ACCESSIBLE SULFHYDRYL GROUPS IN DOUGH[1]

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ABSTRACT

The rapid reaction of iodate ions with sulfhydryl groups was applied to the determination of the accessibility of these groups in flour-water doughs. The maximum amount of iodate that reacts in a dough was obtained graphically, from amperometric titrations of residual iodate, by plotting residual iodate vs. iodate added and extrapolating to zero residual iodate. Reasonable agreement with calculated values for the number of sulfhydryl groups was obtained for doughs which contained added glutathione or thiolated gelatin, and no reaction with iodate was obtained when sulfhydryl groups were blocked with N-ethylmaleimide. Increases in accessible sulfhydryl groups were obtained for doughs prepared from flours of decreasing particle size, doughs subjected to prolonged mixing, and doughs treated with guanidine hydrochloride to break hydrogen bonds. These results indicate that iodate ions react primarily with accessible sulfhydryl groups. Illustrative results for doughs from eight different flours ranged from 4.0 to 7.5 μeq. per g. of protein. Within a single class of flour, the number of accessible sulfhydryl groups increased with increasing protein content. The method was also used to determine the number of accessible sulfhydryl groups in flour-water slurries for one sample of flour.

Recent studies have shown that sulfhydryl groups of flour proteins play a major role in determining the mixing properties of dough (16). In addition, it now seems certain that oxidative improvement of flour quality involves the sulfhydryl group (5,10, and papers cited therein). Current studies in this and the Western Regional Laboratories also

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suggest that it is the sulfhydryl groups that are initially accessible, or subsequently made accessible by prolonged mixing, that are oxidized (4,21). Accordingly, a method for the determination of the *accessible* sulfhydryl groups in dough would probably be more useful from a practical viewpoint than current methods that measure the *total* sulfhydryl content of flour or of various protein fractions obtained from it (2,8,14,15,17–20). References by Mecham, Pence, and coworkers (14,20) which deal with the application of an amperometric titration method to the determination of total number of sulfhydryl groups in flour slurries are most pertinent to the present study.

This paper describes a method for the determination of accessible sulfhydryl groups in dough. The method, based on the rapid reaction of protein sulfhydryl groups with iodate ions, is an extension of the observation made in this Laboratory several years ago that flours of different type react with characteristic amounts of iodate (9). Usefulness of the method is demonstrated by a study of some of the important factors that control the accessibility of sulfhydryl groups and by a determination of the accessible sulfhydryl contents of doughs from eight different flours and of water-flour slurries from one flour.

Materials and Methods

The apparatus, reagents, and methods used for preparing the dough, and extracting and measuring residual iodate, are the same as used in earlier studies of the bromate reaction in dough (3,6,7); however, the procedure will be described again to facilitate the use of the method without referring to several other papers.

Apparatus. (a) The dough mixer must be adapted for mixing under nitrogen; the GRL mixer (12) is used in this Laboratory. (b) Magnetic stirrer with an external speed-control rheostat. (c) Polarizing and current-detection unit which may be constructed according to the circuit diagram given by Kolthoff and Lingane (13). In the unit used, a double-throw, double-pole switch was installed so that the galvanometer could be used for measuring both positive and negative currents. The galvanometer used has a sensitivity of 0.1 μamp. per unit scale division (100 scale divisions = 60 mm.). (d) The indicator is a platinum electrode (Beckman 39271) and the reference is a calomel electrode (Beckman 39270). The platinum electrode was cut down to the same size as the calomel electrode and both are mounted on a holder about 15 mm. apart.

Reagents. (a) Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 155.1 g. per liter. (b) Sodium hydroxide, 26 g. per liter. (c) Sodium thiosulfate, 0.001*N*. (d) Potassium iodate, 0.001*N*. (e) Potassium iodide, 30%. (f) Sulfuric

acid, 10%. (g) n-Octyl alcohol.

Method. The method involves determination of residual iodate in flour-water doughs immediately after mixing. The analysis is repeated with replicate doughs containing increasing amounts of iodate until three or four points representing doughs containing residual iodate are obtained. The maximum amount of iodate that reacts in the dough prepared under a definite set of conditions from a given flour is determined by plotting residual iodate against iodate added and extrapolating to zero residual iodate.

The dough is prepared from 100 g. (or less if a suitable mixer is available) of flour by mixing under nitrogen for 5 minutes at a practical absorption (60% was used in the present study except where indicated otherwise). In the standard procedure, the iodate is added at the beginning of the 5-minute mixing. The flour should be stored overnight under nitrogen to remove oxygen, which competes with iodate for the sulfhydryl group. Oxygen is removed from the liquids used to mix the dough by bubbling nitrogen through for 5 minutes.

