum	3	12	6	32	40	7
	Over	6	6	6	34	41	7
		Slot (Glutenin)	Federation A	B	C	D	More Mobile
Glutenin	Under	76	18	3			4
	Optimum	68	24	8			
	Over	46	31	19	4		
Group A	Under	8	78	12	2	0.3	
	Optimum	6	83	9	2		
	Over	4	86	9	2		
Group B	Under	4	28	56	11	1	1
	Optimum	3	29	60	7	0.5	
	Over	4	28	60	6	1	1
Group D	Under	6	4	5	22	59	4
	Optimum	3	4	4	21	63	5
	Over	2	6	5	21	63	3

buffer for electrophoresis, whereas the mixed flour II (and doughs) was extracted, leaving a residue of undissolved material. It might be expected that the main component of this undissolved material would be glutenin, which remains in the starting slot and is the least soluble of the protein fractions. Calculations from the data based on this assumption further suggest that some of the group A material also remains in the unextracted portion. For these reasons it becomes important to compare the data on the dough extracts to those from the extracts of flour with the labeled fraction added rather than extracts of the isolated labeled material. Unfortunately, in the case of the Federation wheat, the extract of labeled flour was

not counted, so that these data can be examined only as to changes occurring between very brief and longer periods of mixing.

Results in terms of percentage radioactivity in the various fractions before and after dough mixing for three periods of mixing time are given in Table II. Inspection suggests only a few cases where a real change may have occurred. From the data for the glutenin fraction as a labeling material it appears there may have been some change of the material remaining in the slot to faster-migrating molecules as mixing time was extended. When proteins of band group A were used to label the flour there was a similar trend. Calculations indicate that these changes are greater than would be accounted for by the changes in extractability shown in Table I. Although the experiments were not replicated sufficiently for statistical analysis, it seems doubtful if any other changes in the data are significant, including the unrealistically large change in activity of group A proteins when group D material supplied the label.

The smallness of the changes indicated here do not necessarily conflict with the SS bond interchange hypothesis. Since the process of dough development and breakdown is thought to be one of simultaneously creating and breaking bonds (23), it is conceivable that no great change in molecular weight ever occurs, even though there is a change in protein structure. Also, unless similarly charged subunits become bound, net charge would not change either. Hence, gel electrophoresis would not necessarily show a protein change. Meredith and Wren (22) showed essentially no molecular weight (MW) redistribution of protein fractions between flour and flour-water dough, the only change being a marginal increase in extractability in the glutenin fraction after 6 and 20 min. of mixing. This could be explained as a rearrangement of SS bonds in glutenin, without involvement of these bonds in the lower-MW proteins. As indicated above, our extraction data substantiate some change in the nature, principally of glutenin and group A proteins during dough mixing, supporting data accumulated by application of the tracer techniques to study the possible changes in gel-electrophoretic mobility.

Interestingly enough, dough made from the weaker Federation flour has a lower initial percentage of glutenin, which decreases more rapidly with mixing than is the case with Thatcher flour. There is a corresponding increase in the more water-soluble components A, B, and C, in agreement with the findings of Smith and Mullen (24), who reported that short-mixing flours contained more water-soluble protein initially and produced more during dough mixing. This strengthens the case for believing that the observed changes in the glutenin fraction are related to the mixing properties.

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