

Changes in Flour Proteins during Dough-Mixing. III. Analytical Results and Mechanisms¹

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ABSTRACT

Chemical analyses showed that the number of sulfhydryl (SH) groups decreased during mixing in all doughs except in the controls mixed in nitrogen. The rate of loss was slightly different for three varieties of flour. There was no change in the number of disulfide groups with mixing in the doughs investigated. The proteolytic activity and the content of amino groups of the three flours increased with decreasing strength. However, the number of amino groups was not affected by mixing time. There was no change in the amount of free lipid during mixing. A possible mechanism for the breakdown of doughs by excessive mixing (with or without SH reagents) is presented.

The first two articles in this series (1,2) described the changes in solubility, gel filtration chromatographic, and electrophoretic properties of flour proteins during dough-mixing. These studies indicated that under some conditions of dough breakdown, glutenin proteins can undergo depolymerization. The possibility of disulfide and peptide bonds being involved in this depolymerization was investigated. The results are described herein.

MATERIALS AND METHODS

The flours and doughs used for this study were described in the first article (1).

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Proteolytic Activity Determination of Flour

Proteolytic activities of the flours were determined by a modified Ayre-Anderson method (3). The activity was expressed as μ moles of tyrosine formed per min. per g. of flour, dry basis.

Determination of Amino Groups

The content of amino groups of the proteins that could be solubilized in 0.17N acetic acid was determined for all dough and flour samples as described by Hwang (4). According to this procedure, the proteins (of flour or freeze-dried dough) are exhaustively extracted at 4°C. using a flour:solvent ratio of 1:10. Total amino groups are determined spectrophotometrically after reaction with ninhydrin.

Determination of Sulfhydryl (SH) and Disulfide (SS) Contents

SH and SS contents of the flour and samples were determined by amperometric titration method as developed by Sokol et al. (5) and modified by Tsen and Anderson (6). SH and SS contents are expressed in μ eq. per g. of dry flour.

Extraction of Free Lipid

Free lipids were extracted from 3 g. of dry ground dough or flour with 50 ml. of petroleum ether in a Soxhlet extraction apparatus (8 hr.). The amount of free lipids was weighed after the solvent was evaporated and expressed as percent of dry flour.

RESULTS AND DISCUSSION

SH and SS Groups

SH contents of the flours and doughs mixed for 5 and 15 min. in air and in nitrogen with *N*-ethylmaleimide (NEMI) and iodate added at 2.0 μ eq. per g. flour

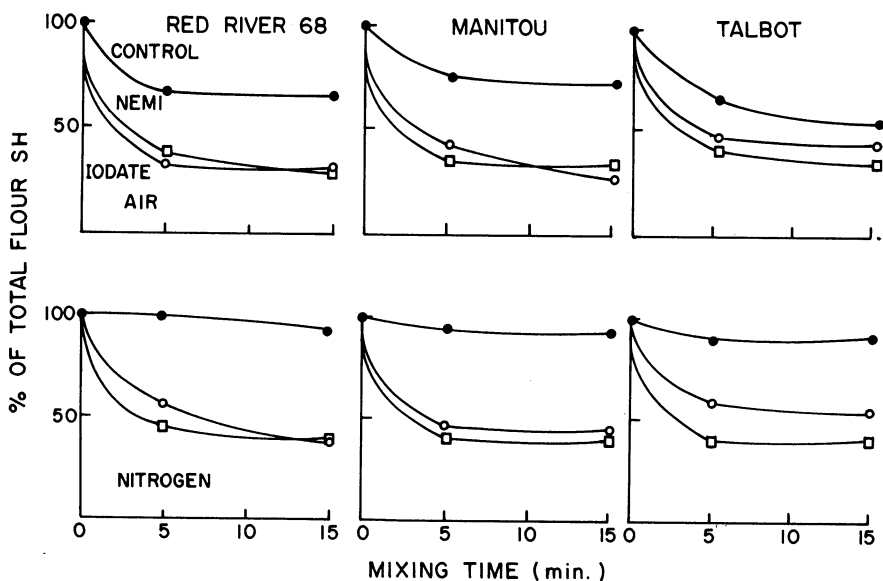


Fig. 1. Changes in SH content of doughs during mixing.

for the three varieties are shown in Fig. 1. For doughs of each variety, SH content decreased rapidly during the first 5 min. of mixing in air. Addition of iodate or NEMI enhanced this decrease. Mixing for an additional 10 min. produced a further but very small decrease in SH content. The effects of atmospheric oxygen and of iodate or NEMI on SH loss appear to be additive. The effect of iodate or NEMI is greater in the stronger doughs than in the weaker dough (Talbot). The results obtained in the present study are generally consistent with the findings of others (7-9).

