Studies with Radioactive Tracers. XV. Some Observations on the Sulfhydryl Groups in Dough[1]

C. C. LEE and TZEN-SON LAI, University of Saskatchewan, Saskatoon, Saskatchewan

ABSTRACT

The oxidation of sulfhydryls in flour-water dough by iodate or bromate and the changes in sulfhydryls during dough mixing in air or under nitrogen, with or without the presence of bromate or glutathione, have been investigated by a radiochemical method for determining sulfhydryls. The SH contents were examined in both the water-soluble and water-insoluble fractions. Oxidative destruction of some of the readily accessible SH was reconfirmed. During mixing in the presence of GSH, some SH apparently became incorporated into the water-insoluble fraction through sulfhydryl-disulfide interchange. Considerations of the present results together with known data in the literature led to the suggestion that a mechanism involving equilibrations via SH-SS exchange processes between a disulfide pool and two types of sulfhydryls may be playing an important role in the chemistry of breadmaking.

A great deal of work has been done on the chemistry of sulfhydryl-disulfide groups in relation to breadmaking. An extensive discussion on this topic has been included in a recent review by Pomeranz (1). While it is generally agreed that SH-SS groups must play important roles, especially in the action of improvers, the detailed nature of these roles remains to be clarified.

From the observation that only part of the total sulfhydryl appeared to be reactive and that maximum improvements in the rheological properties of the dough and in the resultant bread were obtained when only a fraction of the reactive sulfhydryls has reacted with improvers or with SH-blocking agents, Sullivan and co-workers (2) have postulated that, since partial removal of the free SH groups strengthens the dough matrix, a dynamic equilibrium exists between the SH groups of nongluten proteins and the SH and SS groups of the gluten proteins:

$$\underset{\substack{\vdots \\ \text{contributes more} \\ \text{to dough strength}}}{Pr^1SH + Pr^2SSPr^2} \rightleftarrows \underset{\substack{\vdots \\ \text{contributes less} \\ \text{to dough strength}}}{Pr^2SH + Pr^1SSPr^2}$$

Pr^1 = nongluten protein; Pr^2 = gluten protein.

It was suggested that the Pr^1SH may be preferentially reacted because of greater

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accessibility or solubility, thus shifting the equilibrium to the left, enhancing the contribution to dough strength. This reversible reaction would occur during mixing and fermentation, varying with time, temperature, manipulation of the dough, and the amount of SH-blocking agent or improver. The optimum amount of improver is considered to be that which would provide the proper balance of free sulfhydryls and gluten disulfides to give the precise disulfide bridges necessary for best stabilization of the gluten structure.

To assess the validity of the above reversible equilibrium, it would be of interest to obtain more information regarding the behavior of sulfhydryls in the nongluten and gluten proteins. While much work has been done on the behavior of sulfhydryls during dough mixing or upon treatment with improvers (1), further studies on their fate, separately in the nongluten and gluten proteins, would be desirable. Recently we described a sensitive radiochemical method for determining sulfhydryls in flour and dough (3). The assay is based on the radioactivity of the S-succinyl-L-cysteine obtained from hydrolysis after the sulfhydryls of the flour protein have reacted with an excess of N-ethylmaleimide-1-^{14}C (NEMI-^{14}C). When this method was applied, the SH groups readily accessible to reaction with NEMI-^{14}C in the water-soluble and water-insoluble fractions of the dough could be determined routinely (3). Since the water-soluble and water-insoluble sulfhydryls should be related to the sulfhydryls of the nongluten and gluten proteins, the present work was carried out to study the effects of improvers or of mixing on the water-soluble and water-insoluble sulfhydryls of dough.

MATERIALS AND METHODS

As much of the experimental work was done in conjunction with the earlier study (3) which included an investigation on the correlations between sulfhydryls and baking quality of different flours, four flours, all derived from HRS wheat, were used in the present work. Their protein and ash contents, on a 14% moisture basis, are listed below:

Flour	*Protein* %	*Ash* %
A	13.9	0.44
B	15.4	0.42
C	14.2	0.43
D	14.7	0.45

Flours A, B, and C are straight flours milled from plant breeders' samples, and these are the same flours designated as Nos. 10, 19, and 12, respectively, in the earlier work (3). D is a commercial flour obtained from the mill of the Saskatchewan Wheat Pool and has not been treated with maturing or bleaching agents.