Residual iodate is extracted from a 20-g. portion of the dough by dispersing in a Waring Blendor for 3 minutes at low speed with 90 ml. of water, 12.5 ml. of zinc sulfate solution, and 12.5 ml. of NaOH solution; 5 drops of octyl alcohol are added to prevent excessive foaming. Two 50-ml. portions of the dispersion are centrifuged for 10 minutes at 1,500 $\times$ *g*, and duplicate 20-ml. aliquots of the supernatant are titrated.

The titration medium comprises 20 ml. of supernatant, 5 ml. of 0.001*N* thiosulfate, 5 ml. of 10% sulfuric acid, and 3 ml. 30% potassium iodide. Excess thiosulfate is titrated with 0.001*N* potassium iodate, and the end point is established amperometrically. A reagent blank is run daily on 5 ml. of 0.001*N* sodium thiosulfate. The titration medium is stirred with a magnetic stirrer throughout the titration.

The polarizing unit was checked in the usual manner. Polarograms of Fig. 1, A, show that with the electrodes used the amperometric titration can be conveniently made at 0 volts applied potential. Figure 1, B, shows that at this potential the relationship between the current and the volume of titrant is linear. The sensitivity of the electrode used is about 39 μamp. per ml. of 0.001*N* iodate.

The maximum amount of iodate that reacts in a particular dough is determined as follows: Residual iodate is first determined for a series of replicate doughs containing increasing increments of potassium iodate. The residual iodate (expressed in ml. of titrant) is then plotted against iodate added (in p.p.m. of flour) and the maximum amount that reacts, or the iodate saturation value (ISV), is obtained

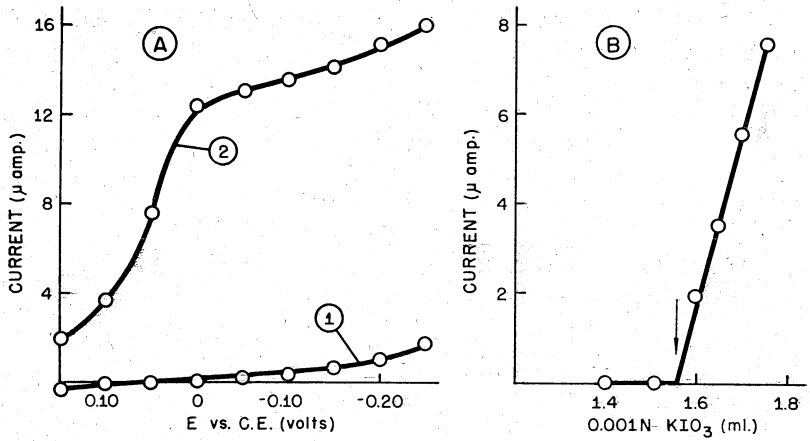

Fig. 1. A, current-potential curves at the platinum electrode for the titrating medium comprising 20 ml. of water, 2 ml. of 0.001*N* thiosulfate solution, 5 ml. of 10% sulfuric acid, and 3 ml. of 30% potassium iodide solution. 1, residual current; 2, 0.36 ml. of 0.001*N* KIO_3 in excess of the thiosulfate. B, current-volume of titrant curve at 0 volts applied potential.

by extrapolation. Typical results for a dough from a straight-grade flour of 13.2% protein content milled from hard red spring wheat are shown in Fig. 2. The ascending limb of the curve is linear, so that a good extrapolation can be made with three or four points. Since the ascending limb will have a slope of one (if the same ordinate and abscissa units are used), a reasonably good estimate of the ISV can be obtained from a single determination at an iodate level higher than ISV.

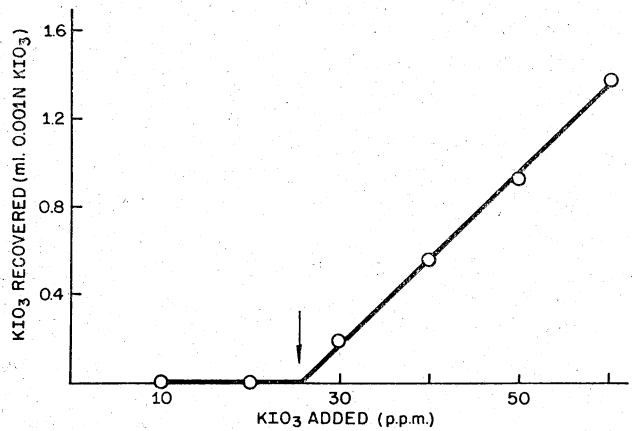

Fig. 2. Iodate recovered vs. iodate added curve for doughs from a straight-grade flour of 13.2% protein mixed in nitrogen for 5 minutes. Iodate saturation value (indicated by arrow) is 25.5 p.p.m.