TABLE I. DISULFIDE CONTENTS OF FLOURS AND DOUGHS MIXED FOR 15 MIN. UNDER VARIOUS CONDITIONS

		Red River 68	Manitou $\mu\text{eq./g. flour}$	Talbot
Flour		10.6	10.4	9.8
Control	Air	10.4	10.3	9.7
	Nitrogen	10.4	10.4	9.5
NEMI (2.0 $\mu\text{eq./g.}$)	Air	10.3	10.2	9.4
	Nitrogen	10.3	10.4	9.6
Iodate (2.0 $\mu\text{eq./g.}$)	Air	10.3	10.4	9.6
	Nitrogen	10.3	10.2	9.4

On the basis of several preliminary analyses, which gave negative results, it was decided not to analyze all of the dough samples for SS content. Accordingly, only the control doughs and the doughs that showed greatest breakdown during mixing were analyzed. The results showed that under conditions of mixing investigated, the SS content was not affected (Table I). There was a suggestion of a slight decrease in SS with mixing when the values for flour and for the corresponding doughs containing the higher concentration (2.0 $\mu\text{eq. per g.}$) of iodate or NEMI are compared. For two of the flours, the differences were just outside the experimental error. If this small loss of SS is in any way responsible for the breakdown observed in the farinograph, then these few SS groups would have to be extremely critical in their functional (rheological) role. As suggested recently by Bloksma (10), this could indeed be the case. However, highly precise analytical and rheological measurements would be required to verify this possibility.

The actual SS contents obtained in the present study are consistent with published values for similar flours. Again, it should be noted that the data are expressed on a dry flour weight basis, and that the protein contents of the flours used, especially the hard red spring wheat flours, are lower than the usual average values.

Proteolytic Activity

The proteolytic activities of the three flours used in this study are given in Table II. Although the activities were low and the absolute differences among the three varieties small, the ranking of varieties according to proteolytic activity followed the order of mixing strength, with the weakest dough being from the flour having the highest activity. The results for the three varieties used in this study fit the hypothesis that mixing strength might be inversely related to proteolytic activity.

TABLE II. PROTEOLYTIC ACTIVITIES AND AMINO GROUP CONTENTS OF THE FLOURS

Flour	Activity X 10 ³ μeq. tyrosine/ min./g. flour	Amino Groups X 10 ² μeq./mg.
Red River 68	1.14	2.3
Manitou	1.25	2.8
Talbot	1.36	3.4

Free Amino Groups

If proteolytic enzymes are active under the mixing conditions used, then it should be possible to follow this activity by an increase of amino groups. The content of amino groups was therefore determined for the flours and is shown in Table II. Talbot flour and doughs had the highest, Manitou intermediate, and Red River 68 doughs the lowest amino group contents. These results rank the three varieties in the same order as the proteolytic activities. However, for doughs of any one variety, the amino group content remained constant during mixing up to 15 min. (data not shown). Mixing in nitrogen instead of air or in the presence of iodate or NEMI also failed to affect the amino group content. Presumably the level of proteolytic activity in the flour is insufficient to produce any change in the amino group content that could be detected by the analytical procedure used.

Free Lipid

The free lipid (lipids extractable with petroleum ether) contents in the various doughs used in this study are given in Table III. For doughs mixed in air, there was a slight increase in the amounts of free lipid, although most of the increases observed are within the experimental error for the analysis used. Addition of iodate or NEMI, which markedly increased the rate of dough breakdown by mixing, had no effect on lipid-binding in dough. Free lipid content decreased slightly in doughs mixed in nitrogen, but again this decrease was within experimental error. These results are generally consistent with the more extensive data of Daniels et al. (11). The absolute quantities extracted agree with published values for doughs from similar flours. It is concluded that the slight increases in free lipid obtained in the present study for doughs mixed in air are not sufficient to account for the observed decrease in dough consistency.

GENERAL DISCUSSION

Results of the exhaustive extraction experiments (1) showed that the increase in the equilibrium solubility of flour proteins in 0.05N acetic acid solution during mixing depends on the intrinsic mixing strength of the flour. Comparison of the flour and the control-air dough results showed that the effect of mixing is greatest for the weakest flour (Talbot). With the two stronger flours, significant additional increases in the steady-state solubility were obtained only by the addition of cysteine or NEMI in doughs mixed in air or in nitrogen, or by the addition of iodate in doughs mixed in air. Iodate in doughs mixed in nitrogen, even at the high level of 2.0 μeq. per g. of flour, produced only a slight increase in protein solubility measured by the exhaustive extraction procedure.