Oxidation of Sulfhydryls by Iodate and Bromate

Six 100-mg. samples of flour A were weighed out in test tubes; two of them served as controls; two were treated with iodate and two with bromate. The treatments were carried out by adding 50 p.p.m. of potassium iodate or potassium bromate in 100 λ of water to the flour. The doughs were hand-mixed for about 1 min., allowed to rest for 3 hr., and then mixed for 10 min. with 25 λ of solution

containing 0.48 μmole of NEMI-^{14}C, together with, or without, 36 mg. of urea. For the controls, the same amount of NEMI-^{14}C in 125 λ of water, with or without urea, was used to make the dough. All the doughs were allowed to stand overnight and then extracted successively with 1.0 and 1.5 ml. of water. The extracts of each sample were combined and mixed with the same volume of 12N HCl; the residue was made into a suspension with 5 ml. of 6N HCl. Aliquots of these acidic solutions or suspensions were assayed for SH content radiochemically as described previously (3).

Changes in Sulfhydryls of Dough Mixed in Air

In the first set of experiments, 35 g. of flour B and 21 ml. of water were mixed in the bowl of the mixograph. At various times during the mixing, a piece of dough weighing about 400 mg. was sampled and immediately put into a glass-stoppered test tube containing 0.68 μmole of NEMI-^{14}C in 2.0 ml. of water. After the weight of the sample was ascertained, the dough was thoroughly dispersed into the NEMI-^{14}C solution. The resulting mixture was allowed to stand overnight and centrifuged, and the residue was further extracted with 3.0 ml. of water. The SH content of the combined extract and of the residue was determined radiochemically in the usual way.

In the second set of experiments, the above procedure was repeated with 35 g. of flour C and 21 ml. of water. In additional studies, instead of water, we used 21 ml. of solution containing potassium bromate (30 p.p.m.) or reduced glutathione (GSH) (0.20 μmole per g. dry flour).

Changes in Sulfhydryls of Dough Mixed under Nitrogen

One-hundred-gram samples of flour D were placed in desiccators which were repeatedly evacuated and flushed with nitrogen. Each sample was allowed to remain under nitrogen for 6 hr. before being used for mixing studies. After removal from the desiccator, a sample was immediately put into the mixing bowl of the air-tight GRL mixer (4)[2], degassed, and purged with nitrogen. Sixty milliliters of water or GSH solution (0.35 μmole GSH per g. dry flour) was introduced under vacuum through the outlet of the mixer, and mixing was then allowed to proceed under a constant flow (about 1 liter per min.) of nitrogen which had been humidified by bubbling through water. Each dough was mixed for a definite length of time and then samples of the dough were taken for sulfhydryl determination in the same way as described under mixing in air.

RESULTS AND DISCUSSION

All sulfhydryl contents were expressed in μmole per g. of dry flour (3). In the studies on dough mixing, the weight of the dough sample was taken as 1.6 times the weight of the flour used.

Effects of Iodate and Bromate on the Water-Soluble and Water-Insoluble Sulfhydryls

The results from the studies with dough, either untreated or treated with 50 p.p.m. of KIO_3 or $KBrO_3$, are summarized in Table I. As pointed out by Pomeranz

[2]Designed by the Grain Research Laboratory, Winnipeg, Manitoba. The mixer used in these experiments was on loan to us through the courtesy of I. Hlynka.

TABLE I. OXIDATION OF FLOUR SULFHYDRYLS (per g. DRY FLOUR) BY 50 p.p.m. KIO_3 OR $KBrO_3$

Oxidizing Agent and Type of SH Determined	Water-Soluble			Water-Insoluble		
	Initial SH[a]	After oxidation	SH oxidized[b]	Initial SH[a]	After oxidation	SH oxidized[b]
	μmol.	μmol.	%	μmol.	μmol.	%
KIO_3						
Accessible, masked[c]	0.18	0.03	83	0.97	0.43	56
Accessible[d]	0.18	0.03	83	0.68	0.18	73
$KBrO_3$						
Accessible,masked[c]	0.18	0.07	64	0.97	0.61	37
Accessible[d]	0.18	0.07	64	0.67	0.30	56

[a]From the control experiment without iodate or bromate treatment.

[b]Initial SH content taken as 100%.

[c]Determined with the presence of urea.