The accessible sulfhydryl content in μeq. per g. of protein, of a dough prepared under a particular set of conditions, is calculated from the equation:

$$\mu\text{eq. SH/g protein} = \frac{100\ \text{ISV}}{\text{P} \times 35.67}$$

where ISV = iodate saturation value in p.p.m. of flour, 14% m.b.;
P = protein content %, 14% m.b.; and
35.67 = equivalent weight of potassium iodate.

For the data of Fig. 2 this equation gives:

$$\frac{100(25.5)}{13.2 \times 35.67} = 5.4\ \mu\text{eq. SH/g protein}$$

This calculation assumes that iodate ions react with sulfhydryl groups according to the reaction

$$6\text{RSH} + \text{IO}_3^- \rightarrow 3\text{R} - \text{S} - \text{S} - \text{R} + \text{I}^- + 3\text{H}_2\text{O},$$

in which the molar ratio of sulfhydryl groups oxidized to iodate at the equivalence point is 6.

The reproducibility of the method was checked by a series of determinations on doughs from the same flour. Standard error obtained was about ± 0.1 μeq. per g. of protein.

When iodate is added to a dough, the amount equal to the ISV reacts very rapidly; however, if a long reaction time is allowed there is a further progressive reaction of iodate. This seems to involve the less accessible sulfhydryl groups reached by diffusion of iodate into the hydrated flour particles. The rate of this reaction is constant for a wide range of iodate concentrations and is equal to 0.04 p.p.m. of potassium iodate per minute or 0.2 p.p.m. for the 5 minutes of mixing used in the standard procedure. The error introduced by this secondary reaction of iodate is generally less than 1% and is not considered as serious.

Results and Discussion

Specificity. Two types of experiments were made to check the specificity of the iodate reaction in dough. In the first type, the sulfhydryl groups were blocked by incorporating into the dough excess N-ethylmaleimide, mixing for 5 minutes, and then adding the iodate and mixing for an additional 5 minutes. Complete recoveries of iodate were obtained at potassium iodate concentration of 10, 15, and 20 p.p.m.; and the straight line through these points extrapolated to zero ISV. Accordingly, under the specified conditions, iodate does not react in significant amount with any group that is not blocked by N-ethylmaleimide.

In the second type of specificity experiment, the sulfhydryl content

of the dough was increased by additions of glutathione (reduced) and of thiolated gelatin (Schwartz, Thiogel A; molecular weight = 10,000, –SH content 0.93 per mole). Since, for this experiment, the actual source of sulfhydryl groups is immaterial, the –SH compound and the iodate were added to the flour at the same time. The results obtained are given below.

		SH Content	
Glutathione	*ISV*	*Experimental*	*Calculated*
mg/100g flour	*ppm*	*μeq/g protein*	*μeq/g protein*
0	25.5	5.4	...
12	40.5	8.5	8.4
24	58.0	12.2	11.3
Thiolated Gelatin			
375	41.7	8.7[a]	7.8[b]
750	59.5	12.0[a]	10.1[b]

[a] The amount of protein added was included in this calculation.
[b] Calculated on the basis of 0.93 —SH groups per 10,000 molecular weight and total protein content of 13.2% + added thiolated gelatin, i.e. 13.58 and 13.95%.

The agreement between the experimental and the calculated values for the sulfhydryl contents given above is reasonably satisfactory. Two reasons can be given to account for the higher experimental values. First is that addition of sulfhydryl compounds to dough might increase the number of accessible sulfhydryl groups in the dough. This does not seem unlikely for glutathione, which has a marked effect on the handling properties of dough; however, it may not be the case with thiolated gelatin, which does not affect the handling properties of dough under the conditions used. On the other hand, it might be that the molar equivalence ratio of sulfhydryl groups to iodate of 6 is too high. Lower ratios would yield lower experimental values. A lower molar ratio would imply that sulfhydryl groups are oxidized by iodate to a state higher than that of the disulfide group. Recent studies by Hird and Yates (11) on solutions of reduced gluten indicate that the molar ratio for sulfhydryl groups oxidized to iodate added is about 4 and depends on the relative concentration of sulfhydryl groups to iodate. Further study is necessary to determine if this ratio applies also to doughs. If this is found true, then the values of accessible sulfhydryl content given in this paper would have to be decreased by one-third; however, the relative positions of the various doughs studied would remain unchanged.