TABLE III. LIPID EXTRACTED BY PETROLEUM ETHER FROM FLOURS AND DOUGHS MIXED UNDER VARIOUS CONDITIONS

Mixing Time (min.)	Red River 68				Manitou				Talbot			
	Air		Nitrogen		Air		Nitrogen		Air		Nitrogen	
	5	15	5	15	5	15	5	15	5	15	5	15
Flour	0.91				0.87				1.04			
Control	0.35 ^a	0.37	0.37	0.34	0.26	0.31	0.27	0.26	0.34	0.35	0.30	0.28
NEMI												
0.4 μ eq./g. flour	0.38	0.41	0.34	0.36	0.26	0.30	0.29	0.28	0.30	0.33	0.30	0.29
2.0 μ eq./g. flour	0.40	0.40	0.38	0.39	0.28	0.30	0.27	0.28	0.33	0.35	0.35	0.33
Iodate												
0.4 μ eq./g. flour	0.36	0.38	0.41	0.39	0.29	0.29	0.30	0.33	0.36	0.37	0.35	0.32
2.0 μ eq./g. flour	0.38	0.40	0.43	0.38	0.28	0.32	0.28	0.27	0.34	0.38	0.31	0.33

^aStandard deviation of the lipid analysis is 0.03%.

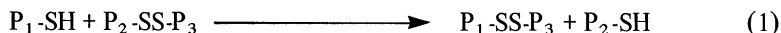
Previous workers, especially Tsen (12), attributed the increase in protein solubility during dough-mixing to disaggregation of protein complexes. However, results of our exhaustive extraction experiments (1) suggest that some of the increase may be a result of some form of depolymerization. In making this conclusion, it was assumed that the action of the exhaustive extraction was sufficient to break down all of the aggregates that might be present in the hydrated flour or freeze-dried dough samples that would affect the solubility of the flour protein. Since the increases in protein solubility observed under some conditions are similar to those obtained by mixing in the presence of cysteine, it appears likely that SS bonds are involved in the depolymerization.

Results of the solubility fractionation (1) and the gel filtration (2) experiments are consistent with the depolymerization hypothesis. Furthermore, these two experiments showed that it is the amount of the high molecular weight (MW) or the most insoluble component of the gluten protein that suffers a major decrease under mixing conditions which produced the most extensive breakdown. Concomitantly, increases were observed in the lower-MW components, or those that are soluble in 70% ethanol solution or in 0.05N acetic acid solution. The marked decrease in the amount of the insoluble residue protein is particularly significant from the technological viewpoint, since loaf volume appears to be directly correlated with this component (13).

The depolymerization that occurs during mixing produced only a minor change in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of the various solubility fractions (2). The alcohol-soluble fraction of doughs that suffered extensive mixing breakdown, e.g., those containing 2.0 μ eq. NEMI, had several "new" bands in the high-MW region. Bands of similar MW were observed in the patterns of the reduced acetic acid-soluble and the insoluble residue proteins. The SDS-PAGE patterns of the reduced acetic acid-soluble proteins were identical to the patterns of the insoluble residue protein. Accordingly, it appears that the components of these two protein fractions are built up from the same subunits. The solubility of these proteins therefore depends on the number of subunits (MW) and on their tertiary structure.

The most plausible mechanism that explains the observed results, and one that is consistent with the depolymerization hypothesis, is based on the SH-SS interchange reactions originally suggested by Goldstein (14). If the SH compound that initiates

the SS interchange is a low-MW peptide such as glutathione (15) or cysteinyl glycine (16), then the interchange would result in a decrease in the average MW. This can be written in the form of an equation as follows:



If $P_1\text{-SH}$ is considerably lower in MW than $P_2\text{-SH}$, then the contribution of the two components on the left of the equation to a physicochemical property such as viscosity (consistency) would be much greater than that of the two components on the right. According to macromolecular theory, viscosity of a polydisperse polymer system depends on the weight average MW of the polymer components. The SS interchange would be particularly facilitated by the presence in the original flour or by the addition to the dough of low-MW SH compounds.

The depolymerization that occurs during the mixing period does not involve the scission of peptide bonds. Although the three flours used in the present study had different proteolytic activities, there was no increase in the number of free amino groups during the 15 min. of mixing. It could well be that the enzyme would make a notable contribution to the physical properties of dough during a much longer reaction time as for example in a 5- to 6-hr. fermentation period. Also, the free lipids do not appear to be involved in the depolymerization; results obtained showed that there was no change in the amount of free lipid under the mixing conditions that were investigated.