[d]Determined without the presence of urea.

in his review (1), the disappearance of sulfhydryl after additions of bromate or iodate has been demonstrated; also, the fact that iodate causes sulfhydryls to disappear more rapidly than does bromate. A recent discussion on the relatively slower rate of action of bromate compared to iodate has also been given by Tsen (5). The results obtained in the present work confirm once again the above general conclusions.

From the data given in Table I, however, it is possible to scrutinize in somewhat more detail the fate of various types of sulfhydryls after undergoing oxidation with bromate or iodate. The flour used in these experiments (flour A) contains 0.18 μmole of water-soluble SH per g. which is all accessible to NEMI-^{14}C (no effect with urea). Of the water-insoluble SH (0.97 μmole per g.), 0.30 μmole per g. or 31% is masked or inaccessible to NEMI-^{14}C and can only be determined in the presence of urea. Much of the accessible sulfhydryls, especially those in the water-soluble fractions, are oxidized by the oxidizing agent; the amount oxidized is greater with iodate than bromate[3]. The amount of masked sulfhydryls remaining after oxidization with iodate and with bromate is, respectively, 0.25 and 0.31 μmole per g. (from 0.43 to 0.18 and 0.61 to 0.30 μmole per g., column 7, Table I). These values are quite close to the masked SH content of the unoxidized control (about 0.30 μmole per g. from column 6, Table I), suggesting that the masked sulfhydryls are quite resistant to oxidation. The difference in masked sulfhydryls remaining after iodate and bromate oxidation (0.25 and 0.31 μmole per g.), if significant, would suggest that iodate, a fast-acting reagent, could compete with NEMI-^{14}C for reaction with the masked SH groups as they are liberated by the action of urea. The present results, therefore, demonstrate clearly that the accessible SH groups are

[3]Control experiments have shown that S-succinyl-L-cysteine, whose radioactivity gave a quantitative measure of the original SH content, was not affected by bromate or iodate under the present conditions; hence, the decrease in SH observed was actually due to oxidation of the SH groups and not to fortuitous destruction of the active S-succinyl-L-cysteine.

TABLE II. ACCESSIBLE SULFHYDRYL (per g. DRY FLOUR)[a] IN WATER-SOLUBLE AND -INSOLUBLE FRACTIONS OF DOUGH FROM FLOUR B DURING MIXING IN OPEN AIR

Mixing Time	Water-Soluble	Water-Insoluble
min.	μmol.	μmol.
0[b]	0.24	0.53
0.67	0.14	0.50
1.33	0.09	0.48
2.00	0.09	0.36
2.67	0.07	0.38
10.0	0.02	0.22
60.0	0.02	0.26

[a]Weight of flour was calculated from weight of dough which was made by mixing 1.0 part of flour with 0.6 part of water.

[b]Determined for the original flour as described in ref. 3.

chiefly responsible for the decrease in sulfhydryls upon treatment with oxidizing agents.

Sulfhydryl Changes in Dough during Mixing in Open Air

The results from the dough-mixing studies with flours B and C are summarized in Tables II and III. The data definitely show decreases in SH during mixing in air for both flours. Presumably, oxidation by atmospheric oxygen may play an important role during mixing. After 10 or 20 min. of mixing, only traces of SH remained in the water-soluble fraction, whereas roughly about 50% of the accessible SH (reacts with NEMI without urea) remained in the water-insoluble fraction even after prolonged mixing. These results are in general accord with the suggestion of Sullivan and co-workers (2) that the more soluble sulfhydryls are oxidized and those resistant to oxidation belong to the gluten protein. A comparison of the data in Table II with those in Table III, obtained with no additive, suggests that the rate of decrease in SH during mixing may be different for the two flours employed.

From Table III, it is seen that the presence of 30 p.p.m. $KBrO_3$ did not affect the rate of decrease of the water-soluble sulfhydryls and that a further small amount of the accessible SH in the water-insoluble fraction was lost during mixing in the presence of bromate. Tsen and Bushuk (6) presented data which show that during mixing, fewer SH groups are oxidized by bromate alone (under a nitrogen atmosphere) than by air or oxygen, whereas in the resting dough, more SH groups are oxidized by bromate than by air or oxygen. It was suggested that these results may be due to the limited diffusion of air or oxygen into the resting dough. The present finding, that bromate does influence the loss of water-insoluble but not water-soluble SH, might possibly also be due to limited diffusion of air into the water-insoluble protein components. The water-soluble sulfhydryls probably may be in the more mobile phase of the dough which is readily exposed to atmospheric oxygen during mixing, and thus the effect of oxygen is predominant and the effect of bromate is relatively not significant. On the other hand, the water-insoluble SH groups probably would be in the less mobile phase of the dough which is in limited contact with atmospheric oxygen, and thus the effect of oxygen is reduced, and some further oxidation by the bromate becomes observable.