Physical Accessibility. Four types of experimental doughs were used to check the validity of the presumption that the rapid reaction of iodate is with the physically accessible sulfhydryl groups: doughs prepared from flours of different particle size; doughs that were subjected

to prolonged mixing under nitrogen; doughs of different absorption mixed for 5 minutes; and doughs treated with guanidine hydrochloride (hydrogen bond-breaking reagent). Results for each type of dough will be discussed separately.

The flours of different particle size were milled in the Laboratory from hard red spring wheat. A coarse farina was milled first and portions of it were reduced completely to a finer farina and flour. A fourth sample was made by ball-milling a portion of the flour. The accessible sulfhydryl contents of the doughs from these flours, together with the description of the flours, are given below.

Flour	*Particle Size*	*Protein Content*	*Sulfhydryl Content*
		%, 14% mb	*μeq/g protein*
Coarse farina	thru 30GG, over 72GG	11.7	4.8
Farina	thru 72GG, over 10XX	11.6	5.3
Flour	thru 10XX	11.6	5.7
Ball-milled flour	....	11.6	6.6

The above results are consistent with the hypotheses that the method determines accessible sulfhydryl groups and that the number of them increases with decreasing flour particle size, i.e., with increasing specific surface area. The increase in sulfhydryl content is comparable to the increase in the rate of bromate reaction in doughs from similar flours (4). This type of accessibility, depending on particle size, is different from the molecular accessibility encountered in proteins in solution. From the practical viewpoint, these results suggest that the effects of milling properties of wheat and milling technique on bromate requirement and response of the resulting flour depend on the number of sulfhydryl groups made accessible during the milling process.

The effect of prolonged mixing under nitrogen on the sulfhydryl contents of doughs of 50 and 70% absorption is shown by results given below. In these experiments, iodate was present in the dough during the last 5 minutes of mixing.

Mixing Time	*ISV*		*Sulfhydryl Content*	
	50%	70%	50%	70%
minutes	*ppm*	*ppm*	*μeq/g protein*	*μeq/g protein*
5	27.0	23.0	5.7	4.9
10	42.0	28.5	8.9	6.0
15	51.0	35.0	10.8	7.4

Prolonged mixing produced a definite increase in the number of sulfhydryl groups that are accessible to iodate. The increase produced is higher for the dough of lower absorption. Thus, mixing time and

absorption condition the accessibility of sulfhydryl groups and thus the response to improvers.

Another type of accessibility was observed in doughs treated with guanidine hydrochloride, a commonly used hydrogen bond-breaking reagent. Figure 3 shows the results that were obtained for doughs

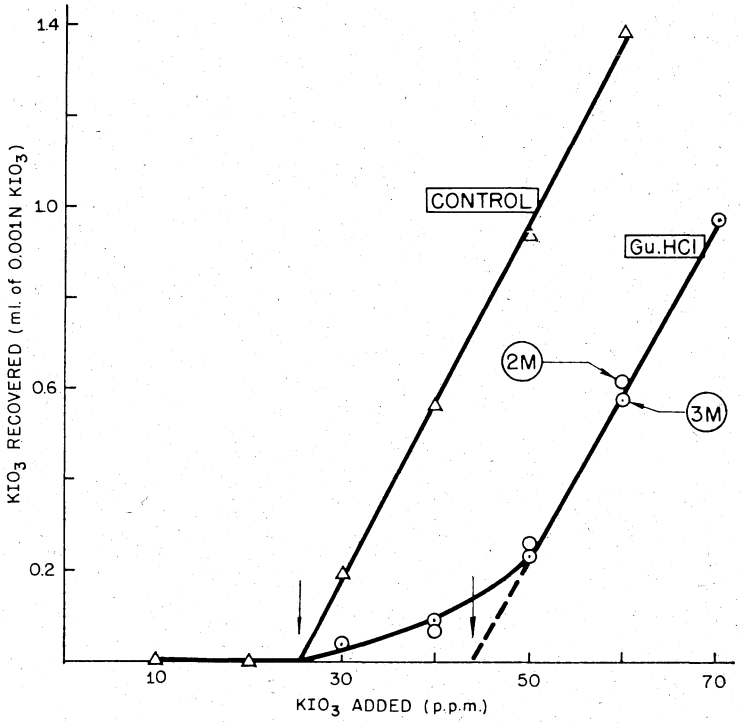

Fig. 3. Iodate recovered vs. iodate added curve for control doughs and for doughs treated with two concentrations of guanidine hydrochloride.

treated with 2*M* and 3*M* guanidine hydrochloride (liquid basis). It seems that the number of sulfhydryl groups reacting depends on the amount of iodate that is added to the dough; but a limit is reached when excess iodate is present. Results were the same for the two concentrations of guanidine hydrochloride used. The sulfhydryl content of the treated doughs obtained by extrapolating the straight portion of the curve (dashed line in Fig. 3) is 9.4 μeq. per g. of protein. Whether this actually represents the total sulfhydryl content of the dough would require a more intensive study.