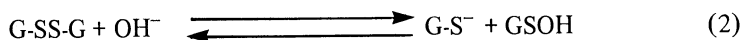
The SS interchange mechanism for the depolymerization of the high MW of the insoluble flour protein is consistent with the analytical data obtained in this study insofar as it would not require any change in the number of SS groups. The rate of this interchange would presumably depend on the concentration and the chemical nature of the low-MW SH compounds initially present in flour or added to the dough. In addition, the number and the nature (accessibility or availability) of the SS groups in the flour proteins and the type of mixing action would also be important in the rate of the interchange reactions.

The SS interchange mechanism of dough breakdown can also account for differences in mixing strength. Tsen and Anderson (6) indicated that one of the differences between weak and strong flours is that the former contain more "available" SS groups that will interchange more readily. The present study suggests that there may be other factors. It was observed that the weak flour (Talbot) contained a considerably higher proportion of low-MW peptides which were lost in the dialysis step used in the separation of the salt-solubles from the water-solubles in the solubility fractionation. The total protein recovery for Talbot was 92.5%, compared with 95.4 and 95.0% for Red River 68 and Manitou, respectively. If the low-MW peptides contain SH groups, they would be extremely effective in initiating the SS interchange reactions. The content of amino groups and the gel filtration results (2) are consistent with the solubility fractionation recovery data in that both suggest that the average MW of the proteins, examined by each method, is lower for the weaker flour than for the two stronger flours. Accordingly, it appears that the content of low-MW SH peptides could be another important factor in determining mixing strength. This suggestion is in general agreement with the "availability" hypothesis of Tsen and Anderson (6), since these SH groups would be readily titratable and would be quite mobile in dough systems. By way of further

work, it would be of interest to compare the chemical nature of the low-MW peptides from flours of different mixing strength.

The SS interchange mechanism of mixing breakdown presents some difficulty in explaining the drastic effects of SH-blocking agents (NEMI) and of strong oxidizing agents such as iodate in air-mixed doughs. Because of their ability to remove SH groups, it would appear that these agents should decrease the rate of SS interchange and thereby slow down dough breakdown. It should be noted that the large amounts of NEMI and iodate added to doughs in the present study were selected so as to accentuate dough breakdown. These amounts are in excess of the SH content. Accordingly, it is presumed that under these conditions NEMI and iodate (in the presence of air) can enhance the cleavage of a few critical SS bonds to produce an effect that is not dissimilar from the effect of reducing agents such as cysteine. Presumably, the SS bonds involved in this action could be those that are extremely critical functionally (rheologically) as postulated by Bloksma (9). Again, by analogy with the effect of cysteine on dough-mixing strength and flour protein solubility, it would appear that the number of SS bonds that would have been cleaved to produce a relatively major physical effect could indeed be too small to be detected by the present analytical procedures. The doughs used in the present study, even those that showed extensive breakdown, did not show any definite changes in SS content that could be measured by the analytical procedure used.

Previous measurements of SS content in doughs mixed under various conditions by Tsen and Bushuk (8) also failed to show any significant trends with mixing time. NEMI could enhance the cleavage of SS bonds in flour proteins by the mechanism advanced by Spackman et al. (17) to explain the results obtained with oxidized glutathione. In this case, the suggested reactions are:



The equilibrium constant for reaction 2 is quite low so that under slightly acidic conditions the amount of GS^- is extremely small. However, in the presence of NEMI, the mercaptide ion would be removed as soon as it is formed, and this would lead to a gradual decrease of SS. It is presumed that iodate and oxygen, if present in amounts in excess of the SH content, could react similarly by an oxidation mechanism. If this suggestion is correct, then it should be possible to detect a loss of SS groups under extreme breakdown conditions in the presence of NEMI or iodate. It is perplexing that no one has yet succeeded in obtaining confirmatory analytical evidence of the suggested loss of SS.

The effect of SH-oxidizing or -blocking agents might also be explained by implicating a central role for the SH group in the quaternary structure of the rheological unit of gluten. This possibility was first suggested by Tsen (18) on the basis of the reported effect of *p*-hydroxymercuribenzoate on the quaternary structure of some enzyme systems (19). On the basis of the hydrogen-bonding tendency of the SH group, it is difficult to explain the substantial effect of this group on the rheological properties of doughs through the blocking mechanism unless the groups play a determining role in stabilizing a specific structure through a cooperative action of a large number of secondary bonds.

The results obtained in the present study extend previously published work, particularly that of Mecham (20) and Tsen (12,18). These workers emphasized the disaggregation mechanism of dough breakdown during mixing, whereas the present study places a greater emphasis on the depolymerization mechanism. Perhaps both mechanisms are important. Further work is required to determine the contribution and the technological significance of each.

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