TABLE III. ACCESSIBLE SULFHYDRYL (per g. DRY FLOUR)[a] IN WATER-SOLUBLE AND -INSOLUBLE FRACTIONS OF DOUGH FROM FLOUR C DURING MIXING IN OPEN AIR[a,b]

	No Additive		$KBrO_3$ Added[b]		GSH Added[c]	
Mixing Time	Water-soluble	Water-insoluble	Water-soluble	Water-insoluble	Water-soluble	Water-insoluble
min.	μmol.	μmol.	μmol.	μmol.	μmol.	μmol.
0[d]	0.13	0.46	0.13	0.47	0.34	0.47
3	0.06	0.45	0.06	0.42	0.13	0.48
6	0.06	0.42	0.06	0.40	0.07	0.47
20	0.02	0.29	0.02	0.22	0.03	0.34

[a]See footnote a in Table II.

[b]Mixing carried out in the presence of 30 p.p.m. $KBrO_3$.

[c]Mixing carried out in the presence of 0.20 μmole GSH per g. dry flour.

[d]Determined for the original flour as described in ref. 3.

When the dough was mixed in air in the presence of added GSH (Table III), the water-soluble sulfhydryls, including those derived from the added GSH, were rapidly oxidized, whereas, on the other hand, the SH in the water-insoluble fraction showed a small but real increase during mixing. Since a relatively large amount of water was used to extract a small sample of dough, most of the residual free GSH probably would be removed in the water-soluble fraction. In view of our recent finding that simple SH compounds such as GSH could readily undergo interchange reactions with the disulfide groups of flour proteins (7), it seems reasonable to account for the increase in water-insoluble SH during mixing in the presence of GSH as arising from SH-SS interchange rather than from any residual GSH held in the water-insoluble fraction of the dough:

$$GSH + PrSSPr \rightarrow PrSSG + PrSH$$

Sulfhydryl Changes in Dough during Mixing under Nitrogen

Results from studies with dough mixed under nitrogen (Table IV) are in general agreement with earlier observations that SH contents increase slightly in doughs mixed under nitrogen (6,8,9). A major cause for the increase is believed to be exposure of inaccessible SH groups by mechanical work. Mecham and Knapp (9) found that SH groups became more accessible to titration in the absence of urea as dough mixing was allowed to proceed. However, other factors, such as mechanical scission or enzymatic reduction of the disulfide groups and sulfhydryl formation from labile groupings such as thiol ester, cannot be excluded. Note, from the present results (Table IV), that the increase in water-soluble SH is as great as the increase in the water-insoluble fraction. Apparently this cannot be explained only by the exposure of inaccessible or masked sulfhydryls, since it has been found (3) that there is no masked sulfhydryl in the water-soluble fraction. The rise in water-soluble SH during mixing under nitrogen thus suggested the possibility that some of the water-insoluble proteins become more water-soluble as a result of the mechanical work. This is supported by the observation that for the flour used in

TABLE IV. ACCESSIBLE SULFHYDRYL (per g. DRY FLOUR)[a] IN WATER-SOLUBLE AND -INSOLUBLE FRACTIONS OF DOUGH FROM FLOUR D DURING MIXING UNDER NITROGEN

Mixing Time	No Additive		GSH Added[b]	
	Water-soluble	Water-insoluble	Water-soluble	Water-insoluble
min.	μmol.	μmol.	μmol.	μmol.
0[c]	0.17	0.53	0.52	0.53
3	0.19	0.56	0.36	0.74
5	0.21	0.58	0.37	0.78
10	0.22	0.60	0.40	0.79
20	0.25	0.63	0.42	0.80
40	0.32	0.68	0.53	0.88

[a]See footnote a in Table II.

[b]Mixing carried out in the presence of 0.35 μmole GSH per g. dry flour.

[c]Determined for the original flour as described in ref. 3.