Accessible Sulfhydryl Groups in Doughs from Various Flours. The method described in this paper was used to obtain data illustrating

differences in the accessible sulfhydryl contents of doughs prepared from flours of different type and quality. Results given below were obtained for doughs of 60% absorption mixed in nitrogen for 5 minutes at 30°C.

Flour	*Protein Content* %, 14% *mb*	*Sulfhydryl Content* *μeq/g protein*
Hard red spring		
Baker's special	12.5	4.0
Straight grade	13.2	5.4
Straight grade	13.3	5.4
Baker's strong	15.4	6.7
Garnet	11.4	7.4
Hard red winter (air-classified)[a]		
Low-protein fraction	10.8	4.2
High-protein fraction	20.4	7.2
Durum	12.9	7.5

[a] Supplied by The Pillsbury Co.

The above results show up two interesting features that deserve comment. Sulfhydryl contents for the first four hard red spring and the two hard red winter flours indicate that accessible sulfhydryl content depends on the protein content of the flour. These results provide a fundamental basis for the observation that bromate requirement for optimum loaf volume for high-grade flours seems to increase with increase in protein content (1). By contrast, an attempt to establish a correlation between the total sulfhydryl content and response to oxidation did not appear particularly fruitful (22). The second feature is the high accessible sulfhydryl content of the poor-quality Garnet flour of relatively low protein content, compared with the values for the other hard red spring flours which were milled from a high-quality bread wheat. More comprehensive studies of a wide range of varieties may well show that *accessible* sulfhydryl content of dough plays a significant role in determining baking quality.

Application of the Iodate Method to Flour Slurries. To provide a more direct basis for comparison between results by the iodate procedure and published results, the method described in this paper was used to determine accessible sulfhydryl contents of one flour in slurries of much higher water:flour ratios than that of dough. The experimental procedure used with flour slurries is as follows: The flour is blended with 95 ml. water in a Waring Blendor running at slow speed; 5 ml. of 0.0056*N* potassium iodate are added and given a 5-minute reaction time with the blender running, and finally the zinc sulfate and sodium hydroxide are added and blending continued for additional 2.5 minutes. The slurry is centrifuged and 20-ml. aliquots are

titrated for residual iodate. Usual precautions are taken to eliminate atmospheric oxygen during all operations.

The results that were obtained for a straight-grade flour of 13.2% protein for five dispersion times and four water:flour ratios are given below.

Total Dispersion Time	*Sulfhydryl Content*			
	50:1[a]	20:1[a]	10:1[a]	5:1[a]
minutes	*μeq/g protein*	*μeq/g protein*	*μeq/g protein*	*μeq/g protein*
7.5	10.4	7.2	5.6	4.5
12.5	10.4	7.6	5.6	4.7
17.5	10.2	7.6	6.1	5.0
27.5	...	8.0	6.7	5.4
37.5	...	8.0	7.4	5.6

[a] Water:flour ratio.

For all water:flour ratios, the number of accessible sulfhydryl groups increased with increasing dispersion time. This effect is analogous to the effect of prolonged mixing on the accessibility of sulfhydryl groups in dough (see above). The effect of prolonged mixing (dispersion) time decreased as the water:flour ratio increased. Although for the lowest water:flour ratio used, the accessible sulfhydryl content is slightly less than that obtained for a dough from the same flour by the standard procedure, the higher water:flour ratios gave considerably higher results. The results for the most dilute slurry indicate that a saturation point is reached in the accessible sulfhydryl content (10.4 μeq. per g. of protein) after 7.5 minutes' dispersion time. This value is slightly higher than the value of 8.1 μeq. per g. of protein obtained by Sokol, Mecham, and Pence (20) by amperometric titration of flour dispersions with silver nitrate. The present results indicate that accessibility of sulfhydryl groups to iodate ions in water-flour slurries increases with increasing dispersion time and water:flour ratio. Accordingly, if the iodate procedure is used to estimate the relative sulfhydryl content of a number of flours, the same conditions must be used for all samples.

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