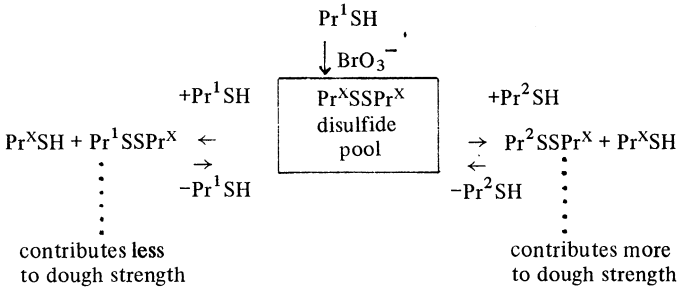
these experiments (flour D), the amount of water-soluble crude protein (N X 5.7) rose from about 2 to about 3% after 40 min. of mixing, either in air or under nitrogen.

A comparison of the SH changes in doughs mixed under nitrogen, with and without added GSH, indicates that an additional rise of about 0.17 to 0.20 μmole of SH per g. dry flour was found in the water-insoluble fraction when the mixing was carried out in the presence of GSH. This additional rise in SH amounts roughly to about 50% of the GSH added (0.35 μmole per g. dry flour). As discussed in the preceding section, the incorporation of more SH into the water-insoluble proteins during mixing in the presence of GSH probably is attributable to SH-SS interchange reactions.

GENERAL DISCUSSION

The results obtained in the present work have again demonstrated that only a part of the SH groups of flour and dough is reactive, and this fits in with the basic ideas that led Sullivan and co-workers (2) to postulate a dynamic equilibrium between various SH and SS groups. Since the reactive sulfhydryls are present not only in the water-soluble but also in the water-insoluble fractions, the discussion might be modified so as not to restrict the dynamic equilibrium as occurring only between nongluten and gluten SH and SS groups. In our previous work (3), it was shown that a high degree of correlation exists between the maximum loaf volume obtained with an optimum amount of bromate and the masked SH contents for 21 different flours, the masked SH being the difference between the total SH determined in the presence of urea and the accessible SH (water-soluble and -insoluble) determined in the absence of urea. Any explanation of the roles of SH and SS groups in breadmaking should take into account this correlation between masked sulfhydryls and baking quality.

It is proposed that the following scheme may play an important role in the chemistry of breadmaking:



In this scheme, Pr^1SH and Pr^2SH represent the sulfhydryls in two types of proteins, the first being presumably of lower molecular weight and including much of the accessible sulfhydryls, whereas the second may be of high molecular weight and would include the masked sulfhydryls. The disulfide pool, Pr^XSSPr^X, would encompass all the disulfide linkages of the flour proteins, including those that might be formed from direct oxidation, although this latter process may be of relatively low probability. The chief reactions would arise from interchange or exchange processes between the various SH and SS groups. While interchange between SH and inter- or intramolecular SS bonds could release strain and contribute to the development of the dough, the main effects of the above scheme may be discussed in terms of intermolecular disulfide cross-linking. It is assumed that interchain cross-linking to Pr^1 via exchange with Pr^1SH may lead to a weakening of dough strength, whereas formation of intermolecular disulfide bonds through exchange with Pr^2SH will give rise to enchanced dough strength. It is worth pointing out again that Pr^2SH is presumed to include the "masked" sulfhydryls, and one might wonder how these "unreactive" sulfhydryls could become involved in exchange reactions. It should be recalled, however, that the "masked" SH used in the present context refers to that portion of the flour sulfhydryls which could not react with NEMI-^{14}C in the flour-water or unleavened dough-water slurry unless the slurry also contained urea to denature the proteins. Under the usual conditions of breadmaking, including such processes as mixing, fermentation, and proofing, the physicochemical environments of the hydrated gluten could be very different from the environments of a freshly formed slurry of flour and water. It is quite probable that during dough development, some exchange processes could take place between disulfides and both the accessible and masked types of sulfhydryls.

Again referring to the proposed scheme, oxidative destruction of some of the Pr^1SH or the blocking of such sulfhydryls with specific reagents will prevent excessive exchange with Pr^1SH to give the dough-weakening Pr^1SSPr^X, and the over-all equilibrations will shift in favor of the formation of more of the dough-strengthening Pr^2SSPr^X. These reactions would occur during mixing, fermentation, and proofing, varying with time, temperature, manipulation of the dough, and the amount of improver or SH-blocking agent that may be present. As pointed out by Sullivan and co-workers (2), the optimum amount of improver is considered to be that which will provide the proper balance of sulfhydryls and disulfides to give the precise extent of SS bridges necessary for the best stabilization of the gluten protein network of the dough.

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